

# Euophysiology 2018



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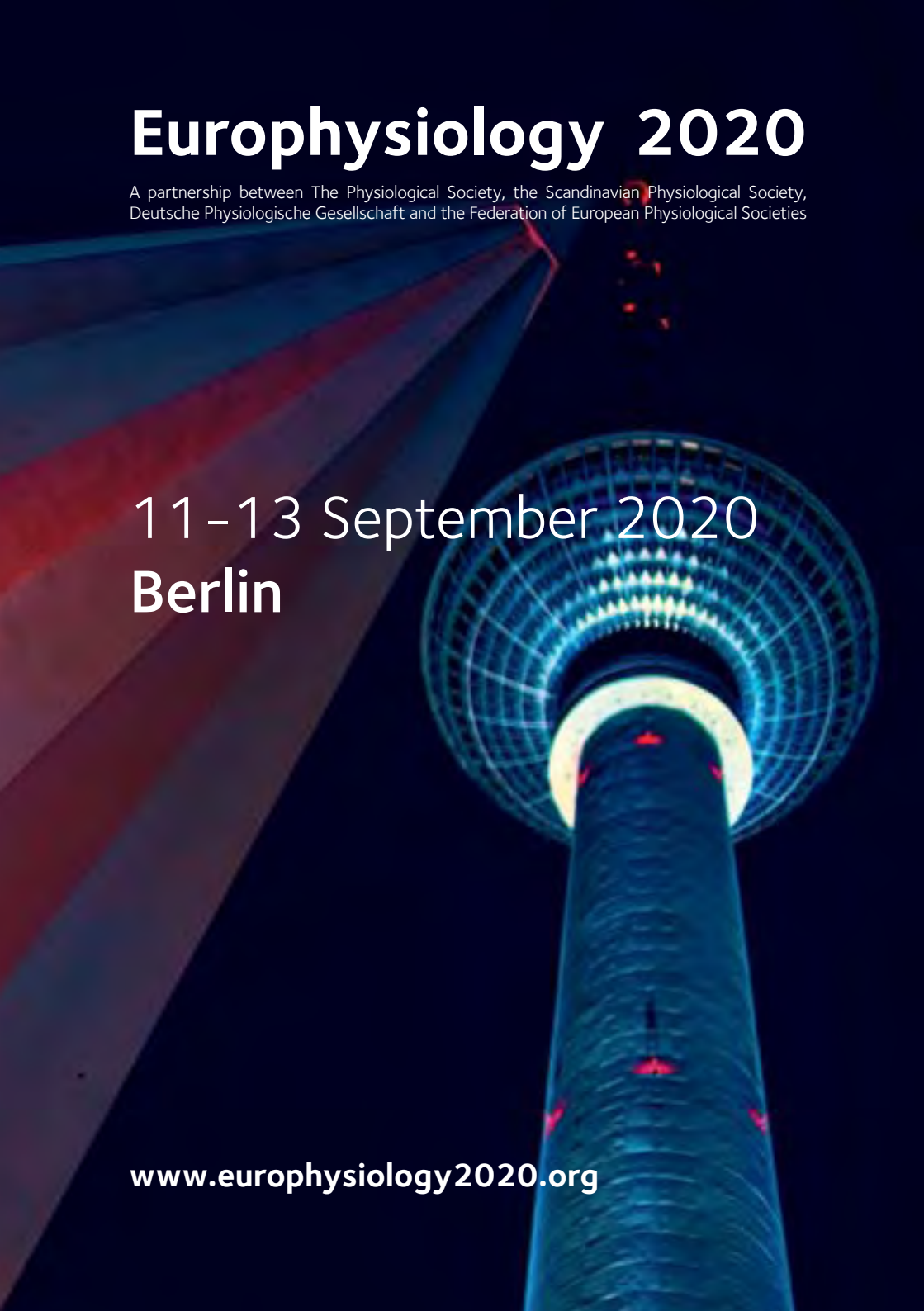
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# **Europhysiology 2020**

A partnership between The Physiological Society, the Scandinavian Physiological Society, Deutsche Physiologische Gesellschaft and the Federation of European Physiological Societies

**11–13 September 2020**  
**Berlin**

**[www.europhysiology2020.org](http://www.europhysiology2020.org)**



|                              |             |
|------------------------------|-------------|
| <b>Prize Lectures</b>        | <b>1P</b>   |
| <b>Plenary Lecture</b>       | <b>4P</b>   |
| <b>Keynote Lecture</b>       | <b>5P</b>   |
| <b>Research Symposia</b>     | <b>7P</b>   |
| <b>Oral Communications</b>   | <b>38P</b>  |
| <b>Poster Communications</b> | <b>105P</b> |

### **Ethical Requirements**

It is a requirement that all vertebrates (and *Octopus vulgaris*) used in experiments are humanely treated and, where relevant, humanely killed.

### **Experiments on animals or animal tissue**

For work conducted in the UK all procedures must conform with current UK legislation.

For work conducted elsewhere all procedures must accord with current national guidelines or, in their absence, with current local guidelines.

### **Experiments on humans or human tissue**

All procedures must accord with the ethical standards of the relevant national, institutional or other body responsible for human research and experimentation, and with the principles of the World Medical Association's Declaration of Helsinki.

**Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.**

## PL001

**Harnessing digital technologies to enhance student learning**

L. Robson

*Biomedical Science, University of Sheffield, Sheffield, UK*

As a Physiologist with over twenty years' experience in learning and teaching one of the major challenges I have experienced is how to address the fear that undergraduate students have around mathematical skills, a recognised issue across the sector (Koenig, 2012). By its very nature Physiology is a mathematical discipline, and for students to develop an advanced understanding of Physiological principles they need to be able to use mathematics to develop data handling skills across a range of techniques. Understanding how to analyse data, and more importantly being able to apply it to solve problems is a key graduate skill. As academics aiming to produce the very best Physiology graduates we therefore need to support and enhance student learning in this area. At the University of Sheffield I run three final year modules, covering ion channel physiology and pathophysiology. These modules develop knowledge and understanding, and also enhance student data handling skills around patch clamp, Ussing chamber and renal clearance approaches. Although students find such areas challenging, I utilise a blended approach to their learning, matching traditional approaches with digital technologies. This allows them to get to grips with the work, and me to fulfil my learning and teaching ethos, which is to always push and challenge students to achieve their best.

Traditional lectures introduce the calculations and analysis that underpin the different methods, with lectures recorded, so that students can review and strengthen their understanding. We also record the more traditional topic based sessions, and integrate these areas into our problem solving sessions (application). The use of lecture capture has led to a vigorous debate over the years around the potential impact on student attainment and attendance (Pursel and Fang, 2012). However, within my modules it is clear that the use of lecture capture is of extreme benefit to students. In the current academic year 97% of the students on my modules have accessed the lecture captures, and all students who completed a survey agreed (19%) or strongly agreed (81%) that lecture capture was useful. An analysis of student attainment in essay and problem based exercises taken two years before and after the introduction of lecture capture in one of my modules shows a clear uplift in student performance, with a higher number of students awarded upper second and first class grades.

Support for students is also provided using short "dynamic" mathematical videos, made using the iPad app Explain Everything. Previous work has highlighted the value of such recordings in enhancing student learning (Hsin and Cigas, 2013). These videos highlight the key mathematical steps the students must master, with students indicating they value this approach to explaining the calculations. These approaches are supported with problem solving sessions where students complete a formative exercise (the best way to learn is to do), that links to lecture content and provides additional guidance. This allows students to develop their skillset in a low risk environment. Personal feedback on the work in group problem solving sessions allows students to feed forward and improve for the summative assessments.

The final module session helps them test their knowledge and understanding of the module, using Lecture Tools to run an interactive session. Students use their electronic devices to answer formative questions that test knowledge and understanding. Within the question set some free text questions allow the students to assess higher level skills around synthesis, and these answers are downloaded and feedback provided to the group via the virtual learning environment. Of course there are always student questions, often sent via e-mail, and sometimes the same question is asked by several different students (adding to the academic workload). To address this issue in Biomedical Science at Sheffield we have brought in "communities of learning" using the Google+ community app. This app allows us to post e-mailed questions and answers to the whole community, meaning that e-mail traffic (and time spend on answering e-mails) is reduced. We also encourage students to participate by posting their own questions, and answers.

The blended approach I have taken to delivery of my final year modules has provided an enhanced learning experience for the students, allowing them to strengthen their knowledge and understanding of physiological experimental approaches, and develop an ability to apply this in scientific problem solving exercises. Their enhanced experience is evidenced by module feedback, which rate my modules and my teaching at the very top within my department. In my talk I will cover how I brought in this blended approach, identifying some of the issues and challenges, and also the benefits to myself as well as the students.

Hsin, W.-J., & Cigas, J. (2013) *Short videos improve student learning in online education*.

*Journal of Computing Sciences in Colleges* 28, 253-259.

Koenig, J (2012). *The mathematics landscape in bioscience undergraduate and postgraduate UK higher education*. The Higher Education Academy. Available from: <http://www.heacademy.ac.uk/>

Pursel, B., & Fang, H. N. (2012). *Lecture capture: Current research and future directions*. Schreyer Institute for Teaching Excellence, Pennsylvania State University.

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## PL005

**From mountains to the bedside: Lessons learnt from Everest**D. Martin<sup>1,2</sup>

<sup>1</sup>*Critical Care Unit, Royal Free Hospital, University College London Division of Surgery and Interventional Science, London, UK and*

<sup>2</sup>*Institute of Sport Exercise and Health, UCL Centre for Altitude Space and Extreme Environment Medicine, London, UK*

Humans are extremely resilient organisms and have successfully adapted to inhabiting and exploring almost every corner of the planet and out beyond its vicinity. Numerous environmental conditions threaten human survival, including heat, cold, microgravity and through a reduction in atmospheric pressure, the lack of oxygen experienced on ascent to high altitude. As we began to explore the mountainous regions of the world, one of the greatest barriers to success was the thin air at great heights which made the smallest of tasks feel like a Herculean effort. Hand in hand with the desire to conquer the Earth's surface came a wish to work out what caused this overwhelming exhaustion. We now know that it is due to the progressive decline in oxygen that is experienced as one ascends and understanding how the body alters and

acclimatises to this lack of oxygen has fascinated physiologists for decades. Sir Joseph Barcroft, Griffith Pugh, John West and Jim Milledge are just a few names that have contributed significantly to this field over the last century.

Disease can lead to internal physiological challenges, arguably in a manner comparable to those that our external environment can generate. Hospitalisation is a significant threshold in the development of any disease, representing deterioration that requires focused investigations and interventions. Failure to respond to treatment and further deterioration can result in a patient requiring admission to an intensive care unit (ICU) to support failing organs and provide complex specialist interventions to the most severely compromised patients in the hospital. Patients in an ICU have a wide range of underlying conditions and therefore present a great heterogeneity of both pathology and treatments. One factor that is common amongst these patients is a lack of oxygen (hypoxia), much like that seen in those at high altitude; the commonest drug given to patients on ICU is therefore oxygen. Despite this, we know surprisingly little about how this lack of oxygen affects our patients, whether they are able to adapt to it and how to optimise the use of oxygen as a therapeutic agent. Research at high altitude may offer an opportunity to study the effect of hypoxia in a more scientifically controlled manner than on an ICU and act as an alternative to animal models of disease. Using a natural model of environmental hypoxia and measuring the responses of healthy volunteers to a graded exposure to high altitude allows us to focus on pertinent physiological pathways. In turn this creates a platform from which we can then return to the bedside and evaluate important mechanisms identified at altitude in critically ill patients.

Through a series of expeditions to high altitude we have studied numerous components of human physiological responses to altitude and are building a picture of a successfully adapted phenotype. Our approach has been to tackle questions at a systemic (whole body), organ, tissue and cellular level, to give us the most comprehensive answers that are feasible from an austere environment. Studying individuals living at sea level and those who have lived at altitude for generations provides us with the ability to differentiate between immediate and long-term strategies to tolerate hypoxia. Our findings may lead to the discovery of novel ways to tackle hypoxia in critically ill patients.

Other extreme environmental conditions may also enrich our understanding of human physiology and lead to clinical benefits that would otherwise not have been discovered. The close relationship between exploration of the universe in which we live and how we change as organisms whilst pushing the boundaries of our physiology may be crucial to the survival of our species.

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PL007

### **Leucine rich repeat kinase 2: From pathology to physiology and back again**

P.A. Lewis<sup>1,2</sup>

<sup>1</sup>*School of Pharmacy, University of Reading, Reading, UK and*  
<sup>2</sup>*Institute of Neurology, University College London, London, UK*

Coding mutations in the *LRRK2* gene on chromosome 12 are the most common genetic cause of Parkinson's disease, and have been the subject of intense experimental scrutiny since

their identification in 2004. Despite these efforts, we are only just beginning to understand how these mutations lead to cell death in the *substantia nigra*, with much of this progress deriving from insights into the physiological function and role of *LRRK2*. In this lecture, the journey from the biochemical and cellular impact of mutations in *LRRK2* to a greater understanding of what the normal function of *LRRK2* is, and how this has fed back into efforts to dissect the role of *LRRK2* in the aetiology of Parkinson's and to targeting this protein in patients, will be described.

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PL008

### **Skeletal muscle mediators and exercise-induced adaptations governing insulin sensitivity in type 2 diabetes**

J. Zierath<sup>1,2</sup>

<sup>1</sup>*Department of Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden and* <sup>2</sup>*Center for Basic Metabolic Research, University of Copenhagen, Copenhagen, Denmark*

Type 2 diabetes mellitus is a life threatening metabolic disease reaching epidemic proportions. Although the molecular basis for this pathology is incompletely understood, genetic and environmental factors, probably in a synergistic manner, contribute to the risk of developing Type 2 diabetes. Type 2 diabetes shares many features of "accelerated aging" including insulin resistance, defective oxidative metabolism/mitochondrial function and loss of muscle mass. Strikingly, long-term participation in exercise training programs prevents insulin resistance and maintains functional muscle mass. The overarching goal of our research is to identify and validate molecules, pathways and ultimately new treatments that confer the benefits of exercise to improve glucose homeostasis and attenuate loss of strength and power throughout the lifespan. The circadian clock, an intrinsic molecular system in virtually all cells, is a key element in homeostatic regulation that controls a large array of metabolic genes. Thus, we are also addressing whether synchronizing exercise and nutrient interventions to the molecular circadian clock will maximize the health promoting benefits of exercise to enhance insulin sensitivity and prevent Type 2 diabetes. This lecture will present new evidence regarding exercise-responsive treatment targets and optimal intervention strategies to prevent metabolic disease.

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PL009

### **Nox4 - multiple functions of a NADPH Oxidase in health and disease**

K. Schröder

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NADPH oxidases have been discovered as enzymes for the first line host defense. They form reactive oxygen species (ROS), such as superoxide anions or H<sub>2</sub>O<sub>2</sub>. Within the last years it became clear, that different NADPH oxidase family members specifically interfere with cellular signaling. The specificity is reached by the mode of activation, the type of ROS produced



and the localization of the individual members of the family. Different to all other members of the family, Nox4 is constitutively active and produces directly  $H_2O_2$ . This enables Nox4 to contribute to long term processes rather than acute signaling. The identification of the physiological function of Nox4 was our matter of interest in the last years. We identified the anti-inflammatory potential of Nox4, discovered it's anti-apoptotic role in endothelial cells as well as it's impact on cellular differentiation. The talk will highlight some of our main findings, which contribute to the conclusion, that Nox4 is the "good NADPH oxidase".

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PL013

### **From toads and sheep to chronotherapy: A melatonin story**

J. Arendt

*University of Surrey, Guildford, UK*

In 1958 the dermatologist Aaron Lerner reported the isolation of N-acetyl-5-methoxytryptamine, the most potent amphibian skin lightening factor, from bovine pineal glands. He called it 'melatonin' from its ability to cause contraction of pigment in amphibian melanophores. This effect provided the first bioassay, but detailed studies awaited the more practical methodology of RIA and later GCMS. In 1965 as I finished my PhD (on tryptophan and its indolic analogues) there were 65 publications with the keyword melatonin in Pubmed. At the time of writing there are 23,549. At one time this small molecule acquired a popular reputation as a universal panacea and elixir of life. So where does the truth lie?

Melatonin is secreted with a marked circadian rhythm, high at night in virtually all species in a 'normal' environment. Light of sufficient intensity and suitable spectral composition (short blue-green wavelengths are the most powerful) will suppress its secretion and is the most important factor maintaining synchrony with the 24h day-night cycle - as it is for the circadian system in general. The duration of secretion depends on the length of the night and this reliable indicator of daylength is used by photoperiodic species such as sheep and hamsters, to time seasonal functions such as reproduction and coat growth. This timing can be controlled using melatonin treatment to maximise benefits to producers. Even humans can show duration changes in melatonin in suitable circumstances but a clear link to human reproduction has yet to be demonstrated.

The melatonin rhythm, timing and sometimes the amplitude, has proved to be the best index of human circadian function to date. Both plasma and saliva melatonin and urinary 6-sulphatoxymelatonin have been extensively used to follow states of circadian desynchrony in, for example, jet lag and shift work. It serves as a marker rhythm in much basic research on sleep and also metabolomics. Diagnosis of circadian rhythm sleep disorders such as delayed/advanced sleep phase and non-24h sleep wake disorder of the blind may need confirmation by melatonin measurement. Abnormally timed sleep in extreme environments such as Antarctica can partly be explained as circadian desynchrony evidenced by the timing of the melatonin rhythm.

Melatonin is often referred to as the 'sleep hormone', whereas in fact it is a darkness hormone. It is, for example, high at night in nocturnal species. In humans surgical removal of the pineal may not be associated with sleep changes, however sleep propensity closely follows the night time rise and low dose exogenous melatonin can induce sleep during 'biological day' i.e. when the endogenous rhythm is low. More importantly exogenous melatonin can shift the timing of the circadian system by advance or delay when correctly timed, and correct timing is ideally calculated from the endogenous melatonin rhythm itself. Thus it has both chronobiotic and hypnotic properties -an ideal combination for the treatment of circadian rhythm sleep disorders. Meta-analyses have concluded that when correctly timed it is an effective therapy and to this end several analogues have been developed and are currently commercialised.

Very many other properties have been attributed to this charismatic molecule and studies of its receptors and their variants are of exceptional interest. From its ability to set the timing of the circadian system and probably to reinforce 'coupling' it is not surprising to find effects on multiple systems. Our studies have considered it as a clock, a calendar, a circadian marker rhythm and a chronobiotic.

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## PL002

**Sweetness and light: Impaired regulation of insulin secretion in diabetes**

F.M. Ashcroft

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Type 2 diabetes is a major global health problem that afflicts >450 million people worldwide. It is characterised by chronic hyperglycaemia that results from insufficient insulin release from pancreatic beta-cells. Both a genetic predisposition and lifestyle factors are involved, which explains why T2D develops with age and is associated with a progressive decline in beta-cell function. Rare genetic forms of diabetes that cause diabetes shortly after birth (neonatal diabetes) can offer insight into the aetiology of type 2 diabetes.

We have studied neonatal diabetes caused by activating mutations in the genes encoding the subunits of the ATP-sensitive potassium ( $K_{ATP}$ ) channel. This channel plays a critical role in insulin secretion by coupling changes in cell metabolism to membrane electrical activity, calcium influx and thereby insulin exocytosis. It consists of pore-forming Kir6.x subunits and regulatory sulphonylurea receptor (SURx) subunits, both of which participate in metabolic regulation by binding adenine nucleotides. ATP binding to Kir6.2 inhibits channel activity whereas Mg-nucleotide interaction with SUR1 stimulates channel activity. About 50% of cases of neonatal diabetes are caused by gain-of-function mutations in Kir6.2 or SUR1. We found that these mutations impair ATP inhibition, locking the channel open and preventing insulin secretion. Using a novel FRET-based method of measuring ATP binding we showed some mutations directly interfere with ATP binding; others act indirectly by increasing the intrinsic channel open probability. In many cases, anti-diabetic sulphonylurea drugs are able to close the mutant channels, thereby stimulating insulin secretion. This has enabled >90% of patients with neonatal diabetes caused by  $K_{ATP}$  channel mutations to transfer from insulin injections to oral tablet therapy, which greatly improves their clinical condition and quality of life.

Transfer is less effective, however, in patients with diabetes of longer duration. To understand why this is the case, we generated a mouse model of neonatal diabetes. We found as little as two weeks of diabetes led to a dramatic metabolic rewiring of beta-cell metabolism. This was characterised by marked changes in gene expression, protein levels and glucose metabolite concentrations, which led to reduced glucose-stimulated ATP production and insulin release. It also caused substantial glycogen storage, impaired autophagy and beta-cell apoptosis. If the diabetes was of short duration, these effects were largely reversed following restoration of euglycaemia with sulphonylurea therapy. However, the ability to effect reversal declined with diabetes duration. These data may help explain why older neonatal diabetes patients find it more difficult to transfer to drug therapy, and why the drug dose decreases with time in many patients.

Similar changes in gene expression and metabolism were found in insulin-secreting INS-1 cells exposed to 25mM glucose for 48 hours; and in a mouse model in which diabetes develops gradually with age (due to deletion of fumarate hydratase). Thus they appear to be a consequence of chronic hyperglycaemia, rather than hypoinsulinaemia or enhanced  $K_{ATP}$  channel activity. This suggests that by causing metabolic rewiring chronic hyperglycaemia may contribute to the progressive

loss of beta-cell function in type 2 diabetes as well as neonatal diabetes. We propose that a small increase in plasma glucose (e.g. due to age, pregnancy or insulin resistance) may reduce mitochondrial metabolism and insulin secretion so increasing glycaemia further and producing a vicious cycle that drives the progression from impaired glucose tolerance to diabetes.

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## PL011

**Elucidation of oxygen sensing pathways in human and animal cells: Implications for physiology**P.J. Ratcliffe<sup>1,2</sup>*<sup>1</sup>University of Oxford, Oxford, UK and <sup>2</sup>The Francis Crick Institute, London, UK*

Animal cells deploy a set of 2-oxoglutarate dependent dioxygenases to signal oxygen levels in cells by modulating the catalytic rate of post-translational hydroxylation of specific amino acids in hypoxia inducible factors (HIFs). HIF prolyl hydroxylation targets HIF- $\alpha$  polypeptides for destruction by the von Hippel-Lindau (pVHL) ubiquitin E3 ligase, whilst HIF asparaginyl hydroxylation inhibits co-activator recruitment and reduces transcriptional activation. In hypoxia these processes are suppressed, allowing HIF- $\alpha$  to escape destruction and form an active transcriptional complex. HIFs bind to DNA at hypoxia response elements and activates several thousand direct transcriptional targets across the genome, which transduce extensive cellular and systemic responses to hypoxia and play a major role in the defence of physiological homeostasis. This lecture will review advances and challenges in the field including insights into the interface between the biochemistry of HIF hydroxylation, the physiology of oxygen homeostasis, and the potential for therapeutic manipulation in disease.

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## PL003

**Diversity of peptide signalling in the brain: of whispered secrets and public announcements**

M. Ludwig

*Centre for Discovery Brain Sciences, University of Edinburgh, Edinburgh, UK*

The brain uses more than 100 different peptides as chemical signals to communicate information, and these have a role in information processing that is quite unlike that of conventional neurotransmitters. Neuropeptides are released from all parts of a neuron, including the axon, soma and, especially, the dendrites, and so are not restricted spatially by synaptic wiring. The neuropeptides oxytocin and vasopressin, are released from dendrites in response to diverse physiological stimuli and dendritic release can be regulated independently from axon terminal release. Oxytocin and vasopressin function as autocrine or paracrine signals at their site of origin, but can also act at distant brain targets to evoke long-lasting changes in behaviour. Thus, diffuse spread of neuropeptides in the extracellular fluid following dendritic release, in addition with focal release from axonal terminals, contributes to regionally and temporally varying combinations of actions providing a large diversity in interneuronal signalling.

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## PL004

**The ins and outs of protein trafficking - a “complex” story?**

R.A. Fenton

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The urinary concentrating mechanism in the mammalian kidney is essential for maintenance of body water homeostasis and thus critical for life (1). One of the major mechanisms in urine concentration is initiated by the binding of the antidiuretic hormone vasopressin (AVP) to its type-2 receptor (AVPR2), resulting in increased abundance of the water channel aquaporin 2 (AQP2) at the apical membrane of collecting duct principal cells. This rate-limiting step for controlling the renal reabsorption of water is highlighted by the disease nephrogenic diabetes insipidus (NDI), where there is a lack of responsiveness of the collecting duct to the antidiuretic actions of AVP, resulting in marked polyuria and polydipsia in patients (2). In recent years, application of modern technologies has accelerated our understanding of AQP2 trafficking, and uncovered that a complex interplay of regulated and constitutive AQP2 exocytosis and endocytosis determines its plasma membrane levels. In addition to AVP, these processes are modulated via various regulatory factors, such as AVP, prostaglandins, secretin or calcitonin. Together they modulate intracellular signalling cascades in the principal cells leading to post-translational modifications within AQP2 and alteration of unique protein:protein interactions (3). In particular, site-specific phosphorylation of AQP2 can determine the rate of its exocytosis and endocytosis. Regulated exocytosis is dependent on a single phosphorylated serine residue (Ser256), which destabilizes actin thin filaments to open the apical actin cortex and facilitates fusion of AQP2 vesicles with the apical membrane. Endocytosis of AQP2 is controlled by concerted effects of Ser256 and Ser269, which when phosphorylated

inhibit interaction of AQP2 with various components of the endocytic machinery and may stabilize AQP2 within plasma membrane lipid-rich domains. In contrast, K63-linked polyubiquitylation of AQP2 at Lys270 can mediate its endocytic retrieval from the plasma membrane and degradation, but site specific phosphorylation of AQP2 is able to over-ride this internalization signal, providing a novel cell biological concept for membrane protein trafficking. AQP2 protein:protein interactions are also important for modulating the proteasomal or lysosomal degradation of AQP2 to maintain AQP2 cellular homeostasis. Recent work indicates an important role for the C terminus of Hsc70 Interacting Protein (CHIP) for both of these processes and thus renal water handling (4). Ultimately, our increased understanding of the intracellular molecular mechanisms of AQP2 trafficking has led to promising new strategies for bypassing defective AVPR2 signalling and restoring AQP2 function in NDI and other water balance disorders (5).

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## PL006

**Therapeutic potential of vascular growth factors**

K. Alitalo

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Antiangiogenic therapy has been a success in the treatment of age-related macular degeneration, but most cancer patients are either refractory or acquire resistance to anti-angiogenic therapeutics. A combination of angiogenesis inhibitors based on knowledge of the major interacting angiogenesis signaling pathways could be used to advance the efficacy of tumor therapy. - Impaired angiogenesis has been implicated in adipose tissue dysfunction and the development of obesity and associated metabolic disorders. New experimental findings indicate that vascular endothelial growth factors can activate the thermogenic program in adipose tissue and even increase the basal metabolic rate, thus preventing diet-induced obesity and related metabolic complications. Several attempts have been made to stimulate angiogenesis and arteriogenesis in tissue ischemia, with limited success. One of the obstacles has been the property of angiogenic growth factors to promote vascular leakage, leading to tissue edema and fibrin deposition, which however can be counteracted by angiopoietins. This could lead to control of vascular leakage in sepsis and other critically ill patients. However, so far, growth factors suitable for angiogenic therapy have not yet provided significant help for patients with cardiovascular disease, but here, better understanding of the biology of the



vascular growth factors may facilitate therapeutics development. The growth of lymphatic vessels, lymphangiogenesis, is involved in a number of pathological processes including tissue inflammation and tumor dissemination, but is insufficient in patients suffering from lymphedema, a debilitating condition characterized by chronic tissue edema and impaired immunity. A lymphangiogenic growth factor is currently moving to phase 2 clinical trial in human lymphedema. The recent discovery of meningeal lymphatic vessels may extend the therapeutic potential of lymphangiogenic growth factors and their inhibitors to neurodegenerative and neuroinflammatory diseases.

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## PL010

### TRP ion channels – Multimodal sensors and guardians of homeostasis

J. Siemens

*University of Heidelberg, Heidelberg, Germany*

Transient receptor potential (TRP) ion channels have been identified as versatile, multimodal molecular sensors. Particularly, several members of the extended TRP ion channel family detect temperature changes in the somatosensory nervous system. Pharmacology and genetic deletion experiments have shown that TRP channels are necessary for mediating responses to painfully hot or cold temperatures and that they become sensitized under inflammatory conditions leading to exacerbated nociceptive signals. TRPs have therefore emerged as targets for analgesic therapy.

Besides constituting a warning system alerting us about noxious thermal conditions, temperature detection in the innocuous range serves another important feat: Mammalian organisms possess the remarkable ability to maintain internal body temperature ( $T_{\text{core}}$ ) within a narrow range close to 37°C despite wide environmental temperature variations. The brain's neural "thermostat" is made up by central circuits in the hypothalamic preoptic area (POA), which orchestrate peripheral thermoregulatory responses to maintain  $T_{\text{core}}$ . How the POA detects and integrates temperature information to achieve thermal balance is largely unknown.

Here, in the first part of the lecture, I will focus on the capsaicin receptor TRPV1, its function as a peripheral temperature sensor and its modulation in the context of inflammatory signals. In the 2nd part I will discuss our recent findings that implicate TRPM2, another thermo-sensitive TRP channel, in hypothalamic thermoregulation.

I will conclude by presenting some preliminary results and an outlook about future experiments geared to address key questions concerning internal (deep brain) temperature detection and thermoregulation.

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## PL012

### Tuning the heart beat through cytoskeletal regulation

B.L. Prosser

*University of Pennsylvania, Pennsylvania, PA, USA*

The pumping of the heart is controlled by the coordinated contraction of around 9 billion cardiomyocytes, muscle cells that stretch and contract with each heart beat. Internal cytoskeletal networks must somehow accommodate these large and rapid geometric changes while maintaining their structural integrity. We have found that with each beat, cardiomyocyte microtubules buckle into short wavelength sinusoids, providing an almost-spring like resistance to cardiomyocyte contraction (Robison et al., *Science* 2016). This buckling behavior depends on the post-translational "detyrosination" of microtubules, which influences the buckling mode by cross-linking microtubules to other cytoskeletal elements and increasing microtubule stability. This cross-linked cytoskeletal network provides a viscous resistance to myocyte motion, stiffening the myocyte during both contraction and diastolic stretch.

Further, we identified a stark upregulation and stabilization of microtubules and intermediate filaments as a conserved signature of end-stage human heart failure. This cytoskeletal upregulation increases the viscous impediment to myocyte motion and contributes to contractile dysfunction in patient cells. Using pharmacologic and genetic approaches to manipulate microtubules and specifically to reduce detyrosination, we can robustly lower the stiffness and improve the contractility of failing human cardiomyocytes (Chen et al., *Nature Med* 2018). Together, this work identifies detyrosinated microtubules as a novel mechanical element within the beating heart cell and a promising new target for the treatment of human heart failure.

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## SA001

**A developmental switch in the activity-dependent plasticity of axo-axonic synapses along the axon initial segment**J. Burrone<sup>1,2</sup><sup>1</sup>Centre for Developmental Neurobiology, King's College London, London, UK and <sup>2</sup>MRC Centre for Neurodevelopmental Disorders, King's College London, London, UK

The axon initial segment (AIS) is an axonal structure close to the soma with a high density of sodium and potassium channels that defines the site of action potential generation. In principal cells of the cortex, it is also innervated by inhibitory synapses formed by a specific type of GABAergic interneuron, the Chandelier cell. Previous work in the lab has focused on activity-dependent forms of plasticity of the AIS and its synapses (Grubb and Burrone, 2010; Wefelmeyer et al., 2015). Here, we present data characterising the changes these structures undergo during postnatal development in the rodent neocortex and the role that local cortical activity plays in this process. We used a recently developed inducible Cre mouse line, *Nkx2.1-CreER*, to label Chandelier cell interneurons (Taniguchi et al., 2013). By visualising Chandelier cell axons and their synaptic boutons in the somatosensory cortex in vivo, as well as in fixed brain preparations, during development, we uncovered a narrow temporal window of synapse formation at the AIS (from P14-P16). We then manipulated the activity levels of either pyramidal neurons or individual Chandelier cells during this period of synapse formation (P12-P18), using a chemogenetic approach. We found that increases in the activity of cortical networks results in a reversible decrease in the length of the AIS as well as the number of axo-axonic synapses it received. Increasing activity specifically in Chandelier cells mirrors the synaptic effect, suggesting this plasticity is cell autonomous. However, when network activity was increased in adult animals (P40-P46) we saw the opposite effect: an increase in axo-axonic synapses along the AIS. This puzzling switch in the direction of axo-axonic synapse plasticity can be explained in the context of homeostatic plasticity. To explore this, we used a genetically-encoded voltage indicator expressed in pyramidal cells to show that ChCs transition from being excitatory at P12-P18, to inhibitory at P40-P46, in agreement with previous findings (Rinetti-Vargas et al., 2017). We propose that this switch in synapse polarity during development is paralleled by a switch in the direction of axo-axonic synapse plasticity that acts to stabilise network activity.

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Taniguchi, H., Lu, J., and Huang, Z.J. (2013). The spatial and temporal origin of chandelier cells in mouse neocortex. *Science* 339, 70-74.

Rinetti-Vargas, G., Phamluong, K., Ron, D., and Bender, K.J. (2017). Periadolescent Maturation of GABAergic Hyperpolarization at the Axon Initial Segment. *Cell Rep* 20, 21-29.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## SA002

**A role for the axon initial segment in rapid modulation of neuronal input-output parameters in mouse barrel cortex**N. Jamann<sup>1</sup>, D. Dannehl<sup>1</sup>, J. Maurer<sup>2</sup>, R. Wagener<sup>3</sup>, M. Kaiser<sup>2</sup>, C. Corcelli<sup>1</sup>, C. Schultz<sup>1</sup>, J. Staiger<sup>4</sup> and M. Engelhardt<sup>1</sup>

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Our classical understanding of neurons emphasizes the role of dendrites for synaptic signal integration and plasticity. In contrast, the axon has been recognized as a rather static output device. This view is rapidly changing, with recent research revealing a much more active role for axonal microdomains in neuronal signal processing. A key regulator in this regard is the axon initial segment (AIS), strategically positioned at the proximal axon. Its molecular architecture, particularly a high density of voltage-gated ion channels, is the anatomical substrate for action potential (AP) initiation. Recent studies have identified the AIS as a significant contributor to the modulation of neuronal excitability. In fact, the AIS exhibits striking structural and functional plasticity, depending on network activity. By regulating AIS length and position, neurons can modulate their excitability and therefore contribute to maintain the functional stability of neuronal circuits. However, the in vitro and in vivo models used often follow rather drastic, non-physiological strategies. Yet if the AIS is to represent another cellular microdomain involved in fine-tuning neuronal activity, a central and presently unanswered question is whether AIS plasticity is actually taking place within a normal physiological range of neuronal function, and which time-frames are required to trigger its remodelling. So we asked whether sudden changes in network state can elicit rapid AIS plasticity (time-scale within hours) for neurons to adapt to global changes. We studied AIS plasticity in the rodent whisker-to-barrel system, specifically investigating AIS-related changes in cortical pyramidal cells of layers II/III and V in mouse primary somatosensory cortex, barrel field (S1BF). Adult mice (P28 and older, n=6 for each group) were subjected to unilateral whisker-trimming and exposed to enriched environment (EE) conditions for 1, 3 and 6 hours, triggering increased stimulation of the remaining whisker-to-barrel pathway. AIS modulations and cellular responses were analysed using multichannel immunofluorescence (against AIS scaffolding proteins ankyrinG and  $\beta$ IV-spectrin, sodium channels, and the immediate-early gene c-fos; n=6 per group), confocal microscopy, and whole-cell patch-clamp recordings in acute slices. Increased stimulation of the barrel network was indicated by upregulation of c-fos in layer II/III pyramidal neurons within 3 hours of EE exposure. The same neurons further showed a significant AIS length reduction in the over-stimulated S1BF compared to controls (mean and S.D.  $20.52 \mu\text{m} \pm 1.98 \mu\text{m}$  EE vs.  $23.54 \mu\text{m} \pm 1.96 \mu\text{m}$  control; n=600 AIS from 6 animals, one-way ANOVA,  $p < 0.05$ ). AIS length then normalized to control level 6 hours after EE (mean and S.D.  $23.82 \mu\text{m} \pm 1.96 \mu\text{m}$  EE vs.  $23.31 \mu\text{m} \pm 1.56 \mu\text{m}$  control; n=600 AIS from 6 animals, one-way ANOVA,  $p < 0.05$ ). Cellular excitability also changed significantly in this 3 hour time-frame: EE-exposed S1BF neurons had a significantly elevated AP threshold (mean and S.D.  $480.4 \pm 36.79$  pA EE vs.  $375.5 \pm 70.5$  pA control; n=9, t-test,  $p < 0.05$ ) and

showed reduced excitability to appropriate stimuli (mean and S.D.  $30.00 \pm 1$  Hz EE vs.  $25.33 \pm 1.03$  Hz control;  $n=9$ ,  $t$ -test,  $p<0.05$ ). Our data indicates that AIS undergo rapid structural plasticity, correlated with functional consequences rendering overstimulated cells less excitable, a mechanism that seems reversible. We therefore propose that rapid AIS plasticity could serve as a fast modulator of neuronal activity in excited networks until a homeostatic balance is achieved.

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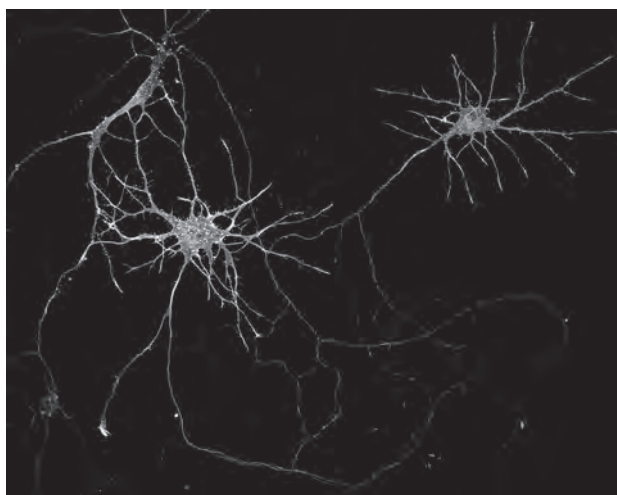
### SA003

#### The axonal cytoskeleton at the nanoscale

C. Leterrier

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The intricate connectivity and molecular identity of axons is maintained for decades, but also adapts to changes in neuronal activity and environment. The axon fulfills these paradoxical demands thanks to a compartmented cytoskeletal architecture that ensures the transport, anchoring and mobility of axonal components. We can now directly visualize these molecular assemblies in situ, thanks to the development of optical super-resolution microscopy techniques (Leterrier et al., 2017). We have used STochastic Optical Reconstruction Microscopy (STORM) to map the nano-architecture of cytoskeletal and scaffold assemblies within the axon. In the axon initial segment, a key compartment for the maintenance of neuronal polarity, we quantitatively resolved a highly organized actin/spectrin/ankyrin scaffold (Leterrier et al., 2015). In addition, we localized several new components within this scaffold (Huang et al., 2017; Berger et al., 2018). Finally, we are developing new versatile labeling, acquisition and processing strategies to characterize the nanoscale architecture of the axon, bringing insights on how it organizes axonal transport (Culley et al., 2018; Almada et al., 2018).



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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

### SA004

#### Submillisecond rapid $\text{Ca}^{2+}$ signalling via $\text{Ca}^{2+}$ permeable axonal voltage-gated $\text{Na}^{+}$ channels

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Cytoplasmic calcium ( $\text{Ca}^{2+}$ ) exerts a wide range of important physiological roles in excitable cells and glia. In neuronal pre-synaptic terminals nanomolar elevation of the cytoplasmic  $\text{Ca}^{2+}$  concentration  $[\text{Ca}^{2+}]_i$  generated by the voltage change during the action potential opens voltage-gated  $\text{Ca}^{2+}$  channels and confers supralinear scaling between  $[\text{Ca}^{2+}]_i$  and transmitter release. Action potentials also generate  $[\text{Ca}^{2+}]_i$  changes in the primary axon including the axon initial segment (AIS) and node of Ranvier but their precise source and role remain poorly understood. Here, taking advantage of the high sensitivity of one-photon epifluorescence imaging we investigated  $\text{Ca}^{2+}$  in somatosensory neocortical layer 5 axons filled with  $\text{Ca}^{2+}$  indicators during somatic patch-clamp recordings in acute brain slices from young-adult rats. Using the high-affinity indicator OGB-1 and correcting fractional fluorescence change ( $\Delta F/F$ ) to background fluorescence revealed that a brief subthreshold depolarization (5 EPSPs with a  $\sim 17$  mV peak amplitude) generated a substantial and transient increase in  $[\text{Ca}^{2+}]_i$  at the AIS and nodes of Ranvier ( $\Delta F/F$   $12.2 \pm 1.2\%$  and  $9.3 \pm 0.9\%$ , respectively,  $n = 9$ ) but not in the internodal domains ( $1.18 \pm 0.35\%$ ; ANOVA with multiple comparisons,  $P = 0.0001$  and  $P < 0.0001$ ,  $n = 9$ ). As expected, action potentials ( $\sim 100$  mV in peak amplitude) evoked larger  $[\text{Ca}^{2+}]_i$  elevations in the AIS and nodes, up to a  $\Delta F/F$  of  $35.9 \pm 4.7\%$  and  $22.2 \pm 3.2\%$  ( $n = 6$  axons) which subsequently spread into the internodes, soma and dendrites. Surprisingly, pharmacological experiments with a wide range of voltage-gated  $\text{Ca}^{2+}$  channel toxins and blockers revealed only a  $\sim 10\%$  contribution during the AP-evoked  $[\text{Ca}^{2+}]_i$  transient in the AIS without an effect on the subthreshold-evoked  $[\text{Ca}^{2+}]_i$  ( $-3\%$  with the T-type blocker TTA-P2 [ $1 \mu\text{M}$ ],  $P > 0.42$ ,  $n = 4$ ). Based on  $> 8$  compounds tested, the activity-dependent  $[\text{Ca}^{2+}]_i$  was only reduced with the voltage-gated  $\text{Na}^{+}$  channel blocker tetrodotoxin (TTX,  $1.0 \mu\text{M}$ ). TTX blocked both the subthreshold-depolarization evoked  $\text{Ca}^{2+}$  ( $91.5 \pm 2.3\%$  block,  $P = 0.0001$ ,  $n = 4$ ) and completely abolished AP-induced  $[\text{Ca}^{2+}]_i$  elevations ( $96 \pm 1\%$  block paired  $t$ -test,  $P < 0.0009$ ,  $n = 4$ ). Based on these data we hypothesized that  $\text{Ca}^{2+}$  enters the axon through the native voltage-gated  $\text{Na}^{+}$  channels, present at very high densities in the AIS ( $\sim 400$  channels  $\mu\text{m}^{-2}$ ). Such unconventional route has been reported in a few cell types and species, including the squid giant axon [1]. To test this we used a recently developed strategy combining a low-affinity indicator OGB-5N with one-photon imaging at the highest possible frame rate of 20 kHz [2] and estimated the time course of  $\text{Ca}^{2+}$  current based on optically recorded and temporally differentiated  $\Delta F/F$ . Results from multiple experiments ( $n = 9$  axons) suggest a microsecond precise alignment of the  $\text{Ca}^{2+}$  current with  $\text{Na}^{+}$  current ( $\sim 200 \mu\text{s}$  activation time constant at room temperature at  $-35$  mV). Taken together, we propose that in the neocortical pyramidal neuron axon initial segment native axonal  $\text{Na}^{+}$  channels are partially permeable

to  $\text{Ca}^{2+}$ , endowing  $\text{Na}^+$ -channel enriched microdomains with a submillisecond rapid  $\text{Ca}^{2+}$  entry pathway and, in concert with amplification from intracellular organelles, enables the tuning of cytoplasmic  $\text{Ca}^{2+}$  to the fast axonal action potential dynamics.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

SA005

### Mechanisms underlying the influence of estrogen on cardiovascular health in women.

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During their fertile stage, women show a lower incidence of cardiovascular diseases than aged matched men, benefit that disappears after menopause. Although age- and sex-related differences point to sexual hormones as possible cardiovascular protective factors, there is controversy surrounding the clinical use of estradiol as a therapeutic agent for cardiovascular disease in women.

Most of the cardiovascular effects of estrogens are attributed to  $17\beta$ -estradiol (E2), and is related to vascular endothelium functions (Sobrinho *et al.*, 2009).  $17\beta$ -estradiol promotes the release of vasoactive compounds, such as endothelial-derived nitric oxide (NO) through an increased expression and activity of endothelial NO synthase, and prostacyclin and thromboxane A2, through the regulation of cyclooxygenases expression and activity, and also shifts the angiotensin axis towards an angiotensin 1-7 production (Sobrinho *et al.*, 2017). Mechanisms underlying estradiol vascular function also include anti-inflammatory and anti-oxidative effects, and epigenetic modifications. Recently, the demonstration of a change in the transcriptomic profile of endothelial cells in response to  $17\beta$ -estradiol and in particular, the involvement of different miRNA in the regulatory pathways of vascular function, extends our expectations on estrogenic vascular actions (Vidal-Gomez *et al.*, 2018).

The wide range of vascular actions mediated by estrogens is mainly exerted by estrogen receptors (ER) through rapid and/or genomic mechanisms. Vascular cells, both endothelial and smooth muscle, express the classic ER,  $\alpha$  and  $\beta$ , and also GPER, a G-protein coupled membrane ER. Likewise, a number of ER isoforms have been described so far. In addition, estrogens differently interplay with other sex hormones by modifying the expression of ER and progesterone receptors and by regulating the hormone levels.

Therefore, the current cardiovascular approach in women health is to resolve discrepancies between the cardiovascular beneficial profile exhibited by estrogens in experimental and some clinical studies and the large randomized trials that could be explained, at least, by two, non-exclusive evidences

(Novella *et al.*, 2012). On the one hand, the “Timing Hypothesis”, which states that estrogen-mediated vascular benefits occur only before the detrimental effects of aging are established in the vasculature. On the other hand, changes in the expression of ER, associated to aging and/or hormone status, that could lead to a deleterious balance of ER $\beta$  over ER $\alpha$ , generally associated with higher oxidative stress and atherosclerotic plaque formation.

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Sobrinho A *et al.* (2017). *Biochem Pharmacol* 129, 67-72.

Vidal-Gomez X *et al.* (2018). *Cell Physiol Biochem* 45, 1878-1892.

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SA006

### Is the estrogen-related receptor responsible for cardiovascular adaptations to exercise in postmenopausal women?

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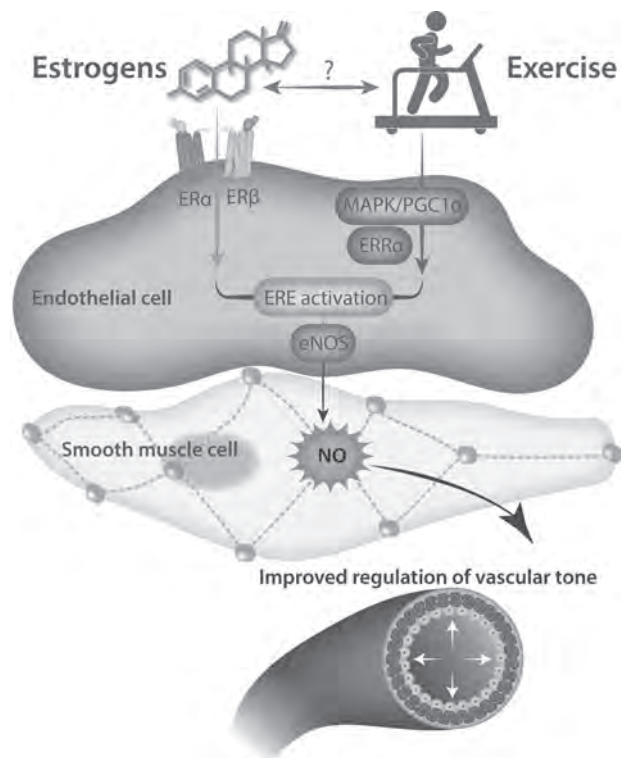
The loss of estrogen at menopause is associated with an accelerated risk of cardiovascular diseases in women. Estrogen is known to impose protective effects on the cardiovascular system and until the menopausal transition, women have a lower prevalence of cardiovascular events compared to aged matched men. One functionally important impairment in the cardiovascular system with menopause is a reduced ability to regulate the vascular tone of peripheral arterioles, an impairment which, is associated with a reduced function of the nitric oxide and prostacyclin systems.

Based on evidence from studies on men, it is generally accepted that exercise training leads to improved vascular function, in part by improved regulation of vascular tone of peripheral arterioles. Moreover, the nitric oxide and prostacyclin systems are *trainable* and are likely, in part, responsible for the beneficial effects of exercise training on vascular function. In men, even at older age, exercise training at a broad range of intensities and modalities improves vascular function whereas older women have been shown to be less responsive to exercise training, and thereby have poorer odds for improving their vascular health via physical activity. However, recent observations indicate that exercise intensity and time after menopause may be of importance for the efficacy of exercise training. The controversial observations that late post-menopausal women do not improve their vascular function by physical activity whereas vascular function is augmented to the same extent in pre-menopausal and early post-menopausal women gives rise to the *exercise timing hypothesis* which suggest that the timing of the initiation of exercise training after menopause determines how much vascular function is improved by a given training intervention.

One potential mechanism behind the *exercise timing hypothesis* is the dynamics of the transcription factor estrogen-related receptor alpha (ERR $\alpha$ ). From cell studies we know that skeletal muscle contraction activates the estrogen response element via a MAPK-PGC1 $\alpha$ -ERR $\alpha$  pathway which ultimately



can induces changes to vascular function by upregulation of endothelial nitric oxide synthase and improved antioxidant defense. In skeletal muscle biopsies from humans,  $ERR\alpha$  expression is higher in recent post-menopausal compared to late pre-menopausal women after a training intervention suggesting that the  $ERR\alpha$  pathway may become more important for the training response after the loss of estrogen. This talk will discuss the putative significance of the  $ERR\alpha$  pathway in mediating the positive effects of physical activity on vascular function in post-menopausal women with a special focus on the nitric oxide and prostacyclin system. Data from topical highly invasive human studies on vascular function are included to highlight the current evidence for a connection between the estrogen-related receptor, estrogen deprivation and exercise training.



Activation of estrogen response element (ERE) by estrogens and muscle contraction. ERE can be activated by muscle contraction via the MAPK - PGC1 $\alpha$  -  $ERR\alpha$  pathway. This pathway leads to upregulation of eNOS which may improve regulation of vascular tone by increasing NO bioavailability. Estrogens activates ERE directly via estrogen receptor (ER) activation and may also lead to improved vascular regulation. The interaction between estrogens and physical activity, in the pre-, early post- and late post-menopausal phase is not fully understood. ERE, estrogen response element; MAPK, Mitogen-activated protein kinase; PGC1 $\alpha$ , peroxisome proliferator-activated receptor gamma coactivator 1 $\alpha$ ;  $ERR\alpha$ , estrogen-related receptor  $\alpha$ ; eNOS, endothelial nitric oxide synthase; ER, estrogen receptor; NO, nitric oxide.

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SA007

### Role of estrogen in exercise training-induced improvements in vascular function

D.R. Seals

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Cardiovascular diseases (CVD) remain the leading causes of morbidity and mortality for men and women in developed societies. Aging is the primary risk factor for CVD in both sexes, as CVD prevalence increases with advancing age and >90% of CVD occurs in middle-aged and older men and women. However, sex hormone deficiency, particularly reductions in circulating levels of estrogen, as occurs during and after menopause, also plays an important role in age-associated increases in CVD risk in women.

Aging increases CVD risk in part via direct effects on the heart and increases in systolic blood pressure, but much of the age-related elevation in risk is mediated by arterial dysfunction. Several types of arterial dysfunction contribute, but two of the most important changes are large elastic artery (aortic and carotid artery) stiffening and the development of endothelial dysfunction, as indicated by impaired nitric oxide (NO)-associated endothelium-dependent dilation. Although the exact mechanisms underlying age-related arterial dysfunction remain under investigation, it is clear that excessive superoxide-induced oxidative stress and chronic low-grade inflammation are the major processes involved.

Regular aerobic exercise (AE) is one of the most effective strategies for reducing the risk of CVD with aging. Although AE lowers CVD risk in part by favorably modifying conventional risk factors such as blood pressure, fasting glucose and circulating lipids, much of benefits of AE are likely transduced through the maintenance of good arterial function and health<sup>1</sup>. Indeed, there is considerable evidence the AE inhibits large elastic artery stiffening and preserves vascular endothelial function with aging by maintaining NO bioavailability and suppressing vascular oxidative stress and inflammation<sup>1</sup>. For example, regular AE is associated with lower carotid-femoral pulse wave velocity (aortic stiffness) and enhanced carotid artery compliance with aging in both men and women<sup>1,2,3</sup>. Importantly, aortic stiffness and carotid compliance are improved in both middle-aged/older men and in postmenopausal women in response to an AE intervention<sup>1,2,3</sup>. There is some evidence to suggest that AE enhances carotid artery compliance in postmenopausal women by suppressing oxidative stress<sup>1</sup>.

Regular AE has a strong vascular-protective effect on endothelial function with aging, at least in men<sup>1</sup>. Both macrovascular (e.g., brachial artery flow-mediated dilation, FMD) and microvascular (e.g., increases in forearm blood flow in response to brachial artery infusion of acetylcholine) endothelial function are preserved in late middle-aged/older men who regularly perform vigorous AE<sup>1,4,5</sup>. Consistent with such cross-sectional observations, AE training restores microvascular and macrovascular endothelial function to or close to levels observed in young healthy males<sup>4,5</sup>. These benefits are mediated by enhanced NO bioavailability and reduced vascular oxidative stress and inflammation<sup>1</sup>.

Although some studies have reported AE-associated enhancement of vascular endothelial function in postmenopausal women, other studies, including our own, indicate a notable



lack of effect in this group as a whole<sup>1</sup>. In a large cross-sectional comparison of healthy adults, we found that brachial artery FMD was ~50% greater in aerobically exercising vs. non-exercising middle-aged and older men, but did not differ in exercising vs. non-exercising (estrogen-deficient) postmenopausal women<sup>5</sup>. After an 8-week AE intervention, we observed a 50% improvement in brachial artery FMD in the men, but no change in the postmenopausal women<sup>5</sup>. A recent investigation by our laboratory confirmed these observations for macrovascular endothelial function, and extended our findings to show that microvascular endothelial function also does not differ between endurance exercise-trained and untrained healthy estrogen-deficient postmenopausal women<sup>6</sup>. To determine the role of estrogen deficiency in these observations, we studied the effects of AE training on endothelial function with and without prior chronic estrogen replacement<sup>7</sup>. We found that AE-induced improvements in brachial artery FMD only occurred in estrogen-replaced postmenopausal women, suggesting a critical permissive role of estrogen in transducing improvements in endothelial function with AE in this group.

In summary, AE is associated with reduced arterial stiffness with aging in both men and women, and enhanced vascular endothelial function with aging in men. Further research is needed to understand the factors and physiological mechanisms, including those related to sex hormone bioavailability, underlying the inconsistent effects of AE on endothelial function in postmenopausal women.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

SA008

### Challenging metabolic tissues with fructose: tissue-specific and sex-specific responses

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Excessive consumption of free sugars (which typically includes a composite of glucose and fructose) is linked to an increased risk of developing chronic metabolic diseases including obesity, non-alcoholic fatty liver disease (NAFLD), type 2 diabetes, and cardiovascular disease. Although current knowledge of fructose metabolism has primarily come from studies involving liver metabolism, where it is implicated in impaired insulin sensitivity, increased fat accumulation and dyslipidaemia, we still have a limited understanding of how consumption of fructose, as part of a mixed meal, may alter hepatic fatty acid synthesis and partitioning. Moreover, the effect of dietary fructose in subcutaneous adipose depots, the largest human fat depot, has not been studied.

A consistent finding in the literature is the effect of fructose on hepatic *de novo* lipogenesis (DNL). By undertaking a randomised cross-over study, we have investigated the effect of a mixed meal, high and low in fructose, in healthy men and women. Hepatic DNL was assessed using stable-isotope tracers. We found after consumption of the low fructose meal, there was no difference in fasting or postprandial hepatic DNL between men and women. In contrast, after consumption of the high fructose meal, despite fasting hepatic DNL being similar, postprandial DNL was significantly higher in women compared to men (effect of sex  $p < 0.05$ , time x sex interaction  $p < 0.05$ ). The potential implications of this observation will be discussed.

Moreover, we have started *in vitro* cellular studies to investigate the effects of fructose on subcutaneous adipocyte metabolism. Our preliminary findings suggest that fructose is metabolised by human adipocytes and is used as a substrate for DNL. This talk will discuss our recent findings and provide evidence for tissue-specific and sex-specific responses to fructose challenges.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

SA009

### Fructose for endurance exercise performance and recovery

C. Fuchs

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Carbohydrate intake is important for optimal performance during prolonged bouts of moderate- to high-intensity exercise. The primary form of carbohydrate that is typically ingested during and after exercise is glucose. However, when ingesting glucose in large amounts ( $>1.2$  g/min), the predominant intestinal glucose transporter SGLT1 will become saturated, thereby limiting further glucose uptake into the circulation. Fructose, which is another form of carbohydrate, will be primarily absorbed via a different intestinal transport protein (GLUT5) and, when co-ingested with glucose, can further increase total exogenous carbohydrate availability and oxidation rates. Subsequently, it has been shown that ingesting a combination of glucose and fructose can improve

exercise performance when compared to equivalent amounts of glucose only.

One mechanism by which carbohydrate ingestion during exercise delays fatigue is thought to be the sparing of endogenous (liver and muscle) glycogen stores. We recently showed that the ingestion of relatively large amounts (1.7 g/min) of carbohydrates (glucose or sucrose) during exercise can completely prevent liver, but not muscle glycogen depletion during prolonged exercise. Furthermore, we observed that ingestion of sucrose (which is composed of glucose and fructose) results in fewer gastrointestinal complaints when compared to glucose only.

After exercise, it can also be of relevance to ingest carbohydrates for replenishing liver and muscle glycogen stores. Normally, glycogen stores can be fully replenished within 24 h after ingesting a carbohydrate rich diet. However, during some events (e.g. Tour de France) the available recovery period between subsequent exercise bouts is less than 24 h. Under such circumstances it is key to utilize strategies that accelerate muscle and liver glycogen repletion. Fructose co-ingestion has been shown to be particularly relevant as it not only increases total exogenous carbohydrate availability, but is also predominantly metabolized in the liver where it can be stored as liver glycogen. Indeed, we have shown that ingesting sucrose after exercise will potentially accelerate liver (but not muscle) glycogen repletion when compared to the ingestion of an equivalent amount of glucose (polymers) only. In addition, sucrose ingestion also results in less gastrointestinal distress when relatively large amounts of carbohydrates (1.5 g/kg/h) are ingested after exercise, when compared to the ingestion of the same amount of carbohydrate in the form of glucose (polymers) only.

In conclusion, combining fructose with glucose is beneficial for trained athletes aiming for optimal performance during prolonged moderate- to high-intensity exercise sessions as well as rapid short-term endogenous (liver) glycogen repletion.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

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SA010

### **Fibroblast growth factor 21 (FGF21) decreases consumption of sugar and sweet-tasting compounds in mice.**

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Health is influenced by the composition of diet as well as the total energy intake, and poor dietary choices, like excessive consumption of fat and carbohydrates, have been shown to cause health complications. Just as there are mechanisms to promote carbohydrate intake, there likely exists unknown pathways that reduce carbohydrate intake, and such interoceptive pathways have the potential to influence the quality of our food choices. In 2013, two genome-wide association studies (GWAS) both identified SNPs in the FGF21 locus associated with increased carbohydrate and decreased protein and fat consumption (Chu, 2013; Tanaka, 2013). This suggested a role for fibroblast growth factor 21 (FGF21) as a modulator of macronutrient intake. FGF21 is a peptide hormone, which mainly, but not exclusively, is produced by the liver, an organ which is uniquely positioned to sense circulating levels of

nutrients and excrete signalling molecules to change ingestive behaviour. We, therefore, hypothesized that FGF21 might affect food preferences in mouse models.

We increased circulating levels of FGF21 by either injecting or infusing human recombinant FGF21 or using a transgenic mouse model that overexpresses endogenous FGF21. We also developed whole-body and liver-specific FGF21 knockout mice. We made use of different choice paradigms to assess sugar preference. Mice were either given a choice between chow and a high-sucrose diet (HSD); or subjected to a two-bottle choice between water and a nutrient solution, for example, fructose.

When endogenous FGF21 is knocked out, we observe a 2-fold increase in consumption of sugars (Suc, Fru, and Glu). Even the liver-specific FGF21 KO strain displays strong preference for sucrose-enriched diet and water, demonstrating that liver-derived FGF21 is responsible for regulating sugar intake. When increasing circulating levels of FGF21 levels, we observe decreased consumption of sugars. A preference towards other macronutrient classes, like fat and protein, was not found in two-bottle choice studies. Furthermore, no preference was found for complex carbohydrates, which warrants a specificity of FGF21 regulation to sugars and sweet-tasting compounds. Importantly, upon ingestion of sugars, we observe a physiological induction of FGF21 transcript and protein levels in WT mice.

The FGF21 co-receptor, beta-klotho (KLB), has previously been shown to be expressed in different brain regions. To examine which brain regions FGF21 is signalling through to regulate sugar intake, KLB was knocked out in either the PVN, SCN or NTS. The FGF21-mediated reduction in sugar intake was attenuated only when KLB was deleted in the PVN. In addition, high plasma levels of FGF21 resulted in increased immunoreactivity of c-fos in the PVN, confirming the involvement of the hypothalamus in sucrose-specific appetite regulation.

Due to the induction of circulating FGF21 by sugar intake, we propose a negative feedback loop, where the liver senses the amount of consumed sugar and down-regulates further intake through expression of FGF21. Knockout of hepatic ChREBP abolishes the induction of FGF21 transcription, suggesting that sugar activate the transcription factor ChREBP to stimulate FGF21 production. This study raises the possibility that sugar intake could be manipulated through molecular therapies, which could have promise in combating obesity and diabetes by enforcing the nutritive quality of dietary choices. Chu A *et al.* (2013). *Hum Mol Genet.* 22(9), 1895–1902.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

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SA011

### **Metabolic effects of high fructose intake in humans**

L. Tappy

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Epidemiological studies consistently show an association between sucrose or high fructose corn syrup intake and metabolic diseases. It is proposed that this relationship is mainly due to the fructose component of sugars. Prospective cohort studies show an association between consumption of added sugars containing fructose and body weight gain, and intervention trials suggest that the relationship is due to a fructose-induced increase in energy intake. The mechanisms

through which fructose alters energy intake remain debated. In addition, many short term studies also indicate that dietary fructose exert specific metabolic effects in humans independently of changes in body weight. It increases fasting and postprandial blood triglyceride concentration, impairs hepatic insulin sensitivity, and increases uric acid and intrahepatic fat concentrations. It is proposed that excess fructose associated with excess energy causes a hepatic metabolic overload which may contribute to the development of non-alcoholic fatty liver disease, insulin resistance and cardiovascular diseases.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

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## SA012

### **Physiology online: expanding knowledge and uptake of physiology through a Massive Open Online Course (MOOC)**

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Massive Open Online Courses (MOOCs) are university-led, distance-learning courses that are freely available to the general population via the internet. The introduction of MOOCs around a decade ago generated considerable excitement and expectation, with the potential for free access, unlimited participation and low running costs promising to extend a university's educational activities beyond its bricks and mortar.

The Physiological Society recently decided to develop a MOOC aimed generally at raising the profile of physiology and specifically at supporting the progression of students into physiology and related disciplines at University. Following a competitive tender process, the University of Liverpool was selected as the content provider, with the course hosted online by FutureLearn, one of the leading MOOC platforms.

The MOOC content was developed during 2016-17 and focused on three core topics in physiology, the heart and circulatory system, the respiratory system and the nervous system, delivered sequentially over the three weeks of the course. Opportunities to explore these topics were provided through video content, interactive discussion boards, collection and analysis of physiological data and quizzes.

The resultant MOOC is entitled 'Physiology: The Science of Life' and, to date, the course has run twice (Autumn 2017 and Spring 2018). A total of 11,328 participants have enrolled on the MOOC and course participants demonstrated a wide geographical spread of 143 countries, with 44% from UK. This uptake profile indicates that the MOOC has been successful in highlighting the discipline of physiology across the globe. The course was aimed primarily at 16-18 year olds considering their next step into higher education, but was open to people of all ages and educational backgrounds. Of those who provided details of their age in the first run of the MOOC (n=835), there was a relatively even distribution across age brackets, with only 22% aged 25 or under. Furthermore only 14% were full-time students and 40% already had a BSc degree or higher qualification in Biology or a related subject. The participant demographics were similar for the second run of the MOOC. These data suggest that although the course was successful in attracting learners, the target audience did not engage preferentially with this learning opportunity; however, potential alternative audiences were revealed.

This talk will address the benefits and limitations of using a Physiology MOOC to support transition to university study, and consider the broader impact of the MOOC in promoting the discipline of physiology.

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## SA013

### **Enhancing education through gamification**

I. Turner

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Gamification uses the mechanics of games such as freedom to fail, levels and point scoring in atypical situations. Game based learning (GBL) can incorporate elements of gamification but is more broadly learning through play. Both gamification and GBL have been successfully used in a range of virtual and physical Higher Education settings. The use of GBL or 'serious games' Higher Education can be a really powerful tool for engaging and educate student learner. This talk will provide an overview of how games can work in the HE classroom but will give you tools necessary to design and test your games.

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## SA014

### **cAMP nano-domains enable precise tuning of cardiac contractility**

M. Zaccolo

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cAMP/PKA signalling modulates contractile, metabolic and transcriptional cellular responses. This signalling system is at the core of the neuro-hormonal regulation of cardiovascular functions and is the target of a number of current therapeutics. The pleiotropic effects of cAMP/PKA signals rely on an intricate intracellular network of simultaneously operating pathways that are uniquely regulated to deliver the signal to the appropriate subcellular location. Local control (compartmentalisation) of cAMP/PKA signalling allows for only a section of the network to be active at any given time and determines the specificity and accuracy of the functional response. Disruption of cAMP/PKA signalling compartmentalisation associates with development of disease. By combining real-time FRET imaging, genetic, biochemical and mathematical approaches we are mapping cAMP/PKA subcellular signalling domains in cardiac myocytes, we are establishing their function and regulation and determining how local signalling is altered in pathological conditions. Our ultimate goal is to develop precision medicine strategies to target local cAMP pools, rather than global intracellular cAMP levels, to achieve greater therapeutic efficacy and specificity. Our recent studies in cardiac myocytes reveal a previously unsuspected sub-microscopic heterogeneity of intracellular cAMP signals and provide evidence that the physiologically relevant cAMP is confined to nano-domains immediately surrounding effector-target complexes. These findings subvert the classical notion of cAMP signalling and provide a new framework for the development of targeted therapeutic approaches.



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SA015

### Intercalated Disc Proteins Modulate Cardiac Excitation-Contraction Coupling: Impaired EC-Coupling and Ion Channel Function in Induced Pluripotent Stem Cell Derived Cardiac Myocytes from Arrhythmogenic Right Ventricular Cardiomyopathy Patients

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Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a primary cardiomyopathy associated with sudden cardiac death (SCD) in young patients during high intensity activity<sup>1</sup>. The most common ARVC subtype 9 includes mutations in plakophilin2 (PKP2), affecting the desmosomal integrity<sup>2, 3</sup>. PKP2 directly and indirectly interacts with the cytoskeleton, the voltage gated sodium channel (Na<sub>v</sub>1.5), the inward rectifying potassium channel (Kir2.1), the gap junctions (Connexins) and modulators of excitation contraction coupling (ECC) such as CaMKII<sup>4</sup>. However, the detailed pro-arrhythmic mechanisms are not fully understood. Here we strive to elucidate the pro-arrhythmic changes in Ca handling and ion channel function in ARVC9 and the triggers for adverse development in some mutation carriers.

We generated induced pluripotent stem cells (iPSC) from a patient who survived SCD and was diagnosed with ARVC9 (1bp-Deletion, c.1664delT; p.Phe555SerfsX8, Exon 7; index-patient), an asymptomatic sibling carrying the same mutation and cells from a healthy proband (CTRL). After cardiac differentiation and a 3 month maturation, iPSC-derived cardiomyocytes (hiPSC-CM) were investigated.

Ca transients (CaT; Fig. A) were measured by confocal microscopy using the Ca sensitive dye Fluo-4 AM. Compared to CTRL, CaT amplitude was significantly reduced in the indexpatient but not the sibling, and CaT decay slowed in iPSC-CM from the asymptomatic sibling and the indexpatient (CTRL n=43, sibling n=55, indexpatient n=80).

Spontaneous CaT frequency (Fig. B) was significantly increased in cells from the sibling and the indexpatient. Importantly, we detected more frequency induced pro-arrhythmic events such as CaT alternans in cells from the sibling and the indexpatient (Fig. C).

Action potentials (APs) and Na currents (I<sub>Na</sub> and I<sub>NaL</sub>) were acquired by whole-cell patch clamp. In accordance with increased CaT frequency, the resting membrane potential (RMP) was significantly more positive in cells from the indexpatient (-60 ± 3.7 mV vs CTRL -70 ± 2.4 mV; p=0.05). These changes cannot be explained by the reduced I<sub>Na</sub> or I<sub>NaL</sub> in cells from the indexpatient and the sibling (Fig. D and E).

Thus, we measured protein expression of the inward rectifier Kir2.1 by Western blot technique. Kir2.1 expression was reduced from 0.45 in CTRL to 0.22 in the sibling and 0.23 in the indexpatient (Kir2.1/GAPDH), which might explain the shift in RMP.

The intercellular communication via gap junctions, analyzed by fluorescence recovery after photobleach (FRAP) in cell clusters loaded with calcein, was also significantly impaired in cells from the indexpatient. The recovery rate of the bleached signal was reduced from 0.009 ± 0.0008 1/s in CTRL cell clusters to 0.005 ± 0.0006 1/s in clusters from the indexpatient (p<0.001).

We show for the first time that hiPSC-CM from an ARVC9 patient mutation have reduced intercellular communication and impaired ECC expressed in prolonged CaT and APD.

Changes in Na currents and RMP were only detected in cells from the indexpatient while the impaired Ca cycling was also apparent in the asymptomatic sibling. We assume that early changes detected in Ca handling might set the basis for adverse ECC, but further changes are needed to cause pro-arrhythmic behavior.

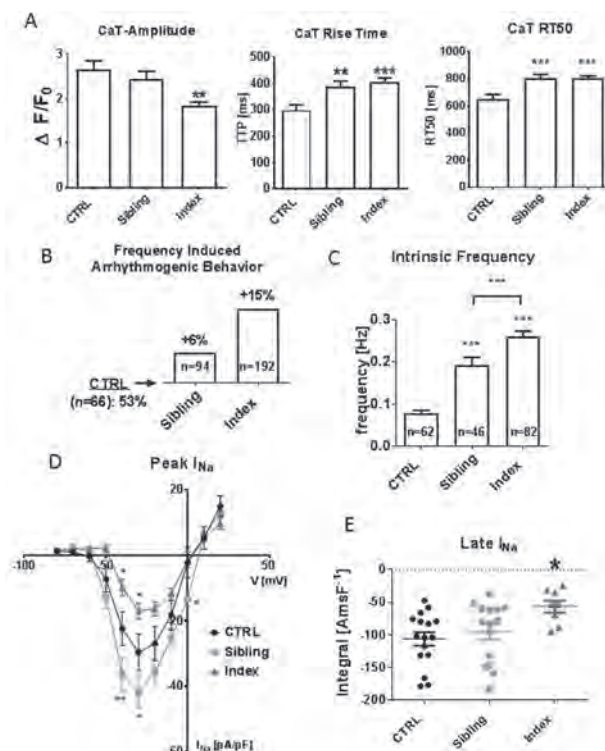


Fig. 1: Impaired Ca handling and ion channel function in an ARVC9 patient. A: Calcium handling in hiPSC-CM (CTRL n=43, sibling n=55, Indexpatient n=80). B: Frequency induced arrhythmogenic behavior is increased in mutation carriers. C: The spontaneous, intrinsic frequency is increased in mutation carriers. D: Peak I<sub>Na</sub> is reduced in the index patient but increased in the asymptomatic sibling. E: Late I<sub>Na</sub> is unchanged in the sibling but reduced in the indexpatient. (\*p<0.05, \*\*p<0.01, \*\*\*p<0.001)

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## SA016

### Revealing Realistic Ryanodine Receptor Organization in Cardiac Myocytes using 3D dSTORM Imaging

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Cardiomyocyte contraction is critically dependent on Ca<sup>2+</sup> release from Ryanodine Receptors (RyRs). While advances in single-molecule localization microscopy have allowed 2D, nanoscale visualization of RyRs at the cell surface, little is known about the geometric arrangement of RyRs in the context of a whole cell. Here, we have developed a novel approach to quantitatively characterize the precise organization of RyRs in isolated adult Wistar rat (male, 250-350 g) cardiomyocytes using 3D direct stochastic optical reconstruction microscopy (dSTORM) (Zeiss ELYRA). We observed that RyR cluster organization at the cell surface was undulatory, resulting in overlap in the z-axis which obfuscated detection by 2D techniques. Non-overlapping clusters were imaged to generate a calibration curve (3.3076 events/RyR, 1740 clusters) for estimating RyR number based on recorded fluorescent events. Employing this method at the cell surface and interior revealed smaller RyR clusters than 2D estimates, as erroneous merging of axially-aligned RyRs was circumvented (mean 3D cluster size = 10.1 ± 1.1 and 13.1 ± 1.0 RyRs at cell surface and interior, respectively). Functional groupings of RyR clusters localized within 100 nm (Ca<sup>2+</sup> release units, CRUs) contained an average of 18 and 23 RyRs at the surface and interior. While smaller than previous reports, this estimate parallels RyR numbers calculated to underlie Ca<sup>2+</sup> sparks. Half of all CRUs contained only a single "rogue" RyR. Internal CRUs were more tightly packed along z-lines than surface CRUs, contained larger and more numerous RyR clusters, and constituted ≈75% of the roughly 1 million RyRs present in an average cardiomyocyte. This complex 3D geometry of internal CRUs was underscored by correlative imaging of RyRs and T-tubules, which enabled visualization and quantification of dyadic and non-dyadic RyR populations. These data provide novel, nanoscale insight into RyR organization in cardiomyocytes critical for understanding excitation-contraction coupling.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## SA017

### Ryanodine receptor clusters in cardiac ventricular myocytes as prototypical calcium signaling nanodomains

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Ryanodine receptors (RyRs) are calcium release channels in the sarcoplasmic reticulum of ventricular myocytes. They provide the molecular basis of the process of calcium-induced calcium release (CICR) that is critical for cardiac excitation-contraction coupling. When in close proximity, adjacent RyRs can activate each other via CICR so that clustering of RyRs can greatly affect the probability with which calcium trigger signals will activate such clusters. For some time, we have therefore investigated the distribution of RyR clusters in rat ventricular myocytes and quantified the size of RyR clusters as well as inter-cluster distances and the distribution pattern of RyRs within clusters. Recently, using the remarkable localization precision of the super-resolution imaging modality DNA-PAINT we visualized punctate labeling within RyR labeling nanodomains, which we confirmed as single RyRs. RyR positions within sub-plasmalemmal RyR clusters revealed that clusters are organized randomly into irregular clustering patterns leaving significant gaps occupied by accessory or regulatory proteins. DNA-PAINT super-resolution imaging supports a quantitative imaging mode termed qPAINT (or quantitative PAINT). We used qPAINT to precisely quantify RyR cluster size and determined that, on average, peripheral clusters contain 8.8 ± 3.6 RyRs (n = 17 cells, 10 animals). RyRs are spaced within clusters at nearest neighbour distances of 40.1 ± 0.9 nm (n = 1802 clusters), similar to recent measurements using electron tomography but greater than the *in vitro* RyR packing distances observed in artificial bilayers. We constructed a Monte Carlo model of protein cluster assembly which reproduced the cluster morphologies observed in the DNA-PAINT image data suggesting that RyRs commonly cluster through a random assembly process into irregular arrays containing significant gaps. Using the chromatic aberration-free Exchange PAINT double-labeling approach we determined the stoichiometry of the junctional protein junctophilin-2 (JPH2), measured through a modified qPAINT counting algorithm, which varied from cluster to cluster (0.5-3.5) with a mean of 1.38 ± 0.5 (n = 250 clusters, 2 animals). Our observations provide a basis for biophysical models of RyR cluster activation. We will also present imaging data on RyR phosphorylation within clusters and discuss some implications of this data.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## SA018

### Differential Roles of Androgens in Acute Kidney Injury in Male and Female Rats: Implications for Humans

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Acute kidney injury (AKI) is a leading cause of morbidity and mortality, and men are more prone to AKI than women implicating androgens as a causative factor whereas estrogens have been thought to be protective. In women with

hyperandrogenemia, a consequence of polycystic ovary syndrome (PCOS), it is not clear if they remain protected from AKI.

In male rats exposed to renal ischemia (30 min, bilaterally), plasma testosterone levels drop, and infusion of testosterone propionate (20 µg/kg iv over 10 min) within 3 hrs of reperfusion protected the rats from elevated levels of plasma creatinine and renal injury. Blockade of the nitric oxide pathway prevents testosterone infusion from being protective in males, suggesting that androgens may be eliciting a vasodilator effect mediated by the NO system to cause protection. Blockade of the androgen receptor with flutamide has no effect on the testosterone-mediated protection from renal injury after renal ischemia-reperfusion (I/R), suggesting that testosterone is not working through the androgen receptor. Using an established female rat model of PCOS, we tested the hypothesis that chronic hyperandrogenemia in females increases the risk of I/R-induced AKI compared to placebo controls. Female SD rats were implanted with dihydrotestosterone (DHT, 7.5mg/90d) or placebo pellets beginning at 6 wks of age; pellets were changed every 85 d. At 6-7 mos of age, female rats were subjected to sham or renal I/R with bilateral clamping of renal vessels (30 min). I/R increased plasma creatinine to a higher level in DHT treated females than in sham controls. Pre-treatment with flutamide for three days prior to I/R had no effect on the plasma creatinine in the DHT treated females. These data show that exposure to chronic low levels of androgens in females causes significantly greater renal injury with I/R AKI than in sham controls, but the renal injury is independent of the androgen receptor, suggesting other mechanisms may be contributing to the higher level of renal injury after I/R AKI in females. The data also suggest that women with PCOS may have higher prevalence of AKI than do age-matched normal women.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

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SA019

### **Sexual dimorphism of KCNQ1:KCNE3 channels affecting colon cancer proliferation and patient survival**

B. Harvey

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Estrogen regulation of epithelial ion secretion and absorption exhibits sexual dimorphism and increased potency in the female estrous cycle. The molecular target of estrogen's anti-secretory actions in the intestine is the KCNQ1:KCNE3 channel. We have recently discovered that this K<sup>+</sup> channel is complexed with β-catenin at the plasmamembrane of colonic epithelial cells and the bidirectional interactions between KCNQ1:β-catenin modulate colorectal cancer (CRC) epithelial to mesenchymal transition (1).

We investigated the molecular mechanisms regulating KCNQ1:β-catenin interactions and their effects on CRC cell differentiation, proliferation and invasion. Molecular and pharmacological approaches were used to determine the influence of KCNQ1 expression on the Wnt/β-catenin signaling pathway and epithelial-to-mesenchymal transition (EMT) in human CRC cell lines of varying stages of differentiation.

The expression of KCNQ1 was lost with increasing mesenchymal phenotype in poorly differentiated CRC cell lines

as a consequence of repression of the KCNQ1 promoter by β-catenin:TCF4. In well-differentiated epithelial CRC cell lines, KCNQ1 was localized to the plasmamembrane in a complex with β-catenin and E-cadherin. The co-localization of KCNQ1 with adherens junction proteins was lost with increasing EMT phenotype. ShRNA knock-down of KCNQ1 caused a re-localization of β-catenin from the plasma membrane and a loss of epithelial phenotype in CRC spheroids.

Over-expression of KCNQ1 trapped β-catenin at the plasmamembrane, induced a patent lumen in poorly differentiated CRC spheroids and slowed CRC cell invasion. The KCNQ1 ion channel inhibitor chromanol 293B caused membrane depolarization, redistribution of β-catenin into the cytosol, a reduced transepithelial electrical resistance and stimulated CRC cell proliferation.

Analysis of human primary CRC tumour patient databases showed a positive correlation between KCNQ1:KCNE3 expression and disease-free survival in females but not in males.

We conclude that the KCNQ1 ion channel is a novel estrogen target gene in colon cancer and a regulator of the Wnt/β-catenin pathway. The estrogen modulation of KCNQ1:KCNE3 underlies the sexual dimorphism of the hormone's protective effects in CRC female patient survival through repression of Wnt/β-catenin, CRC cell proliferation, EMT and tumourigenesis.

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SA020

### **Aetiology of immobilisation vs inflammatory induced muscle atrophy in vivo**

P.L. Greenhaff

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Muscle atrophy is a phenotypic trait of immobilisation and increased inflammatory burden, but it is unlikely that common cellular and molecular mechanisms prevail between the two. The suppression of muscle protein synthesis seems to be the primary driver of muscle mass loss in human immobilisation (De Boer *et al.*, 2007), and that includes blunting of post-prandial increases in muscle protein synthesis (anabolic resistance, Glover *et al.*, 2008). However, there is a substantial gap in the understanding of the mechanistic drivers of this physiological adaptation. The heightened inflammatory states of sepsis and trauma are associated with major and rapid changes in muscle protein turnover and mass in both rodents and humans, which is thought to reflect the suppression of muscle anabolic signalling (Lang *et al.*, 2007), and activation of several pathways important to muscle protein breakdown, including ubiquitin-proteasome pathway, calpain- and lysosome-dependent proteolytic pathways (Voisin *et al.*, 1996). Evidence points to Akt (protein kinase B) signaling and FOXO transcription factors forming part of a common signaling pathway regulating both muscle protein synthesis and breakdown in this scenario (Crossland *et al.*, 2008). However, whilst tracer based measures of protein turnover in the post-absorptive state confirm that lipopolysaccharides (LPS) infusion decreases muscle (rat) and leg (human) protein synthesis, this appears

to be balanced by a decrease in leg breakdown in humans (Vesali *et al.*, 2009). Importantly, given muscle protein synthesis is accepted to be low in the post-absorptive state, it would appear that accelerated muscle wasting in sepsis is most likely attributable muscle cytokine mediated inhibition of nutrient mediated increases in muscle protein synthesis, i.e. LPS infusion induces profound muscle anabolic resistance to nutrition in humans. Given muscle atrophy is an important clinical consequence of sepsis and trauma that impacts negatively on patient survival and recovery, suppression of muscle pro-inflammatory cytokine status may prove beneficial under these conditions.

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## SA021

### Can we 'out-nutrition' physical inactivity? Nutritional countermeasures to attenuate human skeletal muscle disuse-atrophy.

C. McGlory

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Acute skeletal muscle disuse results in the rapid loss of skeletal muscle strength, mass, and the onset of an insulin-resistant phenotype. Whether these initial physiological responses are pro-adaptive or subversive in nature remains contentious; however, what is clear is that a protracted state of skeletal muscle disuse precipitates metabolic disease. Whilst nutritional and resistive exercise are effective non-pharmacological means to attenuate or even prevent muscle disuse atrophy, in many clinical scenarios patients are functionally impaired, and unable to participate in loading activities. Thus, nutritional intervention remains one of the most viable and scalable means to combat muscle disuse atrophy in the clinical setting. A common and reproducible signature of muscle disuse atrophy is the decline in fasted and fed rates of muscle protein synthesis. These declines result in a negative state of net muscle protein balance that ultimately induces muscle loss. Given the anabolic influence of essential amino acids, particularly the branched-chain amino acid leucine, towards rates of muscle protein synthesis, the impact of dietary amino

acid manipulations rich in leucine on muscle protein turnover has received significant experimental attention. There is also data to suggest that supplementation with omega-3 fatty acids enhances rates of muscle protein synthesis in response to amino acid feeding. In this talk, the cellular and molecular mechanisms underpinning muscle disuse atrophy in humans will be summarized followed by the examination of recent data related to how muscle loading, leucine, and omega-3 fatty acids can act alone as well as in concert to enhance muscle anabolism. Specific emphasis will be placed on data generated in humans but the role of these nutritional compounds in regulating muscle protein turnover in complementary pre-clinical and cell-based systems will also be discussed.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## SA022

### Disuse skeletal muscle atrophy in humans: Proteomic and molecular adaptations

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Muscle atrophy is a multifactorial process common in different catabolic conditions including disuse and aging. Muscle atrophy in most conditions is characterized not only by a change in muscle mass, i.e. a change in muscle quantity, but also by a change of the intrinsic capacity of muscle to develop force, i.e. a change in muscle quality, weakness depending on both phenomena. However, the underlying mechanisms widely vary among different conditions and even through disuse models.

We analyzed three human disuse models: Bed Rest of 8, 24 and 35 days (BR), Unilateral Lower Limb Suspension (ULLS) of 21 days and space flight (SF) of 6 months.

Loss of mass occurred at whole muscle level and at single fiber level in all models to a similar extent. CSA of single muscle fibres was 20-30% lower following 35 days of BR, 23% lower following 3 weeks of ULLS and

23-45% lower following 6 months of SF. In all conditions, a significant loss of specific force of individual muscle fibres was observed. The latter phenomenon was accounted for in BR and ULLS by a disproportionate loss of myosin content compared to CSA, i.e. by a lower myosin concentration and a lower number of potential acto-myosin interactions within individual muscle fibres. Interestingly, in all model, proteomic analysis indicated a down-regulation of many other myofibrillar proteins besides a loss of myosin even at early times of disuse when muscle atrophy had not developed yet, i.e. after 8 days of BR. It appears that both muscle quantity and quality are affected by disuse.

Notwithstanding the similar adaptations observed in muscle mass, function and protein pattern, the underlying molecular mechanisms were somewhat different.

Following BR (24 days) and ULLS (21 days) no activation of the ubiquitin proteasome system (UPS) was observed, i.e. no changes of MuRF-1 and Atrogin 1, two major markers of the UPS. On the contrary, following 6 months SF UPS was activated. The present data did not support a significant contribution of the UPS at least in the progression of muscle atrophy at

late stages of BR and ULLS, whereas in SF the UPS system role could be more relevant as it could remain active for long time. Post BR and post SF autophagy was activated, whereas post ULLS it was not. On the contrary, the Akt/mTOR pathway, which control protein synthesis, was downregulated following ULLS, but not following BR (no data post SF). Interestingly, in humans, a decrease in protein synthesis has been suggested to be the major cause of muscle mass loss.

The molecular adaptations observed could be triggered by mitochondrial dysfunction and/or redox unbalance.

Proteomic analysis showed complex adaptations of the metabolic enzymes content that collectively indicate a general derangement of energy metabolism in BR, ULLS and SF. Interestingly, in a mouse model of disuse (hind-limb unloading) similar metabolic impairment was observed and a metabolic program was shown to cause muscle atrophy. Such metabolic impairment was found to be based on PGC-1 $\alpha$  down-regulation and/or mitochondrial dysfunction. PGC-1 $\alpha$  is a master controller of mitochondrial biogenesis. It was down-regulated in BR (after 8, 10, 21 and 24 days) but not in ULLS. Moreover, in BR the metabolic impairment was supported by the alteration of mitochondrial dynamic, namely by the down-regulation of mitochondrial fusion and the up-regulation of mitochondrial fission. On the contrary, in SF PGC-1 $\alpha$  was up-regulated. The latter finding could be due to the impact of countermeasures astronauts had to perform to try to limit loss of muscle mass. Collectively the data suggest that in humans PGC-1 $\alpha$  contribution to trigger muscle wasting is complex and variable through disuse models.

Redox unbalance is considered an important factor involved in muscular atrophy. We observed a different role of oxidative stress in BR and ULLS. In fact, in BR all anti-oxidant defence systems were downregulated in an early stage of disuse (8 days), NRF2, a master controller of antioxidant protein expression, was up-regulated and protein carbonylation occurred following 35 days suggesting that oxidative stress developed. In ULLS antioxidant defence systems were up-regulated, NRF2 was unchanged and no protein carbonylation occurred after 21 days. The latter observations indicate an effective cellular response to a possible redox imbalance and do not support a major role of oxidative stress in ULLS.

The alterations observed suggested that the atrophic stimulus translates in different adaptations in BR, ULLS and SF models although the final outcome, i.e. loss of mass and myosin content, is similar. Since BR, ULLS and SF reproduce conditions of clinical interest, the comprehension of the mechanisms responsible for the specific muscles adaptations could be relevant to designing specific countermeasures or rehabilitation protocols.

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#### SA023

##### **Human skeletal muscle cell interactions to maintain muscle mass throughout the lifespan**

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Skeletal muscle serves fundamental functions, ranging from generating mechanical force to regulating whole-body

metabolic homeostasis, all of which decline with ageing. Both age- and disease-related loss of muscle mass and function are important risk factors for disability and mortality, and over half of all persons >80 years possess insufficient muscle mass and strength to perform daily activities. A major challenge is that often full restoration of muscle mass after periods of disuse is not achieved through regular rehabilitation, especially in elderly individuals, who therefore will more rapidly fall below the threshold for independent living. At opposite ends of the exercise training spectrum, it is well-established that aerobic (endurance) exercise and resistance (strength) training can individually lead to major health benefits, but completely divergent responses are also evident, reflecting the large plasticity of skeletal muscle. The distinct metabolic and molecular signalling responses underpinning these adaptations are the subject of intense research in order to develop strategies to preserve and functional capacity and restore it when it has been lost. Key to this understanding is insight into the interplay between resident and infiltrating cells, which is tightly regulated temporally and spatially to orchestrate optimal tissue maintenance, adaptation and damage repair throughout the lifespan. The main cells involved are satellite cells, fibroblasts, vascular cells, immune cells and their relative influence on the muscle fibre cells themselves at rest and during adaptation to exercise and damage stimuli. Non-steroidal anti-inflammatory medication and Angiotensin II type I receptor blockers will also be considered as potential pharmacological interventions in the context of muscle adaptation at the cellular level.

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#### SA024

##### **Regulation by intracellular pH: from protein dynamics to cancer cell behaviors**

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Although cancer is a diverse set of diseases, cancer cells share characteristics distinct from normal cells, including sustained proliferative signaling, resistance to cell death, and active invasion and metastasis. While less recognized, dysregulated pH is also a distinct feature of most cancers, regardless of tissue origin or genetic background. Compared with normal adult cells, cancer cells have a higher intracellular pH (pHi) of ~ 7.6 and a lower extracellular pH of ~ 7.1. Constitutively increased pHi promotes cell proliferation, limits apoptosis, and is obligatory for efficient directed cell migration. However, how a higher pHi enables disease progression and the molecular mechanisms mediating pHi-dependent cancer cell behaviors are understudied and mostly not understood. One challenge is that increased pHi enables cancer progression through multiple mechanisms. A second challenge is that the molecular basis for pHi-dependent cell behaviors is difficult to determine because it is dependent on protonation/deprotonation of proteins, which cannot be resolved by mass spectrometry or antibodies. We are addressing both of these challenges by bridging protein structure dynamics and cell biology to determine the design principles and functions of pH sensors, defined as proteins with activities or ligand-binding affinities regulated by cellular changes in pH<sup>1</sup>. To characterize pH sensors we use biochemistry, cell biology, molecular dynamics



simulations and NMR. Relevant to cancer, we determined the molecular basis for increased pHi promoting cell migration by revealing the structural dynamics and functional properties of the cytoskeleton-associated pH sensors cofilin<sup>2</sup>, talin<sup>3</sup> and focal adhesion kinase<sup>4</sup>. We are currently identifying pH sensors for additional cancer cell behaviors, including tumorigenesis, metabolic reprogramming and retention of recurring charge-changing mutations. For tumorigenesis, we found that beta-catenin is a pH sensor, with higher pHi increasing beta-catenin degradation, which is mediated by deprotonation of a conserved histidine (His 36 in human beta-catenin) for increased binding of the E3 ligase beta-TrCP1. For metabolic reprogramming we are determining pH sensing by the glycolytic enzyme phosphofructokinase-1 (PFK1). Based on our recently resolved crystal structure of PFK1<sup>5</sup> we found that in the human muscle isoform, His242 is necessary for increased enzyme activity at higher pH. For charge-changing mutations, we found that Arg>His substitutions, which are enriched in cancers, can confer a gain in pH sensing that enables cancer behaviors that we confirmed for p53-R273H and EGFR-R776H<sup>6</sup>. These findings provide new insights on molecular mechanisms enabling cancer progression that can inform therapeutic approaches. Because constitutively increased pHi is a distinct, conserved feature of cancer cells, pH sensors hold promise as therapeutic targets to achieve efficacy with high specificity.

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SA025

### Characterising the acid-handling and acid-sensing phenotype in a large panel of colorectal cancer cell lines

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Microenvironment acidity is a well-established chemical signature of solid tumours. It arises from upregulated cancer metabolism which loads the extracellular space with large quantities of CO<sub>2</sub> and lactic acid, in combination with aberrant blood perfusion which fails to adequately wash away these acids (1). It is now recognised that tumour acidosis is not merely an accumulation of end-products, but a source of feedback that can influence disease progression by selecting phenotypes compatible with the ambient pH (2). Whilst this somatic evolution process has a precedent in, for instance,

hypoxia-driven carcinogenesis, the mechanisms of acid-selection remain unclear. The ability to survive and thrive at any given milieu pH is determined by the ensemble pH-sensitivity of proteins, most of which have multiple protonatable sites. I will talk about our ongoing efforts to characterise acid-sensing and acid-handling in colorectal cancer (CRC). To gain new insights, we are screening a large panel of over 100 CRC lines for phenotypes related to pH. These lines have been sequenced for genotype (e.g. mutations) and gene expression at mRNA level (by microarray), thus allowing us to correlate genetics with function. The size of this panel of cell lines provides adequate statistical testing power and encompasses most permutations of the common mutations found in human colorectal tumours (3). The readouts we use to interrogate "pH phenotype" include cell survival, lumen formation and steady-state intracellular pH measured using a high-throughput plate imaging system over a range of medium pH. Using these criteria, we can categorise CRC cell lines as acid-sensitive or acid-resistant. Most cancer cells are believed to be devoid of cell-to-cell diffusive coupling through connexin-assembled gap junctions. Thus, it is commonly assumed that cancer cells function independently of one another, and do not exchange signals and metabolites across a cytoplasmic syncytium. By imaging the diffusive spread of fluorescent dyes, we can test for diffusive coupling in monolayers. We find that some CRC lines or even their distinct subpopulations manifest strong diffusive coupling. Such heterogeneity may influence the physiological behaviour of cancer cells in tumours, which inherently feature standing gradients that can drive fluxes through such a syncytium (4). The pH landscape of the tumour milieu is not solely shaped by cancer cells and vasculature but also by numerous stromal components that can engage in acid-base transport. Myofibroblasts are the major stromal component in the colon, and our findings show that these assemble into a syncytium with a powerful capacity to absorb acids secreted by cancer cells. Furthermore, this stromal function can be enhanced by cytokines secreted by CRC cells, thus providing a means by which cancer cells can subordinate myofibroblasts (5). In summary, there are multiple players involved in establishing the acidic tumour microenvironment, and complex mechanisms through which cancer cells respond to this chemical selection pressure.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

SA026

**Mitochondrial dynamics in metabolism regulation**S. Diano<sup>1,2,3,4,5</sup>

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Our research has been focusing on deciphering intracellular mechanisms that enable hypothalamic cells to sense and respond to changes in circulating nutrient and hormone levels in the control of systemic energy and glucose metabolism. Our recent findings have unmasked mitochondria as critical intracellular organelle in detecting changes in peripheral metabolism. By altering their size, shape and function, mitochondria enable cells to adjust their activity, which in turn alter behavior and peripheral tissue functions to fine tune systemic metabolism. This presentation will highlight these cellular biological process in the hypothalamic regulation of energy and glucose homeostasis.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

SA027

**Brain amino acid sensing in the control of food intake**

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There is compelling evidence indicating that protein is the macronutrient that has the biggest impact on how much energy we eat. Across diets with various protein contents, total protein intake is tightly regulated, and this is achieved via wide variations in total energy intake. However, how the brain detects dietary protein to regulate appetite and metabolism remains poorly understood. Some specific amino acids, including the branched chain amino acid leucine, can reproduce some of the effects of protein on satiety. We hypothesize that postprandial leucine levels signal protein abundance to specialized brain sensors present in regions relevant to the control of energy balance. Using a multidisciplinary approach, our aim is to establish the neurochemical and molecular identity of these sensors, and characterize the downstream neurocircuits mediating central protein representation and controlling energy balance.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

SA028

**The role of glucose sensing by glucokinase in the arcuate nucleus in the regulation of energy and glucose homeostasis**

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Glucokinase is a member of the hexokinase family of enzymes. However, its main function is not in the production of cellular energy but to act as part of the glucose sensing mechanism of cells.

Glucokinase is widely expressed in the brain including within the hypothalamus and functions as part of the glucose sensing mechanism in an analogous manner to its role in the pancreatic  $\beta$ -cells [1-2].

Within the hypothalamus glucokinase is expressed in several nuclei including the arcuate nucleus and the ventromedial nucleus (VMN). In the VMN glucokinase has an important role in the regulation of the counter regulatory response [3-4]. Until recently its role in the arcuate nucleus was less well characterised.

We have recently characterised the role of arcuate nucleus glucokinase using recombinant adeno-associated virus to both increase and decrease glucokinase activity specifically in the arcuate nucleus. In addition we have used pharmacological agents to increase glucokinase activity in the arcuate and also alter activity of  $K_{ATP}$ .

We found that glucokinase within the arcuate nucleus has important roles in the regulation of both energy [5] and glucose homeostasis [6].

Arcuate nucleus glucokinase regulates food intake and this effect is specific for glucose with intake of other sugars unaffected. This effect is mediated via the  $K_{ATP}$  and voltage sensitive calcium channels.

Arcuate nucleus glucokinase also regulates glucose stimulated insulin release and this effect is mediated via the  $K_{ATP}$ . Importantly this response is maintained in a model of type 2 diabetes suggesting targeting this effect could be a therapeutic approach to treating this disease.

Together these findings suggest that arcuate nucleus glucokinase acts to stimulate intake of glucose rich foods and then ensure that this glucose will be efficiently utilised and stored.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

SA029

### Energetics of Torpor and Hibernation: Implications for Human Space Travels

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Many small mammals and birds have developed specific mechanisms of energy saving, achieved by active and controlled reduction of metabolic rate (MR) and hence body temperature ( $T_b$ ), *i.e.* torpor and hibernation [1]. The state of torpor involves controlled and tight regulations within the organism of many bodily systems (for a recent review, see [2]). However, despite two centuries of research on torpor, physiological mechanisms that regulate this overwintering strategy are still poorly understood.

Polyunsaturated fatty acids (PUFA) are one of the main factors affecting torpor and hibernation performance. Heterothermic mammals fed diets containing plant oils that are rich in n-6 PUFA, notably linoleic acid (LA, C18:2 n-6), show a higher propensity of torpor use, lengthen their torpor bout duration, lower their minimum  $T_b$  and hence increase their energy savings (for review, see [3]). Conversely, there are evidences for adverse effects of n-3 PUFA on hibernation performance [4,5].

The heart plays a pivotal role in maintaining the body homeostasis, and its activity and metabolism are tightly regulated during torpor [6,7]. As previously hypothesized [8], the well-known influence of PUFA may be mediated via effects of the membrane fatty acid composition on the sarcoplasmic reticulum (SR) calcium ATPase (SERCA) in the heart of hibernators. This trans-membrane pump is responsible for removing calcium into the SR and hence for continued cardiac function even at extremely low  $T_b$  in torpor.

We tested the hypotheses that high proportions of n-6 PUFA in general, or specifically high levels of LA, in SR phospholipids (PL) are associated with increased cardiac SERCA activity, and allow animals to reach lower minimum  $T_b$  in torpor. SERCA activity and SR PL fatty acid composition were assessed from hearts of hibernating and non-hibernating Syrian hamsters (*Mesocricetus auratus*), a granivorous, food-storing hibernator, and of hibernating garden dormice (*Eliomys quercinus*), an insectivorous, fat-storing hibernator.

In both species, we found that SERCA activity was strongly up-regulated by increased proportion of LA in SR PL, but was negatively affected by the content of docosahexaenoic acid (DHA; C22:6 n-3) [9,10]. In hibernating hamsters, high levels of LA and low proportions of DHA were found in SR PL [9]. As a result, SERCA activity was significantly higher during entrance into torpor and in torpor compared to inter-bout arousal, *i.e.* phase of high MR and euthermic  $T_b$  between two torpor bouts. A subgroup of hamsters, which never entered torpor but remained euthermic throughout winter, displayed a phenotype similar to animals in summer, *i.e.* lower levels of LA and increased proportions of DHA in SR membranes [9]. Similarly, a group of dormice, which delayed their mean onset of hibernation by almost 4 days (range 0-12 days), showed extremely high levels of DHA prior to hibernation [10]. Both hamsters and dormice with increased SERCA activities reached lower  $T_b$  during torpor [9,10]. Interestingly, SERCA activity in torpor was three-times higher in garden dormice than in Syrian hamsters at similar DHA proportions in SR PL [10].

We conclude that (1) fatty acid composition of SR membranes modulates cardiac SERCA activity, hence determining the

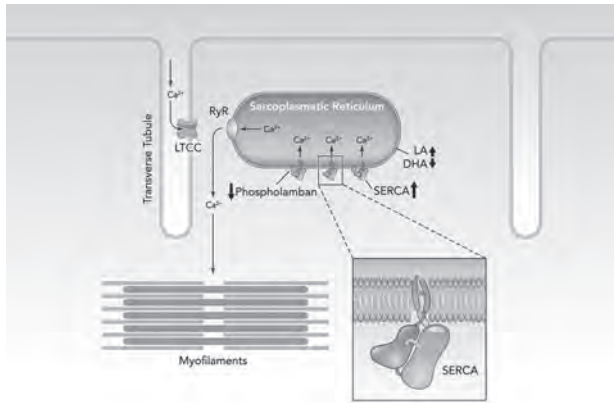
minimum  $T_b$  tolerated by hibernators, and (2) high DHA levels prevent hibernators from entering into torpor, but the critical levels differ substantially between species. These specific roles of PUFA in regulating key organs, such as the heart, hence body homeostasis during torpor and hibernation might shade light on the possibility for humans to enter a hibernation-like state for long-term space travels.



Hibernating Syrian hamster (top) and hibernating garden dormice (bottom)

Photos: Arjen Strijkstra (Syrian hamster) and Sylvain Giroud (garden dormice)





Model of  $\text{Ca}^{2+}$  handling in cardiomyocytes of hibernators (from Arnold et al. Physiology 30: 232-240, 2015). Contraction of myofilaments is caused by  $\text{Ca}^{2+}$  release from the sarcoplasmic reticulum (SR) through ryanodine receptor channel proteins (RyR), following depolarization of the cell membrane and  $\text{Ca}^{2+}$  entry through L-type  $\text{Ca}^{2+}$  channels (LTCC). During hibernation, rapid  $\text{Ca}^{2+}$  reuptake into the SR is enhanced by increased expression of SERCA (the SR  $\text{Ca}^{2+}$  ATPase) and decreased expression of the SERCA inhibitor phospholamban (black arrows). SERCA activity during hibernation is additionally increased due to high linoleic acid (LA) and low docosahexaenoic acid content (DHA) in phospholipids of the surrounding SR membrane. The transport of two  $\text{Ca}^{2+}$  ions per cycle into the SR involves strong conformational changes in the transmembrane domains, which are affected by the fatty acid composition of the bilayer.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

SA030

## Hibernation as a spectrum – hypometabolism in large mammals

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Until recently, the status of black and brown bears as hibernators has been debated. The drop in metabolic rate in brown bears of around 25%, although dramatic in its own right, is far from hibernation metabolic rates down to 5% of normothermic values in small hibernators (Geiser, 2004). However, results from certain species of “non-hibernators” illustrate that hibernation and hypometabolism are not a yes/no phenomena, but are rather a gradient. Both hibernators and non-hibernators have a variety of different strategies for hibernation, torpor and seasonal hypometabolism. The badger, for example, is reported to have cyclic periods of torpor at lower latitudes (Harlow, 1981), but a period of hibernation (October to March) at higher latitudes (Fowler and Racey, 1988).

We used biologgers measuring heart rate and body temperature, both of which are tightly connected to metabolic rate. Indeed, heart rate has often been used as a proxy for metabolic rate. This comparison was done in collaboration with a number of established ecological research projects on free-ranging species including brown bears, moose, wolverine, Svalbard reindeer and the European beaver. In most cases, the loggers were retrieved or the data was downloaded 1-2 years after implantation.

Brown bears of medium size (60-75kg, N=6) decreased their heart rate 75% in winter (from  $75 \pm 10$  bpm in September, to  $20 \pm 7$  bpm in December). Bears of this size had their highest daily mean temperature in September ( $37.7 \pm 0.4^\circ\text{C}$ ), with the lowest daily means in December ( $33.35 \pm 0.82^\circ\text{C}$ ). Smaller bears had higher summer heart rates than larger bears, but across sizes (19-233 kg, N=42) bears had similar heart rates in winter.

Ibex in central Europe have a reduction in heart rate of up to 60% during winter (Signer et al., 2011), while moose in a coastal, mild climate (N=11) only exhibited a 13% drop in heart rate (from  $75 \pm 19$  to  $64 \pm 19$ ) and a  $0.6^\circ\text{C}$  drop in body temperature (from  $38.6 \pm 0.4$  to  $38 \pm 0.2$ ). Svalbard reindeer, living in an extreme environment, dropped the heart rate to around 40 beats per minute in winter, 33% of the summer time heart rate mean of 120 beats per minute.

The mean  $\pm$  SD daily body temperature ( $T_b$ ) of moose in then northernmost areas (N=26) was during the month of July ( $38.65 \pm 0.38^\circ\text{C}$ ) and lowest during February – March ( $37.89 \pm 0.33^\circ\text{C}$ ) and the daily means ranged between  $36.16^\circ\text{C}$  and  $39.38^\circ\text{C}$  during the year. This is a drop of  $0.76^\circ\text{C}$ , slightly more than the  $0.6^\circ\text{C}$  mean drop in  $T_b$  of the moose in the coastal climate. Unfortunately, heart rate data was not available for the northern population.

Beavers (N=2) had a reduced heart rate from summer rates of  $103 \pm 3$  bpm down to  $79 \pm 11$  bpm at the coldest ambient temperatures, a 23% reduction. Body temperature was



36.2±0.44 in mid-summer decreasing to around 35.6±0.43°C at the coldest temperatures.

Wolverine (N=14) displayed constant temperatures throughout the year, with the mean ranging from 38.37±0.14 in December to 38.57±0.13 in June. Individuals displayed a wide daily variation in body temperature and high flexibility in activity patterns.

The moose, in contrast to the wolverine's undramatic shifts in body temperature and the badger's strategy of switching from torpor to hibernation, displays a gradient of hypometabolism depending on the environment, with less dramatic shifts in the moose at the milder coast than in the northern mountains. Although primarily studied in the north, the brown bear also likely has a gradient, to some extent in its phenotypical reactions to its environment. The Svalbard reindeer represents a special case with a dramatic seasonal hypometabolism, falling in-between the moose and the bear.

Space research has long been interested in hibernation because of its potential to solve some of the problems associated with space travel. Medical research is seeking possibilities to reduce metabolic rates during certain treatments. The adaptations of the species presented here, illustrate that many species, not considered classical hibernators, have adapted physiological strategies, which may give new insights relevant to space research.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

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### SA031

#### Core body temperature changes under different physical and environmental conditions on Earth and in space

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Heat exchange between an organism and its environment occurs via conduction, convection, radiation, and evaporation. Under terrestrial conditions, heat exchange for an adult occurs mainly by radiation and convection, and the body core temperature fluctuates by only +/- 0.5°C throughout the day, i.e. the "circadian rhythm". In space the heat loss pathways are challenged due to the lack of natural convection which results in the diminished occurrence and/or efficiency of evaporation. We have recently developed a non-invasive heat flux device (Double Sensor, T<sub>core</sub>®) that can be used to monitor core body temperature changes in humans. Three investigations will be presented. They dealt with core body temperature changes under terrestrial and micro-g conditions: i) during rest and exercise in a climate chamber at 10°C, 25°C, 40°C ambient temperature, ii) during long-term bed-rest, and finally iii) during rest and exercise in space. In the first study (study 1), the heat flux sensor methodology was tested in comparison to nasopharyngeal and rectal temperatures in 20 male subjects; in the second study (study 2) 7 males

were monitored during long-term bed rest, and in the last study core body temperature changes were determined in 11 astronauts during a regular VO<sub>2</sub> ergometer testing before flight, several times in space on the ISS, and after spaceflight (study 3). Study 1 showed that the recordings of the Double Sensor differed by -0.16 to 0.1°C from the mean rectal temperature, study 2 proved that the device correlated well (r>0.704) with the rectal temperature recordings, and study 3 revealed that the astronauts had marked and prolonged increases during exercise in core body temperature, sometimes >40°C, in space on the ISS (p<0.01). Changes in body core temperature can be markedly attenuated in space. Further studies on this topic are urgently needed because i) the frequency, length, and intensity of extravehicular activities will increase in future and ii) any alteration of the circadian rhythms might aggravate the physical, psychological, and cognitive performance of humans in space.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

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### SA032

#### Regulation of neutrophilic inflammation by oxygen sensing pathways

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Whilst neutrophils are required for an effective host pathogen response, inappropriate or persistent neutrophilic inflammation is also associated with a number of disease states exemplified by acute lung injury responses and chronic obstructive pulmonary disease. Tight regulation of neutrophil function and survival in the tissues is therefore important in maintaining a balance that benefits the host. During the inflammatory response, tissue environments are frequently characterised by limited oxygen and nutrient availability. We, and others, have described that local and systemic hypoxia is a critical component of the inflammatory response, with oxygen-sensing pathways implicated in the regulation of neutrophil survival. In this talk I will explore how the adaptation of neutrophils to sites of tissue injury and infection is driven by interplay between oxygen and metabolite sensing pathways. The ability to reprogram neutrophil metabolism by hypoxia and activation of the HIF response will also be discussed in the context of acute bacterial challenge. Finally, the therapeutic potential of targeting hypoxia signalling pathways will be considered for the treatment of inflammatory lung disease.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

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### SA033

#### Hypoxia inducible factors (HIFs) in acute colitis

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The lumen of the colon is almost anoxic whereas the oxygen supply of mucosal and submucosal layers is comprehensive. In the healthy organ epithelial tips show hypoxic features which spread to the basal epithelium, mucosa, and submucosa

whenever inflammatory events disrupt the epithelial barrier. Attracted immune cells have to function in a setting of inflammatory hypoxia. How crucial the gene transcription regulators hypoxia-inducible factors (HIFs) hereby drive immune cell function will be the topic of this talk.

Mice (C57Bl/6 background) with a conditional knockout for HIF-1 $\alpha$  in either cells of the myeloid lineage (lyz2-cre) or in dendritic cells (Itgam-cre) and with a double knockout of HIF-1 $\alpha$  and HIF-2 $\alpha$  in myeloid cells have been used in a model of DSS (dextran sodium sulfate)-induced colitis. Mice have been treated with strain specific DSS concentrations to ensure similar extent of inflammation, details are indicated in the results. During the treatment, we assessed weight loss and disease activity index (containing weight loss, stool consistency, and rectal bleeding) of the mice. Colon and mesenteric lymph nodes have been excised from sacrificed mice and analyzed for shortening and weight, mRNA has been isolated and changes in gene expression have been analyzed with qPCR. In addition, histological analysis of the colon was performed. Data have been analyzed by one way or two way ANOVA plus post-test,  $P < 0.05$  has been considered significant. Loss of HIF-1 $\alpha$  hereby reduced signs of colon inflammation (measured by DAI, disease activity index of histologic analysis of the colon) in lyz2-cre<sup>+/+</sup>/HIF-1 $\alpha$ <sup>+/+</sup> mice (DAI of control mice:  $8.7 \pm 1.2$ ; HIF-1 $\alpha$  DF x lyz2-cre:  $6.8 \pm 0.7$ ; 2.5 % DSS for 6 days;  $n = 5-6$ ) due to reduced numbers of proinflammatory macrophages, dendritic cells, and Th17 positive T cells in the inflamed colon. This was consistent with a reduced and delayed gene expression of pro- and anti-inflammatory cytokines such as *tnfa*, *ifng*, and *il-10*. Parts of these findings could be reversed by additional knockout of HIF-2 $\alpha$  as lyz2-cre<sup>+/+</sup>/HIF-1 $\alpha$ <sup>+/+</sup>/HIF-2 $\alpha$ <sup>+/+</sup> mice do not show a DAI different from wildtype mice ( $7.6 \pm 0.7$ ).

In contrast, mice lacking HIF-1 $\alpha$  in dendritic cells lost significantly more weight during acute colitis ( $86.33 \pm 1.39$  for wt,  $81.35 \pm 1.37$  for ko at day 7 of treatment). Interestingly, knockout of HIF-1 $\alpha$  in dendritic cells did not alter overall colon inflammation (DAI of control mice:  $10.8 \pm 0.5$ , itgam-cre<sup>+/+</sup>/HIF-1 $\alpha$ <sup>+/+</sup>:  $10.9 \pm 0.4$ ; 3 % DSS for 7 days;  $n = 9-14$ ). Bone marrow derived dendritic cells lacking HIF-1 $\alpha$  show a significantly reduced ability to produce the anti-inflammatory cytokine IL-10 in response to inflammatory stimulation. This (amongst other factors) might lead to a diminished stimulation of regulatory T cells in the mesenteric lymph nodes of itgam-cre<sup>+/+</sup>/HIF-1 $\alpha$ <sup>+/+</sup> mice which was assessed by *foxp3* and *ox40* mRNA quantification. Furthermore, gut homing of regulatory T cells was impaired in these mice which resulted in a dominantly pro-inflammatory setting with activated dendritic cells secreting proinflammatory cytokines such as IL-6 and IL-23 and a missing counter-regulation by anti-inflammatory Tregs.

Knockout of HIF-1 $\alpha$  in two different proportions of the cellular innate immune defense results in completely diverse outcome in a mouse model of acute DSS colitis. Knockdown of HIF-2 $\alpha$  in addition to HIF-1 $\alpha$  seemed to reverse HIF-1 driven effects. Our findings begin to shed some light on the complexity of inflammatory hypoxia and the role of HIF complexes in colitis.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

SA034

### The hypoxic response in Natural Killer cells

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We observe that Natural Killer (NK) cells, which can kill target tumour cells, infiltrate hypoxic tumour areas. Cellular adaptation to low oxygen is mediated by Hypoxia-inducible transcription factors (HIFs) that play a pivotal role in driving immune responses and angiogenesis. We show that deletion of HIF-1 $\alpha$  in NK cells impairs tumour cell killing *in vitro*. Paradoxically, *in vivo*, NK cell HIF-1 $\alpha$ -deficiency inhibits tumour growth despite impaired immune surveillance. Tumours developing in these conditions were characterized by a high density network of immature vessels devoid of pericytes, severe hemorrhage and increased hypoxia, indicating non-functional angiogenesis. Mechanistically, loss of HIF-1 $\alpha$  in NK cells reduced expression of antiangiogenic factors within the tumour. Hence, in contrast to other cell types, in NK cells the hypoxic response acts as an inhibitor of angiogenesis, yet, this promotes tumour growth by allowing the formation of functionally improved vessels. Furthermore, HIF-1 $\alpha$ -deficiency in NK cells accelerates physiological angiogenesis and non-fibrotic wound healing but compromises the antimicrobial defense. As a consequence, mice with a deletion of HIF-1 $\alpha$  in NK cells show increased susceptibility to sepsis during bacterial skin infections. In summary, we demonstrate that the hypoxic response in NK cells controls tumor immune surveillance, vascular remodeling and antimicrobial defense mechanisms.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

SA035

### CD8+ T-cell depletion abolishes the anti-metastatic effects of Voluntary Running in a mouse model of Breast Cancer

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**Introduction:** Regular physical exercise provides a significant risk reduction for breast cancer and recent studies suggest beneficial effects also on disease specific recurrence and mortality. However, little is known about how exercise exerts its protective effects.

**Aim;** to evaluate the effect of voluntary running on tumor progression and metastasis in the PyMT mouse model of breast cancer.

**Methods:** From 4 weeks of age, female MMTV-PyMT mice on the FVB background were housed with access either to wirelessly recording running wheels or locked control wheels. Tumor growth was monitored continuously, tumor stage and pulmonary metastases were determined histologically at the 12 week endpoint. In a follow up study, pre-trained female FVB mice were injected intravenously with  $2 \times 10^5$  PyMT derived tumor cells (IC3) and after an additional 10 weeks of voluntary running, pulmonary metastases and immune cell infiltration was quantified (histologically and with flow cytometry). The CD8+ T-cell population was deleted using weekly administration of CD8 specific antibodies.

Results: PyMT mice average running distance was  $6.4 \pm 2.4$  km/day. No significant effects of voluntary running on tumor-initiation, volume or stage were found. However, a reduced number of metastases were observed in mice with access to running wheels (Ctrl  $22.0 \pm 6.8$  and Runners  $9.1 \pm 1.7$ ). Significant reductions in pulmonary metastasis frequency were also found in runners after intravenous injections of tumor cells (Ctrl  $5.2 \pm 1.1$  and Runners  $1.9 \pm 0.7$ ) and running mice had a lower number of metastases with a high proliferation score. Metastatic lesions from running mice showed higher content of Granzyme B positive cells (Ctrl  $1.2 \pm 0.5$  and Runners  $4.9 \pm 1.1$ ), indicating an increased infiltration of cytotoxic T-cells. Depletion of CD8<sup>+</sup> cells abolished the reduction in metastatic burden found in running mice compared to non-running mice. All data is presented as Mean  $\pm$  SEM.

Discussion: In this highly aggressive, genetic, breast cancer model, an average of 6 km/day of voluntary running showed little effect on tumor formation and growth. However, the findings suggest that physical activity reduced outgrowth of metastatic lesions through an increased infiltration of cytotoxic immune cells.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

SA036

### Caveolae protect endothelial cells from mechanical damage

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Caveolae are small, bulb-shaped plasma membrane invaginations that account for up to half the membrane surface area of endothelial cells. Mutations that ablate caveolae lead to diverse phenotypes in mice and humans, making it challenging to uncover their molecular mechanisms. Caveolae have been described to function in endocytosis and transcytosis (a specialized form of endocytosis) and in maintaining membrane lipid composition, as well as acting as signaling platforms. New data also support a model in which the central function of caveolae could be related to the protection of cells from mechanical stress within the plasma membrane. I will present in vitro and in vivo experiments confirming a mechanoprotective role, and present recent data on the molecular architecture and function of caveolae.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

SA037

### Impact of mechanical deformation on the cardiac excitation-contraction machinery

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Cardiomyocytes both cause and experience continuous cyclic deformation, with up to  $\pm 10\%$  changes in length compared

to their resting state. The 3 dimensional (3D) organization of intracellular structures will change, in this context, on a beat-by-beat basis – but the exact effects of mechanical deformation on the topology of intracellular organelles are not well characterised. Cardiomyocytes have low free cytosolic volume. Intracellular organelles (such as T-tubules, sarcoplasmic reticulum or mitochondria) are arranged between strands of the contractile filament lattice, and embedded within a network of rigid and elastic non-sarcomeric cytoskeletal elements that bear and transmit mechanical loads throughout the cell. We investigated deformation-induced 3D nano-scale changes in cardiomyocyte ultrastructure, with focus not only on individual organelles, but also on spatial relationships between homo- and heterotypic structures. To determine how mechanical cell activity alters the 3D nano-topography of (primarily membranous) cell compartments, we utilise electron tomography of rabbit cardiac tissue and of isolated cells. Furthermore, using live-cell imaging, we assessed the functional impact of beat-by-beat structural alterations on dynamic intracellular processes such as T-tubular content exchange dynamics. Key findings are that T-tubules deform in a sarcomere-length dependent manner, without notable effects on intra-tubular diffusion. The results presented here provide reference data for spatial contextualisation of functionally-relevant ultrastructures and their mechanical modulation in healthy cardiomyocytes.

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SA038

### Load-sensitive regulation of t-tubular structure

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T-tubule remodeling during cardiac pathologies such as heart failure leads to impaired contractility of the heart. Increased cardiac load has been suggested to play a role, and we previously identified wall stress as an important trigger of these structural alterations. Specifically, we showed that elevated diastolic wall stress induces t-tubule loss and re-organization in areas surrounding a myocardial infarction (MI) in an experimental rat heart failure model (Frisk *et al.*, Cardiovasc Res, 2016). The precise mechanical stimuli and effectors involved, however, still remain to be elucidated. We therefore presently investigated how preload affects t-tubule structure during compensatory and decompensatory remodeling and hypothesized that preload acts as specific and dynamic regulator of the t-tubule network.

This hypothesis was examined using explanted hearts from mouse models of cardiac pathology and patients. The effects of increased preload were first investigated in mice following aortic shunt. At one week post-surgery, t-tubule density was increased while after 20 weeks (where preload was more markedly elevated) t-tubule density was decreased. Similarly, stretching isolated papillary muscles to reproduce a



modest increase in diastolic stress increased t-tubule density, whereas excessive stretch beyond the optimal muscle length for force generation resulted in loss. In mice with dilated hearts and highly increased diastolic wall stress due to MI or aortic banding, t-tubules were also degraded, whereas compensatory increases in t-tubule density were observed at lower loads. A similar pattern of t-tubule loss was observed in explanted hearts; patients with dilated cardiomyopathy exhibited reduced t-tubule density while t-tubule proliferation was observed in patients with hypertrophic cardiomyopathy and aortic stenosis where diastolic wall stress was only slightly increased.

Taken together, these results indicate that there is a bell-shaped relationship between t-tubule density and preload, with the healthy heart positioned on the rising phase of the curve. Moderately increased preload is associated with a compensatory increase in t-tubule density, likely aimed at adapting to increased contractile demand. However, excessive preload, such as during heart failure development, is detrimental as triggered t-tubule loss contributes to declining cardiac performance.

Frisk, M.; Ruud, M.; Espe, E.K.S.; Aronsen, J.M.; Røe, A.T.; Zhang, L.; Norseng, P.A.; Sejersted, O.M.; Christensen, G.; Sjaastad, I.; Louch, W.E (2016). Elevated ventricular wall stress disrupts cardiomyocyte t-tubule structure and calcium homeostasis. *Cardiovascular Research*. Volume 112 (1), Pages 443-451.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

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#### SA039

### Frank-Starling Law of the heart: myofilament length dependent activation

P.P. de Tombe<sup>1,2,3,4</sup>

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The cellular basis of the Frank-Starling mechanism is sarcomere length modulation of myofilament Ca<sup>2+</sup> sensitivity. The molecular mechanisms that underlie length sensitivity are unknown, but recent evidence has implicated the giant protein titin as possible sarcomeric strain sensor responsible for the phenomenon by an, as of yet unidentified, signal transduction pathway. Our studies over the past decades have employed various biophysical approaches to elucidate the underlying mechanisms of the problem at the level of: multicellular muscle bundles, single cardiac contractile cells, and sub-cellular single myofibrils. Our studies were performed both at steady state as well as during rapid activation/relaxation dynamics, while structure was assessed by utilizing fluorescent probe and x-ray diffraction analysis. Our results indicate that myofilament length dependent activation involves structural changes in both thick and thin filaments that are mediated by titin strain that may involve length dependent as well as phosphorylation dependent interaction of regulatory light chain, troponin-I, and myosin binding protein C interacting with actin and myosin to mediate the length sensing property of the cardiac sarcomere.

de Tombe PP, Mateja RD, Tachampa K, Mou YA, Farman GP, Irving TC. Myofilament length dependent activation. *J Mol Cell Cardiol*. 2010; 48(5):851-858.

Ait-Mou Y, Hsu K, Farman GP, Kumar M, Greaser ML, Irving TC, de Tombe PP. Titin strain contributes to the Frank-Starling law of the heart by structural rearrangements of both thin- and thick-filament proteins. *Proc Natl Acad Sci*. 2016. 113.8 (2016): 2306-2311.

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#### SA040

### Chemogenetic activation of leptin receptor expressing neurons shows that leptin indirectly acts upon dopamine neurons to affect food reward seeking

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Leptin is an adipocyte-derived hormone which links peripheral metabolic state to central control of energy balance. In addition to its role in maintaining energy homeostasis, leptin affects motivation for food reward and nutrient choices by modulating the mesolimbic dopamine system. Although leptin directly reduces activity of ventral tegmental (VTA) dopamine neurons, most of these dopamine neurons do not project to the accumbens which is the dopamine projection implicated in driving food reward seeking. So how does leptin then reduce motivation for food reward? Leptin receptors (LepR) are also expressed in GABA neurons in the ventral tegmental area (VTA) and lateral hypothalamus (LH). There are still gaps in knowledge that we address here: 1) which leptin-receptor expressing neurons connect to the dopamine system, 2) how does leptin modulate their activity and 3) which of these neurons is implicated in regulating the motivation to obtain food reward. We used optogenetic-assisted circuit mapping approach to show cell-type specific effects of leptin on reward circuitry. We demonstrate functional connectivity between LepR-expressing neurons in the LH and VTA neurons as well as LepR-expressing GABA neurons in the VTA and DA neurons. Chemogenetic activation of LH neurons increased the motivation to press lever to obtain a sucrose reward likely by decreasing VTA GABAergic input onto VTA dopamine neurons. We also find that VTA GABA neurons expressing leptin receptors project onto VTA dopamine neurons. Activation of leptin receptor expressing neurons in the VTA using chemogenetics reduced the motivation to press lever for a sucrose reward in food-restricted mice. We conclude that leptin indirectly targets multiple inputs to the dopamine system to reduce food reward seeking.

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#### SA041

### Towards a nervous system compendium of likely etiologic brain cell types for obesity

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Despite the prevalence of obesity – every third US adult is obese – its pathogenesis remains largely unknown. Obesity, defined as body mass index (BMI) >30, is caused by a sustained



positive energy balance and an incremental resetting of the body weight set point. From studies of extreme obesity in humans and rodent studies, it is known that energy homeostasis in part is regulated by hypothalamic melanocortin signaling, however, mutations in genes encoding these brain circuits only account for a modest number of obesity cases. Integration of results from genome-wide association studies (GWAS) for BMI with relevant gene expression data has proven to be an agnostic and powerful approach to identify known and novel biological cell types underlying a range of traits and disease. Drawing on recent GWAS for BMI from >700,000 individuals and murine single cell transcriptomics data covering >500,000 cells throughout >300 cell types across the mouse nervous system, we have developed an approach to identify novel cell types and cell type-specific biological pathways likely etiologic to obesity.

During the talk, I will discuss conceptual and methodological issues, present preliminary results and relate these to findings from the hypothalamic melanocortin system.

Hales CM et al. JAMA. 2018 Apr 24;319(16):1723-1725

Schwartz MW et al. Endocr Rev. 2017 Aug 1;38(4):267-296

Pers TH et al. Nat Commun. 2015 Jan 19;6:5890

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

SA042

### Regulation of anxiety behaviors through targeting of centromedial amygdala inhibitory synapses.

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Abnormalities in inhibitory synaptic transmission are intricately linked to the pathophysiology of psychiatric disorders, but the underlying mechanisms are poorly understood. While synaptic inhibition is crucial throughout the brain, a number of key nodes exist that are particularly heavily controlled by inhibitory inputs, and one of these is the centromedial amygdala (CeM). The CeM represents the major output nucleus of the amygdala complex, through which anxiety responses and other psychiatrically relevant behaviors are processed. CeM neurons receive numerous inhibitory inputs from both afferent projections and local interneurons, which play a pivotal role in gating CeM projections and hence amygdala output. Understanding the biology of these inhibitory synapses is therefore essential in evaluating their vulnerability to pathogenic mutations and their potential as therapeutic targets. In the present study, we investigate the role of two psychiatrically relevant components of the inhibitory postsynapse, the synaptic adhesion molecules Neuroligin 2 (Nlgn2) and IgSF9b, in the CeM anxiety circuitry in mice. Using WT, Nlgn2 KO, IgSF9b KO and double KO mice, we show that deletion of IgSF9b normalizes aberrant anxiety-related behaviors in the open field test and elevated plus maze in Nlgn2 KO mice. This behavioral effect was accompanied by a normalization of neuronal activation in the CeM as assessed using cFos immediate early gene assays and local field potential recordings during exposure to an open field apparatus. To assess the consequences of local deletion of IgSF9b in CeM, we stereotactically injected AAV-IgSF9b-shRNA under Avertin anaesthesia (20 ml/kg).

We show that local knockdown of IgSF9b has prominent anxiolytic consequences in Nlgn2 KO mice. To investigate the mechanism behind the anxiolytic effect of IgSF9b deletion in the CeM, we investigated inhibitory synaptic transmission and inhibitory synapse number in acute slices. We find that deletion of IgSF9b results in increased inhibitory synaptic transmission and an increase in the number of inhibitory synapses in the CeM. These findings support a model in which the anxiety-related CeM overactivation observed in Nlgn2 KO mice is counteracted by the increased inhibition resulting from additional deletion of IgSF9b. Together, our data provide the first description of IgSF9b function *in vivo* and uncover a novel role for IgSF9b in anxiety-related behavior and amygdala inhibitory synapses. Moreover, our findings highlight that IgSF9b-expressing neurons in the CeM may represent an important common target for anxiolytic treatments that is independent of individual upstream mutations.

This study was supported by the European Commission (Marie Curie IRG, D.K.-B.), the Brain & Behavior Foundation (NARSAD Young Investigator Grant, D.K.-B.) and the Alexander von Humboldt Foundation (Research Fellowship, D.K.-B.). O.B. was a student of the Göttingen Graduate School of Neurosciences and Molecular Biosciences (GGNB) and was funded by a PhD fellowship from the Minerva Foundation.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

SA043

### Chemogenetic activation of discrete 5-HT<sub>2C</sub> receptors impacts food reward

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Obesity is a primary healthcare challenge of the 21st century. Medications increasing the bioavailability of the neurotransmitter serotonin (5-hydroxytryptamine; 5-HT) have historically been used for obesity treatment. 5-HT primarily influences appetite via action at the 5-HT<sub>2C</sub> receptor subtype (5-HT<sub>2C</sub>R); the clinical significance of which has recently been realized with the launch of the 5-HT<sub>2C</sub>R agonist lorcaserin for obesity treatment in the USA. Efforts to delineate the underpinnings of 5-HT<sub>2C</sub>R appetite suppression have largely focused upon action within the hypothalamus, a crucial brain region modulating energy homeostasis. However, another neuroanatomical population of 5-HT<sub>2C</sub>Rs is located within the ventral tegmental area (VTA), a primary node within reward circuits. To examine the physiological significance of 5-HT<sub>2C</sub>Rs within the VTA, we utilized designer receptors exclusively activated by designer drugs (DREADD) technology to probe the discrete function of VTA 5-HT<sub>2C</sub>Rs in the reward value of food. Designer Gq receptor (AAV8-hSyn-DIO-hM3Dq-mCherry) was bilaterally injected into the VTA of 5-HT<sub>2C</sub>R-Cre mice producing 5-HT<sub>2C</sub>R-Cre:hM3Dq-expressing neurons exclusively within the VTA. The selective activation of these neurons by designer drug clozapine-N-oxide (CNO) significantly suppressed home cage laboratory chow intake. Further analysis indicated that this was related to a reduction in food reward. Specifically, discrete stimulation of 5-HT<sub>2C</sub>Rs within the VTA significantly suppressed operant responding for food reward (chocolate pellets), both when mice were motivated to eat through food restriction and in the free feeding condition. To evaluate the translational significance of this finding, we next examined whether human obesity

medication 5-HT<sub>2</sub>CR agonist lorcaserin influences the reward value of food. The same concentrations of lorcaserin that reduced ad libitum home cage food intake also significantly reduced operant responding for food reward. Thereby, these data suggest that a component of lorcaserin-induced appetite suppression is due to a reduction in food reward and identify that the little studied population of VTA 5-HT<sub>2</sub>CRs are sufficient to mediate this effect.

Work was supported by the Wellcome Trust (WT098012) and BBSRC (BB/N017838/1).

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

SA044

### **Illuminating mitochondrial architecture and autophagy in vivo**

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Mitophagy, the autophagy of mitochondria, is thought to be an essential quality control (QC) mechanism of pathophysiological relevance in mammals, with dysfunctional mitophagy being implicated diseases such as Parkinson's, cancer and ageing. However, if and how mitophagy proceeds within specific cellular subtypes in vivo has remained unclear, largely due to a lack of tractable tools and models. To address this, we developed "mito-QC", a transgenic mouse with a pH-sensitive fluorescent mitochondrial signal. This allows the assessment of mitophagy and mitochondrial architecture in vivo. mito-QC revealed that mitophagy is a significant process in most tissues, even under basal conditions. However, within tissues it can be spatially restricted to distinct cell types, particularly those with high metabolic demands such as dopaminergic neurons in the brain, proximal tubule cells in the kidney, or cardiomyocytes within the heart. The reporter also revealed the striking differences between mitochondrial morphology in different tissue cell types and enabled easy visualization of the mitochondrial reticulum in skeletal muscle. Thus mitophagy displays a pervasive nature under basal conditions, yet how this changes under physiological stimuli and stress, such as during exercise, remains to be determined - as does the consequences of this process. We hope that mito-QC will be a powerful tool in this endeavour.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

SA045

### **Effects of exercise on mitochondria respiratory supercomplex assemblies**

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Skeletal muscle mitochondria are highly adaptable and respond to exercise training by multiple mechanisms. In humans, exercise modulates the different electron transport chain complexes distinctively. Among the molecular adaptations, exercise favors the shift of free complexes into functional supercomplexes assemblies. These observations

suggest that free and superassembled complexes co-exist and are recruited in response to energy demand. The content of supercomplexes is positively related to muscle respiration and exercise efficiency.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

SA046

### **Optimising the exercise prescription to promote mitochondrial adaptations**

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A sedentary lifestyle has been linked to a number of metabolic disorders that have been associated with sub-optimal mitochondrial characteristics and an increased risk of premature death. Endurance training can induce an increase in mitochondrial content and/or mitochondrial functional qualities, which are associated with improved health and well-being and a longer life expectancy. It is therefore important to better define how manipulating key parameters of an endurance training intervention can influence the content and functionality of the mitochondrial pool.

This presentation will focus on mitochondrial changes taking place following a series of exercise sessions (training-induced mitochondrial adaptations), and it will provide an in-depth analysis of the effects of exercise intensity and training volume on changes in mitochondrial content and mitochondrial respiratory function. Evidence will be presented that indicates manipulation of different exercise training variables promotes specific and diverse mitochondrial adaptations. Specifically:

- a) Training volume appears to be an important determinant of training-induced increases in mitochondrial content (an effect that may be driven by training duration), whereas exercise intensity appears to be a key factor of training-induced increases in mitochondrial respiration.
- b) Training-induced changes in mitochondrial content and respiratory function seem to be differentially regulated, and they are not necessarily associated to one another.
- c) High-intensity interval training at a relative exercise intensity  $\geq 90\%$  of the maximal aerobic power provides the greatest absolute increase in mass-specific mitochondrial respiration, whereas all-out sprint interval training appears to be the most efficient type of exercise to improve mitochondrial respiratory function in terms of total training volume and/or time.
- d) Mitochondrial adaptations to exercise training are rapidly reversed following different types of detraining; however, these can be maintained when a sufficient training stimulus is provided.

Our recent results also indicate that the early molecular events in response to a single bout of exercise differ between high-intensity and high-volume exercise, and this may help to explain the different training responses. However, exercise-induced changes are not always predictive of training-induced adaptations, and possible explanations these discrepancies will also be discussed.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

SA048

### The Dynamics, Machinery and Function of the Lysosome-Endosome Kissing, Fusion and Regeneration Cycle

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Lysosomes are dynamic, terminal organelles of both the endocytic and autophagic pathways which play a role in macromolecule degradation, nutrient sensing, signalling to the cell nucleus, and plasma membrane repair<sup>1,2</sup>. They are classically described as having an acidic lumen which enables efficient function of their resident acid hydrolases; however many studies indicate heterogeneity of lysosomal pH within individual cells, including lysosomes with a neutral luminal pH. The endocytic delivery of macromolecules from the mammalian cell surface for degradation by lysosomal acid hydrolases requires traffic through early endosomes to late endosomes, followed by transient (kissing) or complete fusions between late endosomes and lysosomes<sup>1</sup>. Transient or complete fusion results in the formation of endolysosomes<sup>3</sup>, which are hybrid organelles from which lysosomes are re-formed<sup>1,3</sup>. Through the use of fluorescent reporters of cathepsin activity and acid phosphatase cytochemistry, combined with immunolocalization of endosomal and lysosomal markers, we have found that acid hydrolase activity occurs predominantly in the endolysosomal compartment<sup>4</sup>. Thus, endolysosomes are the principal organelles in which acid hydrolase substrates are cleaved. Endolysosomes can be distinguished from re-usable terminal lysosomes by their accumulation of membrane-permeable cathepsin activity reporters and fluorescent acidotropic probes, neither of which accumulate in more neutral terminal lysosomes. These neutral lysosomes contain inactive acid hydrolases that can be detected by immunoelectron microscopy, suggesting that they may function as a re-usable storage compartment for these enzymes. Using live cell microscopy of NRK (normal rat kidney) fibroblasts, we have demonstrated that transient fusion events, resulting in the formation of endolysosomes, precede the onset of acid hydrolase activity<sup>4</sup>. Thus, kissing may be regarded as nucleating acid hydrolase activity. By means of sucrose and invertase uptake experiments, we have also shown that acid hydrolase-active endolysosomes and acid hydrolase-inactive, terminal lysosomes exist in dynamic equilibrium. Thus, fluid phase endocytosis of sucrose by NRK cells resulted in the accumulation of sucrose-laden osmotically swollen endolysosomes (sucrosomes) and depletion of the terminal lysosome pool within the cells. These cells were subsequently allowed to endocytose invertase, enabling the hydrolysis of sucrose, which resulted in tubulation from the sucrosomes and eventual reformation of the normal proportion of re-usable terminal lysosomes<sup>4</sup>. The sucrose treatment of the NRK cells did not cause an increase in autophagosomes, nor increased translocation of transcription factor EB to the nucleus<sup>4</sup>.

Many questions remain about the lysosome-endosome fusion and regeneration cycle. These include, but are not restricted to:

whether the machinery of fusion determined mainly from cell-free assays is sufficient to explain fusion in living cells. We have recent evidence for SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) compensation in cells in which individual SNAREs are knocked out;

the relationship between the transient pores required for kissing and the expansion of pores to allow full fusion - we suggest a role for tether clearance to achieve full fusion; the regulation of luminal acidity of organelles in the lysosome-endosome fusion and regeneration cycle. The acidic lumen of these organelles requires the proton pumping V-ATPase activity and cation channels and a Cl<sup>-</sup>/H<sup>+</sup> antiporter have been implicated in the necessary charge compensation. Alteration of luminal acidity may involve regulation of any of these components or other factors such as passive (leak) permeability to protons. Using fluorescent protein-tagged constructs of V-ATPase subunits we are currently exploring the dynamics of the V-ATPase during lysosome reformation in the sucrosome/invertase cell model described above; the molecular machinery of reformation of re-usable terminal storage lysosome reformation from endolysosomes and whether it is the same or different to that reported for lysosome reformation from autolysosomes<sup>5</sup>; the relationship between the molecular machineries for lysosome reformation and subcellular localization. This is relevant because others have reported that, in several cell types, peripheral lysosomes are less acidic than juxtanuclear lysosomes, as a consequence of their subcellular location<sup>6</sup>.

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SA049

### Connecting endo-lysosomes with the endoplasmic reticulum

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Membrane contact sites are regions of close apposition between organelles that have emerged as platforms to transfer small molecules. Many compartments of the endo-lysosomal system form contacts with the endoplasmic reticulum (ER), but little is known about how they are regulated. Here I discuss novel mechanisms underlying contact site form and function. Contact between endosomes and the ER requires the calcium



mobilising messenger NAADP, its target endo-lysosomal ion channel, TPC1 and associated local calcium fluxes. Reducing endosomal contacts perturbed endo-lysosomal morphology and impaired EGF-mediated signalling pathways. Additionally, I discuss the role of VAP proteins in regulating lysosome-ER contacts and calcium signalling through TRPML1, another endo-lysosomal ion channel defective in the lysosomal storage disease mucopolipidosis IV. Therefore, membrane contact sites between the endocytic pathway and ER emerge as calcium signalling hubs.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## SA050

### New tools to functionally investigate endolysosomal cation channels

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Lysosomal dysfunction can result in endolysosomal storage disorders such as mucopolipidoses or mucopolysaccharidoses but it is also implicated in development and progression of neurodegenerative diseases such as Alzheimer's and Parkinson's disease, metabolic diseases, infectious diseases, retinal diseases and pigmentation disorders, trace metal deficiencies such as iron deficiency, and cancer. Highly critical for the proper function of lysosomes, endosomes, and lysosome-related organelles is the tight regulation of various fusion and fission processes and the regulation of proton, calcium and other cation concentrations within the endolysosomal system (ES). TRPML cation channels (TRPML1, 2 and 3) as well as two-pore channels (TPCs) have recently emerged as important regulators of such processes within the ES and appear to be essential for a proper communication between the various endolysosomal vesicles. We use endolysosomal patch-clamp techniques, molecular and cell biology techniques as well as genetic mouse models to study the physiological roles and activation mechanisms of these ion channels in-depth. While TRPML1 causes mucopolipidosis type IV when lost or mutated, resulting in severe neuro- and retina degeneration, relatively little is known about the other two relatives, TRPML2 and TRPML3. Here, we present novel data relating to the physiological role of TRPML2 in the innate immune response. For the direct modulation of TRPML2 in endogenously expressing immune cells, we have developed a highly selective and potent agonist for TRPML2, ML2-SA1, which we applied and validated not only in endolysosomal patch clamp experiments, but also in a number of cellular assays to assess the role of TRPML2 in chemokine/cytokine release in more

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## SA051

### TRPML1-mediated Modulation of Dense-core Granules Tunes Functional Potential in NK Cells

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Discrimination of self from non-self through the continuous selection of effector specificity is the backbone of effective immunity. For natural killer (NK) cells this specificity is achieved by unique combinations of variable germ-line receptors that recognize self-MHC antigens. Inhibitory interaction between NK cell receptors and self-ligands is the key determinant in functional potentiation of pre-primed effector responses, a process termed NK cell education. However, the molecular mechanisms connecting homeostatic surface receptor signalling to an NK cell's intrinsic functional potential have remained a key knowledge gap. We have found that NK cells expressing self-MHC specific inhibitory killer cell immunoglobulin-like receptors (KIR) show a greater accumulation of dense-core secretory granules, converged closer to the centrosome in resting NK cells. Upon activation, interference of signaling from acidic  $\text{Ca}^{2+}$  stores reduced both target-specific  $\text{Ca}^{2+}$  flux, degranulation and cytokine production. Furthermore, inhibition of PI(3,5)P2 synthesis or genetic silencing of the PI(3,5)P2-regulated lysosomal  $\text{Ca}^{2+}$ -channel TRPML1 in primary NK cells led to an increase in granular load and enhanced functional potential. These results suggest a model where continuous unopposed signaling through activating receptors render NK cells hypofunctional through TRPML1-mediated modulation of acidic  $\text{Ca}^{2+}$  stores.

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## SA052

### Olfir78 is not required for oxygen regulation of breathing in mice

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The carotid body is essential for the adaptation of mammals to environmental or pathological conditions that result in hypoxemia. The carotid body contains neuron-like  $\text{O}_2$ -sensitive glomus cells. In response to hypoxia, these cells release neurotransmitters that rapidly activate afferent sensory fibers stimulating the respiratory center and inducing hyperventilation. The mechanisms that glomus cells utilize to detect changes in blood  $\text{O}_2$  tension have remained unclear. Single dissociated glomus cells can respond robustly to hypoxia when superfused with standard, lactate-free hypoxic solutions. As such, it was surprising that Chang et al. Nature



2015 claimed that lactate activation of an odorant receptor (Olfr78), which is expressed in glomus cells, is required for oxygen regulation of breathing. Here we report that we are unable to replicate these findings. We show that Olfr78-null mice (Bozza et al. Neuron 2009) have a normal hypoxic ventilatory response. In addition, we show that the physiological responses of single glomus cells to hypoxia and lactate are indistinguishable between wild-type and Olfr78-null mice.

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## SA053

### Neutrophil GPCRs as novel therapeutic targets

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Each year in the United States, 2 million people are infected with antibiotic-resistant pathogens, such as methicillin-resistant *Staphylococcus aureus* (MRSA), with 23,000 patients dying from such infections. The increase in antibiotic resistance and slow development of antibiotics have created an urgent need for new approaches to treat infections, including "Immune boosting" approaches that target the host. One target of such approaches may be G protein-coupled receptors (GPCRs) expressed by neutrophils, critical first-line effectors of innate immune defense against pathogens. To date, a relatively limited number of GPCRs have been studied in the context of neutrophil function. Using qPCR-based gene expression arrays and RNAseq, we have found that neutrophils express ~190 (of the ~360 known) non-chemosensory (i.e., other than visual, odorant or taste) GPCRs. Many of these GPCRs have unknown or poorly understood roles in neutrophil function. We propose targeting such GPCRs to enhance the antimicrobial response of neutrophils via modulation of chemotaxis/phagocytosis and the production of neutrophil extracellular traps (NETs). Such approaches may provide a novel way selectively boost the innate immune response to pathogens.

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## SA054

### Uncovering a novel role for olfactory receptors in glucose handling

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Olfactory receptors (ORs) are G protein-coupled receptors which serve to detect odorants in the nose. It is now appreciated that these receptors have roles beyond the canonical smell response and serve important functions in a number of different tissues. The epithelial lining of the kidney must carefully monitor the composition of the tubular fluid as it moves through the different segments of the nephron; thus, it is well

suited to take advantage of sensory receptors such as ORs in order to maintain homeostasis. Among the growing list of ORs known to be expressed in the kidney is olfactory receptor 1393 (Olfr1393). We recently determined that Olfr1393 is found on the apical membrane of the proximal tubule where it contributes to renal glucose handling as a novel regulator of sodium glucose co-transporter 1 (Sglt1) localization. Glucose reabsorption in the proximal tubule (via Sglt1 and Sglt2) has emerged as an important contributor to the development of diabetes. Inhibition of Sglt2 is now accepted as a viable therapeutic treatment option for patients with type II diabetes and has been shown to delay diabetic kidney disease. We hypothesized that Olfr1393 may contribute to the progression of type II diabetes, particularly the development of hyperfiltration which has been linked to increased Na<sup>+</sup> reabsorption in the proximal tubule via the Sglts. To test this, Olfr1393 wildtype (WT) and knockout (KO) C57BL6 mice were challenged with a high fat diet (HFD) to induce early stage type II diabetes. After 16 weeks on the HFD, WT mice displayed increased fasting blood glucose values and impaired glucose tolerance. Both of these effects were significantly blunted in the male KOs. In addition, both male and female WT mice presented with an increased glomerular filtration rate (via transcutaneous measurement of FITC-Sinistrin clearance) over time indicating that they developed diabetes-induced hyperfiltration, and this response was attenuated in the Olfr1393 KO mice. Collectively, this data indicates that renal Olfr1393 can contribute to the progression of type II diabetes. Outside of the kidney, Olfr1393 is expressed in several other tissues including the liver, the major site of glucose metabolism. Given this, it is possible that Olfr1393 and other ORs function as regulators of glucose homeostasis in other tissues as well. Using a TaqMan real time PCR assay, we identified a total of 17 murine ORs with detectable expression in the liver including Olfr1393. Efforts are currently underway to determine how these receptors contribute to liver physiology and glucose homeostasis in both health and disease.

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## SA055

### Chemogenetic approaches to define G protein-coupled receptor mediated mediated effects of Short Chain Fatty Acids

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The primary means of production of short chain fatty acids in the body is by fermentation of non-digestible carbohydrates by the microbiota in the lower intestine. Such short chain fatty acids have wide ranging functions in the body both locally in the gut and after entry into the systemic circulation. It is increasing clear, as for many other metabolic products of ingested foodstuffs, that short chain fatty acids produce a number of effects via activation of G protein-coupled receptors. Two such receptors, now designated FFA2 and FFA3, were de-orphanised more than 10 years ago. Still, however, in large part due to a limited pharmacological tool box of ligands that activate or block these receptors selectively and with high affinity, there remains considerable confusion as to their specific functions and whether they offer interesting, novel therapeutic targets. Moreover, at least three other poorly characterised G protein-coupled receptors have been shown

to be regulated by short chain fatty acids. To unravel specifically the roles of FFA2 we have adopted a number of chemogenetic approaches. As the only classes of FFA2 antagonist ligands act only at human and other primate orthologues of this receptor and not at rodent orthologues we initially generated a transgenic mouse line in which a humanised sequence of FFA2 was 'knocked-in' to the mouse FFA2 receptor gene locus to replace the mouse orthologue. In an extension to this approach we developed a 'Designer Receptor Exclusively Activated by Designer Drugs' (DREADD) variant of this human receptor which is no longer responsive to short chain fatty acids but instead is activated by either sorbic acid or a benzoic acid derivative we designate 'compound 36' and then used this to generate a transgenic mouse line in which this variant replaces the mouse orthologue of FFA2. In both of these situations, because anti-G protein-coupled receptors antisera are notoriously poorly selective we also added a haemagglutinin (HA) epitope tag to allow effective immunodetection of receptor expression patterns. I will discuss studies performed using these mice and demonstrate the specific roles of FFA2 in the release of the incretin glucagon-like peptide 1 from dissociated colonic crypts and intact colonic preparations, in neutrophil chemotaxis and in the regulation of lipolysis.

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SA056

### **Bile acid- gut microbiota crosstalk induced changes in TGR5-regulated metabolism**

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Bile acids are endocrine molecules that in addition to facilitating the absorption of fat-soluble nutrients regulate numerous metabolic processes, including glucose, lipid, and energy homeostasis. The signaling actions of bile acids are mediated through specific bile acid-activated nuclear and membrane bound receptors. The two major bile acid receptors that regulate host metabolism are the nuclear farnesoid X receptor (FXR) and the membrane-bound Takeda G protein-coupled receptor 5 (TGR5) and they are considered to have a large impact on the development of metabolic disorders. TGR5 is highly expressed in gallbladder, spleen, intestine, liver, placenta, lung, brown and white adipose tissue, skeletal muscle, and bone marrow. TGR5, in contrast to FXR, is activated by secondary bile acids (LCA and DCA) that are formed from primary bile acids (CDCA and CA) by gut microbiota-dependent deconjugation and dihydroxylation reactions. TGR5 is especially expressed in muscle and brown adipose tissue (BAT) where it promotes the conversion of inactive thyroxine into active thyroid hormone which induces thermogenesis and increased energy expenditure. TGR5 activation in intestinal L-cells promotes the secretion of GLP-1. INT-777, which is a derivative of CDCA and a specific TGR5 agonist, ameliorates hepatic steatosis and adiposity and improves insulin sensitivity in mice with high-fat diet-induced obesity. Of note, FXR and TGR5 seem to have opposite effects on GLP-1 signalling. Thus, when providing natural bile acids that are agonists for FXR the gut microbial metabolism may generate

ligands for TGR5, which may result in unpredictable treatment responses.

Rapid metabolic improvement after bariatric surgery is associated with changes in bile acid profiles. These are positively correlated with a number of metabolically active peptides, including adiponectin, peptide YY and in particular, GLP-1, which could be attributed to increased secondary bile acid mediated activation of TGR5. Vertical sleeve gastrectomy (VSG) is a major bariatric surgery procedure that nowadays is performed in about half of the patients with morbid obesity. Studies on VSG in *TGR5*-deficient mice showed that TGR5 is fundamental for the beneficial effect of VSG in terms of improved glucose metabolism, insulin signaling and fat accumulation in the liver, while data on postoperative body weight reduction are controversial.

Studies in conventional, germ-free and gnotobiotic mice lacking either the FXR or TGR5 receptors may help to decipher the interplay between bile acids and microbiota aiming to find specific microbiota that improve metabolism by specifically activating either bile acid receptor.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

SA057

### **Regulation of intestinal inflammation by bile acids**

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Bile acids, classically known for their roles in facilitating lipid digestion and absorption, have become appreciated as a family of enterocrine hormones that regulate many aspects of intestinal physiology, including epithelial barrier permeability, immune function, apoptosis, and inflammation. Dysregulation of intestinal epithelial barrier function is closely associated with the development of several diseases, most notably inflammatory bowel disease (IBD), a condition where compromised epithelial barrier function allows the contact of luminal contents with mucosal immune cells, thereby promoting inflammation. Our research focusses on understanding the roles that bile acids play in regulating intestinal epithelial barrier function, how they contribute to disease pathogenesis and, ultimately how they can be targeted for the development of new treatments. Ursodeoxycholic acid (UDCA) is a secondary bile acid normally produced by bacterial metabolism in the colon. UDCA has a long history as a safe and effective therapeutic, first in Traditional Chinese Medicine and later in Western medicine as a treatment for cholestatic liver diseases. Based on its known cytoprotective and anti-inflammatory actions, we have investigated the role of UDCA in regulation of intestinal barrier function and its potential for development as a new approach to treat IBD.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

SA058

**Relationship between microbes, bile acids and FXR signalling in gestational metabolic switching**

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Normal gestation is associated with a gradual change in lipid, bile acid and glucose metabolism that results in relative hypertriglyceridaemia, hypercholesterolaemia, hypercholanemia and impaired glucose tolerance by late pregnancy. Endocrine signals are known to contribute to this gestational switch, and it is likely to be an adaptation to ensure adequate nutrient transfer to the developing embryo. In susceptible women, the gestational alterations in metabolism can be more marked resulting in diseases, e.g. gestational diabetes mellitus or intrahepatic cholestasis of pregnancy.

The gut microbiota contribute to bile acid metabolism via deconjugation and enzymic modification of luminal bile acid species. It has been shown that transplantation of faeces from women in the third trimester of uncomplicated pregnancy to germ free mice results in enhanced weight gain and insulin resistance compared to transplantation of samples from women in the first trimester of pregnancy. There is an alteration in gut microbes in normal gestation, with modification of the bile acid pool and entero-endocrine signals that influence bile acid and lipid metabolism. The lecture will focus on the impact of these alterations and the relevance to gestational metabolic switching in health and disease.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

SA059

**Allosteric tuning of myosin force generation: New avenues towards therapeutical treatment**

A. Houdusse

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Force production by myosin motors plays major roles in muscle contraction, intra-cellular trafficking and maintenance of critical cellular structures such as microvilli, stereocilia and invadopodia. Deficit in different myosin motors can lead to a number of serious disease, thus myosins are important potential targets for therapeutical treatment. Structures of myosins in complexes with small molecules reveal unsuspected allosteric sites and provide valuable insights for the design of specific modulators. These reveal the mechanistic control of motor transitions by inhibitors and activators and provide novel understanding of the rearrangements controlling the force producing lever arm swing. Current progress and outstanding questions regarding the important sequential rearrangements that lead to force production by myosins will be presented in light of recently solved X-ray structures of myosin/drug complexes. New insights into the mechanism of allosteric tuning of myosin force generation is thus anticipated to lead the way in the development of new myosin-directed therapeutics.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

SA060

**Motor Pharmacology: novel inhibitors for different myosin-2 isoforms**M. Gyimesi<sup>1</sup>, S. Kumar Suthar<sup>3</sup>, A. Szabo<sup>4</sup>, M. Penzes<sup>1</sup>, L. Vegner<sup>1</sup>, M. Kovacs<sup>2,1</sup> and A. Malnasi Csizmadia<sup>2</sup>

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We have designed and synthesized over 100 different compounds targeting the blebbistatin site of myosin 2. Based on their inhibitory properties on six myosin-2 isoforms including skeletal, cardiac, smooth and NM2A/B/C, we have made a detailed SAR analysis. We found that the maximal ATPase inhibition can be modulated between 0-100% by the chemical structure of the drug. This unique property of the series of these drugs provides a great pharmacological advantage because the physiological effect can be modulated by the maximal ATPase inhibition and not only by the drug dosage. In order to correlate the compounds' inhibitory profiles with their effects on cell migration, cell shape formation and neurite outgrowth, the compounds were tested on several human neuronal and fibrocyte cell lines. Besides the general characterization two disease indications are being elaborated. (1) A highly specific drug to skeletal muscle myosin 2 was found to be an efficient muscle relaxant without causing any effect on other physiological processes including heart function and smooth muscle related functions. The specificity relies on a residue that is Leu490 in human skeletal muscle myosin-2s where all the other myosin-2 isoforms have Phe. (2) We developed a compound which can be administered into the ischemic focus of stroke in rat brain and significantly increased ischemic regeneration visualized by MRI, SPECT and PET-CT. The drug treatment drastically improved the general and focal symptoms of stroke compared to the control. These compounds demonstrate that the blebbistatin site of myosin-2 is a pharmacologically highly relevant target.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

SA061

**In situ study of the coupling of the working stroke with the release of the hydrolysis products in muscle myosin**

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The contraction of striated muscle is due to cyclical, ATP-driven interactions of the motor protein myosin II with actin. In each sarcomere, the ~2 µm long structural unit of striated muscle, two bipolar arrays of myosin motors emerging from the thick filament overlap with the thin, actin containing, filaments originating from the ends of the sarcomere. A structural working stroke in the myosin head, accompanied by the release of the ATP hydrolysis products (γ-phosphate (Pi) and ADP), generates force and relative filament sliding pulling the actin filament towards the centre of the sarcomere. In each half-sarcomere, the myosin motors are mechanically coupled as parallel force generators via their attachment to



the thick filament, and the collective motor formed by the array of myosin motors, the interdigitating actin filaments and other cytoskeleton and regulatory proteins is the basic functional unit of muscle. When the external load is smaller than the isometric force generated by the motor array, the actin filament slides past the myosin filament and the sarcomere shortens at a velocity that is larger at lower loads (force-velocity relation), accompanied by the increase in the rates of ATP-driven detachment-attachment cycles and energy liberation. Power output is maximum when muscle shortens under a load  $\sim 1/3$  the isometric force, at which the ATP is split  $\sim$  four times faster than in isometric contraction and macroscopic efficiency is 40% (1).

*In vitro* X-ray crystallography and cryo-EM studies have provided a high resolution description of the structure of the actomyosin complex, leading to a model of the working stroke that consists in a  $70^\circ$  tilting of the light chain domain of the myosin that links the actin-attached catalytic domain to the tail and the myosin filament (2). *In situ* such a model accounts for the 11-nm filament sliding following a sudden drop of the load to zero (3). However, both *in situ* and *in vitro* evidences that the motor compliance is relatively low (0.35 nm/pN) suggest that, based on energetic considerations, the working stroke must be a multi-step structural transition with only the first few steps responsible for the generation of the isometric force.

One relevant, still unsolved question emerges about the coupling between structural and mechanical events accompanying the release of the ATP hydrolysis products under different loads. The question whether the working stroke is associated with Pi release has been addressed by *in situ* measurements of the working stroke and its kinetics under the loads the myosin motor experiences as a part of a collective motor. In those experiments, fast-sarcomere-level mechanics on demembranated fibres from rabbit psoas was used to determine how the Pi concentration ([Pi]) modulates the number and force of actin-attached myosin motors during isometric and isotonic contractions. In isometric conditions the increase in [Pi] reduces the isometric force because of a proportional reduction in the number of attached myosin motors without reduction of the force per motor (4). The Pi-dependent reduction in isometric force is not accompanied by a proportional reduction in the rate of ATP hydrolysis (5), suggesting that under high load a motor that has already undergone the force generating transition can detach at an early stage of the ATPase cycle, then rapidly release the hydrolysis products and bind another ATP. In isotonic conditions the velocity transient following a force step has an early rapid shortening component, which represents the mechanical manifestation of the working stroke. By determining the effect of [Pi] on the velocity transient it was found that the working stroke is not affected by the increase in [Pi], while the subsequent transition to the steady shortening velocity is accelerated and the steady power at high loads is reduced (6).

A chemo-mechanical model in which the working stroke and Pi release are orthogonal processes has been demonstrated to be able to reproduce the load dependence of velocity transient, as well as steady shortening and power. In the model biochemical and mechanical steps are not tightly coupled: (i) the release of the hydrolysis products from the catalytic site of a myosin motor can occur at any stage of the working stroke, though progression in the working stroke increases the rate constants of product release, and (ii) a myosin motor, in an intermediate state of the working stroke, can slip to the next actin monomer away from the center of the sarcomere before terminating the biochemical cycle. The model provides

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SA062

### Structure-function relations in the muscle myosin family: Isoforms and myopathies.

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The muscle myosin family comprises  $\sim 12$  major isoforms each expressed from a different gene and each used for a distinct physiological role (Schiaffino & Reggiani, 2011) (Golomb et al., 2004). In a striated skeletal muscle, for example, the maximum velocity of contraction is a property of the myosin isoform expressed in a muscle fibre (Pellegrino et al., 2003). Therefore the distinct mechanical properties of each myosin isoform and how they are suited to specific tasks is of broad interest from a structure-function viewpoint. Similarly it is of interest how naturally occurring non-lethal mutations in the myosin motor alter the biochemical and mechanical properties of the motor to cause a range of human myopathies. Using mouse C2C12 cell lines we have been able to express the motor domain of most of the human muscle isoforms and complete a biochemical kinetic analysis of the actin-myosin cross bridge cycle (Deacon, Bloemink, Rezavandi, Geeves, & Leinwand, 2012) (Bloemink et al., 2014). This study has revealed that for individual myosin isoforms the basic cross bridge cycle is adjusted to alter the speed of contraction (by altering the rate of ADP release), the load sensitivity of the cycle and economy of ATP usage while maintaining a low duty ratio (the fraction of the cycle time spent strongly attached to actin) and the intrinsic force/step size of individual myosin motors (Mijailovich et al., 2017). The results emphasise the importance of balancing each primary event in the cross bridge cycle to maintaining an efficient cycle. Our data on has been used to model the complete cross bridge cycle for four major muscle myosin isoforms; fast muscle-2a, slow or



$\beta$ -cardiac,  $\alpha$ -cardiac and embryonic myosin. This reveal how speed and power maybe increase but at the cost of a greater rate of ATP usage. Exactly how the amino acid sequence changes between isoforms bring about the changes in behaviour is a current interest.

Our biochemical analysis is useful when studying mutations in myosin associated with human disease. For example, mutations associated with major heart disease such as hypertrophic or dilated cardiomyopathy are relatively mild since the heart carrying such mutations can often continue to function well for 30 or 40 years. Understanding how the mutation alters the overall cycle is important to understand changes in the mechanics and economy of contraction. Recent data will be presented utilising human  $\beta$ -cardiac myosin carrying one of 10 distinct mutations; 5 associated with hypertrophic and 5 dilated cardiomyopathies.

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SA063

### Renal nerves, renal inflammation, and hypertension: is there a link?

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Human essential hypertension is associated with increased activity of the sympathetic nervous system (SNS). Drugs targeting the SNS globally are effective antihypertensive agents but are also associated with numerous side effects that limit their use. The development of catheter based renal nerve ablation (CBRNA) to ablate nerves to the kidney specifically in humans shows promise as an anti-hypertensive treatment that avoids unwanted side effects. Clinical trials of CBRNA will be briefly reviewed as well as the current limitations and challenges. Beneficial unexpected “off target” effects of CBRNA have raised questions regarding the mechanism by which CBRNA decreases arterial pressure. Specifically, the contibution of ablation of renal *efferent* nerves that control renal function, and renal *afferent* nerves that modulate SNS activity, in mediating the cardiovascular responses to CBRNA is unclear. Our laboratory recently developed a novel method for targeted ablation of renal afferent nerves to address this question in preclinical models. Comparison of the cardiovascular responses to *total* renal denervation to *afferent* specific ablation suggest the underlying mechanisms are model specific.

Moreover, our studies suggests an important relationship between renal nerves, renal inflammation, and hypertension. The significance of these preclinical findings to the efficacy of CBRNA in the treatment of human hypertension and other renal based cardiovascular diseases will be discussed.

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SA064

### The afferent activation hypothesis of hypertension

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For decades, the treatment of cardiovascular disease has been to target end organs. However, autonomic imbalance has been poorly controlled by frontline medications (Chant et al. 2018). This is important as reduced parasympathetic drive to the heart and excessive levels of sympathetic activity contribute to both the development and maintenance of cardiovascular disease (Fisher & Paton 2012). Recently, we proposed an afferent activation hypothesis of autonomic imbalance (Ford et al. 2015; Koeners et al. 2016) by which visceral afferents that produce reflex increases in sympathetic activity become sensitised and generate aberrant tone. My lecture will describe the mechanisms and functional consequence of sensory neurone hyperexcitability as it contributes to hypertension, heart failure and disordered breathing in animals and humans. I will demonstrate that both the elevated reflex sensitivity and hypertonicity of petrosal afferents serving the carotid body chemoreceptor reflex are mediated by upregulation of purinergic P2X3 receptors (Pijacka et al. 2016). Importantly, blockade of these receptors abolished both the hyperreflexia and aberrant tone, normalised afferent excitability thereby restoring physiological reflex function and autonomic balance. Thus, P2X3 receptors have surfaced as a novel target for treating cardiorespiratory diseases. This invokes a paradigm shift away from targeting end organs to visceral sensory neurones for the management of diseases in which autonomic imbalance prevails.

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SA065

### Calcium channels and microvascular reactivity: possible novel therapeutic targets

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Endothelial cell dysfunction is an early and ubiquitous feature of cardiovascular disease, which includes a loss of vasodilator capacity resulting in increased vascular reactivity. Vasodilator capacity is due to the synthesis of nitric oxide (NO) and the activation of endothelium-dependent hyperpolarization (EDH). In myogenically active cerebral arteries, mimicking dysfunction by loss of endothelial nitric oxide leads to intense vasospasm (Toda *et al.*, 1991).

In small resistance arteries and arterioles, vascular smooth muscle (VSM) contraction is predominately the result of  $\text{Ca}^{2+}$  influx through L-type voltage-gated calcium channels (VGCCs). These vessels provide the bulk of the peripheral vascular resistance, direct tissue blood flow and are targeted therapeutically by  $\text{Ca}^{2+}$  channel blockers, particularly the dihydropyridines (Nelson *et al.*, 1990). VSM also contain T-type channels, which appear to provide a larger contribution to myogenic tone in small arteries, relative to L-type VGCCs, although remaining of secondary importance (Hansen, 2015). A role for T-type VGCCs may at first seem surprising, as the channels have a low activation threshold and inactivate rapidly. Consistent with this property, in cerebral arteries the functional contribution of T-type VGCCs relative to L-type appears greater at low intraluminal pressure, when the membrane potential is more hyperpolarized. However, in isolated cerebral artery VSM T-type window currents peak at around -40mV in the absence of L-type channels, and also contribute to the combined window currents when both forms are active, supporting a significant but modest role for these channels in myogenic tone (Harraz *et al.*, 2014). The pore-forming  $\alpha_1$  subunits,  $\text{Ca}_v3.1$  and  $3.2$  have been found in VSM, with  $\text{Ca}_v3.2$  forming a microdomain with ryanodine receptors and large-conductance Ca-activated K channels in cerebral arteries, serving to attenuate vasoconstriction (Harraz *et al.*, 2015). However, this may be a characteristic of cerebral arterioles, as similar microdomains do not appear operate in peripheral arterioles (Mullan *et al.*, 2017).

Membrane potential measurements in intact cerebral arteries revealed that block of NO synthesis was followed by the appearance of depolarizing spikes, apparently mediated by a *de novo* involvement of T-type VGCCs. These events modified myogenic tone giving rise to vasospasm (McNeish *et al.*, 2010). The emergence of T-type VGCC in cerebrovascular VSM has also been demonstrated using patch-clamp recording in isolated cells and suggested to be mediated through cGMP/PKG signalling (Harraz *et al.*, 2014). However, the overall effect of NO in cerebral arteries is complex, as it also inhibits L-type VGCCs and activates RyRs, the latter suppressing vasoconstriction via  $\text{BK}_{\text{Ca}}$  (Yuill *et al.*, 2010).

Clinically, combined block of T- and L-type VGCCs appears to be more effective in treating hypertension than L-type block alone (Hansen, 2015), which at least in part may indicate an action against raised vascular reactivity. The possibility T-type channels underlie vasospasm in resistance arteries outside of the cerebral circulation has not been addressed, although it is known that T-type VGCCs are recruited in mouse resistance arteries once endothelial cell NO-signalling is compromised (Howitt *et al.*, 2013). Other considerations include the extent to which an increase in T-type VGCC input might contribute

to the vasoconstrictor response of non-myogenic arteries and possible interactions with endothelial signalling. In terms of vasoconstriction, a reduction while sustaining myogenic tone for autoregulation would be a desirable outcome. With the endothelium, bidirectional signalling involving VSM VGCCs can modulate endothelial cell microdomains, suppressing vasoconstriction and possibly involving T-type VGCCs, (Garland *et al.*, 2017). Discovering how and to what extent T-type VGCCs contribute to small artery vasospasm may therefore provide novel targets to treat cardiovascular disease.

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SA066

### Migraine associated mutation in the $\alpha 2$ isoform $\text{Na}^+, \text{K}^+$ -ATPase leads to disturbance in neurovascular coupling

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Background: It has been suggested from clinical studies that migraine is associated with changes in neurovascular coupling (NVC) although the underlying mechanism is unclear. We addressed this question by studying heterozygous mice bearing a mutation (G301R) of the  $\alpha 2$  isoform  $\text{Na}^+, \text{K}^+$ -ATPase, which is known to be associated with familial hemiplegic migraine type 2 (FHM2) in humans. We have previously shown that this mutation leads to increased contractility of cerebral arteries associated with increased Src-kinase dependent  $\text{Ca}^{2+}$ -sensitivity in vascular smooth muscle cells. Moreover, we found that pre-constricted cerebral arteries from FHM2 mice dilated stronger to increased  $\text{K}^+$  concentrations (8 – 12 mM) than arteries from wild types. This suggests stronger vasodilation to elevation of interstitial  $\text{K}^+$  in vivo, i.e. NVC, in FHM2 mice.

Methods: NVC was assessed using confocal microscopy in brain slices loaded with CalciumGreen/AM. Parenchymal arteriole diameter and  $[Ca^{2+}]_i$  changes in astrocytes and smooth muscles were measured ex-vivo in response to neuronal excitation induced by electric field stimulation. Change in cerebral blood flow in response to whisker stimulation was measured noninvasively using Laser Speckle Imaging.

Results: Neuronal excitation ex vivo led to  $Ca^{2+}$  waves in astrocytic endfeet and relaxation of adjacent arterioles. Arterioles from FHM2 mice increased the diameter more than arterioles from wild type mice ( $7.83 \pm 0.87\%$ ,  $n=8$  vs.  $4.03 \pm 0.39\%$ ,  $n=7$ ;  $P=0.001$ ). The decrease in smooth muscle  $[Ca^{2+}]_i$  associated with this relaxation was the same in both groups but  $[Ca^{2+}]_i$  recovery in astrocytes was significantly slower in FHM2 brains. Whiskers stimulation increased blood flow in the corresponding part of the sensory cortex and this response was significantly stronger in FHM2 mice.

Conclusion: In vivo and ex vivo experiments suggested NVC disturbances in a mouse model for FHM2. Cerebral arterioles from FHM2 mice showed increased dilations to excitation of nearby neurons even though the changes in smooth muscle  $[Ca^{2+}]_i$  was similar to that in wild types. We suggest that increased dilation of cerebral arterioles from FHM2 mice consequent to neuronal activity is due to altered  $Ca^{2+}$ -sensitivity of smooth muscle cells, likely caused by increased Src-kinase activity.

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Much more narrow paravascular spaces exist around arteries that penetrate the brain. Most likely, arterial pulsations induce mixing of the fluid in these spaces. This facilitates exchange between the interstitial fluid and CSF, and thereby aids the removal of waste products from the brain.

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SA067

### **Fluid flow in the brain, role of paravascular spaces and hypertension**

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Clearance of waste products from the brain is of vital importance. Recent publications suggest a potential clearance mechanism via paravascular channels around blood vessels. However, the anatomy, driving forces, and flow pattern into and out of the brain along these channels remain poorly characterized. In this study, we directly observed paravascular flow through a thinned-skull cranial window in anesthetized mice (ketamine 75 mg/kg, dexmedetomidine 0,5 mg/kg). In this model, we observed that microspheres moved preferentially in the paravascular space of arteries rather than in the adjacent subarachnoid space or around veins. Paravascular flow was pulsatile, generated by the cardiac cycle, with net antegrade flow along leptomeningeal arteries. Confocal imaging confirmed that microspheres distributed along these arteries, while their presence along penetrating arteries was limited to few vessels. Smaller tracers (4 kD and 500 kD) injected into the CSF of mice and rats revealed paravascular spaces around arteries that penetrated the brain and mostly followed the cisterns and clefts between brain territories. Tracers injected directly into the hippocampus dispersed inhomogeneous, with accumulation at border zones between brain parenchyma and cerebrospinal fluid, and accumulation and spreading along arteries. This suggests that interstitial fluid drains into the CSF while larger solutes are retained by sieving. Distribution of tracers was drastically enhanced in spontaneously hypertensive rats, but removal from the brain was not affected. Collectively, these data suggest that paravascular spaces around leptomeningeal arteries form low resistance pathways on the surface of the brain that facilitate cerebrospinal fluid flow.

C001

# **Asymptomatic and symptomatic long QT 2 syndrome disease model using iPSC-CM from patients with L552S mutation**

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Mutations in the KCNH2 gene; encoding Potassium ion channel IKr, represents one of the most frequent causes of Long QT 2 syndrome. This study is focused on a heterozygous missense mutation L552S in the S5 subunit of the ion channel KCNH2, which has been identified as founder mutation in Finland. The same genetic mutation in a family often shows different phenotype. Current treatment is directed towards symptom relief and prevention of sudden cardiac death and no specific therapy or treatment for LQT2 exists. Studying the disease mechanism with a human iPSC-CM model would be necessary for better treatment and making improved drugs. Aim of this study was to model LQT2 with iPSC-CM from asymptomatic and symptomatic mutation carriers from the same family and gain insight into the disease causing mechanism induced by the genetic mutation L552S. The results from this study is proof that a functional iPSC derived LQT2 model from both asymptomatic and symptomatic patients can be made and it shows differences. Electrophysiological differences on HERG block, and adrenergic stimulation were of found. HERG current was measured with Voltage clamp and the calcium kinetics was studied with calcium imaging. The allelic imbalance was studied by quantitative polymerase chain reaction. To uncover the mechanism of the disease causing mutation, and the HERG protein was expressed in HEK cells and their expression and localization was analyzed using confocal imaging. This is the first study till date which has analyzed at cellular level with an aim to understand similarities or differences at genetic, protein and electrophysiological level between cardiomyocytes from asymptomatic and symptomatic LQT2 patient cell lines. Our model does not only recapitulate major phenotype characteristics as observed in LQT2 patients but also is a proof of concept that this model can be used to study the disease mechanisms further and can serve as a platform for pharmacological screenings. The results would potentially help in personalized treatment of LQT2.

The study was approved by the Ethics Committee of Pirkanmaa Hospital District to establish, culture, and differentiate hiPSC lines (R08070). A signed informed consent was obtained from individuals participating in donation of skin biopsies along with oral and written descriptions of the study. We would like to acknowledge the granting agencies CIMO and Finnish Cultural Foundation. Henna Lappi and Markus Haponen for the assistance in stem cell culture and cardiac differentiation. Kim Larsson in the MEA data analysis. Kirsty Haddow for help during cloning and confocal imaging. And the Tampere Facility of Electrophysiological Measurements for their service.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

C002

# **Postnatal development of t-tubules and alterations in triggered calcium release in the sheep atria**

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Transverse (t)-tubules are invaginations in the membranes of cardiac myocytes that facilitate synchronous calcium release and thus contraction. In diseases such as heart failure, atrial t-tubules are lost which contributes to reduced triggered calcium release and contractile dysfunction. Because of this the restoration of t-tubules is therapeutically desirable; however the process of t-tubule formation is not fully understood. T-tubules are absent at birth in the ventricles of small mammals, where they develop postnatally with subsequent changes in calcium handling. Nothing is currently known about the development of t-tubules and changes in calcium release in the atria.

1 week, 1 month, 3 months and adult sheep (~18 months) were euthanased with 200 mg/kg intravenous pentobarbitone and cells isolated from the left atrial appendage. T-tubule density was assessed using di-4-ANEPPS and confocal microscopy. Perforated patch clamp experiments were performed in Fluo-3 loaded cells to assess spatial calcium release. Data is presented as mean  $\pm$  SEM, for n animals per group, compared by SPSS linear mixed models or two way repeated measures ANOVA where appropriate.

T-tubule density increased during development with the fractional area of t-tubules increasing from  $0.022 \pm 0.004$  at 1 week,  $0.045 \pm 0.004$  at 1 month to  $0.087 \pm 0.004$  at 3 months where density was unchanged vs adult ( $0.067 \pm 0.01$ ,  $p < 0.001$ ,  $n = 6-11$ ). As t-tubule density increased postnatally, there were improvements in the uniformity of calcium release throughout the cell. Whilst calcium transient amplitude was reduced in the cell centre compared to the surface by  $24.0 \pm 10.9\%$  in 1 week animals, this discrepancy was reduced to  $15.4 \pm 11.4\%$  at 1 month with no surface vs centre differences observed in 3 months and adult ( $p < 0.01$ ,  $n = 7-10$ ). When compared to the ventricle, triggered calcium release in the atria is patchy and occurs in discrete locations on the cell surface and in the cell centre – likely on t-tubules. In neonates where t-tubule density is less, the amplitude of calcium release at these discrete surface sites was  $47.2 \pm 25.4\%$  greater than release sites in the centre ( $p < 0.001$ ,  $n = 8$ ).

T-tubules are present at birth in the sheep atria and develop until ~3 months of age where there is no further t-tubule development. Our data suggests that reduced t-tubule density results in reduced central calcium release in neonates that is compensated for by enhanced surface activity. In later life the synchronicity of calcium release throughout the cell is improved, coinciding with t-tubule maturation.

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C003

### Small conductance calcium-activated potassium (SK) channel regulation by protein kinase A (PKA) in atrial fibrillation

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SK channels are unique as they sense changes in intracellular  $\text{Ca}^{2+}$  concentration and convert it to  $\text{K}^{+}$  conductance. In 2010, GWAS studies showed association with SK3 coding gene (*KCNN3*) with lone atrial fibrillation (AF) and in 2017 with *KCNN2* gene. In pharmacological studies, blockade of SK channels has been efficacious in converting AF to sinus rhythm by prolonging the atrial effective refractory period (AERP) [1]. Being more prominent in atria than in ventricles elevates its importance as atrial-selective target for AF by reducing the risk of adverse toxicities of current anti-arrhythmic drugs; however, the molecular mechanism involved is not clear yet. PKA had shown to regulate surface expression of SK2 channel in hippocampal neurons [2]. This study aims to decipher PKA regulation on SK channel in AF. Mean  $\pm$  S.E.M. are stated and analysed by ANOVA. Whole-cell currents were acquired in HEK293-hK<sub>Ca</sub>2.3 cells which showed that activation of PKA by forskolin (FSK) 10  $\mu\text{M}$ , an adenylyl cyclase activator inhibits SK3 current density and inhibition by PKA inhibitor, KT5720 0.1  $\mu\text{M}$  results in increased SK3 current density ( $-69 \pm 13$ ,  $n=12$  vs  $-298 \pm 31$ ,  $n=16$ ,  $p<0.0001$ ). Immunofluorescence in HEK cells transfected with rat K<sub>Ca</sub>2.3 showed that SK3 surface expression was reduced in FSK 10  $\mu\text{M}$  treated cells but KT5720 0.1  $\mu\text{M}$  increased it, which suggests role in SK channel trafficking. Langendorff experiments were performed using female Dunkin Hartley guinea pigs (350-450g) anesthetized with intraperitoneal injection of pentobarbital (200mg/ml), lidocaine hydrochloride (20mg/ml) at a dose of 0.1ml/100g body weight, after which tracheotomy was performed and the guinea pigs were ventilated using a rodent ventilator. The hearts were then excised and perfused via cannulated aorta in Langendorff retrograde setup (all groups  $n=6$ ). All animal studies were performed in accordance with the Danish guidelines for animal experiments according to the European Commission Directive 86/609/EEC. Application of FSK 1  $\mu\text{M}$  increased the intrinsic rhythm ( $339 \pm 12$  vs  $216 \pm 18$  bpm,  $p<0.01$ ) compared to KT5720 0.1  $\mu\text{M}$ . Also FSK decreased atrioventricular Wenckebach point ( $100 \pm 2.7$ ), whereas KT5720 showed vice-versa ( $126 \pm 3.3$ ,  $p<0.01$ ) with no significant change in AERP ( $56 \pm 1.7$  vs  $58 \pm 3.2$ ,  $p>0.05$ ) and rheobase ( $0.28 \pm 0.02$  vs  $0.18 \pm 0.02$   $p>0.05$ ) between both groups. AF was induced by addition of 1  $\mu\text{M}$  acetylcholine with 40X burst pacing (50 Hz, 10 V) or until sustained AF of  $>3$  min was observed. FSK showed protective role in duration and number of AF events compared to KT5720 that showed vulnerability to AF. Thus, *in vitro* studies show SK channel regulation mediated via PKA and *ex vivo* acute AF model shows activating PKA to be protective in acute AF and KT5720 inhibition of PKA making it more vulnerable to AF. Further studies are being carried out with SK blocker along with PKA regulators. This study provide evidence for the first time about PKA regulation in guinea pig explanted heart acute AF model.

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[2] Abiraman K *et al.* (2016). *J Mol Biol* 428, 2521–2537.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

C004

### The opioid oxycodone use-dependently inhibits the cardiac sodium channel Nav1.5

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Oxycodone is a potent semi-synthetic opioid that is commonly used for the treatment of severe acute and chronic pain. However, oxycodone is also one of the opioids most implicated in drug abuse and addiction and contributes significantly to what is now being called the opioid epidemic in the US. Treatment with oxycodone can lead to cardiac electrical changes, such as long-QT syndrome, potentially inducing sudden cardiac arrest. Here, we investigate whether the cardiac side effects of oxycodone can be explained by modulation of the cardiac sodium channel Nav1.5.

We stably expressed human Nav1.5 in HEK293 cells and used these for whole-cell patch-clamp electrophysiology. A variety of voltage-clamp protocols was used to test the effect of oxycodone on different channel gating modalities. To test the effect of oxycodone on cardiomyocyte beating, we used commercially available human stem cell-derived cardiomyocytes in combination with a CardioExcyte 96 device.

Oxycodone concentration-dependently inhibits Nav1.5 with an  $\text{IC}_{50}$  of 483.2  $\mu\text{M}$ . We show that this block is use-dependent, leading to a continuous run-down in Nav1.5 inward current over time, which is not the case in vehicle recordings. In addition, oxycodone slows both fast and slow time constants of recovery of Nav1.5 from fast inactivation. When we also tested slow inactivation of Nav1.5, we found that during vehicle treatment, approximately 25 % of channels entered the slow inactivated state with a slow time constant (mean  $\pm$  SEM  $16.8 \pm 2.1$  s;  $n = 8$ ). During application of oxycodone, we observed an increase in the amount of slow inactivation as well as a speeding of the time constant. In this condition, 46 % of channels displayed slow inactivation with a significantly faster time constant (mean  $\pm$  SEM  $5.0 \pm 0.5$  s;  $n = 9$ ). Finally, application of oxycodone even at 100  $\mu\text{M}$  (far below the  $\text{IC}_{50}$ ) to spontaneously beating human stem cell-derived cardiomyocytes leads to a reduced beat rate and to arrhythmia. The basal beat rate of  $58 \pm 1$  beats per minute was reduced by  $-21.5 \pm 3.6$  % (mean  $\pm$  SEM in  $n = 5$  wells) by oxycodone 100  $\mu\text{M}$ , but not by vehicle (mean  $\pm$  SEM  $+4.9 \pm 1.7$  % in  $n = 8$  wells). The slope of the early downstroke of the extracellular field potential was strongly reduced by oxycodone, confirming a Nav1.5-mediated mechanism of action. Furthermore, the duration of the extracellular field potential was reduced by oxycodone, indicating an additional block of hERG potassium channels.

Oxycodone leads to an accumulation of Nav1.5 in inactivated states with a slow time course. While the concentrations

needed to elicit cardiac arrhythmia *in vitro* are comparably high, some patients under high-dose treatment with oxycodone as well as drug abusers might suffer from cardiac side effects induced by the slow effects of oxycodone on Nav1.5 and potential effects on other cardiac ion channels.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

C005

# **Impaired $\text{Ca}^{2+}$ -mediated $\text{Cl}^-$ secretion *in vitro* is confirmed by nasal potential difference (NPD) measurements in patients with chronic rhinosinusitis (CRS)**

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Chronic rhinosinusitis (CRS) is one of the most common chronic disorders of the upper airways that is frequently observed in patients with cystic fibrosis (CF), a rare genetic disease caused by abnormal epithelial ion transport. CRS is a heterogeneous disease which may be linked to impaired mucociliary clearance and sinonasal inflammation. However, other underlying CRS pathophysiologic mechanisms still remain unspotted. Here, we aimed to study whether the epithelial ion transport, in particular the  $\text{Ca}^{2+}$ -mediated  $\text{Cl}^-$  secretion is affected in patients with CRS. We further determined the epithelial expression of cytokines being both, associated with  $\text{Cl}^-$  transport and implicated in CRS.

First, cultured nasal epithelial primary cells of healthy individuals (n=10-20) and CRS patients (n=10-22) were utilized to study epithelial ion transport and transcript levels of the  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channel TMEM16A and CFTR. Levels of cytokines were also assessed (IFN- $\gamma$ , IL-13, IL1- $\beta$ ). Effects of IL-13 on gene expression and epithelial  $\text{Cl}^-$  transport were investigated. Second, nasal potential difference (NPD) measurements were carried out in healthy individuals (n=16) and CRS patients (n=8). Results, expressed as mean $\pm$ SEM were compared by ANOVA.

Bioelectric studies revealed significantly reduced basal short-circuit current ( $I_{sc}$ ) and  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  secretory responses (p<0.05), albeit mRNA levels of the  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channel TMEM16A were significantly increased (Control: 1.0 $\pm$ 0.2 vs. CRS: 2.0 $\pm$ 0.3, p<0.01). mRNA and protein expression of the Th2-inflammation marker IL-13 were strongly increased in CRS cultures. Whilst mRNA and protein expression levels of the Th1-inflammation marker IL1- $\beta$  were significantly decreased (protein: Control: 19.5 $\pm$ 9.7 vs. CRS: 0.2 $\pm$ 0.1, p<0.05). By acute treatment with IL-13,  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  secretion by means of UTP-mediated  $I_{sc}$  can be partially restored in CRS cultures (untreated: 0.3 $\pm$ 0.1 vs. IL-13 treatment: 1.8 $\pm$ 0.5, p<0.05). Levels of TMEM16A expression were also further increased by 3-fold (p<0.05). *In vivo* nasal PD measurements confirmed a significant reduction of  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  secretion in CRS patients compared to healthy individuals (p<0.05).

Our data suggest a complex dysregulation of epithelial  $\text{Cl}^-$  transport dominated by reduced basal  $I_{sc}$  values and  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  secretion in nasal epithelia of CRS patients. Changes in  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  secretion were further confirmed *in vivo* by NPD measurement. The epithelial inflammation in CRS cultures is also significantly evident in the signature of cytokines being implicated in epithelial  $\text{Cl}^-$  transport. These data indicate that TMEM16A-mediated  $\text{Cl}^-$  secretion may play an important role in the pathogenesis and TMEM16A may serve as a therapeutic target in CRS.

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C006

# **Breathing with neuromuscular disease: insights from pre-clinical studies**

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Duchenne muscular dystrophy (DMD) is characterised by severe skeletal muscle weakness, which extends to the striated muscles of breathing. Hypoventilation, carotid body hypo-activity (sensory deficit) and diaphragm dysfunction have been described in the *mdx* mouse model of muscular dystrophy (Burns et al., 2017), however there is a paucity of information concerning neuromechanical control of the respiratory muscles. Since inflammation is a major pathological feature of DMD, we assessed the potential of a combined anti-inflammatory and anti-stress interventional strategy in ameliorating respiratory deficits in *mdx* mice.

Young (8-week-old) wild-type (WT; n=41) and *mdx* (n=37) mice were studied. Respiratory stability and ventilatory capacity were determined. Diaphragm EMG activity and inspiratory pressure generating capacity was examined in anaesthetised (1.7 g/kg i.p. urethane) spontaneously breathing mice *in vivo*. Diaphragm muscle structure and force-generating capacity were assessed. Monoamine concentrations, density of glial cells, and pro-inflammatory gene expression was determined in the spinal cord (C3-C5), containing the phrenic motor nucleus. Interventional studies consisted of a combinational treatment of a neutralizing interleukin-6-receptor antibody and urocortin 2 (corticotrophin releasing factor receptor 2 agonist), or saline in WT (n=32) and *mdx* (n=32) mice for 2 weeks. Ventilation and diaphragm muscle form and function were determined. Data were statistically compared by unpaired Student's *t* test.

Poincaré analysis of respiratory timing revealed no evidence of perturbed respiratory rhythm in *mdx* mice, which retained a capacity to enhance ventilation despite diaphragm weakness and structural remodelling. Peak inspiratory oesophageal pressure generation was preserved during basal breathing and was greater during augmented breaths in *mdx* (P<0.05) compared to WT mice, probably due to recruitment of accessory muscles of breathing. Diaphragm EMG activity was not

potentiated compared with WT across ventilatory behaviours. Serotonin and noradrenaline concentrations were elevated in *mdx* ( $P < 0.05$ ) spinal cord. There was no evidence of spinal neuroinflammation. Combinational therapy in *mdx* mice completely restored ventilation during normoxia, improved diaphragm muscle force-generating capacity and restored myosin heavy chain complement in diaphragm muscle.

We reason that compensatory neuroplasticity in accessory motor pathways preserves ventilatory capacity in *mdx* mice despite profound diaphragm muscle weakness. Interventional studies indicate that strategies aimed at preserving muscle fibre complement and promoting muscle fibre quality in *mdx* respiratory muscles can alleviate breathing and muscle functional deficits. These data may have relevance to the development of interventional strategies designed to alleviate respiratory insufficiency in the human dystrophinopathies.

BURNS, D. P., ROY, A., LUCKING, E. F., MCDONALD, F. B., GRAY, S., WILSON, R. J., EDGE, D. & O'HALLORAN, K. D. 2017. Sensorimotor control of breathing in the *mdx* mouse model of Duchenne muscular dystrophy. *J Physiol*, 595, 6653-6672.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## C007

### Nuclear envelope disruption drives myocardial 'Inflammageing' in cardiomyopathies

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Accumulation of prelamin A, the precursor to the nuclear intermediate filament protein lamin A, is known to initiate the onset of premature senescence in various tissue types. In addition, mutations in the *LMNA* gene, encoding the lamins A & C, are known to cause a specific subset of cardiomyopathies known as *LMNA* cardiomyopathy. An unresolved question is whether prelamin A accumulation is relevant to primary heart disease. That there are mutations known to abolish prelamin A processing suggests there is a potential role for prelamin A amongst familial cardiomyopathy cases. Additionally, there is an active debate as to whether highly active anti-retroviral therapy (HAART), employed in the treatment of HIV patients, contributes to the presentation of HIV cardiomyopathy. Evidence suggests that certain HAART regimes impair prelamin A processing. We hypothesised that prelamin A may be a mediator of cardiac pathology in both of these settings and sought to investigate mechanisms. To confirm the presence of prelamin A in diseased tissue we performed Western blotting and immunostaining on Human heart tissue samples. To investigate potential cause and effect we generated a novel line of cardiomyocyte specific prelamin A (csPLA) transgenic mice and performed in vivo physiology, ex vivo histology and investigated changes on the molecular level. We discovered

that prelamin A accumulated in a subset of human dilated cardiomyopathy samples. csPLA mice died of heart failure at 5 weeks post-natal. At 4 weeks, echocardiography was performed with a fast induction of 5% isoflurane inhalation anaesthesia followed by maintenance of 1-1.5%, which was vaporized in 100% oxygen delivered at 1.5-2 liters/min. A marked decline in cardiac function in vivo was observed, e.g. ejection fraction was substantially depressed in transgenic mice ( $20.9 \pm 3.6\%$ ) compared with wildtype ( $56.7 \pm 11.2\%$ ,  $P = 0.01$ ,  $n=6/\text{group}$ ). Cardiac histology showed marked cardiomyocyte disarray, profound fibrosis and necrosis. CD45 and CD68 expressing cells were evident in the myocardium and genes for pro-inflammatory cytokines were upregulated. DNA damage accumulation and expression of senescence markers suggested that prelamin A accumulation initiated an immuno-senescence or 'Inflammageing' response within the myocardial milieu that was regulated by activation of nuclear factor kappa-light-chain-enhancer of activated B cells signaling, instigated by persistent DNA damage. Identifying a link between the csPLA model and HIV associated cardiomyopathy phenotypes, we confirmed that prelamin A accumulated in heart tissue of patients presenting with HIV cardiomyopathy. In conclusion, prelamin A accumulation in cardiomyocytes resulted in myocardial inflammageing. These results provide novel insights for a subset of cardiomyopathies in which prelamin A processing is affected, in particular HIV cardiomyopathy, and may also have translational potential in this setting via the modulation of HAART regimes.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## C008

### Exercise training increases myocardial capillary density rather than coronary artery size or function in the healthy and diabetic heart

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Introduction: Exercise training (EX) is postulated to improve myocardial vascular structure and function, but this idea has not been consistently verified. On the other hand, diabetic metabolic derangement (DMD) is often associated with impaired vascular function. Here we tested the hypothesis that EX enhances myocardial capillary densities, and improves coronary small artery function and produces an increase in epicardial artery diameter thereby opposing DMD-induced coronary structural and functional abnormalities. Methods: DMD was produced in adult male Göttingen miniswine by streptozotocin injections and high fat diet, while control animals (CON) remained on normal pig chow. At 3-month (mo) follow-up, swine were randomized into 2-month exercise training (EX) on a treadmill, 3-times per week or remained untrained (UT), ultimately resulting in 5mo of study and four study groups ( $N=8$  in CON-UT and CON-EX and  $N=9$  in DMD-UT and DMD-EX groups). Results: Robust DMD developed, evidenced by hyperglycemia, hypercholesterolemia and hypertriglyceridemia. Under local nitroglycerin infusion, angiographically assessed mean diameter of left anterior descending, circumflex, or right coronary arteries were not affected by DMD or by EX in either group. Moreover, EX did not improve endothelium-dependent relaxation to bradykinin



in coronary small arteries, whereas it increased bradykinin-induced relaxation in skeletal muscle small arteries. Also, EX did not restore the DMD-induced impairment in endothelium-independent relaxation to nitroprusside of coronary small arteries. Finally, myocardial capillary density was not affected by DMD, but it was increased significantly by EX (capillaries/mm<sup>2</sup>, mean±SEM: 1358±85 CON-UT, 1510±123 DMD-UT, 2084±258 CON-EX and 1929±209 DMD-EX,  $p=0.0036$ ). Protein levels of VEGF were not different between the groups, but EX groups showed higher HIF levels ( $p=0.03$ ), which correlated with myocardial capillary density ( $r=0.44$ ,  $p=0.01$ ). Conclusion: Both in health and DMD, short term (2 months) exercise training appears to induce more prominent changes at the capillary level at this stage, as compared to epicardial or small artery function, suggesting that capillary changes precede changes in large and small arteries.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## C009

### Modulation of Sympathetic Tone in Heart Failure: a Role for neuronal Nrf2

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Nrf2 is a key transcription factor involved in modulating redox signaling by regulating the expression of a number of antioxidant genes. It is known that both sympathetic hyperactivity and increased oxidative signaling are hallmarks of chronic heart failure (HF). Previous data from our group has shown that enhanced oxidative signaling in the RVLM contributed to the sympatho-excitation in rabbits with HF. We hypothesized that Nrf2 expression would be downregulated in sympathetic neural tissue in HF, and that overexpression of Nrf2 in the RVLM would suppress HF associated increased sympathetic tone. To test the role of RVLM Nrf2 over expression in HF 30 C57BL/6 male mice were randomized into 4 groups : Sham-Lenti GFP ( $n = 7$ ); Sham-Lenti GFP Nrf2 ( $n = 7$ ); HF-Lenti GFP ( $n = 8$ ); and HF-Lenti GFP Nrf2 ( $n = 8$ ). HF was induced by coronary artery ligation (under 2% isoflurane), or sham surgery as control. Nrf2 overexpression was selectively targeted to glutamatergic neurons of the RVLM by utilizing a Ca<sup>2+</sup>/calmodulin-dependent protein kinase-II (CaMKII) promoter to drive Nrf2 expression, a corresponding GFP vector was used as control. We found that Nrf2 protein was significantly downregulated in the RVLM of HF mice, which was restored by Nrf2 gene transfer. That the increased urinary NE excretion in HF was attenuated by Nrf2 overexpression (Sham-GFP:  $575.2 \pm 265.0$ , HF-GFP,  $1119.6 \pm 270.5$ , HF-Nrf2:  $812.1 \pm 133.0$  ng/24hr,  $p < 0.05$ ). Radio telemetry experiments (in the conscious state) showed that Nrf2 overexpression enhanced spontaneous baroreflex gain and increased PE induced bradycardia in HF (Decline of HR: Sham-GFP:  $-53.7 \pm 4.8$ ; HF-GFP:  $-4.7 \pm 5.1$ ; HF-Nrf2:  $-25.1 \pm 2.3$  % of Max.  $P < 0.05$  HF-GFP vs Sham-GFP and HF-Nrf2 vs HF-GFP). Moreover, no difference in baseline blood pressure and heart rate were observed between groups. Acute experiments (2 % isoflurane) revealed a decrease in basal renal sympathetic nerve activity in heart failure with Nrf2 over expression in the RVLM (Sham-GFP:  $28.6 \pm 5.2$ , HF-GFP:  $64.7 \pm 8.3$ , HF-Nrf2:  $44.0 \pm 6.5$  % of Max.  $P < 0.05$  HF-GFP vs Sham-GFP and HF-Nrf2 vs HF-GFP). From this data we conclude that upregulating Nrf2 expression in glutamatergic neurons of the RVLM reduces

sympathetic activity in mice with HF. In addition to the mouse model we observe a reduction in Nrf2, and its downstream target NQO1 in the stellate ganglia of HF rats (10 weeks post coronary artery ligation - 2% isoflurane,  $n = 5$ , sham and HF), reduction in stellate ganglia Nrf2 is correlated with infarct size and ejection fraction. This indicates that Nrf2 may be globally reduced within sympathetic tissue in HF and could open the potential for future therapeutic targets. The role of stellate ganglia Nrf2 in cardiac sympathetic drive and redox signaling in HF remain to be determined.

All experiments were performed under the approved IACUC at the University of Nebraska Medical Center. IACUC #. 13-015-05, 15-045, and IBC#: 16-11-026 ABL2

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## C010

### Intra-tissue specific gene and miRNA changes in response to myocardial infarction in fetal compared to adolescent sheep

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Animal models indicate that there are critical molecular mechanisms that can be activated to induce myocardial repair at specific points in development. Neonatal mice and fetal sheep have the capacity to regenerate heart tissue after myocardial infarction (MI). We investigated the immediate response to injury within specific regions of the infarcted cardiac tissue in fetal and postnatal hearts to characterise the differences between proliferative and quiescent hearts in a large animal model. We used sheep, which like humans complete most cardiomyocyte maturation before birth, to model the effect of age on the molecular response to cardiac damage by ligating the second diagonal of the left anterior descending coronary artery. Surgery was performed on fetuses (at ~102 days gestation when all cardiomyocytes are mononucleated and proliferative) and postnatal sheep (at 6 months of age when all cardiomyocytes contribute to heart growth by hypertrophy). Ewes underwent surgery using general anesthesia induced by the intravenous injection of diazepam (0.3mg/kg) and ketamine (7mg/kg) and maintained with inhalation of isoflurane (1-2%) in oxygen and received antibiotics and analgesia post-operatively. Total RNA was extracted for qRT-PCR, gene array and miRNA array analyses and immunohistochemistry was performed on fixed sections. Specific gene and miRNA targets were filtered from significantly upregulated or down-regulated probes in the infarct area compared with sham tissue and remote-zone tissue. Identified target miRNAs were tested *in vitro* in H9c2 cardiomyoblasts under hypoxic and low substrate conditions to simulate MI. Proliferation of H9c2 cells was assessed using MTS assay and Ki67 staining. Gene ontology analysis revealed several enriched pathways that were differentially regulated in the fetal compared to adolescent sheep that appear to be important in the immediate response to infarction. These included; *E2F Targets*,



**Cardiac Muscle Hypertrophy, WNT and MTORC1 signaling.** Specific miRNAs that were oppositely regulated in the fetal compared to adolescent sheep were identified based on their expression profile across the infarcted tissue regions. miR-150 and miR-1538 were upregulated in the adolescent infarct but downregulated in the fetal infarct compared with remote-zone tissue. In H9c2 cell culture, miR-558 and miR-1538 inhibitors significantly increased cell proliferation in normoxia and hypoxia (Figure 1). These results indicate that fetal hearts have a 'resistance' to cardiac damage. This tissue-wide response indicates a prioritisation within the fetal heart to increase proliferation/replacement of cardiomyocytes within the border and remote-zones, and retain a normal developmental pattern in the infarct area. Further investigation of specific gene pathways and miRNAs that are uniquely modulated in the fetal heart after infarction may allow for development of new targeted therapies of human heart disease.

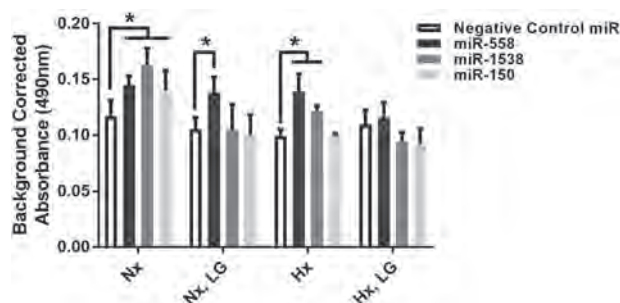


Figure 1. MTS assay for H9c2 cardiomyoblast proliferation after treatment with miRNA inhibitors. Nx, normoxia; LG, low glucose; Hx, hypoxia.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## C011

### Different low-level lead exposure profiles induce long-lasting physiological changes

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Lead (Pb) is a toxic metal, and its widespread use has resulted in environmental contamination and significant public health problems. Exposures to lead during developmental phases can alter the normal course of development, with lifelong health consequences. Permanent Pb exposure leads to behavioural changes, cognitive impairment, sympathoexcitation, tachycardia, hypertension and autonomic dysfunction. However, the effects of an intermittent Pb exposure, increased in the past years, have not been studied. We aimed to describe Pb health effects along various profiles of exposure, including a new animal model of intermittent low-level lead exposure. For that, the tap water of seven-day pregnant *Wistar* females was replaced by 0.2% (p/v) lead acetate solution. After being weaned at 21 days, rat pups, both sexes, were divided into 3 groups of lead exposure (n=10/group/timepoint): long-term - PbP (exposure from foetal period until 28 weeks of age), short-term - PbS (exposure from foetal period until 12 weeks) and intermittent - PBI (exposure from foetal period until 12 weeks, lead-free period until 20 weeks and a second exposure between 20 and 28 weeks of age). At 12, 20 and 28 weeks of age, animals were anaesthetised with sodium pentobarbital solution (60mg/kg, ip) and maintained by 20% (v/v)

anaesthetic solution. Blood pressure (BP), electrocardiogram, heart rate (HR) and respiratory frequency (RF) were recorded. Baro and chemoreceptor reflexes were stimulated by phenylephrine (0.2ml, 25µg/mL, iv) and lobeline (0.2ml, 25µg/mL, ia) injections, respectively, and baroreflex gain and chemoreflex sensitivity calculated. Low frequencies (LF) and high frequencies (HF) were determined for sympathetic and parasympathetic activity estimation. An age- and sex-matched control group was also used (n=10/timepoint). One-way ANOVA was used (significance p < 0.05) for statistical analysis.

Our data showed a clear association between lead exposure, hypertension and baro and chemoreflexes impairment, RF increase, without HR changes. At 28 weeks, PBI group showed a less pronounced hypertension when compared to PbP group. Moreover, we showed that only a longer lead-free period is capable to reverse baroreflex impairment (PbS), without significant changes in chemoreflex function. Regarding the autonomic data, the overactivity of the sympathetic nervous system, evaluated by LF band, was concomitant with baroreceptor reflex impairment and/or hypertension in all groups. Nevertheless, a lead-free period of 8 weeks was sufficient to restore sympathetic activity toward LF values obtained in healthy control rats. In summary, this study brings new insights on the environmental factors that influence nervous and cardiovascular systems during development, which can help create public policy strategies to prevent and control the adverse effects of Pb toxicity.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## C012

### Differential expression profile of microRNAs in patients with atrial dilatation and atrial fibrillation

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Atrial fibrillation (AF) is a multi-factorial disease, supported by changes in electrophysiological and structural properties. Abnormal substrates are commonly encountered in different conditions predisposing to AF, whereby atrial stretch plays a key mechanistic role. Despite accumulating evidence supporting the role of stretch in AF [1,2], the precise molecular determinants of stretch-induced changes remain to be fully elucidated. In particular, although the role of microRNAs (miRs) in AF is well-recognized [3], their involvement in stretch-induced remodeling has not been clarified yet.

This study aimed to address the issue by quantifying the differential expression profiles of 12 miRs, known for their involvement in cardiac disease and AF, in patients with atrial dilatation and/or AF. Tissue samples from the right atrial appendage were excised from 28 patients (6 females, median age 72.5 years) undergoing cardiac valve replacement. Samples were flash-frozen in pre-chilled liquid isopentane, and stored at -80°C until RNA isolation. After sample processing and homogenization, total RNA was extracted by miRNeasy mini kit (Qiagen, Italy) and reverse-transcribed by mercury LNATM Universal cDNA Synthesis kit II (Exiqon, Denmark). qPCR assays based on SYBR Green I were performed according to the manufacturer's protocol (Exiqon, Denmark) on the

selected miRs. miR expression was normalized to SNORD48 [4]. For the purpose of the study, patients were divided into three groups: control group with normal or mildly dilated left atrium (LA) and no history of AF; atrial dilatation (AD) group with moderately to severely dilated LA and no history of AF; AD+AF group with moderately to severely dilated LA and history of AF >6 months.

miR profiling in the three groups pointed out different expression profiles of miRs in atrial stretch and AF. AD was associated with significant up-regulation of miR-328 and miR-133b (fold-change versus control of 1.6 and 2.2, respectively), albeit up-regulation persisted in AD+AF patients only for miR-328 (fold-change 1.9). Further alterations in miR expression were observed in presence of AF, with up-regulation of miR-1, miR-21, miR-29a, miR-208b, and miR-590 (fold-change ranging from 3.8 for miR208b to 10.1 for miR29a).

Our results showed that atrial stretch per se was associated with alterations in miR expression in humans. Given the role of miR-328 in L-type  $\text{Ca}^{2+}$  channel regulation [5], its up-regulation may be implicated in  $\text{Ca}^{2+}$  current reduction leading to atrial remodeling, suggesting the involvement of a post-transcriptional mechanism in stretch pro-arrhythmic effects.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

C013

### Anti-inflammatory factor Del-1 protects from angiotensin II-induced hypertension and cardiovascular remodelling

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Hypertension and cardiovascular diseases are the leading causes of mortality worldwide. Angiotensin II (ANGII), T-cell inflammation, along with TGF- $\beta$ 1 and interleukin 17 (IL-17) have been implicated as key factors in the pathophysiology of hypertension and cardiovascular diseases. Therefore, anti-inflammatory approaches have been proposed as a potential therapeutic strategy.

We tested the role of an endogenous anti-inflammatory factor, developmental endothelial locus-1 (Del-1), in prevention of ANGII-induced hypertension and cardiovascular remodeling. Transgenic mice overexpressing endothelial Del-1 (Del1 Tg) and an interventional study in WT mice repetitively injected with soluble Del-1 (50  $\mu$ g) were used. Hypertension and cardiovascular remodeling was induced via ANGII infusion using osmotic minipumps (4-weeks, Alzet®), implanted under anesthesia with 3% isoflurane. Systolic blood pressure (SBP) was measured via tail-cuff. Mice were sacrificed with a Ketamine overdose (90 mg/kg plus 10 mg/kg Xylazine) and cardiac remodeling was evaluated by histology, whereas

flow cytometry for quantification of inflammatory cells and Mulvany myograph for assessment of endothelial function. After 4-weeks of ANGII infusion, Del-1 Tg mice had significantly ( $P<0.01$ ) lower SBP compared to WT littermates. Left ventricular lumen-to-wall ratio was significantly higher ( $P<0.01$ ) and cardiomyocyte area smaller ( $P<0.01$ ) in Del-1 Tg mice compared to WT. Interstitial and perivascular coronary collagen area was significantly ( $P<0.01$ ) less in Del-1 Tg mice than in WT. Del-1 Tg mice had significantly ( $P<0.01$ ) less aortic medial thickness, less adventitial collagen ( $P<0.01$ ) and more elastin ( $P<0.01$ ) area compared to WT mice. Endothelium-dependent relaxation of aorta was significantly ( $P<0.01$ ) stronger in Del-1 Tg mice than in WT. Injections of soluble Del-1 completely protected from ANGII-dependent development of hypertension and cardiovascular remodeling. Del-1 injected mice had significantly lower ( $P<0.05$ ) SBP compared to vehicle treated mice. Del-1 injections resulted in higher ( $P<0.05$ ) left ventricular lumen-to-wall ratio, lower cardiomyocyte area and less interstitial fibrosis. Del-1 injections also resulted in less aortic medial thickness and adventitial collagen area ( $P<0.05$ ). Endothelium-dependent relaxation was significantly ( $P<0.01$ ) better preserved in Del-1 injected mice. Both Del-1 Tg and soluble Del-1 injected mice had less ( $P<0.05$ ) CD45+ leukocytes, CD3+ T-cells, as well as CD4+ T-helper and CD8+ T-cytotoxic cells, as well as less ( $P<0.01$ ) active MMP2 and TGF- $\beta$ 1 in heart and aorta compared to WT mice. CD3+/IL-17+ double positive T-cell count was also significantly ( $P<0.05$ ) less in Del-1 Tg than in WT mice.

Our findings demonstrate that Del-1 protects from ANGII-induced hypertension and cardiovascular remodeling via limitation of inflammation and MMP2/TGF- $\beta$ 1 activation. Its proof of efficacy via exogenous injections presents Del-1 as a potential therapeutic agent.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

C014

### Immune modulatory role and cardio stimulatory effect of the adenosine 2b Receptor in a murine model of myocardial infarction

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In previous work we demonstrated that the adenosine A2b receptor (A2bR) was specifically upregulated on cardiomyocytes (CM) three days after MI as well as on immune cell after infiltrating the infarcted myocardium (Borg et al. (2018)). This study therefore aimed to investigate in more detail the anti-inflammatory role of A2bR in the healing processes after MI. A2bR knockout mice, when subjected to 50 min cardiac ischemia by left descending coronary artery ligation (anaesthetized with 1.5% isoflurane in medical respiratory air) followed by four weeks of reperfusion, did not show altered cardiac function in the post MI phase as determined by MRI at 9.4 T. Flow cytometric analysis (n=5), however, revealed that the number of B cells per mg heart tissue was increased in A2bRKO mice (WT:  $57 \pm 15$  vs. KO:  $99 \pm 26$ ,  $p<0.05$ ). Similarly, NK cells (WT:  $30 \pm 4$  vs. KO:  $61 \pm 12$ ,  $p<0.01$ ), CD8 T cells (WT:  $28 \pm 7$  vs. KO:  $56 \pm 18$ ,  $p<0.05$ ) and CD4 T cells (WT:  $44 \pm 18$  vs. KO:  $80 \pm 28$ ,  $p<0.05$ ) including FoxP3<sup>+</sup> regulatory T cells (WT:  $3 \pm 0.9$  vs. KO:  $8 \pm 3$ ,  $p<0.01$ ) were elevated. A2bR deficient T cells isolated from the infarcted heart (n=7) showed elevated

mRNA expression of pro-inflammatory  $\text{IFN}\gamma$  (WT:  $0.09 \pm 0.05$  vs. KO:  $0.2 \pm 0.1$ ,  $p < 0.05$ ); IL2 (WT:  $0.04 \pm 0.02$  vs. KO:  $0.07 \pm 0.02$ ,  $p < 0.05$ ) as well as increased cytokine secretion ( $n=4-6$ , pg/ml) of  $\text{IFN}\gamma$  (WT:  $157 \pm 856$  vs. KO:  $501 \pm 284$ ,  $p < 0.05$ ), IL2 (WT:  $242 \pm 141$  vs. KO:  $812 \pm 361$ ,  $p < 0.05$ ) and  $\text{TNF}\alpha$  (WT:  $2 \pm 0.7$  vs. KO:  $5 \pm 2$ ,  $p < 0.05$ ). In contrast relative mRNA levels ( $n=4$ ) of pro-inflammatory IL1 $\beta$  (WT:  $18 \pm 6$  vs. KO:  $5 \pm 2$ ,  $p < 0.01$ ) and IL6 (WT:  $0.03 \pm 0.02$  vs. KO:  $0.007 \pm 0.01$ ,  $p < 0.05$ ) in granulocytes and CM (IL1 $\beta$ : WT:  $0.79 \pm 0.3$  vs. KO:  $0.3 \pm 0.2$ ,  $p < 0.05$ ; IL6: WT:  $0.6 \pm 0.2$  vs. KO:  $0.3 \pm 0.07$ ,  $p < 0.01$ ) were significantly reduced when A2bR was lacking. Since the A2bR was shown to be up-regulated after MI on CM we analyzed cardiac ejection (EF) fraction 6 hours after injection of the A2bR agonist BAY60-6583 ( $80 \mu\text{g/kg}$  BW) in healthy and infarcted WT mice. EF was found to be increased by 16% only in infarcted mice ( $n=3$ ) (control:  $104 \pm 6$  vs. infarcted:  $120 \pm 5$  % of values pre-injection,  $p < 0.05$ ). Values are means  $\pm$  SD, all significances were determined by Student's T test.

In summary, deletion of the A2bR does not alter post MI cardiac function but increased the pro-inflammatory character of T cells infiltrating the infarcted myocardium. Other compensatory mechanisms include repression of pro-inflammatory signals in granulocytes and CM and increased number of anti-inflammatory regulatory T cells. Pharmacologic stimulation of A2bR, upregulated in the ischemic heart, temporarily increases cardiac function post MI.

Borg N et al. (2017) *Circulation*. 18;136(3):297-313

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

C015

#### Circadian rhythm in heart rate is due to an intrinsic circadian clock in the sinus node

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There is a circadian rhythm in the resting heart rate (HR), which is slower at night. In addition, bradyarrhythmias primarily occur at night. We hypothesised that an intrinsic mechanism in the pacemaker of the heart, the sinus node (SAN), is involved. In conscious C57BL/6J mice maintained during a 12 h light-12 h dark cycle, there was a circadian rhythm in HR measured by telemetry: the HR was 61 beats/min slower at the start of sleep (light) period (Zeitgeber time, ZT 0), as compared to the start of awake (dark) period, ZT12 ( $P < 0.05$ ). There was no correlation between HR and activity by telemetry measurement. As a further test, a 1 h light pulse was given at the start of the awake period; activity ceased but the HR remained relatively high. We conclude that the circadian rhythm in HR is not activity dependent. Furthermore, there was a circadian rhythm in the intrinsic HR measured either using the isolated Langendorff-perfused heart or the isolated SAN: the HR was 97 and 71 beats/min slower at ZT 0 as compared to ZT12 ( $P < 0.05$ ) respectively. This suggests that an intrinsic mechanism in the heart is at least contributing to the

circadian rhythm in HR. This is likely to be either the  $\text{Ca}^{2+}$  clock or the membrane clock responsible for pacemaking. To study the  $\text{Ca}^{2+}$  clock, the number of  $\text{Ca}^{2+}$  sparks and the diastolic  $\text{Ca}^{2+}$  level were significantly reduced at ZT 12 compared with ZT 0. Furthermore, in isolated SAN preparations, the change in HR caused by 2 mM ryanodine (incapacitates the  $\text{Ca}^{2+}$  clock) was not significant between ZT 0 and ZT 12. To study the membrane clock, patch clamp experiments were conducted on SAN cells isolated at the two time points. The key pacemaker current,  $I_f$  (funny current), was measured during 5 s hyperpolarizing pulses from a holding potential of -35 mV.  $I_f$  (at -125 mV) at ZT 2 was  $17.8 \pm 2.8$  pA/pF, whereas at ZT 14 it was  $35.0 \pm 6.3$  pA/pF ( $P < 0.05$ ). This change could explain or at least contribute to the circadian rhythm in HR. Consistent with this, on application of 2 mM  $\text{Cs}^+$  to block  $I_f$ , the circadian rhythm in HR in the isolated SAN was abolished. Furthermore, in the conscious mouse, injection of 6mg/kg ivabradine to block  $I_f$  abolished the circadian rhythm in HR. HCN channels are responsible for  $I_f$ . SAN biopsies were collected at ZT 0 and ZT 12 and the expression level of HCN4 (principal ion channel responsible for  $I_f$ ) was measured at the mRNA level by qPCR and the protein level by Western blot. HCN4 mRNA was 89% higher at ZT 0 compared to ZT 12, whereas HCN4 protein was 49% lower ( $P < 0.05$ ); although the changes are discordant, a time lag of hours is expected between mRNA and protein, and the protein change is consistent with the change in  $I_f$  density. Further experiments showed that HCN4 is directly regulated by a local circadian clock in the SAN. It is concluded that a circadian rhythm in HCN4 and  $I_f$  driven by an intrinsic circadian clock in the SAN at least contributes to the circadian rhythm in HR.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

C016

#### Optogenetic hyperpolarization of cardiomyocytes to terminate ventricular arrhythmia

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**Introduction:** Defibrillation to terminate lethal ventricular arrhythmia is currently performed by applying high energy electrical shocks which induce simultaneously depolarization as well as hyperpolarization in different areas in the ventricles. The importance of hyperpolarization for the success of defibrillation has been suggested by *in silico* modeling but could yet not be determined experimentally. Here we report selective optogenetic hyperpolarization in cardiomyocytes of transgenic hearts expressing the light-activated proton pump Archaeorhodopsin-T (ArchT) to terminate episodes of ventricular tachycardia and fibrillation (VT/VF).

**Methods:** Transgenic mice with cardiac expression of ArchT were generated by crossbreeding  $\alpha\text{MHC-Cre}$  mice with a mouse line expressing ArchT-eGFP from the CAG promoter after Cre-mediated excision of a floxed stop cassette. Histological analysis was performed to detect transgene expression in slices and after dissociation. Hearts were explanted, perfused in Langendorff configuration and stimulated electrically with silver electrodes on the right ventricle while ECG recording. Action potentials were recorded by sharp electrode impalement during pharmacological contraction inhibition ( $10 \mu\text{M}$  Blebbistatin). Perfusion with low  $\text{K}^+$  (2 mM) and the  $\text{K}_{\text{ATP}}$ -channel activator Pinacidil ( $100 \mu\text{M}$ ) allowed induction



of sustained VT/VF by electrical burst stimulation to explore optogenetic arrhythmia termination. Precisely timed light pulses (525 nm) were applied with a high power LED coupled to a microscope (1x objective, 0.25 NA).

**Results:** Membrane-bound eGFP signals confirmed ArchT expression in >90% of ventricular cardiomyocytes. Action potential measurements proved light-induced (525 nm) hyperpolarization resulting in faster upstroke and shorter duration of action potentials as well as faster conduction velocities. Illumination (6.5 mW/mm<sup>2</sup>) focused on the epicardial surface of the anterior ventricular wall (24 mm<sup>2</sup>) was able to terminate VT/VF episodes with an average success rate of 53.3±7.1% in ArchT expressing hearts (n=11) which was significantly higher (p=0.0007, Kruskal Wallis test) than spontaneous termination rate within the same time period in illuminated control hearts (25±4.5%, n=14) or ArchT expressing hearts without illumination (14±6.3%, n=5).

**Conclusions:** We herein provide the first evidence that hyperpolarization is able to terminate ventricular arrhythmia and could play an important role for the success of defibrillation by electrical shocks. Therefore this study confirms the value of optogenetic approaches to understand electrical defibrillation and to potentially improve current defibrillation strategies. All data displayed as mean±standard error of the mean.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

C017

# **Inward rectifier current downregulation in heart failure promotes triggered activity in a novel model of rat ventricular electrophysiology and calcium handling**

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Aberrant intracellular calcium (Ca<sup>2+</sup>) handling contributes to both the mechanical and electrical dysfunction seen in heart failure, promoting arrhythmogenesis and potentially resulting in sudden cardiac death. The underlying mechanisms for these processes, occurring across different temporal and spatial scales, can be difficult to dissect with experimental techniques, however computational modelling approaches can provide quantitative and mechanistic insight in their place. Previous experimental data from our laboratory (1) has shown that inward rectifier (*I<sub>K1</sub>*) channel expression is reduced by 55% in failing rat myocytes compared to control (p = 0.01, two-way ANOVA; n = 12) and spontaneous Ca<sup>2+</sup> release is increased, yet we have been unable to use existing computational models of rat ventricular electrophysiology to explore this pro-arrhythmic behaviour as they are unable to reproduce sub-cellular spatio-temporal Ca<sup>2+</sup> handling dynamics. Thus, we have been unable to explore the hypothesis that this more frequent release may result from increased sarcoplasmic reticulum (SR) load combined with decreased membrane stability. A new computational model was therefore developed by integrating a recent rat ventricular electrophysiology model (2) with a novel model of stochastic spatio-temporal Ca<sup>2+</sup> handling dynamics developed in our laboratory (3). The newly-developed model was used to dissect and quantify changes in Ca<sup>2+</sup> homeostasis caused by remodelling of key ion channels. A 50% reduction in *I<sub>K1</sub>* (comparable to that observed experimentally) resulted in a 57% increase in action potential duration (APD) in our simulations, from 58.1 to 91.4 ms. This prolonged APD resulted in greater loading of the SR, raising [Ca<sup>2+</sup>]<sub>SR</sub> to

super-threshold levels and giving rise to more frequent spontaneous release events. These, in turn, triggered forward-mode (depolarising) sodium-calcium exchange (*I<sub>NaCa</sub>*), leading to triggered action potentials; a pro-arrhythmic phenomenon. Resting membrane potential was also depolarised by 3.3 mV in HF myocytes in our simulations, thus a smaller Ca<sup>2+</sup> release (ergo, a smaller concomitant *I<sub>NaCa</sub>* current) was required to elicit triggered activity.

The newly-developed model has reproduced experimental findings from the laboratory and provided insight into the mechanisms of spontaneous Ca<sup>2+</sup> release in HF; that a reduced repolarising *I<sub>K1</sub>* current prolongs APD sufficiently for [Ca<sup>2+</sup>]<sub>SR</sub> to exceed the threshold for spontaneous release, promoting spontaneous activity and providing a trigger for arrhythmia development in failing myocytes. Thus, the model provides a supplementary and stand-alone research tool to explore how sub-cellular changes in electrophysiology and Ca<sup>2+</sup> handling result in arrhythmias at the tissue- and organ-levels.

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Gattoni S et al. (2016) *J Physiol* 594, 4193-4224

Colman MA et al. (2017) *PLOS Comput Biol* 13, 34

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

C018

# **The pro-arrhythmic effect of adenosine A<sub>1</sub>-receptor activation in the atrium**

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G protein-coupled Inwardly Rectifying K<sup>+</sup> channels (GIRK) play a key role in the physiological regulation of the heart. GIRKs are responsible for the vagus-induced negative chronotropy and rapid repolarization of atrial action potential (AP) through the activation of muscarinic receptor. In the atrium, GIRKs activation shortens AP duration and the effective refractory period (ERP). Dysregulation of these currents is associated with atrial fibrillation (AF). Evidences have shown that adenosine (Ado), via A<sub>1</sub>-receptor (A<sub>1</sub>-R), increases GIRK channels conductance. Ado naturally occurs from Ado triphosphate breakdown. Both in animal models and humans, extracellular Ado raises under oxygen demand due to CD73 activity that catalyzes the conversion of Ado monophosphate to Ado.

To study the electrophysiological role of A<sub>1</sub>-R in the atrium, rat isolated beating heart preparations were tested in a Langendorff configuration with a selective commercial A<sub>1</sub>-R agonist (50 nM CCPA) or with a potent commercial A<sub>1</sub>-R antagonist (40 nM PSB36). In addition, to interfere with CD73 activity, the commercial inhibitor AMPCP (500 μM) was perfused. To translate the results from rodents to humans, AP measurements were conducted on isolated contracting muscles in right atrial appendages (hRAAs) superfused with 1 μM CCPA or 1 μM PSB36 from sinus rhythm patients. Data are summarized using the mean ± SEM compared by ANOVA. Animal experiments were performed in accordance with the Danish guidelines according to the European Commission Directive 86/609/EEC. Wistar rats were anesthetized with sodium pentobarbital (40 mg/kg PI) and sacrificed subsequently. Investigation on



human samples conforms to all principles outlined by the Declaration of Helsinki. According to the guidelines of the Medical Association Ethical Review Committee (Hamburg, Germany), patient data were used anonymized and donors gave informed consent.

A<sub>1</sub>-R agonist CCPA shortened AP duration at 90% of repolarization (APD<sub>90</sub>) and refractoriness both in rats and humans. Thus, we showed for the first time the activation of A<sub>1</sub>-R in hRAAs. By contrast, the A<sub>1</sub>-R antagonist PSB36 prolonged APD<sub>90</sub> and ERP both in rats and humans, suggesting that an intrinsic release of Ado has an effect on atrial electrophysiology. Confirmatory, AMPCP significantly prolonged refractoriness in rats. Rat hearts treated with CCPA revealed a profound increase in AF inducibility (control 54.5% ± 10.7 vs CCPA 82.7% ± 0.8, P<0.05). In contrast, PSB36 and AMPCP significantly prevented AF events (PSB36 20.1% ± 0.8, P<0.05; AMPCP 25.6% ± 12.0) and reduced AF duration (control 11.5s ± 2.5; CCPA 27.3s ± 10.0, P<0.005; PSB36 2.9s ± 1.8, P<0.005; AMPCP 3.0s ± 1.6, P<0.005). In conclusion, in these experimental models, A<sub>1</sub>-R activation, probably due to a local Ado release, promotes AF vulnerability.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

C019

### Epithelial sodium channels (ENaC) containing the $\delta$ -subunit are highly sensitive to extracellular acidification in *Xenopus laevis*

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The epithelial sodium channel (ENaC) is a sodium-selective ion channel within the apical membrane of various vertebrate epithelial cells. ENaC consists of three subunits ( $\alpha$ ,  $\beta$ ,  $\gamma$ ), however, an additional  $\delta$ -subunit can replace  $\alpha$ -ENaC. We recently demonstrated that incorporation of the  $\delta$ -subunit into *Xenopus* ENaC changes channel activity from a constitutively-open into a transient form<sup>(1)</sup>, yet, regulators of this transient activity remain unknown. Based on the evolutionary relationship between  $\delta$ -ENaC and acid-sensing ion channels within the degenerin/ENaC protein family, we hypothesized that protons regulate the activity of this ENaC isoform.

Activity of *Xenopus* ENaCs was measured by two-electrode voltage-clamp and cell-attached Patch-Clamp electrophysiology using the *Xenopus* oocyte expression system. Data are presented as mean ± SEM. Reduction of extracellular pH from pH 8.0 to 6.0 increased amiloride-sensitive transmembrane currents of oocytes expressing  $\delta\beta\gamma$ -ENaC by a factor of  $7.8 \pm 0.9$  (n=15) whereas currents mediated by  $\alpha\beta\gamma$ -ENaC were increased by a factor of  $1.1 \pm 0.04$  (n=12; Student's unpaired t-test: p<0.001). Patch-Clamp recordings indicated that changes in the extracellular pH affected the open probability (P<sub>o</sub>) of  $\delta\beta\gamma$ -ENaC. Compared to channel P<sub>o</sub> at pH 7.4 ( $0.28 \pm 0.04$ , n=18) alkaline pH 8.0 reduced P<sub>o</sub> ( $0.11 \pm 0.02$ , n=15) whereas acidic pH 6.0 increased P<sub>o</sub> ( $0.52 \pm 0.04$ , n=15; ANOVA: p<0.001). Transmembrane currents mediated by  $\delta\beta\gamma$ -ENaC are sensitive to sodium self-inhibition (SSI), the fast

inhibition of ENaC in the presence of high extracellular [Na<sup>+</sup>]. Changes in extracellular pH influenced the magnitude of SSI as %-inhibition was maximal at pH 8 ( $55.7 \pm 3.5$  %, n=10) and pH 7.4 ( $53.8 \pm 3.5$  %, n=17) but declined with extracellular acidification (pH 7:  $39.5 \pm 4.9$  %, n=13; pH 6:  $24.7 \pm 2.3$  % n=10; ANOVA: p<0.001). A pH-sensitive aspartate of a putative Na<sup>+</sup>-sensing site within the extracellular domain of  $\delta\beta\gamma$ -ENaC was replaced by asparagine via site-directed mutagenesis ( $\delta_{D296N}$ ). Compared to wildtype  $\delta\beta\gamma$ -ENaC ( $\delta_{wt}$ ), this mutation had a reduced SSI (%-inhibition of  $\delta_{wt}$ :  $54.5 \pm 3.4$  %, n=18,  $\delta_{D296N}$ :  $24.7 \pm 1.3$  %, n=11; Student's unpaired t-test: p<0.001) and a reduced maximal pH-mediated channel activation between pH 8-6 (fold-activation of  $\delta_{wt}$ :  $5.1 \pm 0.7$ , n=8;  $\delta_{D296N}$ :  $2.6 \pm 0.2$ , n=8; Mann-Whitney U-test: p<0.001). Furthermore, human  $\delta\beta\gamma$ -ENaC which does not display SSI, showed a markedly reduced fold-activation by extracellular acidification ( $1.1 \pm 0.02$ , n=12).

These results demonstrate that *Xenopus*  $\delta\beta\gamma$ -ENaC is highly sensitive to changes in the extracellular pH whereas channels containing the  $\alpha$ -subunit are not. This further supports the hypothesis that *Xenopus*  $\delta$ -ENaC represents a functional example for ENaC at the interface of stimulus-activated and constitutively open ion channels in the degenerin/ENaC protein family.

(1) Wichmann L et al. (2018). J Biol Chem 293, 6647–6658.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

C020

### Complicated receptors in the collecting duct: regulation of ENaC-mediated Na<sup>+</sup> transport by corticosteroids.

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ENaC-mediated Na<sup>+</sup> reabsorption by principal cells (PC) in the renal collecting duct is stimulated by aldosterone and contributes importantly to Na<sup>+</sup> and blood pressure homeostasis<sup>1</sup>. PCs express both mineralocorticoid (MR) and glucocorticoid receptors (GR) but the role of the GR is uncertain since 11 $\beta$ HSD2 is thought to metabolise active glucocorticoids within the cell. *In vivo* studies challenge this concept and in immortalised PC models, the GR agonist dexamethasone strongly stimulates ENaC. In this study we used the mouse-derived mCCD<sub>cl1</sub> cells<sup>2</sup> which respond to physiological concentrations of aldosterone<sup>3</sup> to examine the relative roles of the MR and GR in mediating ENaC-mediated Na<sup>+</sup> transport in response to the corticosteroids: aldosterone, dexamethasone and corticosterone.

Transepithelial ion transport was assessed across polarised mCCD<sub>cl1</sub> cells using epithelial-volt-ohm meter measurements of transepithelial voltage (V<sub>t</sub>) and resistance (R<sub>t</sub>) allowing calculation of equivalent short-circuit current (I<sub>eq</sub>) by Ohm's law. Amiloride (10 $\mu$ M) was added at the end of experiments to assess ENaC-mediated Na<sup>+</sup> transport and RNA was then isolated from cellular lysates to confirm expression of MR, GR and 11 $\beta$ HSD2 by qPCR. Data are shown as mean±S.D., statistical significance was determined by one-way ANOVA, with Tukey's post-hoc test.

Basal I<sub>eq</sub> in cells was  $-11.2 \pm 4.2 \mu\text{A}/\text{cm}^2$  (n=164), ~95% of which was amiloride-sensitive: I<sub>eq</sub> predominantly reflects ENaC-mediated Na<sup>+</sup> transport. Application of aldosterone (3nM, 3h)

stimulated  $I_{eq}$  by  $2.8 \pm 1.7$  fold ( $n=6$ ) compared to  $1.3 \pm 0.1$  fold in cells treated with solvent vehicle ( $n=8$ ). Pre-incubating cells with a non-steroidal MR antagonist PF-03882845 ( $1 \mu M$ , 30min) abolished aldosterone-induced  $I_{eq}$  ( $n=5$ ,  $p<0.001$ ). Pre-incubation with mifepristone, a GR antagonist with no affinity for MR, inhibited aldosterone-induced  $I_{eq}$  by  $\sim 55\%$  ( $n=6$ ,  $p<0.001$ ). Dexamethasone, a synthetic glucocorticoid, stimulated  $I_{eq}$  by  $3.9 \pm 0.5$  fold ( $n=8$ ). This response was inhibited by  $\sim 70\%$  by mifepristone ( $n=8$ ) and  $\sim 20\%$  by MR inhibition ( $n=5$ ). Corticosterone ( $100 nM$ , 3h) did not alter  $I_{eq}$  ( $n=8$ ), consistent with the expression of  $11\beta HSD2$ . When cells were pre-incubated with carbenoxolone ( $10 \mu M$ , 30min), an inhibitor of  $11\beta HSD2$ , corticosterone increased  $I_{eq}$  by  $3.6 \pm 0.5$  fold ( $n=8$ ,  $p<0.001$ ) and this was inhibited  $\sim 65\%$  by MR blockade ( $n=5$ ,  $p<0.001$ ) and  $\sim 20\%$  by GR antagonism ( $n=8$ ,  $p<0.001$ ). The key findings of this study are that the GR plays a permissive role in the regulation of ENaC by physiological concentrations of aldosterone. In contrast to dexamethasone, corticosterone predominantly engages MR rather than GR and necessitates inhibition of  $11\beta HSD2$  to stimulate ENaC. Thus, for endogenous corticosteroids, the interplay between MR and GR is complex and further work is required to unpick the role of the GR in the regulation of ENaC in the distal nephron.

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Kidney Research UK

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## C021

### Induced nephrotic range glomerular proteinuria in mice is associated with amiloride-sensitive urinary plasmin formation and sodium retention

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**Introduction:** In several proteinuric conditions, aberrantly filtered serine proteases, e.g. plasmin, can activate the epithelial sodium channel (ENaC) through proteolytic interaction with the gamma subunit. This may lead to increased sodium reabsorption and subsequent expansion of the extracellular volume. This supports that nephrotic edema is a primary "overfill" situation founded in an intrarenal mechanism that mediates sodium and water retention, independently of systemic factors.

**Aim:** The study characterizes sodium balance in a murine model of pure glomerular nephrotic syndrome and tests the hypothesis that nephrotic range glomerular proteinuria in

mice leads to plasmin-mediated sodium and water retention abolished by amiloride.

**Methods:** We used a previously established mouse model of focal and segmental glomerulosclerosis (FSGS) by inducible conditional inactivation of the podocin gene in adult mice creating podocin knock out mice (Pod KO,  $Nph2^{lox,lox}; Cre^{+}$ ). Wild type (WT,  $Nph2^{lox,lox}; Cre^{-}$ ) littermates served as controls. Gene knockout was induced by intraperitoneal injection with tamoxifen at a dose of  $0.05 \text{ mg/g BW} \times 1/\text{day}$  for 5 days during a short anesthesia with isoflurane. Beginning 10 days after induction, urine and feces were collected in metabolic cages for a total of 10 days. Daily body weight was measured and sodium balance was calculated. Urine plasminogen was analyzed by ELISA and western blotting, and serine protease activity was identified by zymography and the ability to evoke inward current by whole-cell patch clamp of single M-1 cells. In a separate series, Pod KO mice were treated with amiloride ( $2.5 \text{ mg/kg}$  for 2 days followed by  $10 \text{ mg/kg}$  for 2 days) or vehicle by intraperitoneal injection twice daily beginning at day 13 after induction.

**Results:** From day 12 after induction Pod KO mice revealed significantly increased urinary albumin excretion and body weight when compared to WT, which was associated with ascites and suppressed plasma renin. Zymography demonstrated protease activity that co-migrated with active plasmin. Plasminogen was identified in the urine from Pod KO mice and correlated with albumin excretion. Compared to WT, the urine from Pod KO mice induced a greater and amiloride-sensitive inward current in M1 cells. Administration of amiloride promoted significant weight loss, increased Na excretion, normalized sodium balance, and prevented activation of plasminogen to plasmin in urine.

**Conclusion:** Proteinuria in a mouse model of FSGS is associated with ENaC-dependent sodium accumulation and suppression of renin which supports an overfill model of nephrotic syndrome in mice.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## C022

### pH-dependent regulation of pancreatic stellate cell activation and proliferation

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Pancreatic stellate cells (PSCs) are stromal cells of the pancreas that can become activated in various diseases such as chronic pancreatitis and pancreatic cancer. Upon activation, PSCs secrete a dense fibrous, so-called desmoplastic stroma. In the healthy pancreas, PSCs are exposed to intermittent changes in the interstitial pH. When pancreatic ductal cells secrete  $HCO_3^-$  into the pancreatic duct, they extrude an equimolar amount of  $H^+$  through their basolateral membrane domain, thereby acidifying the interstitium of the pancreas. Upon the tumorous transformation of the pancreatic ductal cells, the impaired ductal  $HCO_3^-$  secretion eventually leads to a relative alkalization of the interstitial pH of the pancreas. In this study we aim to elucidate in what manner changes in extracellular pH ( $pH_e$ ) affect the intracellular pH ( $pH_i$ ) homeostasis and activation of pancreatic stellate cells.

We isolated PSCs from healthy adult wild-type C57/BL6 mice. Following isolation, we cultured PSCs in culture for up to 120 hours in media buffered to pH 6.0, 6.6, 7.0 or 7.4. To

investigate the impact of  $pH_e$  on the expression of potential pH sensors, regulators and pH-regulated transporters of PSCs, we performed RT-qPCR measurements. To further evaluate the functional relevance of these transporters, we performed  $pH_i$  measurements using BCECF as fluorophore. As readout for cellular activation, we measured  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) positivity and cellular area using immunocytochemistry. Moreover, we assessed cellular proliferation and performed cell cycle analysis using flow cytometry.

Alkalization of the extracellular milieu substantially increases cell size,  $\alpha$ -SMA positivity as well as cellular proliferation. Moreover, cell cycle progression is also facilitated in an alkaline  $pH_e$  environment. Our results also imply that multiple pH sensors and regulators, especially those that are regulated by HIF-1 $\alpha$ , are differentially expressed in cells cultured in  $pH_e$  7.4 compared to freshly isolated cells and cells cultured in  $pH_e$  6.6. Also, intracellular pH acidifies moderately upon cellular activation taking place at alkaline  $pH_e$ . When cells are cultured in an acidic  $pH_e$  environment, the  $pH_i$  acidifies to  $pH_i \leq 7.0$ . We conclude that alkaline  $pH_e$  is a potent activator of PSCs as it induces cell cycle progression and the HIF1 $\alpha$ -signaling pathway.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## C023

### Role of Transient Receptor Potential Melastatin 2 channel in neutrophil function

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Neutrophils are the most abundant immune cells in the body and provide pivotal defense mechanisms. The role of ion fluxes upon neutrophil activation is increasingly appreciated. TRPM2 is a nonselective cation channel highly expressed in neutrophils and gated by products of oxidative stress, which are predominantly produced by NADPH oxidase (NOX2). Despite this direct link to neutrophil function, TRPM2 activation and its beneficial or detrimental consequences still need to be elucidated.

Using murine (C57BL/6) WT and TRPM2<sup>-/-</sup> bone marrow-derived neutrophils, 3D chemotaxis in gradients of fMLP, KC, LTB<sub>4</sub>, and C5a was analyzed (N $\geq$ 3). Absolute intracellular Na<sup>+</sup> concentration [Na<sup>+</sup>]<sub>i</sub> was measured using sodium-sensitive Asante Natrium Green-2 (ANG-2). [Na<sup>+</sup>]<sub>i</sub> upon fMLP stimulation and its dependence on Na<sup>+</sup>/K<sup>+</sup>-ATPase and Na<sup>+</sup>/Ca<sup>2+</sup> exchanger (NCX) activity was analyzed (N=3 for each condition). Reactive oxygen species (ROS) production was measured in differentiated siTRPM2-HL-60 cells using dihydrorhodamine (DHR) oxidation assay (N=3).

Chemotaxis of TRPM2<sup>-/-</sup> neutrophils in 3D matrix is not significantly altered when compared to WT neutrophils. After fMLP stimulation [Na<sup>+</sup>]<sub>i</sub> rises in neutrophils of both genotypes. It reaches higher levels in TRPM2<sup>-/-</sup> neutrophils (13.1  $\pm$  0.6 mmol/l vs 15.6  $\pm$  0.9 mmol/l). Addition of ouabain leads to a further increase in [Na<sup>+</sup>]<sub>i</sub>. Addition of the NCX inhibitor KB-R7943 lowers [Na<sup>+</sup>]<sub>i</sub> in WT neutrophils, but not in TRPM2<sup>-/-</sup> (16.5  $\pm$  1.2 mmol/l vs 25.4  $\pm$  1.7 mmol/l). Permeabilizing membranes with a Na<sup>+</sup> ionophore in the absence of [Na<sup>+</sup>]<sub>o</sub> reveals intracellular sodium release, which is higher after fMLP stimulation in TRPM2<sup>-/-</sup> than in WT neutrophils.

TRPM2 knock-down in HL-60 cells leads to diminished ROS production. Values are means  $\pm$  S.E.M.

Although chemotaxis is not altered in TRPM2 knock-out neutrophils, the ionic homeostasis in TRPM2<sup>-/-</sup> is disrupted. High [Na<sup>+</sup>]<sub>i</sub> in activated TRPM2<sup>-/-</sup> neutrophils hinders NCX transport. NCX activation normally follows neutrophil stimulation and ROS production. Both NCX and NOX2 are electrogenic and thereby modulate ROS production. Our findings suggest that TRPM2 gating upon neutrophil stimulation regulates not only ionic concentrations but the function of NCX and NOX2 and hence downstream effects depending on these proteins.

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## C024

### Phosphorylation regulates LAT4 function in response to anticipated food intake and dietary protein content

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System L amino acid transporter LAT4 (SLC43A2) is a sodium-independent uniporter that transports branched chain- and few other essential amino acids. Deletion of LAT4 leads to a slight intrauterine growth retardation, postnatal growth failure and early death in mice, possibly due to defective amino acid (re)absorption. Since it is suggested, that essential amino acid uniporters like LAT4 may limit amino acid flux through epithelia, we hypothesize that posttranslational modifications such as phosphorylation could regulate LAT4 function.

We investigated the potential functional role of LAT4 phosphorylation sites, previously detected by others using phosphoproteomics, by expressing (non-)phosphorylation mutants in *X. laevis* oocytes. The dose-dependent uptake of radiolabeled phenylalanine (n = 30), kinetics assessment and immunofluorescence revealed that mimicking the non-phosphorylated state of S274 leads to an increase in LAT4 affinity (p<0.001, ANOVA), surface membrane localization and thus improves transport function; in contrast, the same mutation of S297 severely reduced the surface expression and transport rate of LAT4 (p<0.001, ANOVA).

To investigate these sites *in vivo*, we used new phosphosite-specific antibodies to test the phosphorylation of S274 and S297 by Western blot and immunofluorescence. To determine whether LAT4 function, expression and intracellular localization are subjected to diurnal rhythm and whether they show an adaptive response to changes in dietary protein content, we entrained mice with time restricted feeding using normal (18%, n = 5) and low (8%, n = 5) protein diets and sacrificed them by cervical dislocation either at the start of the active phase (ZT12), when food intake is expected or at the beginning of the passive phase, 4 hours after food removal (ZT0).

We observed that the phosphorylation of intestinal LAT4, but not its expression nor its localization are submitted to diurnal rhythm. At the time of expected food intake, phosphorylation on S274 was low (p<0.001, ANOVA) and restricted to patches of cells along the villi, whilst phosphorylation on S297 was increased (p<0.05, ANOVA). On the other hand, mice maintained under 8% diet had a lower expression level of



intestinal LAT4 ( $p < 0.01$ , ANOVA) that displayed an increased intracellular localization, a higher S274 phosphorylation level ( $p < 0.001$ , ANOVA) with more even distribution along the villi and a decreased S297 phosphorylation ( $p < 0.01$ , ANOVA). These results suggest that under normal protein diet, the expectation of food intake enhances LAT4 function, leading to a higher amino acid absorption capacity. Under low protein diet, LAT4 response to food entrainment is limited and its transport activity appears to be set continuously on a lower level.

In conclusion, we identified phosphorylation as a novel regulatory mechanism for a basolateral amino acid uniporter.

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## C025

### Activator protein 1 complex controls Claudin 1 expression in response to air-liquid-interface conditions in NCI-H441 epithelia

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Tight junctions (TJ) are crucial for the barrier function of lung epithelia. They are essential for establishing and maintaining air-liquid-interface (ALI) conditions in the airways and alveoli and hence they are prerequisite for an appropriate lung function. Breakdown of TJ and ALI results in respiratory distress syndrome. Most important for the specific function of TJ are proteins called claudins, the activator protein 1 complex (AP-1), a dimer consisting of c-Jun and c-Fos, is shown to modulate different claudins in tissues other than lung. However, the role of AP-1 for regulating claudin expression in human airway epithelium in response to ALI is sparsely elaborated.

To elaborate the changes in functional properties of TJ while ALI conditions get established we investigated NCI-H441 cells as a model of the distal lung epithelium cultivated either at ALI or liquid-liquid interface (LLI) conditions. Paracellular permeability was quantified by measuring transepithelial electrical resistance (TEER). Semi-quantitative RT-PCR, western blot and immunofluorescence experiments were performed to identify differentially regulated TJ-proteins and the changes in AP-1 expression. Promoter regions were analyzed *in-silico* for putative binding sites of AP-1 and AP-1 activation of cldn1 promoter was verified using luciferase assays. Formation of AP-1 was detected by proximity ligation assays. To uncover AP-1 controlling of claudin expression, c-Fos was overexpressed in cells using recombinant adenoviruses.

In comparison to ALI cultivated NCI-H441 epithelia, LLI conditions resulted in TEER reduction by approximately 40%. This agreed with the observed downregulation of Claudin 1 (Cldn1), which is known as a claudin that tightens TJ. Based on the *in silico* analysis of Cldn1 promoter region we identified putative binding sites for c-Fos and c-Jun. While there is an upregulation of AP-1 components in LLI versus ALI cultivated epithelia on mRNA level, we observed reduced protein levels for c-Fos and c-Jun and reduced AP-1 formation in LLI cultivated cells. Inhibition of AP-1 with SR11302 reduced Cldn1 promoter activity in luciferase assay experiments. Adenoviral overexpression of c-Fos in ALI and LLI cultivated NCI-H441 epithelia resulted in upregulation of Cldn1 expression levels.

Our results underscore the role of AP-1 as an activating factor for Cldn1 expression in NCI-H441 cells. It modulates Cldn1 expression in response to ALI conditions and therefore it

adjusts functional properties of TJ to maintain ALI conditions. The herein identified mechanisms may contribute to disturbance of epithelial barrier function during lung edema and may facilitate respiratory distress.

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## C026

### Trafficking, localization and degradation of the $\text{Na}^+$ , $\text{HCO}_3^-$ co-transporter NBCn1 in breast cancer and kidney epithelial cells

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The  $\text{Na}^+$ ,  $\text{HCO}_3^-$  co-transporter NBCn1 (SLC4A7) is a major physiological regulator of intracellular pH (1). Consequently, NBCn1 dysfunction is linked to human diseases such as cardiovascular disease and cancer. Specifically, it has been reported that some human breast cancers exhibit increased NBCn1 plasma membrane expression and NBCn1 knockout reduces breast tumor development in mice. NBCn1 trafficking and turnover, however, are essentially unstudied. The aim of this work was to delineate mechanisms of NBCn1 trafficking in epithelial cells. Polarized or non-polarized epithelial MCF-7 and MDCK-II cells were cultured under standard cell culture conditions (5%  $\text{CO}_2$ , 95% humidity and 37°C). By confocal microscopy, endogenous NBCn1 and exogenous GFP-NBCn1 (N-terminally GFP-coupled rat NBCn1-D) were consistently found in the basolateral membrane ( $n=3-5$ ) (2). In congruence with this, endogenous NBCn1 co-immunoprecipitated with the cell polarity protein lethal giant larvae (LLGL1). GFP-NBCn1 membrane localization was abolished by deletion of the full NBCn1 C-terminal tail (C-tail) but unchanged following mutation or deletion of a putative C-terminal PDZ-binding motif in the most distal part of the C-tail ( $n=3$ ). Glutathione-S-Transferase-pulldown of the C-tail followed by mass spectrometry analysis revealed putative interactions with multiple sorting-, degradation- and retention factors, including the scaffolding protein RACK1 (2). Pulldown of FLAG-tagged deletion constructs mapped the RACK1 interaction to the proximal portion of the NBCn1 C-tail ( $n=3$ ). Native NBCn1 and RACK1 interaction was confirmed in a cellular context by Proximity Ligation Assay and co-immunoprecipitation assays ( $n=4$ ). RACK1 knockdown reduced NBCn1 membrane localization without affecting total NBCn1 expression ( $p < 0.05$ ,  $n=3$ ) arguing for a role of RACK1 in NBCn1 trafficking. NBCn1-RACK1 plasma membrane co-localization ( $p < 0.05$ ,  $n=3$ ) was only present in non-confluent cells, consistent with the notion that RACK1 regulates the trafficking of NBCn1 to the membrane (2). One-third of surface NBCn1 was constitutively endocytosed from the basolateral membrane within 60 min ( $p < 0.05$ ,  $n=3$ ) while total NBCn1 degradation, predominantly by a lysosomal mechanism, was slow (half-life  $> 24$ h;  $n=3$ ). Together these data suggest that a fraction of NBCn1 exhibits recycling between the basolateral membrane and intracellular compartment(s). In support of this idea, preliminary data show NBCn1 co-immunoprecipitation with the recycling regulating transport protein Rab4 and that

NBCn1 endocytosis partly depends on activity of the vesicle scission GTPase dynamin. In conclusion our findings shed new light on the trafficking, localization and degradation of NBCn1 in epithelial cells and contribute to a broader understanding of NBCN1 regulation in human physiology.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## C027

### The apical Na<sup>+</sup>/H<sup>+</sup> exchanger 2 regulates intestinal wound healing and barrier function

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The apical Na<sup>+</sup>/H<sup>+</sup> exchanger isoform NHE2 is highly expressed in the gastrointestinal tract. *nhe2*<sup>-/-</sup> mice display a delayed trefoil factor-stimulated gastric epithelial wound healing and impaired small intestinal barrier recovery after ischemia, but the molecular mechanisms behind are still not understood. Previous study on experimental wound healing in the gastric cell line RGM-1 showed that overexpression of NHE2 triggers increased cellular pH<sub>i</sub>, but decreased migratory speed (1). To understand how a reduced migratory speed may result in improved barrier function, we used models where the endogenous expression of NHE2 was deleted (*nhe2*<sup>-/-</sup> mice) or silenced by shRNA interference (Caco2Bbe cells) and studied enterocyte migration, proliferation and differentiation *in vitro* and *in vivo*. NHE2 gene was knocked-down using shRNA in Caco2Bbe cells, resulting in ~70±9.6% decrease of NHE2 expression on mRNA level. Colons from *nhe2*<sup>-/-</sup> and *wt* mice were used for the *in vivo* studies. All *in vivo* experiments were performed using 3 to 6 months old *nhe2*<sup>-/-</sup> and *wt* littermates, congenic on the FVB/N background. Mice were killed by cervical dislocation after light isoflurane anesthesia. The loss/downregulation of NHE2 triggered significant decrease of the pH<sub>i</sub> and alterations in cell proliferation. Colonocyte migration from the crypt base to the surface accessed at different time points after bromodeoxyuridine (BrdU) labeling of the mice was significantly faster in the colon of *nhe2*<sup>-/-</sup> mice compared to the control littermates. *In vitro* wound assay showed significantly faster restitution velocity of the NHE2 silenced Caco2Bbe cells, which was mechanistically supported by altered cell-cell adhesion and assembly of the E-cadherin/β-catenin complex. Further analysis of the *nhe2*<sup>-/-</sup> colon revealed elongated crypts with impaired differentiation. The expression and activity of alkaline phosphatase, as an enterocyte differentiation marker, was significantly reduced in cells lacking NHE2, both *in vivo* and *in vitro*. In contrast, a shift towards the secretory lineage was detected in the *nhe2*<sup>-/-</sup> colon identified by the increased number of MUC2 positive goblet cells. The results imply an important role of NHE2 in the lower/middle segment of the crypt where the progenitor intestinal cells emerge from the stem cell niche and enter enterocyte differentiation. The faster migratory speed in NHE2-deficient cells is associated with a dysregulated enterocyte differentiation pattern including junctional alterations and may explain the observed barrier

disturbance previously observed in the gastrointestinal tract of *nhe2*<sup>-/-</sup> mice. The results suggest that NHE2 expression and activity are necessary for pH<sub>i</sub> regulation during enterocyte migration and differentiation.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## C028

### Characterizing the expression of TRPV4 in the choroid plexus epithelia as a prospective component in the development of hydrocephalus.

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Homeostatic maintenance of cerebrospinal fluid (CSF) secretion and absorption is essential for basic neurologic function. The choroid plexus epithelia, which produce the majority of the CSF, are among the most secretory of all epithelia. However, the control of this secretory process is poorly described. In diseased states like hydrocephalus, where this homeostasis is disrupted, patients often experience symptoms such as cognitive impairment, motor/stability issues, and incontinence. Current treatment for hydrocephalus is limited to surgically invasive shunting procedures which often fail and need revision – particularly in pediatric cases. We have used several preclinical models to study the mechanisms of CSF regulation. In addition to a porcine choroid plexus epithelial cell line, two rodent ciliopathy models were used – the *W-WPK* rat and the *GAS8* mouse. The *W-WPK* rat is analogous to Meckel-Gruber Syndrome, and the *GAS8* mouse has a phenotype similar to Primary Ciliary Dyskinesia. Both of these models present with severe perinatal hydrocephalus in the mutant null pups. It is believed that one of the mechanisms of pediatric hydrocephalus is the overproduction of CSF by the choroid plexus (CP) epithelial cells that line the ventricles of the brain. The protein of interest to this research is the non-specific cation channel, Transient Receptor Potential Vanilloid 4 (TRPV4), which has been shown to be activated by multiple stimuli including osmotic and pressure changes as well as by prostanoid metabolites. Activation of TRPV4 results in Ca<sup>2+</sup> influx through the channel resulting in changes in intracellular signaling including the secondary activation of Ca<sup>2+</sup>-stimulated ion transporters. In both models, TRPV4 is localized to the apical membrane of the CP epithelial tissue in the Wild Type pup. Preliminary data indicate that TRPV4 is overexpressed in CP epithelia, ependymal cells and in the sub ventricular zone in the mutant null pups of both models as their hydrocephalus progresses when compared to the wild type and heterozygous animals. Furthermore, in the rat pups, treatment of an antagonist compound of TRPV4 (RN 1734, administered i.p. once daily for seven days) reduced the hydrocephalus in the affected animals (via MRI ventricular

volume quantification) and diminished TRPV4 expression. This suggests that antagonistic compounds of the TRPV4 channel have the potential to reduce hydrocephalus in these models. Successfully targeting the molecular mechanisms for hydrocephalus in rodent models can provide a promising base for preclinical studies aimed at developing pharmaceutical agents to treat this disease.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## C029

### The bladder mucosa produces the highest level of NADPH oxidase-derived superoxide in the body: pathological significance and therapeutic potential

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Lower urinary tract diseases affect 1/3 of world population, with 546 million having overactive bladder (OAB) in 2018<sup>1</sup>. Despite the exceptionally high prevalence, specific treatments are lacking. The urothelium, the bladder epithelial lining, has generated intense interest for new targets following recent identification of its novel sensory role<sup>2</sup>. The tissue is also highly susceptible to age-related dysfunction<sup>3,4</sup>. The role of oxidative stress and reactive oxygen species (ROS) in several bladder pathologies has been demonstrated, including OAB<sup>5</sup>. However, the sources of ROS are unknown, which may provide therapeutic targets. NADPH oxidases (Nox) have gained increasing interest, as they produce ROS as a sole function and have the advantage over other ROS-generating enzymes with little side effects. We hypothesise that the urothelium is capable of producing high levels of ROS, through Nox enzymes, contributing to age-related bladder pathologies. Tissues from bladder and other organs were isolated from mice (12wk, male C57BL/6) by micro-dissection in accordance with UK/EU regulations. Immunofluorescence and Western blot determined Nox subtype expression. Lucigenin-enhanced chemiluminescence quantified superoxide (SO) release from live tissue. Data are expressed as medians±interquartiles, tested by Wilcoxon's (non-Gaussian data). Western blotting (n=10 bladders) and immunohistochemistry (n=5) demonstrated Nox1, 2 and 4 subtype expression throughout the bladder, with no Nox3 or 5. The mucosa had significantly higher expression than smooth muscle (SM) of NOX2 (mucosa: 4.5[2.9, 6.7] Vs SM: 1.7[0.8, 2.0] p<0.05) and Nox4 (mucosa: 1.02[0.7, 1.9] Vs SM: 0.6[0.4, 0.8] p<0.05), but similar Nox1 expression. Chemiluminescence identified significantly higher SO release from mucosa compared to other bladder tissues and major organs (mean light units (MLU): mucosa[n=102]: 340[182, 539] SM[n=102]: 16[12, 24] Aorta[n=38]: 33[21, 86] Cerebrum[n=34]: 17[10, 29] Kidney[n=39]: 109[63, 146] Liver[n=34]: 97[62, 116] Ventricle[n=34]: 7.4[5.0, 13] Large int.[n=16]: 106[60, 228] Small int.[n=14]: 15 [11, 27] p<0.05 for all mucosa Vs. other tissue). The contributions to mucosal SO from mitochondria were 35% with uncoupling agent (con: 90[58, 106] 1µM FCCP: 56[52, 63] n=9, p<0.05) and 15% with complex1 inhibitor (con: 92[82, 128] 10µM rotenone: 74[56, 86] n=13, p<0.05). Nox contributed 75% of SO production (con: 82[47, 97] 20µM DPI: 7[5, 17] n=7, p<0.05) with Nox1 accounting for 20% (con: 86[72, 104] 5µM NoxA1ds:

66[57, 91] p<0.05). This study identified for the first time an exceptionally high level of ROS from the urothelium, compared to that in other major organs known to produce high levels of ROS. These findings explain the highly inflammatory nature of bladder urothelium and demonstrate that Nox enzymes are a major contributor to age-dependent bladder pathologies and serve as a novel group of drug targets.

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## C030

### Regulation of constitutive cation entry and cell proliferation by TRPM7 channels in human pancreatic stellate cells.

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Pancreatic ductal adenocarcinoma (PDAC) is one of the deadliest cancer with a 5-year survival rate less than 5%. This extremely poor prognosis is mainly due to a late diagnosis, to the occurrence of metastases, and to the absence of efficient treatment. Thus, there is an urgent need to better understand the cellular and molecular mechanisms that drive PDAC progression. Among them, it is now largely admitted that cancer cells interact with their close microenvironment when they invade the stroma. This reaction called “desmoplastic reaction” leads to the activation of pancreatic stromal cells and to the enhancement of cancer cell capacities to form metastasis in the neighboring organs. Indeed, the activated stromal cells or pancreatic stellate cells (PSCs) are studied as potential therapeutic targets in PDAC. Ion channels are involved in cancer cell proliferation, survival, migration, invasion, etc. Recently, we showed that TRPM7 channel expression is increased in human PDAC tissues compared to healthy tissues, and that TRPM7 expression is inversely related to patient survival. Moreover, TRPM7 regulates PDAC cell migration and invasion through constitutive Mg<sup>2+</sup> entry (Rybarczyk *et al.*, 2012; Rybarczyk *et al.*, 2017). In the present work, we aim to assess the role of TRPM7 channels in human PSCs. We found that TRPM7 was expressed as both mRNA and protein level in human PSCs cell lines. By using Western-Blot and immunofluorescence staining, we also found an expression of the activation markers of PSCs including α-SMA, COX-2 and desmin. The inhibition of TRPM7 by siRNA or by a pharmacological blocker NS8593 (10 µM) induced the growth arrest of PSCs. Cell cycle analysis after TRPM7 inhibition showed an



accumulation of PSCs in G0/G1 phase along with a decrease of the cell number in S and G2/M phases of the cell cycle. The number of apoptotic PSCs was also significantly increased by TRPM7 silencing. Finally,  $Mn^{2+}$  quench experiments showed the involvement of TRPM7 in cation constitutive entry in PSCs. In conclusion, we show that TRPM7 is required for PSCs proliferation and survival possibly by maintaining a constitutive cation entry. Further experiments are needed to determine how TRPM7 regulates PSCs growth ( $Ca^{2+}$  and/or  $Mg^{2+}$ -dependent mechanisms?).

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### C031

#### **Spadin selectively antagonises arachidonic acid activation of mTREK-1 channels**

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We recently revealed the mechano-gated two-pore domain potassium ( $K_{2p}$ ) channel, TREK-1, to be a major regulator of murine intestinal contractility (Ma *et al*, 2018). However, verification was complicated by a lack of well-defined  $K_{2p}$  modulators and species specific pharmacology. Here, using heterologous expression in *Xenopus laevis* oocytes and electrophysiological analysis using two-electrode voltage clamp in standard bath solutions, we characterised the pharmacological profile of mouse homologues of TREK-1 and TREK-2 using previously documented human  $K_{2p}$  activators; arachidonic acid (AA, 10  $\mu$ M), BL-1249 (1  $\mu$ M) and cinnamyl-3,4-dihydroxy- $\alpha$ -cyanocinnamate (CDC, 10  $\mu$ M) and inhibitors; spadin (1  $\mu$ M) and barium chloride ( $Ba^{2+}$ , 1 mM). Values are stated as mean  $\pm$  S.E.M. and were statistically compared by ANOVA. Mouse (m) TREK-1 and TREK-2 channel currents were significantly increased by AA, BL-1249 and CDC in a voltage-independent manner. At 0 mV, AA increased mTREK-1 currents by  $2.5 \pm 0.3$ -fold ( $n = 7$ ,  $p < 0.05$ ) and mTREK-2 by  $1.6 \pm 0.1$ -fold ( $n = 7$ ,  $p < 0.05$ ), BL-1249 increased mTREK-1 currents by  $2.9 \pm 0.3$ -fold ( $n = 9$ ,  $p < 0.05$ ) and mTREK-2 by  $1.5 \pm 0.1$ -fold ( $n = 9$ ,  $p < 0.05$ ), and CDC increased mTREK-1 currents by  $2.1 \pm 0.1$ -fold ( $n = 8$ ,  $p < 0.05$ ) and mTREK-2 by  $2.3 \pm 0.2$ -fold ( $n = 6$ ,  $p < 0.05$ ). Under basal conditions, both mTREK-1 and mTREK-2 currents were insensitive to application of spadin at all activating voltages. In contrast  $Ba^{2+}$  induced a significant, voltage-dependent reduction in outward currents of both channels. At 0 mV, mTREK-1 and mTREK-2 currents were inhibited by  $56.1 \pm 1.4\%$  ( $n = 7$ ,  $p < 0.05$ ) and  $55.4 \pm 2.4\%$  ( $n = 7$ ,  $p < 0.05$ ) respectively. Furthermore, spadin did not significantly inhibit either mTREK-1 or mTREK-2 currents following pre-activation by either AA, BL-1249 or CDC ( $n = 8-12$ ,  $p > 0.05$ ). However, we found pre-exposure to spadin significantly perturbed the activation of mTREK-1

currents by AA, voltage-independently. At 0 mV, activation of mTREK-1 by AA was reduced by  $42.5 \pm 1.8\%$  ( $n = 12$ ,  $p < 0.05$ ) compared to AA alone. Activation of mTREK-1 channels by either BL-1249 or CDC was not significantly attenuated by pre-exposure to spadin ( $n = 8-11$ ,  $p > 0.05$ ). Similarly, pre-exposure to spadin was unable to antagonise mTREK-2 channel current activation by either AA, BL-1249 or CDC ( $n = 6-8$ ,  $p > 0.05$ ). These data demonstrate that pharmacological activation of mouse TREK-1 and TREK-2 channels by AA, BL-1249 and CDC is comparable to that observed in their human orthologues. Additionally, our data suggests spadin is not a pore blocker of mTREK-1 or mTREK-2 channels, like  $Ba^{2+}$ , but specifically antagonises the activation of mTREK-1 channels by AA, and not other pharmacological activators. Further studies are required to reveal the precise molecular mechanisms of spadin antagonism.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

### C032

#### **EnNaC regulation: SECS, drugs and Rho/Rac**

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Endothelial cells form the innermost layer of blood vessels. Due to this strategic position endothelial cells control many aspects of vascular function. One of the major characteristics of endothelial function is the ability of the endothelial cells to release vasoactive substances such as nitric oxide (NO), which cause a vasodilatation<sup>1</sup>. Recently, it was shown that stiffening of the endothelial cell cortex, an actin-rich layer 50-150 nm underneath the cell membrane, leads to a reduced NO release and therefore causes an increase in (systemic) blood pressure<sup>2</sup>. We identified the endothelial  $Na^+$  channel (EnNaC) as major regulator of the cortical stiffness in that its membrane abundance stiffens the cortex: An increased abundance of EnNaC in the cell membrane causes a rise in functional stiffness of the endothelial cell cortex which is accompanied by a decreased NO release<sup>3</sup>. Strikingly, EnNaC-mediated endothelial stiffening can be reversed by pharmacological or genetic downregulation of the channel. Since the actin-polymerizing agent Jasplakinolide reverses this softening process, a direct interaction between EnNaC and the subcortical actin cytoskeleton is postulated. To identify the underlying mechanisms of this observation, specific components of the cortical actin cytoskeleton were degraded by adding inhibitors for the Rac1 (NSC23766) and RhoA pathway (CT04) as well as the Arp 2/3 complex (CK548). Under these conditions, the mechanical stiffness of endothelial cells (EA.hy 926) was probed with the Atomic Force Microscope. In parallel, EnNaC membrane abundance was studied using Quantum Dot-mediated immunofluorescence microscopy.

We were able to show that the inhibitor-induced degradation of the actin cytoskeleton leads to a significant reduction in cortical stiffness of 15.0% for NSC23766, 13.0% using CT04 and 18.3% for CK548. This decrease is accompanied by a significant reduction of EnNaC membrane abundance: 35.6% for NSC23766, 25% for CT04 and 38.2% using CK548. We conclude that the structural dynamics of the outer shell of endothelial cells is caused by functional interaction between the subcortical actin cytoskeleton and EnNaC. This interaction results in a change of the cortical stiffness and is thereby one

major component responsible for proper endothelial function. With these results we found a novel mechanism of regulation of nanomechanical properties of endothelial cells. Since this is pivotal for vascular function, our results might lead to novel therapeutical strategies regarding the treatment of endothelial dysfunction and cardiovascular pathologies.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

interact with P2X4 and stabilize its closed state leading to the decrease of ATP-activated P2X4 currents.

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## C033

### Bile acids down-regulate human purinergic receptor P2X4 heterologously expressed in *Xenopus laevis* oocytes

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Human P2X4 receptor belongs to the P2X family of ATP-gated non-selective cation channels. P2X4 is abundantly expressed in the apical membrane of cholangiocytes and may contribute to the local regulation of biliary secretion (1). Recently, we have demonstrated that bile acids stimulate several members of the epithelial sodium channel (ENaC)/degenerin (DEG) ion channel family including the acid-sensing ion channel 1 (ASIC1) (2–4). There are no significant amino acid sequence relationships between ASICs and P2X receptors. However, a comparison of their crystal structures revealed similarities of ASIC and P2X receptor ion channel domains (5). Therefore, we hypothesized that bile acids may also modulate P2X4. In the current study, we investigated whether unconjugated cholic (CA), deoxycholic (DCA) and chenodeoxycholic (CDCA) acid or their tauro-conjugated forms (t-CA, t-DCA, t-CDCA) may modulate human P2X4 heterologously expressed in *Xenopus laevis* oocytes. Whole-cell and single-channel currents were measured using the two-electrode voltage clamp and outside-out patch-clamp technique, respectively. Values are presented as mean  $\pm$  S.E.M. Student's t-test was used for statistical analysis. Application of DCA or t-DCA (250  $\mu$ M) reduced ATP-activated P2X4-mediated currents by 65 $\pm$ 3% ( $p < 0.001$ ,  $n = 22$ ) or 68 $\pm$ 3% ( $p < 0.001$ ,  $n = 22$ ), respectively. In contrast, the inhibitory effect of t-CDCA was only 34 $\pm$ 8% ( $p < 0.05$ ,  $n = 20$ ) and CDCA, CA or t-CA had no significant effect on P2X4-mediated currents ( $n = 20$ ). Inhibition of P2X4 by t-DCA was reversible. In the presence of t-DCA the affinity of P2X4 to ATP ( $EC_{50} = 2.4 \pm 1.3$   $\mu$ M) was similar to that under control conditions ( $EC_{50} = 4.8 \pm 1.2$   $\mu$ M). In outside-out patch-clamp recordings we observed that t-DCA significantly decreased the rate of P2X4 activation by ATP and increased the rate of subsequent channel inactivation. Moreover, the estimated channel open probability was significantly reduced in the presence of t-DCA. This can be attributed to a 3-fold increase of the apparent mean channel closed time from 13 $\pm$ 4 ms ( $n = 7$ ) in the absence of t-DCA to 42 $\pm$ 11 ms ( $n = 6$ ;  $p < 0.01$ ) in its presence. Finally, using site-directed mutagenesis we identified several amino acid residues involved in mediating the inhibitory effect of t-DCA on P2X4. We conclude that t-DCA may specifically

## C034

### Using CRISPR technology to generate models of polycystic kidney disease

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Polycystic kidney diseases (PKD) are characterised by the formation and progressive growth of renal cysts. Autosomal dominant (AD)PKD, the commonest PKD, affects 1 in 1,000 individuals and is caused by mutations in either *PKD1* (85%) or *PKD2* (15%), which encode for polycystin-1 and -2 respectively. These two proteins regulate proliferation, fluid secretion, ciliary function, cell–cell adhesion, and cell–matrix interaction of renal epithelial cells. Mutations of either *PKD1* or *PKD2* result in increased cyclic AMP signalling which promotes cyst formation. Understanding the molecular basis of PKD is essential for developing more effective therapies for patients and this requires clinically-relevant cellular models. To do this, we employed the CRISPR-Cas9 system to generate mutations in exon 36 of *PKD1* in human embryonic kidney (HEK) 293 cells. Two different cell lines were generated: one with a homozygous mutation in *PKD1* (c.10744delCC) and another with a compound heterozygous mutation (allele 1 c.10744delCC and allele 2 c.10732delCGAGCTTCCC), both of which led to a frameshift deletion in *PKD1*. Next, the cells were embedded in Matrigel and compared with HEK293 wild-type cells in 3-dimensional culture. Over a period of five days, seeded single cells of both wild-type and *PKD1* genotypes proliferated to form discrete cell cultures, some containing apparent lumens. Examination of the size of 180 clusters found that those containing wild-type cells were significantly smaller than those composed of either homozygous and compound heterozygous *PKD1* knock-down cells ( $p < 0.05$  in both cases). Next, we exposed the cells to forskolin, a cyclic AMP agonist. In wild-type cells, forskolin significantly increased the area of the clusters ( $p < 0.05$ ). In contrast, in both of the *PKD1* knock-down cells, forskolin did not cause any significant change in the cluster size. In conclusion, we have generated a cellular model of ADPKD using CRISPR-Cas9 technology which may be used to test the effect of different drugs in cystogenesis or examine different biological process involved in ADPKD such as cell proliferation or migration.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

C035

# **FRET-ting about intracellular airway epithelial glucose concentrations and metabolism**

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Normally glucose in the liquid lining the lumen of the airway (airway surface liquid; ASL) is maintained at ~0.4mM, and this is thought to promote sterility of the airways. However elevation of blood/basolateral glucose and inflammation increase glucose in the ASL both *in vitro* and *in vivo*<sup>1</sup>. Glucose crosses the airway epithelium from blood to ASL via paracellular and transcellular routes. For transcellular transport to occur, we hypothesised that intracellular glucose concentrations must exceed that of ASL glucose. Intracellular glucose concentrations are determined by glucose metabolism.

We investigated the effect of extracellular glucose on intracellular glucose concentrations and metabolism to determine whether hexokinase activity is a rate determining step for ASL glucose concentrations. We used the intracellular FRET sensor Gluonic<sup>2</sup>, a glucose binding protein with donor and acceptor fluorophores cyan and yellow fluorescent proteins (CFP and YFP respectively) transfected into H441 airway epithelial cells. Changes in intracellular glucose were measured using CFP/YFP FRET ratio, which decreases with an increase in glucose concentration. A glucose dose response curve for Gluonic, was generated, and values for intracellular glucose were calculated from this. Sorbitol concentrations were measured (a product from the polyol metabolic pathway) by colorimetric assay. Data are shown as mean±S.D.

In H441 cells exposed to an extracellular glucose concentration of 5mM D-Glucose +10mM L-Glucose (osmotic control), intracellular glucose, as measured by FRET ratio was  $4.57 \pm 1.46 \mu\text{M}$  (n=83). An increase in extracellular D-glucose to 15mM decreased intracellular glucose to  $2.17 \pm 0.54 \mu\text{M}$  (n=83,  $p \leq 0.001$ ). H441 cell hexokinase activity was  $32.9 \pm 5.5 \text{ nmol/mg/min}$  (n=5). Bromopyruvic acid (100μM) reduced total hexokinase activity by  $23 \pm 5\%$  ( $P \leq 0.01$ ; n=5) in 5mM glucose, which resulted in an increase of intracellular glucose to  $0.031 \pm 0.0047 \text{ mM}$  (n=83,  $p \leq 0.001$ ) in H441 cells.

In 15mM extracellular D-glucose, there was a trend towards increased intracellular sorbitol concentrations compared to 5mM (n=3) and inhibition of hexokinase had no effect on these values.

These data indicate that under normoglycaemic conditions, intracellular glucose is ~100 fold lower than ASL glucose and that elevating extracellular glucose concentrations decreased intracellular glucose. This would suggest that under both conditions, transcellular transport of glucose is unlikely because the glucose gradients would favour uptake from the ASL over efflux into the ASL, and that the primary flux of glucose into the ASL occurs via the paracellular route.

In addition, we previously showed that hyperglycaemia increased lactate production<sup>3</sup>. This study has provided preliminary data indicating that the polyol pathway may also be activated in hyperglycaemia. We therefore propose that the decrease in intracellular glucose in hyperglycaemia is due to a shift in metabolic processing of glucose.

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Garnett JP *et al*, 2016 Sci Rep 6, 37955

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

C036

# **Hypoxia inhibits K<sup>+</sup>-currents and alters the redox state in murine pulmonary arterial smooth muscle cells**

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Hypoxic pulmonary vasoconstriction (HPV) is a physiological response to localized alveolar hypoxia that is intrinsic to the pulmonary circulation. By hypoxia-induced contraction of pulmonary arterial smooth muscle cells (PASMCs), pulmonary capillary blood flow is redirected to alveolar areas of high oxygen partial pressure (pO<sub>2</sub>), thus maintaining the ventilation-perfusion ratio<sup>(1)</sup>. Although the principle of HPV was recognized decades ago the underlying pathway still remains elusive. Voltage gated K<sup>+</sup>-channels (K<sub>v</sub>-channels) are known to be redox-sensitive and crucial for mediating membrane potential in PASMCs - thereby controlling Ca<sup>2+</sup>-entry and subsequently vascular tone. Here, we investigated whether acute hypoxia alters the redox state of isolated PASMCs from mice and if this impacts K<sub>v</sub>-channel activity, membrane potential and intracellular Ca<sup>2+</sup>-level.

K<sub>v</sub>-currents and membrane potential in response to hypoxia or the oxidizing agent H<sub>2</sub>O<sub>2</sub> (124 nM) were investigated by whole cell patch clamp experiments (voltage and current clamp, *resp.*) on freshly isolated murine PASMCs. Simultaneously, pO<sub>2</sub> was recorded using an optical oxygen sensor and intracellular Ca<sup>2+</sup> was measured by ratiometric FURA-2 imaging. The redox state was monitored by Raman spectroscopy using an excitation wavelength that was in resonance with the heme proteins (532 nm).

Raman-spectra values for reduced myoglobin (1607 cm<sup>-1</sup>) and cytochrome c (1622 cm<sup>-1</sup>) both increased upon exposure of PASMCs to hypoxia (4 % O<sub>2</sub>) and returned to the initial value upon reverting to normoxia (21 % O<sub>2</sub>), thus indicating a shift in the cells' redox state. In accordance, K<sub>v</sub>-currents were dose-dependently inhibited by hypoxia, resulting in a depolarization from  $-37.5 \pm 2 \text{ mV}$  (before) to  $-28.1 \pm 2 \text{ mV}$  after exposure to hypoxia (Mean ± SEM; n = 23;  $p < 0.001$ ; paired t-test) - thereby triggering a rise in intracellular Ca<sup>2+</sup>. The oxidizing agent H<sub>2</sub>O<sub>2</sub> (124 nM) mimicked this effect under normoxic conditions by elevating the membrane potential of the PASMCs from  $-37.4 \pm 3 \text{ mV}$  (before) to  $-25.2 \pm 4 \text{ mV}$  (Mean ± SEM; n = 5,  $p = 0.005$ , paired t-test).

In conclusion, hypoxia induced a shift in the redox state of PASMCs, thereby mediating the inhibition of K<sub>v</sub>-channels. The resulting depolarization and Ca<sup>2+</sup>-entry may account for the contraction of PASMCs – thus initiating HPV.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.



C037

### The strong binding states of the myosin head contribute to the activation of the thin filament in human soleus slow-twitch muscle fibers

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Force development of striated muscle is triggered by the calcium-induced activation of the troponin complex and the subsequent azimuthal displacement of tropomyosin, which exposes the high affinity myosin binding sites on actin. Several experimental models including proteins in solution, reconstituted thin filaments and isometric contracting muscle fibers have suggested that the thin filament is additionally activated by the strong-binding state of the myosin head although some observations made in isometric contracting muscle fibers have questioned this view. Since these studies have been performed either on cardiac or on fast-twitch muscle preparations, it is unknown whether cycling cross-bridges influence the thin filament activation in slow-twitch skeletal muscle. Human soleus fibers express the same myosin isoform as the human heart and constitute an interesting experimental model for the examination of myosin mutations related to familial hypertrophic cardiomyopathy. To probe the calcium and myosin induced activation of the thin filament in isometric contracting human soleus fibers, the endogenous troponin complex was exchanged for a well characterized fast-twitch skeletal troponin complex labeled with the fluorescent dye IANBD (fsTnIANBD). The effectively exchanged amount of troponin was 70%. To dissect the individual contributions of Ca<sup>2+</sup> and cross-bridges on thin filament activation, force-pCa and fsTnIANBD emission-pCa relations were explored in absence and presence of para-aminoblebbistatin (PAB), an inhibitor of the strongly-bound state of myosin. Increasing the calcium concentration from relaxing (pCa 7.5) to saturating levels (pCa 4.5) without PAB induced maximal force development and generated a 32.2% ( $\pm$  1.33%, N = 8) decrease in the emission intensity of fsTnIANBD. 50  $\mu$ M PAB strongly inhibited active force at pCa 4.5 to 6.8% ( $\pm$  1%, N = 8) of the active force without PAB while it decreased the Ca<sup>2+</sup>-induced decrease in emission intensity of fsTnIANBD to only 17.8% ( $\pm$  2.6%, N = 8). At all calcium concentrations, the calcium induced activation of the thin filament was higher than the myosin induced activation. However, the myosin induced activation increased with the calcium concentration (i.e. myosin contribution to the total activation was about 45%, 35% and 20% at saturating, intermediate and low calcium concentrations, respectively). Importantly, when switching from isometric contraction to isotonic contraction at low load, changes in fluorescence indicating the kinetics of the conformational changes of fsTnIANBD were at least ten times faster than kinetics of force development. Thus our kinetic data indicates that the rate of thin filament switching does not limit the rate of force development.

We are grateful to DAAD Central America for funding ALD during the initial phases of this project.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

C038

### Afternoon exercise is more efficacious than morning exercise at improving blood glucose levels in men with type 2 diabetes.

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Exercise is a recommended intervention for prevention and treatment of type 2 diabetes (T2DM). Low-volume High Intensity Interval Training (HIIT) remodels skeletal muscle and the cardio-respiratory system to a similar or greater extent than continuous moderate-intensity training, with reduced time commitment and exercise volume. Even though perturbed circadian rhythms are associated with metabolic dysfunction, time of day at which most robust adaptations to exercise can be achieved is unknown. We compared the efficacy of morning and afternoon HIIT in lowering blood glucose levels in participants with T2DM. Eleven men with T2DM underwent a randomized cross-over trial with either 2 weeks morning or afternoon HIIT (3 bouts/week), followed by a 2-week washout period and a subsequent period of the opposing training regime. Continuous glucose monitoring (CGM) based blood glucose levels were recorded throughout the study and hourly time-point means were compared. Blood samples were collected pre-training and after both training regimens. During week 1 of training, afternoon HIIT reduced CGM-based glucose levels as compared to either pre-training (mean change 0.9 mmol/l,  $p$ <0.05) or morning HIIT (1.1 mmol/l,  $p$ <0.05) at 4 and 10 time-points throughout the day, respectively. Conversely, during week 1 of training, morning HIIT increased CGM-based glucose levels as compared with pre-training (1.2 mmol/l,  $p$ <0.05) at 4 time-points throughout the day. During week 2 of training, afternoon HIIT reduced CGM-based glucose levels as compared to either pre-training (0.9 mmol/l,  $p$ <0.05) or morning HIIT (0.9 mmol/l,  $p$ <0.05) at 4 and 6 time-points throughout the day, respectively. Conversely, during week 2 of training, morning HIIT increased CGM-based glucose levels as compared with pre-training (0.9 mmol/l,  $p$ <0.05) at 2 time-points throughout the day. The elevated glucose concentration with morning HIIT persisted even into the subsequent days after exercise. Additionally, plasma T<sub>4</sub> was decreased and TSH was increased following the training as compared with pre-training, suggesting a hormonal mechanism behind the differing responses, as these changes were potentiated following afternoon HIIT. Plasma triglycerides levels were elevated following morning HIIT as compared with pre-training, suggesting changes in fuel utilization with morning training. This was a field-based study in 'free-living' individuals, thus the specific factors responsible for the differing blood glucose levels between morning and afternoon exercise remain to be elucidated. Afternoon HIIT is more efficacious than morning HIIT in controlling blood glucose levels in men with T2DM whereas morning HIIT may even be deleterious for optimal blood glucose control. Our data highlight the importance of optimizing the timing of exercise bouts to improve glycaemic control in people with T2DM.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

C039

### Skeletal myogenesis can be driven by key growth factors contained in human platelets.

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**Introduction:** Platelet-based applications offer a potentially straightforward, cost-effective and safe, autologous method of delivering growth factors to injured tissue, in an attempt to promote regeneration. However, there is currently contrasting evidence on the role of platelets as biomaterials in skeletal muscle injuries. Given that the molecular mechanisms involved in platelet-mediated regeneration are not well established, the aim of this study was to determine the effect of platelet releasate on skeletal myogenesis *in vitro* and muscle stem cells *ex vivo*.

**Methods:** We studied the effect of platelet releasate on proliferation and differentiation of C2C12 myoblasts, single muscle fibres and isolated satellite cells by means of cell proliferation assays, immunohistochemistry and gene expression. Moreover, we expanded *in vitro* cell culture findings on by establishing the effect of human platelet releasate on murine skeletal muscle stem cells using protein expression profiles for key myogenic regulatory factors.

**Results:** We show that platelet releasate increases myoblast proliferation in a dose-dependent manner. The proliferative effects were prevented by pharmacological inhibition of vascular endothelial growth factor- (VEGF) and/or platelet-derived growth factor- (PDGF) receptors. The cell culture findings were validated with primary skeletal muscle stem cells, where we observed that platelet releasate upregulated MyoD and Scrib expression on and promoted their myogenic potential through the VEGFR and PDGFR-mediated pathways. Application of platelet releasate can either attenuate fusion of myoblasts into myotubes or promote terminal myogenic differentiation in a temporal manner, indicating that the timing of releasate application is crucial. Key components of the platelet releasate were identified by immuno-based assays.

**Conclusion:** Taken together, *in vitro* and *ex vivo* data provide supporting evidence of a positive effect of platelet releasate on myoblast proliferation, with temporal effects on cell differentiation. The effects are mediated through the VEGF and PDGF signalling pathways. Platelet-based methodologies aiming to provide autologous biomaterials have implications for regenerative medicine and muscle healing.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

C040

### The influence of high altitude exposure on sympathetic baroreflex function in humans: a comparison of lowlanders and Nepalese Sherpa

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Muscle sympathetic neural activity (MSNA) contributes to blood pressure regulation. Microneurographic studies have shown sustained high altitude hypoxia is accompanied by heightened MSNA; however, the influence on baroreflex control of MSNA and blood pressure remains unknown. We investigated sympathetic baroreflex function in 10 lowlanders and 7 Nepalese Sherpa. Lowlanders were studied at low altitude (LA; 344m) and high altitude (HA; 5050m), with Sherpa only studied at HA. Indices of sympathetic baroreflex function (i.e. 'operating pressure', 'set point' and 'gain') were determined from beat-by-beat changes in diastolic blood pressure (DBP, photoplethysmography) and corresponding MSNA (microneurography) during supine rest, and during boluses of vasodilator sodium nitroprusside and vasoconstrictor phenylephrine (modified Oxford test). Values are means and 95% confidence intervals, compared using paired and unpaired T-tests. In lowlanders, the sympathetic baroreflex 'operating' DBP was unchanged at HA compared to LA (71 [64-78] vs 68 [64-73] mmHg,  $P=0.56$ ); however, the corresponding MSNA burst frequency (29 [22-36] vs 10 [7-12] bursts/min,  $P=0.0004$ ) and MSNA burst incidence (i.e. 'set point,' 45 [34-56] vs 18 [13-24] bursts/100HB,  $P=0.0007$ ) were increased. Sympathetic baroreflex gain was unchanged (-2.6 [-1.8- -3.5] vs -2.3 [-1.8- -2.8] %/mmHg,  $P=0.30$ ). In lowlanders, breathing 100% oxygen for 5 minutes at HA ( $n=9$ ), to silence peripheral chemoreceptor drive, did not influence DBP (74 [66-82] vs 72 [65-79] mmHg,  $P=0.22$ ), MSNA burst frequency (26 [18-35] vs 30 [22-38] bursts/min,  $P=0.33$ ), MSNA burst incidence (45 [33-57] vs 44 [31-56] bursts/100HB,  $P=0.78$ ), or sympathetic baroreflex gain (-2.5 [-1.7- -3.2] vs -2.8 [-2.0- -3.6] %/mmHg,  $P=0.16$ ). Compared to lowlanders at HA, Sherpa had a similar baroreflex operating DBP (66 [59-73] mmHg,  $P=0.28$ ); however MSNA burst frequency (22 [12-33] bursts/min,  $P=0.05$ ) and MSNA burst incidence (30 [17-43] bursts/100HB,  $P=0.02$ ) were lower. Sympathetic baroreflex gain was similar (-2.6 [-1.8- -3.4] %/mmHg,  $P=0.94$ ) to lowlanders at HA. Our data show that sustained HA exposure in lowlanders is accompanied by an upward resetting of the sympathetic baroreflex, with no difference in the operating pressure, or the responsiveness of MSNA to changes in blood pressure. In Sherpa, the sympathetic baroreflex set point is lower, with a similar operating pressure and reflex responsiveness, compared to lowlanders at HA. Furthermore, the inability of acute hyperoxia to influence sympathetic baroreflex set point, suggests that mechanisms other than the peripheral chemoreflex could play a role in baroreflex resetting in lowlanders at HA. In conclusion, sustained HA exposure modifies sympathetic baroreflex function in lowlanders; however, the underlying mechanism(s) remain to be delineated.

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C041

### The influence of myostatin on sexual dimorphism of human skeletal muscle

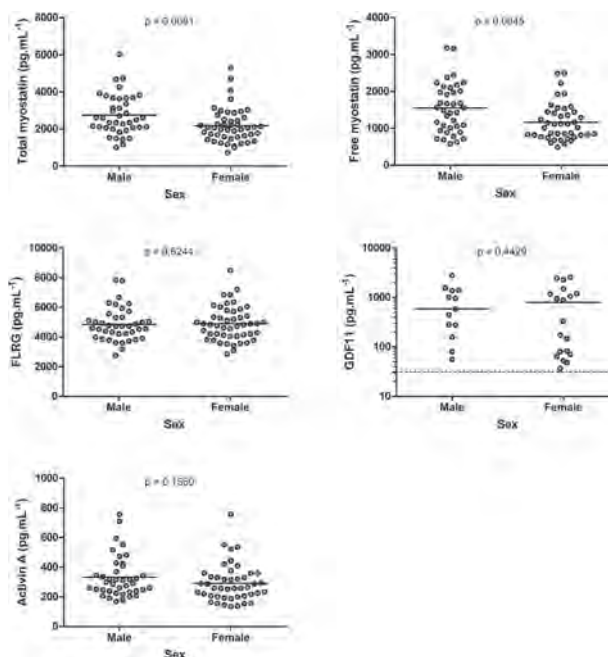
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Sexual dimorphism in the adult human is partially characterised by larger skeletal muscle mass in the male compared to the female (Gluckmann, 1974), which is in part controlled by post-pubertal differences in circulating testosterone, growth hormone and insulin-like growth factor. Myostatin is a muscle-specific negative regulator of muscle mass (McPherron et al., 1997), acts in an endocrine manner, and can be inhibited by circulating peptides, including follistatin related gene protein (FLRG). Whilst animal studies have suggested differences in myostatin content (McMahon et al., 2003) that may be linked to growth hormone activity (Oldham et al., 2009), the role of myostatin in human sexual dimorphism has yet to be investigated. Thus, we aimed to investigate the existence of sex related differences in myostatin levels in humans, as a potential regulator of sexual dimorphism of skeletal muscle. Following written informed consent,  $n = 88$  (39 males, 49 females; sex self-declared) healthy individuals (age range 18-68 years) took part. Participants attended in a rested fasted state. Body composition was measured by stadiometer and air volume displacement. Venous blood samples were collected and immediately centrifuged and with plasma aliquots frozen at  $-80^{\circ}\text{C}$ . Plasma samples were analysed in triplicate by ELISA for total and free myostatin, FLRG and growth and differentiation factor 11 (GDF11). Values are given here as 'mean $\pm$ SE', compared by unpaired t-test (total and free myostatin, FLRG and activin A) or Mann-Whitney (GDF11) as appropriate, with statistical significance set at  $p > 0.05$  throughout.

Males show greater muscle mass than females ( $57.59 \pm 2.03$  vs  $43.16 \pm 1.04$  kg,  $p < 0.001$ ). Total and free myostatin were significantly ( $p = 0.008$  and  $p = 0.005$  respectively) lower in females. Total myostatin was  $578.00 \pm 38.60$  pg.mL $^{-1}$  higher in males, Free myostatin was  $384.28 \pm 31.20$  pg.mL $^{-1}$  higher in males. This difference was no longer present when normalised to fat free mass. No other endocrine factors measured were significantly different between sexes in their absolute concentrations. Similarly, to myostatin, Activin A and GDF11 were not significantly different between males and females, when normalised to FFM. However, FLRG was significantly ( $p = 0.004$ ) lower in males than females, when normalised to FFM.

Myostatin is a negative regulator of skeletal muscle growth and has been found in animal models to be differentially regulated between males and females, possibly accounting for mammalian sex-related differences in skeletal muscle growth. Whilst absolute myostatin (total and free) differed between sexes, normalization to FFM removed this difference, suggesting myostatin's effect on muscle mass is not sex dependent. Despite more FLRG per kg of muscle mass in females, female participants show reduced muscle mass, suggesting a sex dependent sensitivity to FLRG in the adult human.



Concentration of total myostatin, free myostatin, FLRG, GDF11 and Activin A (left to right, top to bottom, each pg.mL $^{-1}$ ) as a function of sex ( $n = 39$  males, 49 females). Individual data points as shown, horizontal lines indicate group means.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

C042

### MUSCLE MEMORY WITH A RAT CLIMBING MODEL

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Previous work from Prof. Kristian Gundersen's lab show that myonuclei obtained during hypertrophy are not lost after atrophy, and we hypothesized that these extra myonuclei aid in later muscle re-growth when re-introduced to hypertrophic stimuli (1). This mechanism is dubbed "muscle memory". Briefly, mice received a supraphysiological supplementation of the anabolic-androgenic steroid testosterone propionate (AAS), leading to an increased myonuclear number and muscle growth. After a washout period without AAS, muscle size was back to baseline, but the myonuclear number was still elevated. When subjected to a second hypertrophic stimulus by overloading the lower leg muscles by synergist ablation, muscles previously treated with AAS had a much more pronounced growth than controls without the history



of AAS treatment. We hypothesize that the muscle memory mechanism is important also during more physiological relevant training, detraining and retraining. Thus, the aim of the present study was to test the muscle memory hypothesis with a rat climbing model.

4-week-old male Sprague Dawley rats were housed in cages with the inner walls and ceiling covered with a climbing mesh, and the food supply in the middle of the ceiling. Thus, the rats had to climb in order to feed and were thereby trained (1. training). After 5 weeks in the climbing cage, rats were housed in normal cages for 10 weeks (detraining). Age-matched rats in standard cages were used as ground controls. After detraining, both detrained and ground-control rats were subjected to one of two different retraining regimes: 1) 2 weeks in climbing-cages or 2) 2 weeks overload of soleus by synergist tenotomy of gastrocnemius in order to maximize the hypertrophic stimuli and thereby potential differences between experimental groups. Rats were sedated with 2-3% isoflurane in air for the tenotomy and terminal operations. The soleus and tibialis anterior muscles were frozen in melting isopentane in liquid nitrogen, cryosectioned, stained with the myonuclei-specific antibody PCM1 (2), dystrophin antibody to visualize the fiber border and DAPI to visualize all nuclei, followed by analysis of myonuclear number and myofiber cross-sectional area.

The 1. training led to an increase in both myonuclear number and myofiber cross-sectional area of both the soleus and tibialis anterior muscles compared to ground-controls. After detraining, cross-sectional area was back to the level of the ground-control, but the myonuclear number was still elevated. Preliminary results indicate that retrained rats had a stronger hypertrophic response compared to age-matched controls performing the training for the first time.

Our results support the muscle memory hypothesis under more training-relevant conditions, indicating that an active lifestyle as juvenile might be beneficial for responses to hypertrophic stimuli later in life due to an increased myonuclear number retained in adulthood.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

#### C043

### Two popular high intensity interval training protocols elicit similar health benefits in a controlled but real world environment.

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**Introduction:** Currently 40% of the UK do not meet the physical activity guidelines with a 'lack of time' the most commonly cited barrier to sufficient physical activity. In laboratory based training interventions, high intensity interval training (HIT) offers a time-efficient alternative to moderate intensity continuous training (MICT), but its success requires expensive specialised cycle ergometers and vigorous encouragement from the researchers. Furthermore, over 100 different HIT protocols have been investigated, potentially leading to

confusion within the literature and a misleading public health message.

**Aim:** To investigate whether two popular HIT protocols, performed using readily available cycle ergometers and without encouragement improve aerobic exercise capacity, arterial stiffness, glycaemic control and body composition.

**Methods:** Seventy nine sedentary males (n=26) and females (n=53) aged 18-65 participated ( $28 \pm 1$  y, BMI  $25 \pm 0.4$  kg.m<sup>-2</sup>) in the study. In a randomised cross-over design, participants completed either 6 weeks of 30HIT (4-8x30s sprint with 120s active recovery) or 60HIT (6-10x60s sprint with 60s active recovery). Participants then completed a 4-week washout period before completing the alternative intervention. Training sessions were completed on a Wattbike, 3 times per week.  $VO_{2peak}$ , body composition (DXA), glycaemic control (oral glucose tolerance test (OGTT)) and arterial stiffness (aortic pulse wave velocity (aPWV)) were assessed pre and post each 6-week training phase.

**Results:**  $VO_{2peak}$  increased post intervention in 30HIT ( $36 \pm 1$  to  $39 \pm 1$  ml.min<sup>-1</sup>.kg<sup>-1</sup>) and 60HIT ( $36 \pm 1$  to  $39 \pm 1$  ml.min<sup>-1</sup>.kg<sup>-1</sup>) ( $P < 0.001$ ). Body fat percentage decreased pre to post training in 30HIT ( $1.0 \pm 0.5\%$ ) and 60HIT ( $1.7 \pm 0.6\%$ ) ( $P = 0.002$ ). Arterial stiffness improved post intervention, aPWV decreased in 30HIT ( $5.9 \pm 0.2$  to  $5.7 \pm 0.1$  m/s) and 60HIT ( $5.9 \pm 0.1$  to  $5.71 \pm 0.1$  m/s) ( $P < 0.003$ ). During the OGTT there was a trend towards a reduction in glucose area under the curve pre to post ( $P = 0.083$ ). No differences between the intervention groups were observed for any variable ( $P > 0.05$ ).

**Conclusion:** 6 weeks of 30HIT and 60HIT induce comparable improvements in aerobic capacity, body composition and arterial stiffness. Importantly, these favourable improvements were achieved using self-selected exercise intensities and without researcher encouragement. Therefore, this data suggests that either 30 or 60 second interval interventions could be used to improve health outcomes in a sedentary population in the real world.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

#### C044

### Altered mTOR localization is associated with decreased protein synthesis and loss of body mass in experimental cancer cachexia

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Cancer cachexia is a debilitating condition associated with marked loss of muscle mass, decreased tolerance to cancer therapies and increased mortality.<sup>1</sup> However, the molecular mechanisms of cachexia-associated muscle wasting are still not fully understood. The mechanistic Target of Rapamycin (mTOR) is one of the key proteins involved in the regulation of muscle protein synthesis. Its localization at lysosomes/late-endosomes has been associated with its activation and possibly also with growth regulation in skeletal muscle.<sup>2</sup> The

aim of this study was to investigate the mechanisms of muscle wasting and its prevention by activin receptor ligand blocking in experimental cancer cachexia.

BALB/c mice were anaesthetised with a mixture of ketamine (60–70 mg/kg, i.p.) and xylazine (9 mg/kg, i.p.) and subcutaneously inoculated with C26 cancer cells (n = 15) or vehicle control (n = 9).<sup>3</sup> Half of the tumour-bearing mice were treated with soluble activin receptor type IIB (sACVR2B-Fc, 5 mg/kg, i.p., twice a week, n = 8) to block myostatin and activins and thus to prevent cancer-induced muscle loss.<sup>3</sup> Tibialis anterior (TA) muscles were collected 11 days after cancer cell inoculation. Protein synthesis was analysed using SUNSET method.<sup>4,5</sup> The colocalization of mTOR and LAMP2 was analysed by immunofluorescence from TA cryosections. C26 and sACVR2B-Fc effects were analysed by Students t-test and correlations by Pearson correlation coefficient. Statistical significance was set at  $P < 0.05$ .

C26 cancer resulted in reduced colocalization of mTOR with LAMP2, a marker of lysosomes/late-endosomes, indicating decreased mTOR activity based on previous studies<sup>2</sup> and supported by our Western blot results of markers of mTOR signaling (S6K1 and rpS6 phosphorylation). Further supporting the role of mTOR localization in cachexia, mTOR-LAMP2 colocalization also correlated with protein synthesis ( $r = 0.751$ ;  $P < 0.01$ ) and with the body mass change of the last day of the experiment ( $r = 0.630$ ;  $P < 0.01$ ) in untreated mice. Interestingly, muscle mass and mTOR localization were restored by sACVR2B-Fc treatment despite unaltered protein synthesis at this time-point. This discordance may be related to the time-point studied and to observed decreases in food intake and physical activity in the tumour-bearing mice.

These results show that cancer-induced muscle wasting is associated with reduced mTOR localization to lysosomes/late-endosomes resulting potentially in attenuated mTOR activation. This provides new insights into the molecular mechanisms underlying decreased protein synthesis and muscle wasting in cancer cachexia. These results also open up new research questions and possible treatment options for cachexia.

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This work was supported by the Academy of Finland, Cancer Society of Finland, and Jenny and Antti Wihuri Foundation.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

C045

## Mechanisms underlying increases in $\text{Ca}^{2+}$ leakage from sarcoplasmic reticulum with prolonged low-frequency force depression in rat skeletal muscle

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We have shown that in fast skeletal muscles,  $\text{Ca}^{2+}$  release of the sarcoplasmic reticulum (SR) was markedly reduced in the early stage of recovery from prolonged low-frequency

force depression (PLFFD), which is defined as a grater loss of force at low than at high stimulation frequencies (1). The contents of  $\text{Ca}^{2+}$  released from the SR depend largely on the  $\text{Ca}^{2+}$  contents contained in the SR lumen (2) that are primarily determined by the balance between uptake and leak of the SR. The purpose of this study was a) to investigate changes in the  $\text{Ca}^{2+}$  uptake and leak properties of the SR in the early stage of recovery from PLFFD and b) to identify the mechanism(s) underlying the changes if recognized. All experimental procedures were approved by the Animal Care Committee of Hiroshima University. Under an anesthesia using the mixture of medetomidine, midazolam and butorphanol with appropriate ratio (3), gastrocnemius muscles from Wistar male rats (8–9 weeks old) were electrically stimulated via the sciatic nerve at 70 Hz until the force was reduced to ~50% of the initial force (fatiguing stimulation) and then allowed to rest for 30 min. Afterwards, mechanically skinned fibres and SR microsomes were prepared from the superficial regions of the gastrocnemius muscles and were used for the assay of SR  $\text{Ca}^{2+}$  uptake and leak properties. The contralateral muscles were used as controls. The obtained values ( $\pm$  SE) were tested by t-test and two-way ANOVA as required. The major results of this study are shown in table 1 indicating that fatiguing stimulation, leading to PLFFD, brought about significant increases in  $\text{Ca}^{2+}$  leak, but no changes in  $\text{Ca}^{2+}$  uptake.  $\text{Ca}^{2+}$  leak was alleviated to the same extent in control and stimulated fibres by treatment with 20  $\mu\text{M}$  2,5-ditert-butylhydroquinone (TBQ: SR  $\text{Ca}^{2+}$ -ATPase inhibitor) suggesting the increased  $\text{Ca}^{2+}$  leakage through the ryanodine receptor (RyR). Conversely, in SR microsome, TBQ-induced  $\text{Ca}^{2+}$  leak is reduced in stimulated muscle ( $46 \pm 5$  for control vs.  $26 \pm 4$  nmol/min/g wet wt for stimulated microsome,  $n=7$  for each group,  $P < 0.05$ ). With regard to the conflicting results, the dihydropyridine receptor (DHPR) might be involved in stimulation-related changes in the SR  $\text{Ca}^{2+}$  leak properties. To examine this point, the  $\text{Ca}^{2+}$  leak properties were measured in the solutions replacing all  $\text{K}^+$  with  $\text{Na}^+$  where the voltage sensor was inactivated. Under this condition, the relative contents of  $\text{Ca}^{2+}$  leaking out of the SR in stimulated fibres approximated to those in control fibres (Fig. 1). These results indicate that in the early stage of recovery from PLFFD, the contents of  $\text{Ca}^{2+}$  leaking through the RyR are increased and suggest that increased SR  $\text{Ca}^{2+}$  leak, which presumably involves the DHPR, is responsible, at least partly, for decreased SR  $\text{Ca}^{2+}$  release that occurs in fatigued skeletal muscles.

Table 1. Main results of this study

| Measurements                       | Samples       | Indicators (units)                                        | Values              |                       |
|------------------------------------|---------------|-----------------------------------------------------------|---------------------|-----------------------|
|                                    |               |                                                           | Control             | Stimulated            |
| Force at low frequency             | GAS muscle    | Torque at 20 Hz (mN)                                      | $95.3 \pm 3.8$ (11) | $49.8 \pm 4.0^*$ (11) |
| SR $\text{Ca}^{2+}$ uptake ability | Skinned fibre | Time to 50% of 120-s loaded area (sec)                    | $22.1 \pm 3.0$ (9)  | $34.1 \pm 1.6$ (12)   |
| SR $\text{Ca}^{2+}$ leak ability   | Skinned fibre | Remaining $\text{Ca}^{2+}$ (% of rest-leak response area) | $45.6 \pm 4.5$ (7)  | $30.6 \pm 3.4^*$ (7)  |

The number of samples were indicated in the brackets. Values are means  $\pm$  SE. The normality of the data sets was examined by Shapiro-Wilk normality test and all data sets showed normal distribution. The statistically significance was examined by two-tailed t-test.  $^*P < 0.05$  vs. control. GAS, gastrocnemius; SR, sarcoplasmic reticulum

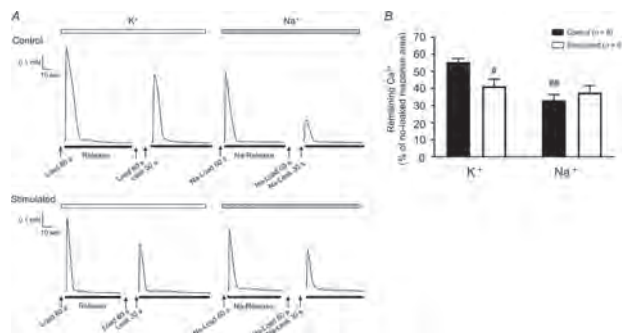


Fig. 1.  $\text{Ca}^{2+}$  leakage of sarcoplasmic reticulum (SR) with inactivation of the voltage sensor.

A: typical examples of force response. The time integral of force response (response area) reflects remaining SR  $\text{Ca}^{2+}$  content with leak treatments. B: remaining SR  $\text{Ca}^{2+}$  content. Values ( $\pm$  SE) were expressed as a percentage of response area without leak treatment in each ion composition.  $n$  shown in bracket indicates the number of fibres for each case. The normality of the data sets was examined by Shapiro-Wilk normality test and all data sets showed normal distribution. Two-way ANOVA for repeat measures was used for examining statistically significance. The “#” shows the significant difference between control and stimulated fibres in  $\text{K}^+$  solutions ( $P < 0.05$ ). The “###” shows the significant difference between  $\text{K}^+$  and  $\text{Na}^+$  solutions in control fibres ( $P < 0.05$ ).

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

C046

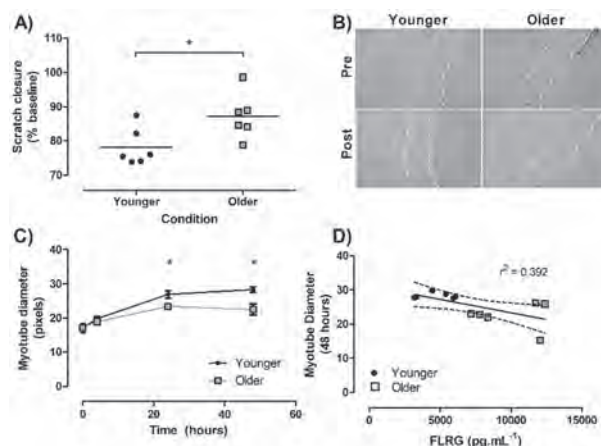
### Differing effects of younger and older human plasma on C2C12 myocytes *in vitro*

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Ageing is associated with a reduction of physiological function including muscle mass and strength. Endocrine factors such as myostatin, activin A, growth and differentiation factor 11 (GDF-11) and their inhibitory peptides influence muscle mass in health and disease. We hypothesised that myocytes cultured in plasma from older and younger individuals would show an ageing effect. Participants provided written, informed consent prior to participation. C2C12 myoblasts were stimulated with media conditioned with 5% plasma from healthy male participants (younger [ $n = 6$ , 18 – 35 years of age]; older [ $n = 6$ , > 57 years of age]). Plasma myostatin (total and free), follistatin-like binding protein (FLRG), GDF-11 and activin A were quantified by enzyme-linked immunosorbent assay, and all results are given as mean [SE]. FLRG and activin A were elevated in older individuals (FLG 4731.6 [522.5] vs 9915.8 [975.1] pg.mL<sup>-1</sup>; activin A 215.9 [20.2] vs 291.6 [21.0] pg.mL<sup>-1</sup>), whilst myostatin (free and total) and GDF-11 were not. Myoblasts *in vitro* showed no difference in proliferation rate between group, however scratch closure was greater in younger vs older plasma stimulated myoblasts (78.2 [2.2] %

vs 87.2 [2.7] % of baseline scratch diameter, respectively). Myotube diameters were larger in cells stimulated with younger plasma than with older at 24 and 48 hours, but not at 4 hours. A significant negative correlation was noted between *in vivo* plasma FLRG concentration and *in vitro* myotube diameter 48 hours following plasma stimulation ( $r^2 = 0.392$ ,  $p = 0.030$ ). Here we show that myoblasts and myotubes cultured in media conditioned with plasma from younger or older individuals show an ageing effect, and further this effect moderately correlates with circulating FLRG concentration *in vivo*. The effect of ageing on muscle function may not be innate to the tissue, but involve a general endocrine environment change. Further work is needed to examine the effect of increased FLRG concentration on muscle function in ageing populations.



Ex vivo plasma from older individuals reduces myocyte potential *in vitro*. A) Scratch injury recovery (% of baseline). B) representative scratch images. C) Myotubes diameter at 4, 24 and 48 hours post stimulation. D) correlation between plasma FLRG *in vivo* (pg.mL<sup>-1</sup>) and myotube diameter change (48 hours) *in vitro*.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

C047

### Identification and characterisation of the senescent phenotype of human primary myogenic precursor cells

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Cellular senescence, and the senescence associated secretory phenotype (SASP), are believed to contribute to the physiological ageing process(1). However, their role within skeletal muscle, a highly regenerative tissue which shows a decline in mass and function with age, is less clear. Inducing replicative senescence in cultured human primary muscle precursor cells (MPCs) is challenging due to i) the amount of time in culture and ii) the likelihood that even cultures with initially high myogenic purity can become overrun with fibroblast cells(2). To overcome these limitations, senescence can be induced using the chemotherapy drug, Doxorubicin(3), but its effect on inducing senescence in human MPCs is unknown. The aim of the present study was to investigate the characteristics of human primary MPCs when senescence was induced via two different approaches: i) passaging to replicative senescence (RS) and ii) 24 hours of Doxorubicin (DOX, 0.2  $\mu\text{M}$ )



treatment. Both approaches were conducted on the same MPC populations isolated from muscle biopsy samples obtained from young healthy male volunteers ( $n=6$ ,  $22\pm 1$  years). Cumulative population doublings (CPD), the presence of senescence-associated  $\beta$ -galactosidase (SA  $\beta$ -gal) and the expression of p16 together with the absence of proliferation marker, Ki67, were monitored to determine cell senescence. Additionally, mRNA levels of established SASP factors (PAI-1, TGF- $\beta$ 1, IL-8 and IGFBP-3) were measured. Three of the six MPC populations maintained myogenic purity through to RS, which was reached at  $25.8\pm 1.9$  CPD. In RS there was a trend for increased p16 expression ( $n=3$ , fold change= $2.2\pm 1.0$ , ns), decreased Ki67 expression ( $n=3$ , fold change= $0.4\pm 0.1$ ,  $p=0.01$ , paired t-test) and increased number of SA  $\beta$ -gal-positive MPCs ( $n=2$ , 75% increase), compared to early passage. Over 35 days after DOX treatment, MPCs showed 99-100% of cells positive for SA  $\beta$ -gal ( $n=2$ ). There was increased p16 expression ( $n=6$ , fold change= $1.6\pm 0.3$ ,  $p<0.05$ , Dunnett's test) and decreased Ki67 expression ( $n=6$ , fold change= $0.1\pm 0.0$ ,  $p<0.05$ , Dunnett's test) in DOX-treated MPCs, compared to pre-DOX treatment. MPC populations which reached RS with maintained myogenic purity were used for comparison against paired DOX-treated MPCs for analysis of SASP factors ( $n=3$ ). Here PAI-1 and IGFBP-3 showed significantly higher mRNA expression levels following RS compared to early passage and DOX treated MPCs ( $p<0.05$ , RM ANOVA). DOX-treated MPCs were not significantly different to early passage for any SASP factor. In conclusion, we have characterised the replicative senescence and DOX-induced senescence phenotype in MPCs. Replicative senescent MPCs showed increased SASP factor expression, whereas DOX-treated senescent MPCs did not. Further analysis should determine the similarities of these two models as regards to their senescent phenotype, as well as whether these models reflect *in vivo* ageing in skeletal muscle.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## C048

### Specific cleavage of the titin springs in situ uncovers titin's role in sarcomere structure and active muscle contraction

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Background. The giant protein titin contributes to muscle force generation. However, titin's mechanical function in muscle is still incompletely understood, in part because no tool has been available to specifically cleave the titin springs in the sarcomere, e.g., during mechanical measurements.

Methods and Results. In a new genetic mouse model, a tobacco etch virus (TEV) protease-recognition site and a HaloTag were cloned into elastic I-band titin. The HaloTag-TEV cassette allowed for in-situ imaging of titin, specific proteolysis during myofibre mechanics and visualisation of successful cleavage on protein gels or tissue sections. Using permeabilized skeletal myofibre bundles dissected from mice homozygous for HaloTag-TEV titin (knock-in; KI), we measured passive force over the sarcomere-length (SL) range 2.2-3.4  $\mu$ m and maximum  $\text{Ca}^{2+}$ -triggered force (pCa 5.0) at 2.6  $\mu$ m SL, in the absence or presence of TEV enzyme. Wildtype (WT) myofibres treated with TEV protease served as controls. Incubation of the skinned myofibers with TEV protease cleaved HaloTag-TEV titin specifically and completely in <1/2 hour (room temperature), but had no effect on other proteins or on titin in WT myofibres, as detected by loose protein gel electrophoresis. Titin cleavage barely affected myofibre ultrastructure, measured by electron microscopy, in the absence of a stretch or  $\text{Ca}^{2+}$ -activation. However, mechanical stressing of TEV protease-treated KI fibres caused sarcomere destabilization and myosin-filament disarray. Interestingly, the Z-discs and M-bands were also affected, getting out of alignment and appearing staggered. In psoas and soleus fibre bundles, passive tension dropped with titin cleavage, on average by ~80% and ~60%, respectively, the remaining tension being attributable to extracellular-matrix proteins, such as collagen. Active force development at pCa 5.0 was reduced, on average by ~50%, in titin-cleaved psoas and soleus fibres, compared to controls. Large inter-sample variability in the proportion of active-force reduction was due to heterogeneity in sarcomere structural preservation.

Conclusion. The HaloTag-TEV mouse allows, for the first time, a direct quantitation of titin's contribution to passive and active forces in muscle. Our findings show that intact titin springs are not only necessary for most of the passive tension generation of skeletal muscles, but also for the regular structure of all sarcomere bands and for maintaining high active  $\text{Ca}^{2+}$ -dependant force.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## C049

### Effect of physical activity on telomere length in elderly subjects

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Background: Physical activity (PA) has been shown to alleviate the telomere shortening (1). Suggested mechanisms include inflammation control and reduction of oxidative stress through exercising (2). The effect of PA intensity on telomere length is unclear suggesting protective as well as damaging effects (3).

The aim of this study is to determine the influence of different intensities of PA and PA history on telomere length in a cohort of elderly subjects.

Methods: 700 human subjects born in Oulu in 1945 (Oulu Cohort 1945) were studied (296 male and 404 female; 68

to 70-years old). DNA was isolated from frozen whole blood using Macherey-Nagel Genomic DNA and quantified using Nanodrop. Relative telomere length (RTL) was determined using a qPCR-based method developed by Cawthon (4,5). PA was recorded with wrist-worn activity meter (Polar Active) from a 2-week period. A questionnaire was used to collect sedentary time, PA intensity and PA history. Statistical analyses were made with IBM SPSS Statistics 21. Pearson correlation coefficient was used to determine correlations and Mann-Whitney test to determine differences between classes. Data was divided into three groups according to age for analysis. Results: Women had longer RTL than men ( $p=0.031$ ). Age was negatively correlated with RTL in men and women ( $-0.210$ ,  $p=0.000$  and  $-0.174$ ,  $p=0.000$ ). Women took significantly more steps during the 2-week period than men ( $p=0.001$ ) but mean steps taken during the 2-week period significantly correlated with RTL only in men ( $p=0.05$ ). In 70-year old subjects RTL was significantly correlated with total steps taken ( $0.202$ ,  $p=0.04$ ) and sedentary time ( $-0.247$ ,  $p=0.007$ ). Moderate PA was the only intensity able to induce significant changes between the quartiles,  $0.023$  between 4<sup>th</sup> and 1<sup>st</sup>,  $0.04$  between 4<sup>th</sup> and 2<sup>nd</sup> and  $0.027$  between 4<sup>th</sup> and 3<sup>rd</sup> (Figure 1). The effects of moderate PA were only visible in 70-year old subjects. Conclusions: Moderate physical activity has telomere protecting effects in old age. However, only the subject's current PA level is important and no differences were found concerning the PA history. Within two years, there is a difference in RTL, which suggests that the telomere attrition rate accelerates in older age. Women had longer RTL. Women took more steps, but in men the mean steps were significantly correlated with RTL. The observed gender difference can partially be explained by the traditional gender roles and differences in health observation and supplementation.

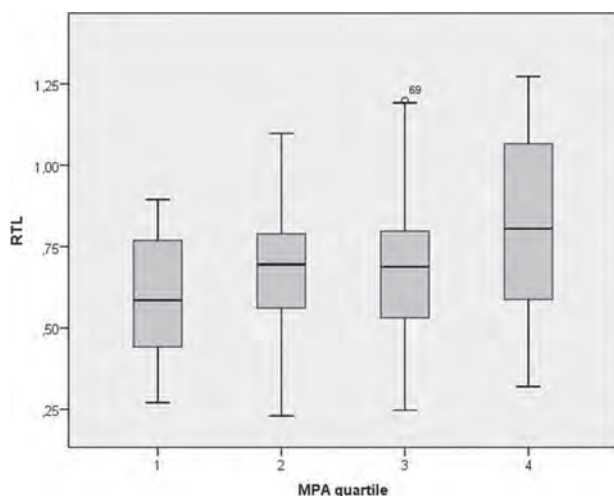


Figure 1. Moderate PA quartiles and RTL. Quartile 1 on the left with the least amount of moderate PA in a week and quartile 4 on the right with the most.

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C050

## Two-weeks of time-efficient high-intensity interval training (HIIT) does not appear sufficient to elicit cardiorespiratory fitness gains in older adults

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### BACKGROUND:

Declines in cardiorespiratory fitness (CRF) and physical function with advancing age are both associated with increased morbidity and mortality *per se* (1), as well as in the peri-operative period (2). Given the increasing number of older adults presenting for surgery (3), and the time-constraints often imposed on the preoperative period, time-efficient high-intensity interval training (HIIT) may be a promising surgical 'prehabilitation' tool (4). However, whether a HIIT protocol that has been shown to induce meaningful increases in CRF in older adults in 4-weeks, can elicit improvements in CRF and physical function in just 2-weeks; a common time-to-treatment window for a number of major intra-abdominal surgeries, is not yet known.

### METHODS:

Seven (3 male: 4 female) independent community-dwelling volunteers aged 73 (70-78) years, with no co-morbidities that excluded them from exercise testing in accordance with the American Thoracic Society (ATS) Cardiopulmonary Exercise Test (CPET) Guidelines (5), undertook six sessions of time-efficient reduced-intensity HIIT over 2-weeks. Each session lasted 16.5 minutes, including five 1-minute efforts at 100-110% watt max. CPET was used before and after the HIIT intervention to assess CRF and exercise performance, with timed up-and-go (TUG) used to assess global physical function. RESULTS:

Neither anaerobic threshold ((AT);  $12.11 \pm 1.83$  vs.  $12.73 \pm 2.84$  ml/kg/min,  $p=0.62$ ) or  $\text{VO}_2\text{peak}$  ( $21.5 \pm 4.2$  vs.  $22.8 \pm 4.0$  ml/kg/min,  $p=0.26$ ) significantly increased following our HIIT intervention, despite over half of our volunteers achieving the previously reported threshold for a clinically meaningful improvement in AT ( $>1.5$  ml/kg/min). There was no relationship between baseline CRF and magnitude of change for either AT ( $r^2=0.2$ ,  $p=0.32$ ) or  $\text{VO}_2\text{peak}$  ( $r^2=0.15$ ,  $p=0.40$ ). Despite the lack of significant improvement in CRF parameters, exercise performance ( $856 \pm 130$  vs.  $973 \pm 132$  seconds,  $p=0.03$ ) and global physical function increased significantly after HIIT (TUG:  $7.38 \pm 1.53$  vs.  $6.13 \pm 1.65$  seconds,  $p=0.005$ ). No adverse events were encountered during the HIIT, with volunteers reporting high levels of acceptability and enjoyment.

### CONCLUSION:

Despite our well-tolerated, time-efficient HIIT programme being able to improve the CRF of older adults in a 4-week period, 2-weeks does not appear to be a long enough duration to elicit comparable gains. Despite these findings, 2-weeks of our HIIT programme can improve aspects of physical function in older adults, and as such has the potential to improve post-operative outcomes. In addition, further research in a larger cohort is required to determine the true utility of this intervention for improving CRF in older adults.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## C051

### The effect of lifelong physical activity on vascular function in postmenopausal women.

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**Introduction:** Cardiovascular function is impaired with aging and, in women, this effect of aging accelerates after the menopausal transition, due to the loss of estrogen. The favorable effect of physical activity on vascular function is well documented in men, but in postmenopausal women the effect has been suggested to be low or absent. The purpose of the present study was to investigate how lifelong physical activity reduces the age- and menopause related reduction in vascular function in women.

**Methods:** Thirty-seven healthy postmenopausal women (61±0,7 years) were included in the study and divided into three groups with different degree of physically active lifestyle. 1) Inactive physically lifestyle (LOW; n=12), 2) moderate physically active lifestyle (MOD; n=11), and 3) very physically active lifestyle (HIGH; n=14), with 245, 411 and 725 METS/day respectively on average over the last 20 years.

We examined vascular function, assessed by vasodilator response to intra-arterial infusion of *acetylcholine*, the prostacyclin analog *epoprostenol* and nitric oxide donor *sodium nitroprusside* (SNP) into the femoral artery. Arterial blood pressure and blood flow were measured continuously and arterial and venous blood samples were collected. Leg vascular conductance (LVC) was calculated. In addition, muscles biopsies were obtained from m. vastus lateralis and are analyzed by Western blot.

**Results:** At baseline weight, BMI and body fat % of HIGH was lower than MOD and LOW. MAP was 20% higher in LOW compared with MOD and HIGH. *Vascular function:* The absolute change in LVC with acetylcholine infusion was larger (p<0,05) in HIGH than in MOD and LOW, (2,5±0,2, 1,9±0,3 and 1,5±0,3 mL min<sup>-1</sup> mmHg<sup>-1</sup> kg<sup>-1</sup>, respectively). In response to infusion of epoprostenol HIGH and MOD showed a larger (p<0,05) change in LVC compared with LOW, (0,7±0,1, 0,6±0,1 and 0,5±0,1 mL min<sup>-1</sup> mmHg<sup>-1</sup> kg<sup>-1</sup> respectively). The increase in LVC with SNP infusion was larger (p<0,05) in HIGH compared with LOW (1,0±0,1 and 0,6±0,1, respectively) but the change in LVC in MOD was not different from either group. Analyses of plasma and muscles biopsies are ongoing and will be presented.

**Conclusion:** The present study indicates, that women with a very active lifestyle have a better vascular function, compared with women with a moderate or inactive lifestyle. Women with a physically moderate active lifestyle show a better response to prostacyclin and have lower blood pressure than women with an inactive lifestyle. The results imply that both a moderate and very active lifestyle is beneficial but also that a greater training load/intensity through life leads to better vascular function in postmenopausal women. This study expands the current knowledge on the favorable effect of physical activity on the vascular system in the aging woman and is useful for lifestyle recommendations.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## C052

### The role of mitochondrial NDUFA4L2 in peripheral artery disease

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Skeletal muscle weakness is part of the pathophysiology of peripheral artery disease (PAD), which is a common comorbidity in patients with obesity and type II diabetes. The molecular mechanism behind PAD-induced muscle weakness is not yet fully understood. Here we elucidate the role of the hypoxia-sensitive nuclear-encoded mitochondrial NDUFA4L2 protein in PAD-induced skeletal muscle weakness. To this purpose unilateral femoral artery ligation (FAL), a mouse model of PAD, was induced in C57BL6 mice on a normal chow diet (ND) and in C57BL6 mice that were kept on a high-fat diet (HFD) for eight weeks prior FAL.

Anaesthesia was induced with 4% isoflurane prior to FAL and maintained with 2% isoflurane. All mice were sacrificed eight days post FAL by cervical disarticulation. In ND mice, NDUFA4L2 gene expression (t-test, p< 0.001, n=14) and protein expression (t-test, p< 0.01, n=11) were increased in quadriceps muscle from the FAL-leg as compared with the control leg (8 days post FAL). Moreover, the increase in NDUFA4L2 expression was associated with an ~50% reduction in muscle force production over a wide range of stimulation frequencies (20-120 Hz) in EDL and soleus muscle from the FAL leg as compared with the control leg (fold change=0.545, SE=0.072, p< 0.001, n=9-10). Both NDUFA4L2 protein and gene expression returned to baseline expression levels in muscle from the FAL-leg 30 days post FAL (n=6). The muscle force also returned to control levels 30 days post FAL (n=9). In HFD mice, the NDUFA4L2 gene expression and protein level was increased in the muscle from the FAL legs, yet the increase was significantly lower than in muscle from ND-FAL leg (8 days post FAL). Furthermore, the force production of EDL and soleus muscle from HFD-FAL legs were significantly lower than in the ND-FAL legs (EDL: fold change=0.013, SE=0.004, p<0.0001, n=6; soleus: fold change=0.120, SE=0.035, p<0.0001, n=6). Thus, the loss of force production was more severe in HFD-FAL muscle than in ND-FAL muscle.

In conclusion, FAL induced NDUFA4L2 gene and protein expression that correlated with the loss of muscle force in EDL and soleus muscle. Eight weeks of HFD prior FAL attenuated the recovery of force loss in skeletal muscles, potentially through inhibiting the activation of NDUFA4L2. Thus, implicating NDUFA4L2 as an integral player in the remodelling process associated with FAL.



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C053

### Exercise-induced fatigue is a consequence of exercise intensity and is independent of task power

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During constant-power cycle ergometry that exceeds the lactate threshold (LT), exercise-induced fatigue decreases the capacity of the exercising muscles to generate power (Cannon et al., 2011). The magnitude of task-specific, exercise-induced fatigue can be quantified using brief ( $\sim 6$  s) isokinetic measurements of maximal voluntary power before and immediately upon cessation of the exercise task (Cannon et al., 2011). During intermittent exercise, task power (power during work phases) can be dissociated from the exercise intensity (physiological and perceptual stress (Whipp, 1996)) (Davies et al., 2017). Thus, a greater task power can be performed during intermittent than intensity-matched constant-power exercise. As a higher task power likely increases recruitment of fatigue-susceptible muscle fibres (Hodson-Tole & Wakeling, 2008), we aimed to determine whether intermittent exercise increases the magnitude of exercise-induced fatigue compared to intensity-matched constant-power exercise.

Eight healthy participants ( $23 \pm 2$  yr) performed ramp-incremental cycle ergometry to the limit of tolerance to determine LT and peak oxygen uptake ( $VO_{2peak}$ ). Participants then completed (1) constant-power exercise and (2) intermittent exercise with work:recovery durations of 10:10 s. Task power for constant-power and intermittent exercise was predicted by a computational model (Benson et al., 2013) to evoke an oxygen uptake ( $VO_2$ ) that was 40% of the difference between LT and  $VO_{2peak}$ . Isokinetic maximal voluntary power was measured pre- and post-exercise at 80 rpm to determine the magnitude of the exercise-induced fatigue. Pulmonary gas exchange and heart rate were measured throughout. Blood lactate concentration and subjective ratings of dyspnoea and leg tiredness were obtained every 5 min and at the end of exercise.

Task power was greater during intermittent than constant-power exercise (mean  $\pm$  SD;  $296 \pm 98$  vs.  $169 \pm 54$  W;  $p < 0.01$ ). There was no difference between constant-power and intermittent exercise:  $VO_2$  ( $2.71 \pm 0.69$  vs.  $2.65 \pm 0.62$  L/min;  $p > 0.05$ ), heart rate ( $171 \pm 13$  vs.  $167 \pm 17$  bpm;  $p > 0.05$ ), dyspnoea ( $6.1 \pm 1.4$  vs.  $5.9 \pm 1.5$ ;  $p > 0.05$ ), leg tiredness ( $6.6 \pm 1.3$  vs.  $6.4 \pm 1.6$ ;  $p > 0.05$ ) or blood lactate ( $4.5 \pm 1.2$  vs.  $4.0 \pm 1.3$  mmol/L;  $p > 0.05$ ). The reduction in isokinetic maximal voluntary power (i.e. exercise-induced fatigue) was not different following constant-power vs. intermittent exercise ( $86 \pm 45$  vs.  $86 \pm 36$  W;  $p > 0.05$ ).

Intermittent exercise allowed for an  $\sim 76\%$  increase in task power compared to constant-power exercise for the same physiological ( $VO_2$ , heart rate, blood lactate) and perceptual (dyspnoea, leg tiredness) stress. Exercise-induced fatigue was not different following intensity-matched intermittent vs. constant-power exercise, suggesting exercise-induced fatigue is a consequence of exercise intensity and is independent of task power.

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C054

### Effects of Mitochondrial Antioxidant (MitoQ) Supplementation and Endurance Exercise Training on Endothelial Microparticles and Endothelial Cell Integrity

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Antioxidants and chronic exercise are viable options to mitigate deleterious reactive oxygen species (ROS) and improve endothelial function, but the efficacy of non-specific antioxidants to elicit beneficial vascular effects, alone or during exercise training, remains inconsistent. MitoQ, a mitochondria-specific antioxidant, has been shown to elicit favorable changes in the vasculature [1, 2]. The impact of chronic exercise with and without MitoQ on training-induced vascular adaptations, and related functional consequences in the endothelium, are not fully understood. Endothelial microparticles (EMPs) are shed from the endothelium in response to activation and apoptosis, facilitating intercellular communication. Thus, using *in vivo* and *in vitro* complementary studies, we investigated the effects of MitoQ on endothelial cell integrity. As previously reported [3], 20 healthy men ( $22.1 \pm 0.7$  yr) performed 3 weeks of aerobic cycle training at 50–70% maximal oxygen uptake ( $VO_{2max}$ ) for 45–60 min, with and without MitoQ supplementation ( $n=10$ /group). For the current study, blood samples were analyzed for plasma levels of EMPs derived from apoptotic endothelial cells ( $CD31^+/CD42b^-$ ) and two subpopulations of activated endothelial cells ( $CD54^+/CD45^-$ ,  $CD62E^+$ ). *In vitro*, ROS were assessed in human umbilical vein endothelial cells pretreated with and without MitoQ before exposure to Antimycin-A for 24hr. Data are presented as mean  $\pm$  SEM and were analyzed using a 2-factor repeated measures ANOVA. A group  $\times$  time interaction was observed for EMPs derived from apoptotic endothelial cells after training ( $P=0.06$ ). MitoQ supplementation reduced  $CD31^+/CD42b^-$  EMPs by 27% after training ( $18.1 \pm 2.7$  to  $13.3 \pm 1.8$  EMPs/ $\mu$ L;  $n=10$ ;  $P=0.06$ ), whereas no change was observed in the placebo group ( $14.0 \pm 2.0$  to  $16.1 \pm 2.4$  EMPs/ $\mu$ L;  $n=10$ ;  $P>0.05$ ). Circulating levels of  $CD54^+/CD45^-$  EMPs, derived from activated endothelial cells, were unaltered by exercise training or MitoQ administration ( $P>0.05$ ). Independent of MitoQ supplementation, circulating levels of  $CD62E^+$  EMPs from activated endothelial cells were reduced by 30% ( $70.6 \pm 9.3$  to  $49.8 \pm 6.3$  EMPs/ $\mu$ L;  $n=20$ ;  $P<0.05$  for main effect of training). *In vitro*, pretreating

endothelial cells with MitoQ reduced ROS production by 15% (n=3;  $P<0.05$ ). In summary, short-term endurance exercise training reduces endothelial cell activation with or without MitoQ supplementation. The addition of MitoQ supplementation during training may increase endothelial integrity by reducing endothelial cell apoptosis. MitoQ reduces Antimycin-A induced ROS production in endothelial cells.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## C055

### Diet-induced obesity impairs muscle stem cell recruitment patterns and induces oxidative stress in skeletal muscle which are mitigated by CD36 deletion

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**Introduction:** Muscle stem cells, called satellite cells (SC), are of crucial importance for muscle regeneration. Preliminary evidence suggests that diet-induced obesity (DIO) may have a negative impact on SC function, muscle redox homeostasis and subsequently skeletal muscle repair. Recent findings indicate that ectopic lipid accumulation not only leads to metabolic deficits but contributes to lipotoxicity and increased oxidative stress. Given the importance of lipid accumulation in the context of impaired muscle metabolism, the fatty acid translocase CD36 has recently received a great amount of attention as a possible target to improve muscle health in obese conditions. However, the underlying protective mechanism of abrogated CD36 signalling is still unknown. Here we investigated the impact of CD36-deficiency in skeletal muscle oxidative stress and stem cell function in response to DIO.

**Methods:** CD36-deficient and wild-type (WT) mice were administered either a standard chow or a high-fat (HF; 45% fat) diet for 12 weeks. Single muscle fibres and muscle tissue were studied for stem cell recruitment (i.e. activation, proliferation and differentiation patterns), fibre type composition and markers of oxidative stress. Lipid accumulation was assessed in skeletal muscle and the liver.

**Results:** We report here that CD36-deficient mice were protected against adiposity, intramuscular lipid accumulation and exhibited reduced expression for markers of oxidative stress. Despite similar baseline SC number among experimental groups, obese mice showed impaired SC proliferation and differentiation. Interestingly, CD36 deficiency rescued SC proliferation, but led to impaired differentiation, independent of diet. Additionally, CD36-deficient mice showed increased lipid infiltration in the liver in response to HF diet.

**Conclusions:** Using the ex vivo model of single muscle fibres we conclude that DIO impairs muscle stem cell proliferation and differentiation which is partially rescued by CD36 deletion. Moreover, loss of CD36 decreases intramuscular lipid accumulation and attenuates skeletal muscle oxidative stress, but leads to hepatic steatosis. It remains to be established whether CD36 inhibition represents a therapeutic target to tackle obesity and related metabolic disorders.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## C056

### Effect of time-restricted feeding and exercise on metabolic diseases in mice

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**Background:** Obesity is a major global threat and risk factor for type-2 diabetes, hypertension, cardiovascular diseases and cancer. Diet and exercise are the two major regulators of adiposity. In addition time-restricted feeding has been shown to improve clock and nutrient sensor functions. **Method:** To test whether high fat diet (HFD) with or without exercise results in decreased metabolic disruption and weight gain, we fed male mice (C57BL6) either time-restricted (t-RF) or *ad libitum* with HFD (60% Kcal fat) or control diet (CD, 10% Kcal Fat) for 12 weeks. Zeitgeber time 0 (ZT0) was designated as lights-on time and ZT12 as lights-off time. The t-RF mice were allowed to eat for 10 hours between ZT15–ZT24. HFD (t-RF and *ad libitum*) fed mice were exercised on a treadmill with a speed of 12m/min for 1 hour every other day. Body weight and food intake were measured every second day. Insulin and glucose tolerance tests were performed after 8 and 12 weeks of feeding. Fasting plasma HDL, LDL, total cholesterol and triglycerides were measured after 12 weeks of feeding. The differences between the groups were analyzed using one-way ANOVA for significance.

**Results:** HFD feeding (t-RF or *ad libitum*) significantly ( $p<0.0067$ ) increased body weight gain compared to CD fed mice. The t-RF significantly decreased body weight gain by 51% in HFD, while no significant changes were observed in CD fed mice. In addition, exercise significantly decreased body weight gain by 34% in *ad libitum* fed mice, while no effects were seen in t-RF mice. The t-RF mice on HFD significantly ( $p<0.019$ ) decreased food intake compared with *ad libitum* fed. Exercise did not change food intake either on t-RF or *ad libitum* fed compared to their respective controls. HFD feeding significantly increased plasma LDL-Cholesterol and total cholesterol compared to CD. Time-restriction significantly ( $p<0.0005$ ) lowered plasma LDL-cholesterol and total cholesterol in HFD fed, but not in CD fed mice. Furthermore, exercise significantly ( $p<0.03$ ) increased plasma HDL-Cholesterol in *ad libitum* fed mice. The t-RF significantly improved glucose tolerance in HFD and CD fed mice ( $p<0.05$ ). In addition, exercise significantly ( $p<0.001$ ) improved insulin sensitivity in t-RF fed mice, while it had no effect on *ad libitum* fed mice.

**Conclusion:** Time-restricted feeding significantly improves the metabolic profiles and reduces adiposity in HFD induced obese mice. Exercise increases the plasma HDL and hence cardio metabolic fitness. Time-restricted feeding in combination with exercise improves metabolic profiles and might decrease obesity and metabolic diseases.

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C057

# High fat diet abolishes the inhibitory effect of centrally administered nesfatin-1 on gastric emptying rate: role of GLP-1 and CCK receptors

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Obesity was shown to be associated with facilitated gastric emptying, which could either be the cause or the consequence of obesity.<sup>1</sup> The anorexigenic neuropeptide, nesfatin-1 (NES-1), when given centrally, suppresses gastric contractions and gastric motility<sup>2</sup>. High fat diet (HFD) in mice was shown to decrease plasma NES-1 levels.<sup>3</sup> However, the impact of HFD on NES-1 induced inhibition of gastric motility, and the peripheral mechanisms underlying the inhibitory effect of NES-1 on gastric emptying were not elucidated yet. In the present study, the involvement of cholecystokinin (CCK)-1, CCK-2 and glucagon-like peptide-1 (GLP-1) receptors in non-caloric liquid emptying and the effect of HFD on NES-1-induced alterations in gastric emptying were investigated. Male Sprague-Dawley rats (10-week-old) were fed with normal diet (ND, 2.7% fat, n=8) or HFD (45% fat, n=8) for 8 weeks. Under ketamine and chlorpromazine (100 and 10 mg/kg, intraperitoneally) anaesthesia, a Gregory cannula was installed in the gastric corpus, and one week later an intracerebroventricular (icv) cannula was placed. After 2 weeks, gastric emptying (GE) rate of saline was determined from the volume and absorbance of fluid recovered 5 min after instillation of phenol red-added saline into the stomach. Gastric emptying was commenced 5 min after icv NES-1 (0.05 µg/0.5 µl) or saline (0.5 µl) injection, which were preceded with subcutaneous injections of either saline or GLP-1 receptor antagonist exendin 9-39 (30 µg/kg) or CCK-1 receptor antagonist devazepide (1 mg/kg) or gastrin/CCK-2 receptor antagonist YM022 (1 mg/kg). At the end of the experiments, verification of icv cannula placement was made using methylene blue. In ND-receiving groups, central NES-1 administration significantly decreased gastric emptying rate ( $2.62 \pm 0.02$  ml/5min) compared to icv saline administration ( $3.06 \pm 0.08$  ml/5min). None of the antagonists changed the GE rate in icv saline administered rats, but NES-1-induced delay in GE rate was abolished by GLP-1 ( $3.06 \pm 0.13$  ml/5 min), CCK-1 ( $3.25 \pm 0.13$  ml/5 min,  $p < 0.05$ ) and CCK-2 ( $3.35 \pm 0.13$  ml/5 min,  $p < 0.05$ ) receptor antagonist. On the other hand, GE rates in HFD-fed rats were not different among centrally NES-1 ( $2.95 \pm 0.26$  ml/5 min) or saline ( $2.95 \pm 0.26$  ml/5 min) injected ones, and were similar to saline-injected ND-fed rats, while antagonists did not alter GE rates of HFD-fed rats injected icv with NES-1 or saline. The findings of current study demonstrate that NES-1-induced delay in gastric emptying was mediated via the involvement of GLP-1, CCK-1 and CCK-2 receptors, while this inhibitory effect of NES-1 on gastric emptying rate was not evident when rats were on HFD, suggesting that prolonged consumption of HFD abolishes the inhibitory role of anorexigenic nesfatin-1 on gastric motility and facilitates gastric emptying.

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C058

# Potentiation of satiety by activation of glucagon-like peptide-1 producing preproglucagon neurons

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Glucagon-like peptide-1 (GLP-1) analogues are anti-obesity treatments, however their clinical utility is compromised by nausea. Chemogenetic activation of GLP-1 producing preproglucagon (PPG) neurons in the caudal brainstem suppresses food intake, however it is unclear whether this represents potentiation of satiety, or is secondary to induction of nausea/malaise. The extent to which PPGs are involved in physiological satiety further remains to be determined.

To address this, transgenic mice expressing Cre recombinase in PPG neurons were anaesthetised with ketamine (75mg/kg, i.m.) + medetomidine (0.5mg/kg, i.m.) and stereotactically injected with Cre-dependent AAVs encoding either hM3Dq or hM4Di DREADD receptors. Sex-matched cohorts (n=8) were used to investigate the effects of 2mg/kg (i.p.) CNO-induced neuronal activation (PPG-Gq) or inhibition (PPG-Gi) on feeding behaviours using within-subjects designs. Food intake was recorded using automated pellet dispensers and video coding of behavioural satiety sequences (BSS). Data are means  $\pm$  SEM and analysed by ANOVA and *post hoc* Bonferroni or t-tests.

In 3hr fasted PPG-Gq mice, CNO reduced dark phase intake ( $2.62g \pm 0.15$  to  $1.47g \pm 0.21$ ;  $p = 0.0027$ ), in a sex-independent manner (Sex x CNO:  $F_{(1,5)} = 0.23$ ,  $p = 0.65$ ). Over 48hrs, intake was reduced during hour 1-5 ( $p < 0.05$ - $0.0001$ ), and cumulative intake from hour 3-48 ( $p < 0.001$ - $0.0001$ ), with no compensatory refeeding observed. cFos immunoreactivity revealed this anorectic effect was due to activation of  $81.3 \pm 6.8$  of hM3Dq-transduced cells vs  $0.8 \pm 0.6$  in saline controls ( $p < 0.0001$ ). To assess effects of PPG activation on satiety in the BSS paradigm, PPG-Gq mice were fasted for 18hrs prior to dark onset. PPG activation reduced 40min intake ( $0.31g \pm 0.06$  to  $0.06g \pm 0.01$ ;  $p = 0.016$ ) and advanced the onset of satiety (15-20min to  $\leq 5$ min), with the typical BSS maintained. Eating duration was decreased ( $3.1min \pm 0.73$  to  $0.44min \pm 0.10$ ;  $p = 0.012$ ), and resting duration increased ( $11.5min \pm 1.8$  to  $23.2min \pm 2.6$ ;  $p = 0.016$ ).

In PPG-Gi mice, CNO-induced PPG inhibition had modest effects on food intake, only readily apparent during high-volume Ensure liquid diet intake. Following a 15min Ensure preload, hour 1 chow intake was increased ( $0.04g \pm 0.02$  to  $0.08 \pm 0.03$ ;  $p = 0.039$ ), driven by reduced latency to meal 1 ( $75.5min \pm 13.1$  to  $53.0min \pm 11.0$ ;  $p = 0.011$ ). With 1hr Ensure access at dark onset, Ensure intake itself was increased by CNO ( $1.84g \pm 0.19$  to  $2.09g \pm 0.17$ ;  $p = 0.014$ ), followed by a compensatory decrease in chow intake during hour 2 ( $0.09g \pm 0.02$  to  $0.07g \pm 0.02$ ;  $p = 0.016$ ).

These anorectic effects of chemogenetic PPG activation are consistent with potentiation of satiety, rather than induction of nausea/malaise. Given their apparently minor role in physiological satiety, PPG neurons thus have the capacity for supraphysiological stimulation to reduce food intake, which may represent an effective anti-obesity strategy.



Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## C059

### The obesity-independent impact of hyperlipidaemia and atherosclerosis on skeletal muscle metabolism and redox biology

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Hyperlipidaemia and atherosclerosis, in the context of obesity, are considered significant risk factors for pathophysiological changes in peripheral tissues, such as the liver and adipose tissue [1,2]. However, both hyperlipidaemia and atherosclerosis can occur in the absence of obesity and their impact on skeletal muscle is not understood sufficiently [3]. In this regard, we used the Apolipoprotein E-deficient (ApoE<sup>-/-</sup>) mouse model, an established model of obesity-independent hyperlipidaemia and atherosclerosis, to determine the impact of Western-type diet (WD) and ApoE deficiency on skeletal muscle morphological, metabolic and biochemical properties. Data were analysed by two-way ANOVA (genotype x diet). We found that individual myofibres were significantly enlarged due to extensive lipid accumulation and most importantly the ex vivo myogenic potential of skeletal muscle stem cells from ApoE<sup>-/-</sup> mice was impaired, showing attenuated differentiation. Ectopic lipid accumulation in skeletal muscle was accompanied by a transition towards more oxidative myofibres and enhanced capillarisation, possibly representing a metabolic adaptation in response to atherogenic diet and ApoE deficiency. However, metabonomic analysis and gene expression of skeletal muscle revealed perturbed fatty acid metabolism and impaired mitochondrial metabolism. Moreover, ApoE deficiency and diet independently induced oxidative stress, as shown by elevated lipid peroxidation and oxidative protein and DNA modifications in the skeletal muscle. Most importantly, pharmacological inhibition of NADPH oxidase 2 (Nox2) with the use of the Nox2ds-tat peptide inhibitor decreased superoxide production, DNA damage and protein carbonylation, one of the most harmful protein modifications, in the muscle of ApoE<sup>-/-</sup> mice. These data indicate that hyperlipidaemia and atherosclerosis alter morphological features, induce oxidative damage and impair mitochondrial metabolism in skeletal muscle independently of obesity that can be mitigated by Nox2 inhibition, highlighting its role as a potential therapeutic target for attenuating oxidative stress in skeletal muscle.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## C060

### The calcium signaling network in murine testis

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The physiological principles underlying spermatogenesis, a crucial step in male fertility, are still largely unexplored. Seminiferous tubules represent the functional units of the testis and orchestrate male germ cell maturation<sup>1</sup>. In a sequence of tightly coordinated events, germ cells develop from immature spermatogonia to spermatozoa<sup>2</sup>.

Here, we investigate physiological signaling events in different testicular cell types using live-cell imaging. A novel intact *in situ* preparation of seminiferous tubule sections allows detailed analysis of spontaneous activity in Sertoli cells, putative peritubular cells, and germ cells at different developmental stages. Employing genetically engineered mice that conditionally express a genetically encoded Ca<sup>2+</sup> indicator (GCaMP6f) in a cell type-specific manner allows investigation of both Sertoli and germ cell-specific Ca<sup>2+</sup> signaling patterns. Sertoli cells generate slow, low-frequency signals. Putative peritubular cells exhibit tonic activity at higher frequencies. Finally, germ cells either display long-lasting Ca<sup>2+</sup> signals that occur at low frequencies, or they show transient high frequency signals. Independent of cell type, signal shape is both modulated by extracellular Ca<sup>2+</sup> and dependent on intracellular Ca<sup>2+</sup> stores. Strikingly, we record some spontaneous Ca<sup>2+</sup> signals in each testicular cell type that display intrinsic periodicity. Moreover, cross-correlation analyses indicate testicular networks that appear to coordinate ensemble activity.

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<sup>2</sup> de Kretser D.M., Loveland, K.L., Meinhardt, A., Simorangkir, D., Wreford, N. (1998) Spermatogenesis, *Human Reproduction*, Volume 13, Issue suppl\_1, 1 April 1998, Pages 1–8.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## C061

### Can the modulation of adenosine and/or ATP signaling in the carotid body be used to treat type 2 diabetes?

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Our group have described that carotid body (CB) is involved in the genesis and maintenance of insulin resistance and glucose intolerance, since carotid sinus nerve (CSN) resection prevents and reverses these pathological features in prediabetes animal models (1,2). We have also showed that CB is overactivated in prediabetes animal models (1,3), which agrees with the observed increase in CSN basal activity in high-fat (HF) rats (3). Knowing that adenosine and ATP are key mediators in the CB, mediating the CSN hypoxic response (4), the aim of this work was to investigate the role of adenosine and ATP in the CB/CSN overactivity induced by HF diet.

Two groups of 8-9 weeks male *Wistar* rats were used: control (chow diet) and the HF group (60% lipid-rich diet, 21 days). After this period, insulin sensitivity was evaluated. To perform the ex vivo recordings of the CSN activity, animals were

anesthetized with pentobarbital sodium (60 mg/kg ip) and the carotid bifurcation was removed and the CB-CSN was dissected and transferred to a recording chamber superfused (37°C) with Tyrode bicarbonate equilibrated with normoxia (20%O<sub>2</sub>+5%CO<sub>2</sub>). CSN activity was identified and confirmed by its response to hypoxia (0%O<sub>2</sub>). The effect of ZM241385 (300nM; A<sub>2</sub> antagonist), SCH58261 (20nM; selective A<sub>2A</sub> antagonist), suramin (50μM; P2 receptor antagonist) and AF-353 (0.3 and 1μM; P2X<sub>3</sub> antagonist) on the CSN activity in normoxia and hypoxia (0 and 5%O<sub>2</sub>)-evoked CSN action potentials was evaluated. Animals were killed by an intracardiac overdose of pentobarbital sodium (60mg/kg i.p.).

HF diet increased basal CSN activity (CTL=1.56±0.24Hz/s; HF=3.11±0.55Hz/s;  $p<0.05$ ). In control rats basal CSN discharge did not change with adenosine antagonists. However in HF animals, CSN basal activity decreased by 34.85 ( $p<0.05$ ) and 48.21% ( $p<0.05$ ) with SCH58261 and ZM241385, respectively. Also, suramin and AF-353 did not change basal CSN activity. ZM241385 decreased the response to hypoxia (0%O<sub>2</sub>) by 43.84 ( $p<0.01$ ) and 52.10% ( $p<0.05$ ), in both control and HF group. Suramin decreased the chemosensory response to hypoxia (0% O<sub>2</sub>) by 59.42 ( $p<0.01$ ) and 59.72% ( $p<0.05$ ), in both control and HF group, respectively. ZM241385 application plus suramin decreased the CSN response to hypoxia (0%O<sub>2</sub>) by 80.57 ( $p<0.001$ ) and 72.73% ( $p<0.05$ ), in both control and HF group. As expected, AF-353 showed a higher effect in decreasing CSN chemosensory in response to 0%O<sub>2</sub> than to 5%O<sub>2</sub>, an effect that was dose dependent [0%O<sub>2</sub>: 0.3μM decreased 71.07 ( $p<0.01$ ) and 1μM ( $p<0.001$ ) decreased 89.75%; 5%O<sub>2</sub>: 0.3μM decreased 57.98 ( $p<0.01$ ) and 1μM decreased 72.92% ( $p<0.01$ )].

We conclude that adenosine, more than ATP, is involved in the CSN overactivation induced by the HF diet, mainly via A<sub>2A</sub> receptors. Additionally, in control animals ATP contribute more than adenosine to generate CSN activity in intense hypoxias. Our results suggest that the modulation of adenosine signaling in the CB can be a therapeutic target for type 2 diabetes.

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## Surviving metabolism: acidity as a selection pressure in colorectal cancer cell lines

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**Introduction:** Extracellular acidity is a chemical signature of tumours. It arises because the raised metabolic rate in cancer cells releases large quantities of lactic acid and CO<sub>2</sub> into the tumour microenvironment (1, 2). Dysregulated pH has been shown to perturb or even kill cancer cells. Although targeting acidity is a good candidate for the therapeutic management of tumour growth (3), so far, none of the major approved therapies are based explicitly on disrupting acid handling and/or signalling. Using a large panel of colorectal cancer (CRC) cell lines, our aim was to identify the molecular processes that provide a survival benefit to cancer cells living under acid-stress.

**Methods:** We investigated pH-related physiology in over 40 CRC cell lines. We measured (i) survival by sulphorhodamine B (SRB) assay, (ii) lumen formation by F-actin staining, (iii) extracellular pH (pH<sub>e</sub>) time courses as a readout of metabolic rate, and (iv) intracellular pH (pH<sub>i</sub>) on a single-cell level by fluorescence imaging. These measurements we performed over a range of pH<sub>e</sub> values using an imaging plate reader (Cytation 5, Biotek).

**Results:** CRC cell lines displayed a wide range of sensitivities to changes in pH<sub>e</sub>. A 50% reduction in survival was associated with pH<sub>e</sub> values of 7.3 (most sensitive: Iscero1) to below 6.5 (least sensitive: C106). pH<sub>e</sub>-sensitivity was also observed in 3D cultures grown in Matrigel. CRC cell lines categorised as pH-sensitive formed smaller colonies under acidic pH<sub>e</sub> (6.76); however, their differentiation status was independent of pH<sub>e</sub>. Furthermore, the cell lines differed in their ability to regulate pH<sub>i</sub> in response to changes in ambient pH<sub>e</sub>, quantified in terms of the slope of their pH<sub>i</sub>-pH<sub>e</sub> relationship. This metric of pH<sub>i</sub>-control ranged from 0.16 (Colo206) to 0.59 (Iscero1), representing strong to weak pH<sub>i</sub> control, respectively.

**Conclusions:** We established a database that provides a comprehensive appraisal of the pH phenotype for individual CRC cell lines. This information allows us to correlate phenotype with mutations and gene expression profiles of these cell lines, and to identify molecular processes involved in acid handling and signalling.

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C063

**Resting metabolic rate in vegetarians and omnivores before and after inhibition of the oral nitrate/nitrite pathway**R. Bescos Garcia<sup>1</sup>, A. Ashworth<sup>1</sup>, C. Easton<sup>2</sup> and L. Liddle<sup>2</sup><sup>1</sup>University of Plymouth, Plymouth, UK and <sup>2</sup>University of the West of Scotland, Paisley, UK

Low energy density of vegetarian diets has been suggested to induce a reduction in the resting metabolic rate (RMR) of individuals following this dietary pattern. This can also be a key factor to explain the impact of these diets in the management of obesity. However, other factors such as dietary nitrate (NO<sub>3</sub>) intake may play a significant role on the modulation of the RMR through the nitrate/nitrite/nitric oxide pathway (1). Thus, the main aim of this study was to estimate dietary energy and NO<sub>3</sub> consumption in a group of vegetarians and omnivores, and its effect on the RMR before and after the inhibition of the oral nitrate/nitrite pathway. Twenty-two healthy subjects following a vegetarian diet (V) for at least a year (16 F + 6 M) and 19 omnivores (O) (11 F + 8 M) of similar age (V= 26 ± 6; O= 26 ± 6 y/o), BMI (V= 22.9 ± 3.8; O= 22.1 ± 2.9), gender and levels of moderate and vigorous physical activity (V= 315 ± 221; O= 334 ± 208 min/w) participated in this study. They recorded all their food consumption for seven days using food diaries while they used a placebo mouthwash (water) to rinse their mouth twice a day (1 min). On the seventh day, a saliva and blood sample were collected under fasting conditions before the RMR was assessed with an indirect calorimetric system connected to a ventilated hood (Jaeger Oxycon Pro) for 30 min. Then, participants were given antibacterial mouthwash (Corsodyl, UK) for seven more days in order to inhibit the oral NO<sub>3</sub>/NO<sub>2</sub> pathway following a single-blind and non-randomized design. They were also encouraged to replicate their food intake from the previous week. The same measurements were taken under fasting conditions on the fourteenth day. Results are expressed as mean ± SD. Energy intake was slightly higher but not statistically different (P>0.05) in omnivores (2,021 ± 560 kcal) than in vegetarians (1,827 ± 526 kcal). Dietary NO<sub>3</sub> intake was greater in vegetarians (97 ± 79 mg/day) than in omnivores (78 ± 47 mg/day), but this was not statistically different (P>0.05). No differences were found (P>0.05) in plasma NO<sub>3</sub> (V= 43 ± 33 µM; O= 40 ± 18 µM) and NO<sub>2</sub> (V= 84 ± 41 nM; O= 74 ± 21 nM) and salivary NO<sub>3</sub> (V= 525 ± 698 µM; O= 542 ± 571 µM) and NO<sub>2</sub> (V= 329 ± 340 nM; O= 319 ± 283 nM) between both groups after using placebo. The RMR was also similar in both groups under placebo conditions (V= 1,396 ± 254 kcal; O= 1,397 ± 304 µM). A significant reduction (P<0.05) of plasma NO<sub>2</sub> (V= 65 ± 15 nM; O= 61 ± 16 nM) and salivary NO<sub>2</sub> (V= 155 ± 171 nM; O= 133 ± 214 nM) was observed in vegetarians and omnivores after using antibacterial mouthwash indicating a significant inhibition of the oral nitrate/nitrite pathway. However, this did not affect the RMR in either group (V= 1,404 ± 255 kcal; O= 1,401 ± 318 kcal). These data suggest that vegetarian diets do not reduce the RMR compared to an omnivore healthy diet. Additionally, the oral nitrate/nitrite pathway does not seem to be a key regulator of RMR in vegetarians and omnivores.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

C064

**Ethnic differences in the pregnant vaginal environment, host response and risk of spontaneous preterm birth**R.M. Tribe<sup>1</sup>, T. Kanno<sup>1</sup>, E. Prosdociami<sup>2</sup>, N. Hezelgrave<sup>1</sup>, E. Chin-Smith<sup>1</sup>, V. Mistry<sup>1</sup>, A.H. Shennan<sup>1</sup> and A.J. Mason<sup>3</sup>

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**Introduction:** We reported inflammation in the female reproductive tract (14-18 weeks') of women at higher risk of spontaneous preterm birth (sPTB) [1-2]. The driver for this innate host immune response was hypothesised to be the vaginal microbiome. This study investigated the effect of the vaginal environment (microbiome composition and metabolites) on the innate host immune response in pregnant women.

**Methods:** Cervico-vaginal samples were obtained at two gestations (13-15<sup>+6</sup> & 16-23<sup>+6</sup> weeks; n=355 women; White n=233, black n=76, other n=34) from a prospective longitudinal cohort of women with written consent (INSIGHT study, NRES London City and East approval 13/LO/0393). Metabolites: measured using Bruker Avance II 700 NMR, with preprocessing/OPLS-DA using MVAPACK. Vaginal microbiome: determined by sequencing the V1-V2 region of the 16S rRNA gene and data analysed using the mothur pipeline. Operational taxonomic units were constructed at 98.5% similarity and classified by comparison to the vaginal microbiome database. Sample composition between groups was compared by AMOVA applied to a theta-YC dissimilarity matrix. Elafin and cathelicidin measured by ELISA.

**Results:** Early pregnancy microbiome composition was significantly influenced by maternal ethnicity (AMOVA, p<0.001) with differences in microbiome, metabolome, host immune responses relationships and sPTB identified for Black and White women. A correlation between microbiome and sPTB (AMOVA, p=0.036 and p=0.004 for early and later pregnancy) was only observed in White women, in parallel with increases in acetate and cathelicidin.

**Conclusion:** Specific bacterial species/community states or metabolites are unlikely to be a global indicator of risk in heterogeneous antenatal populations. Research into mechanisms of preterm birth and biomarkers must consider confounders such as ethnicity. A 'precision medicine' based approach to prediction of sPTB is required.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.



C065

***Waltheria indica* root exhibits male contraceptive effect through inhibition of androgenesis in male Wistar rats**A. BASIRU<sup>1,2</sup><sup>1</sup>VETERINARY PHYSIOLOGY AND BIOCHEMISTRY, UNIVERSITY OF ILORIN, ILORIN, NIGERIA, Ilorin, Nigeria and <sup>2</sup>VETERINARY PHYSIOLOGY AND BIOCHEMISTRY, UNIVERSITY OF IBADAN, IBADAN, Nigeria

Current methods of population control have not yielded significant result in developing countries such as Nigeria. This has been attributed to factors such as lack of acceptability in rural areas, perceived adverse effects, poor penetration of rural communities and mostly female-centered approach. Hence, the need for culturally acceptable and male-centered birth control methods. Traditional use of *Waltheria indica* as male contraceptive has been reported in Nigeria. This study was designed to evaluate the contraceptive potential of *Waltheria indica* root (WIR) in male rats. Twenty males Wistar rats (190-236g) and twelve female rats of proven fertility were used. The males rats were randomly divided into four groups (n=5). Group I served as the control and was administered with distilled water while group II, III and IV were given crude ethanolic extract of WIR (200mg/Kg), synthetic quinoline alkaloid (13.5mg/Kg) and a pure compound (yet to be characterized) isolated from WIR (1000µg/Kg) respectively. The treatment was done for 28 days after which three males from each of the groups were paired with three female rats. The female rats were artificially brought to oestrous according to standard procedure. After the mating, ethyl ether anaesthetized male rats were sacrificed. Epididymal sperm, testicular and serum samples were assayed for sperm parameters, testicular androgenic enzymes [ $\Delta^5$ , 3 $\beta$ -Hydroxysteroid dehydrogenase and 17 $\beta$  Hydroxysteroid dehydrogenase (17 $\beta$ -HSD)] and testosterone respectively. All data obtained (means  $\pm$  standard deviation) were subjected to one way analysis of variance with Dunnett's Multiple Comparison Test used to compare means at  $p < 0.05$  on GraphPad Prism. The crude extract of WIR and compound isolated from WIR significantly reduced the sperm count ( $p < 0.05$ ) and motility ( $p < 0.001$ ). There was also significant reduction in serum level of testosterone for the crude extract of WIR ( $p < 0.01$ ) and the compound isolated from WIR ( $p < 0.05$ ). The serum levels of  $\Delta^5$ , 3 $\beta$ -HSD and 17 $\beta$ -HSD enzymes were significantly reduced by the isolated compound from WIR ( $p < 0.001$ ). The compound, crude extract of WIR and synthetic quinoline alkaloid reduced the fertility index from 100% (control) to 0%, 25% and 75% respectively. These findings support the traditional use of *Waltheria indica* as male contraceptive agent. More so, reduced level of testosterone and subsequent reduction of key androgenic enzymes indicate inhibition of androgenesis. This suggests that contraceptive effect of WIR is through inhibition of androgenesis.

**Key words:** *Waltheria indica* root, contraceptive, androgen, male rats

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

C066

***Sex-dependent hepatic response to high fat diet in male and female C57bl/6 mice***

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Obesity and secondary development of type-2-diabetes mellitus (T2D) is a major healthcare problem. Sex-differences exist in the prevalence of T2D with men being diagnosed at lower age and body mass index than women (1). Increased hepatic glucose production (HGP) is one of the key elements of the pathophysiology of T2D. Glycerol is a precursor for HGP and hepatic glycerol uptake via the glycerol channel AQP9 is regulated in a sex-specific manner. Similarly, glycerol kinase that phosphorylates glycerol into glycerol-3-phosphate is also influenced by sex (2). In this study we investigate the effect of 12 and 24 weeks of high fat diet (HFD) on hepatic glycerol handling in age-matched male (n=10+10, 10+8) and female (n=10+10, 9+10) mice. At the termination of the experiment the mice were anaesthetised using isoflurane inhalation (induction 4%, maintenance 2% in atmospheric air) and the liver was removed for immunoblotting and histology. Values are means  $\pm$  S.E.M. and means are compared by either student t-test or ANOVA. After both 12 and 24 weeks of HFD male and female mice had a significantly increased bodyweight (BW) (g) ( $32.5 \pm 0.3$  vs.  $48.4 \pm 0.9$ ,  $p < 0.001$   $21.9 \pm 0.5$  vs.  $29.5 \pm 1.0$ ,  $p < 0.001$ ) and ( $34.5 \pm 0.8$  vs.  $51.1 \pm 1.2$ ,  $p < 0.001$ ,  $22.3 \pm 0.4$  vs.  $39.2 \pm 2.2$ ,  $p < 0.001$ ) when compared to controls. In male mice blood glucose levels (BG) (mmol/l) were increased after 12 and 24 weeks of HFD ( $7.7 \pm 0.4$  vs.  $10.7 \pm 0.7$ ,  $p < 0.001$ ,  $7.5 \pm 0.03$  vs.  $10.2 \pm 0.5$ ,  $p < 0.01$ ) when compared to controls. In female mice the BG were only elevated after 24 weeks of HFD ( $7.0 \pm 0.4$  vs.  $8.3 \pm 0.3$ , ns,  $5.5 \pm 0.1$  vs.  $8.7 \pm 0.1$ ,  $p < 0.001$ ). Males demonstrated an 80 and 62% increase in liver weight (LW) in response to 12 and 24 weeks of HFD, respectively, when compared to controls. In females a 21 and 40% increase in LW were observed. LW normalized to BW increased after 24 weeks of HFD when compared to controls in male mice, whereas this ratio was decreased in female mice, which suggests a relatively lower hepatic fat accumulation in female than in male mice. This observation was supported by histological analysis of the liver. The hepatic expression of AQP9 remained unaffected by HFD in both male and female mice. However, after both 12 and 24 weeks of HFD female groups demonstrated a markedly higher hepatic AQP9 expression than male control mice ( $1.00 \pm 0.09$  vs.  $0.20 \pm 0.02$ ,  $p < 0.05$ ,  $1.15 \pm 0.09$  vs.  $0.20 \pm 0.02$ ,  $p < 0.01$ ) and ( $1.0 \pm 0.1$  vs.  $0.2 \pm 0.0$ ,  $p < 0.05$ ,  $1.2 \pm 0.2$  vs.  $0.2 \pm 0.0$ ,  $p < 0.01$ ). The hepatic expression of glycerol kinase was increased after 12 and 24 weeks of HFD in female mice ( $1.0 \pm 0.1$  vs.  $1.5 \pm 0.1$ ,  $p < 0.001$ ,  $1.0 \pm 0.1$  vs.  $1.7 \pm 0.1$ ,  $p < 0.05$ ) compared with controls, whereas the expression remained unaffected in males. In conclusion, the response to HFD is sex-dependent in C57bl/6 mice. Female mice respond with less hepatic steatosis than males and with an increased expression of glycerol kinase.

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C067

# THE EFFECTS OF ORAL CHONDROITIN SULFATE ON METABOLISM AND GUT INFLAMMATION INDUCED BY HIGH FAT DIET IN RATS

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Chondroitin sulphate (CS) controls weight gain<sup>1</sup> induced high fat diet (HFD) in mice by restricting fat absorption and increasing incretin hormone secretion<sup>2</sup>, alleviates intestinal inflammation in chronic mild stress models. It is unknown whether anti-inflammatory action of CS has a role in the control of weight gain induced by HFD. Sprague-Dawley rats (10 week old, male, n=6 each) treated with either saline (S, 0.1 ml/100g/rat) or CS (80 mg/kg/day, oral), were fed daily and isocalorically for 4 weeks with chow ND (fat 2.7%) or HFD (45% fat). Rats in ND+S, ND+CS, HFD+S and HFD+CS groups were weighed weekly. Fecal samples were taken at 0-2-4 weeks under sterile conditions to detect *Desulphovibrio piger* (D. piger) by qPCR. Intestinal and colonic motility was recorded at the end of the experiments. Rats were decapitated and trunk blood was obtained for biochemical analysis. Epididymal fat was weighted and myeloperoxidase (MPO) activity studied in the ileal, colonic and gastric samples. Histopathological evaluation and scoring of inflammation were done in H&E stained samples. Data were stated as mean±standard error or median (range), compared with 2-way ANOVA; p<0.05 were considered significant. Daily caloric intake of ND group was found higher than HFD group (F<sub>7,21</sub>=5.02 diet, F<sub>2,3</sub>=19.8 time, p<0.05). CS has decreased the amount of weight gain (F<sub>1,24</sub>=7.658, p<0.05) and also decreased the HFD induced increment of epididymal fat weight (F<sub>1,20</sub>=36.4 diet, F<sub>1,20</sub>=4.4 treatment, p<0.05). In ND group triglyceride levels significantly decreased with CS treatment (F<sub>1,2</sub>=5.25, p<0.05). HFD groups resulted in increased MPO levels, indicating neutrophil infiltration to the tissues, as compared to ND groups in gastric and ileal tissues (F<sub>1,14</sub>=21.3 and F<sub>1,17</sub>=11.57 respectively, p<0.05). Histopathological inflammation score worsened by HFD while ameliorated by CS treatment in ileal (F<sub>1,21</sub>=22.52, diet, p<0.001, F<sub>1,21</sub>=6.56 CS, p<0.05) and gastric (F<sub>1,20</sub>=36.4 diet, F<sub>1,20</sub>=4.4 CS p<0.05) tissue samples. HFD has increased small intestinal transit (F<sub>1,20</sub>=5.7, p<0.05) while decreasing pellet output (F<sub>1,20</sub>=15.12, p<0.001). Time dependent increase was recorded in D. piger bacterial population with HFD (F<sub>2,32</sub>=33, p<0.001). Also D. piger population significantly increased in ND group dependent to time and CS treatment (F<sub>2,30</sub>=40.7, F<sub>15,30</sub>=2.7 respectively, p<0.01). In conclusion, the most likely reason for decreasing levels of weight gain in CS treated rats might be the recovery from intestinal inflammation. Changes

in intestinal microbiota by means of CS treatment might be another reason which requires further elucidation. Current study demonstrates that HFD promotes visceral fat deposition, inflammation in the gastrointestinal tract, triggers changes in gut motility and has a role in the maintenance of microbiota while CS regulates this interaction.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

C068

# A novel ex vivo/in vitro approach to investigate the effects of milk-derived peptides on glut4 translocation

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## Introduction

Traditional *in vitro* experiments using mammalian cell culture have provided valuable insight to skeletal muscle metabolism. The nature of traditional approaches to cell culture, however, lacks direct translocation to *in vivo* physiology (Cantor et al 2017). Following the ingestion of whey protein, a plethora of hormones from several different tissues are released, which stimulate cross-talk between multiple organs to coordinate an integrated systemic response (van Loon et al 2000; Nilsson et al 2004). Due to these hugely dynamic multi-organ responses, direct presentation of intact or hydrolysed proteins to a cell in culture very rarely replicates the nutrient, humoral or bioactives the cell encounters *in vivo*. Consequently, using a novel *ex vivo/in vitro* approach, we investigate the effects of fasting and postprandial human serum on GLUT4 translocation in L6-GLUT4myc myotubes following protein ingestion.

## Methods

L6-GLUT4myc myotubes were first treated with human serum [0.1-20%] collected after an overnight fast (~10 h) to characterise the effect on GLUT4 translocation and signal transduction. After optimising *in vitro* screening conditions, myotubes were incubated with traditional cell culture media supplemented with postprandial human serum collected from male volunteers (n=8) after ingestion of 0.33 g/kg BM of either intact or hydrolysed whey protein, or an isonitrogenous control. Blood samples were collected every 30 min for 2 h post feeding. One and two-way ANOVA with repeated measures (time x treatment), were used to assess statistical differences where appropriate (α level set at p<0.05). For post-hoc testing, Tukey's or Dunnett's multiple comparisons test was used to detect significant differences between treatments and/or time points when a significant main effect or interaction effect was indicated.

## Results

Fasting human serum increased cell surface GLUT4 abundance, p-AKT Tyr<sup>308</sup> and Ser<sup>473</sup> in a dose-dependent manner, independent of changes in p-IRS-1 Tyr<sup>895</sup>. Cell culture media supplemented with postprandial serum collected 60 min after ingestion of a whey protein hydrolysates significantly increased GLUT4 translocation relative to baseline and compared to intact and isonitrogenous controls (mean±SEM; 0.99±0.02 and 1.02±0.02 fold, respectively). Moreover, the increase in GLUT4 translocation was independent of a change in plasma glucose, insulin and incretin hormones, respectively.

## Discussion

The present investigation demonstrates the potential application of investigating fasted versus fed conditioned human serum using a novel *ex vivo/in vitro* approach on GLUT4 translocation and intracellular signaling. Further work is required to identify a mechanistic basis for the potential of a whey protein hydrolysate to modulate GLUT4 translocation.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

C069

## Vitamin B12 deficiency inhibits the lipid lowering effect of metformin in the liver

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### Background:

Metformin is currently the first drug of choice for treatment of type 2 diabetes (T2D). In addition to improving insulin sensitivity and hyperglycaemia, metformin is also known to reduce lipid levels through activation of AMP activated protein kinase-alpha (AMPKα). It is known that metformin administration induces deficiency of vitamin B12 (B12) in patients with T2D. Observational studies in humans also show that low B12 is associated with dyslipidemia (higher triglycerides and low HDL). Therefore, we investigated whether B12 deficiency may impair metformin action from achieving the desired lipid lowering effect in the liver.

### Methods:

Hep G2 cell line was cultured using custom made B12 deficient Eagle's Minimal Essential Medium (EMEM) and seeded in four different concentrations of B12 media such as 500nM (control), 1000pM, 100pM and 25pM (low) B12 until 100% confluence was achieved. The cells were exposed to 24hour treatment with 1mM and 2mM metformin before harvest. Gene expression assays, protein expression and mitochondrial spare respiratory capacity were characterized using real time

PCR (qRT-PCR), western blotting and seahorse XF24 assays respectively.

### Results:

Low B12 (25pM) in HepG2 cell line decreased levels of AMPKα and its downstream target pACC, compared to control. Administration of increasing concentrations of metformin (1mM and 2mM) to low B12 hepatocytes significantly impaired the upregulation of pAMPKα and pACC. In addition, we found that downregulation of nuclear transcriptional factor sterol regulatory element binding protein (SREBF1) and the genes involved in hepatic *de novo* fatty acid synthesis pathway, [fatty acid synthase (FASN), acetyl coenzyme A carboxylase (ACC) and elongation-of very-long-chain fatty acid (ELOVL6)] and TG biosynthesis [glycerol-3-phosphate acyltransferase (GPAT) and diacylglycerol acyl transferase 2 (DGAT2)] were significantly impaired in low B12 cells treated with metformin. In the fatty acid oxidation (FAO) pathway, upregulation of the rate limiting enzyme carnitine palmitoyl transferase 1 alpha (CPT1α) and downstream genes carnitine acyl carnitine translocase (CACT) and Long chain Acyl-CoA dehydrogenase (ACADL) were significantly impaired in low B12 hepatocytes. Finally, the spare respiratory capacity of mitochondria in metformin treated hepatocytes under low B12 was impaired.

### Conclusion:

Our study provides novel evidence that Vitamin B12 deficiency (1) lowers levels of pAMPKα and pACC, and (2) metformin administration in low B12 hepatocytes failed to restore the levels of pAMPKα and pACC, and the genes involved in lipid metabolism. Mitochondrial functional capability in the oxidation of fatty acid was also impaired. This supports that the lipid lowering effect of metformin in vitamin B12 deficiency is compromised. The mechanisms involving regulation via AMPK requires further studies.

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C070

## A novel tool for identifying transcriptional targets in kidney development using the example of Wilms tumor protein (WT1)

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The Wilms tumor transcription factor WT1 is necessary for normal kidney formation. Identification of WT1 downstream target genes may thus allow one to analyze genetic pathways relevant to renal development. Since mice with germ-line knockout of Wt1 are embryonic lethal and lack kidneys, alternative tools are needed to study WT1-dependent gene networks. By combining *vivo-morpholino* induced gene knockdown in metanephric explants with next generation sequencing, we established a novel instrument for identifying WT1 downstream targets in kidney development.

Kidneys were excised from mouse embryos (E12.5) and grown in hanging droplets of nutrient. The explants were treated for 48 h with either Wt1 antisense or mismatch *vivo-morpholino*. WT1 knockdown was assessed by immunoblotting and mRNA from pooled kidney samples was subjected to deep sequencing. The DESeq package was used to identify the differentially expressed genes. Deep sequencing data were validated by quantitative PCR of more than a dozen of arbitrarily picked transcripts.



Silencing of Wt1 by vivo-morpholino treatment significantly reduced 308 and increased 258 mRNAs in metanephric organ cultures. Comparison with previously published ChIP-sequencing data (Motamedi et al., 2014) showed that WT1 binds to 217 of the down-regulated and 194 of the up-regulated genes in murine embryonic kidneys. Among these differentially expressed genes, 184 could be retrieved from the atlas of gene expression in the developing kidney (Brunskill et al., 2008). According to this atlas, 32 of the 184 genes are predominantly expressed in the metanephric mesenchyme, i.e. the site of WT1 expression. The top candidate WT1 target genes were further analyzed. To spread these results from the complex organic system to a cellular system, the endogenous mRNA levels of these top candidates were measured by quantitative PCR in cells treated with WT1 siRNA or non-targeting siRNA. We used the functionally related M15 cell line (murine mesonephros-derived cells), the U2OS cell line (human osteosarcoma cells) as well as the 786-0 cell line (human kidney cancer cells) to analyze gene expression patterns in human cellular systems. Subsequent co-transfection experiments using various reporter constructs indicated that the promoters of the homeobox b9 (Hoxb9), natriuretic peptide receptor 3 (Npr3) and dishevelled associated activator of morphogenesis 2 (Daam2), among other genes, are significantly stimulated by WT1.

With these findings, we identified sets of genes in murine embryonic kidneys that are regulated either directly or indirectly by WT1. In conclusion, combination of vivo-morpholino induced gene knockdown in embryonic kidney explants with deep RNA-sequencing is a useful approach for analyzing transcriptional networks in kidney development.

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using the XF-24 extracellular flux analyser (Seahorse Bioscience, Inc.). Imaging the kinetics of ATP in  $\beta$ -cells was performed on a zoom microscope AxioZoom.V16 (Carl Zeiss) and utilised fluorescent sensor for  $Mg^{2+}$  (Mg-Green, ThermoFisher) as a surrogate for ATP.

In comparison to islets from control mice, islets from diabetic mice showed a significant reduction in the % increase in OCR when ambient glucose was raised from 2 to 20 mmol/l (diabetic =  $48.44 \pm 8.98$  vs. control =  $91.29 \pm 7.26\%$  increase in OCR,  $p < 0.005$ ;  $n = 9-12$ , 6 animals/genotype). Sequential addition of the ATP-synthase inhibitor oligomycin produced significantly less inhibition of OCR in islets from diabetic mice compared to control, indicating hyperglycaemia reduces the activity of ATP-synthase (diabetic =  $88.72 \pm 6.43$  vs control =  $123.05 \pm 5.59\%$  decrease in OCR,  $p < 0.05$ ;  $n = 5-7$ , 3 animals/genotype). Subsequent addition of rotenone and antimycin A, which inhibit complex 1 and 3 of the electron transport chain respectively, suppressed OCR to the same degree in both diabetic and control islets, indicating no difference in the level of mitochondrial leak. ATP increase in response to 20 mmol/l glucose was reduced in hyperglycaemic islets by  $56 \pm 6\%$ .

Our results demonstrate that hyperglycaemia leads to impaired mitochondrial respiration and ATP production in pancreatic islets. Mitochondrial metabolism is essential for the stimulation of insulin secretion by glucose and therefore hyperglycaemia-induced mitochondrial dysfunction is likely to contribute to  $\beta$ -cell failure in T2DM. It remains to be determined if the deleterious effects of hyperglycaemia are limited to  $\beta$ -cells or if they occur in all islet cell types.

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C071

### Hyperglycaemia adversely affects mitochondrial function in pancreatic islets

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Glucose metabolism is essential for glucose-stimulated insulin secretion from pancreatic  $\beta$ -cells. Metabolically generated ATP causes  $K_{ATP}$  channel closure, membrane depolarisation and calcium influx, which stimulates exocytosis. There is accumulating evidence to suggest that  $\beta$ -cell mitochondrial metabolism is impaired in type 2 diabetes mellitus (T2DM), and contributes to the reduced insulin secretion. As hyperglycaemia is common to all forms of diabetes, we aimed to determine if hyperglycaemia adversely affects mitochondrial function and thereby ATP production in pancreatic islets.

In human patients and animal models of T2DM, hyperglycaemia often occurs in conjunction with elevated levels of circulating lipids, making it difficult to distinguish between the deleterious effects of glucose and lipids. We therefore used the  $\beta V59M$  mouse model in which hyperglycaemia is not accompanied by dyslipidaemia. In these mice, tamoxifen-inducible expression of a constitutively open  $K_{ATP}$  channel specifically in pancreatic  $\beta$ -cells inhibits insulin secretion and rapidly elevates blood glucose ( $>20$  mmol/l). Diabetes was induced at 12 weeks of age and islets isolated 2 weeks later. Non-diabetic littermates were used as controls. Cellular Oxygen Consumption Rate (OCR) was monitored in real-time

C072

### The impact of acute exercise upon myokine secretion in rat models outbred for either low or high aerobic capacity in response to treadmill run training

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**Introduction:** Exercise causes skeletal muscles to express and secrete myokines, that in turn stimulate autocrine/paracrine, and endocrine activities. Myokines secreted from muscle are purported to promote adaptations (e.g. mitochondrial biogenesis (1) and trans-organ cross-talk to exert numerous beneficial effects of exercise upon health (2). Nonetheless, the functional role of myokines in governing exercise adaptation is ill defined. To address this, we adopted 'low responder trainer' (LRT) and 'high responder trainer' (HRT) rat models to compare myokine responses to acute exercise. We hypothesised that HRT animals would display greater myokine secretion than LRT reflecting differences in adaptive potential.

**Methods:** LRT and HRT ( $N=8$ ) rats were selectively bred for low/high adaptive capacity to running training (3). Blood was collected at baseline and then immediately, 1h, and 3h after a bout of running exercise. Plasma was analysed using targeted

proteomics by multiplex ELISA to concurrently quantify 12 myokines: BDNF, FGF21, Fractalkine, IL-6, IL-15, Irisin, Erythropoietin, FSTL-1, Myostatin, LIF, SPARC, and Osteocrin. Data were analysed by ANOVA and Bonferroni tests;  $P < 0.05$  being significant.

Results: BDNF and FSTL-1 exhibited no changes in concentration after exercise. However, Myostatin, IL-15, and Fractalkine concentrations declined 3h post-exercise in both LRT ( $P = 0.039$  (30%),  $P = 0.004$  (12%),  $P = 0.012$  (40%) respectively) and HRT ( $P = 0.025$  (20%),  $P = 0.008$  (10%),  $P = 0.004$  (40%) respectively) animals. Similarly, plasma Irisin paradoxically declined by ~20% at 1h post-exercise, only in LRT animals ( $P = 0.032$ ). In contrast, IL-6 and LIF increased at 3h post-exercise in both LRT ( $P = 0.02$  (130%),  $P = 0.027$  (20%) respectively) and HRT ( $P = 0.0004$  (310%),  $P = 0.016$  (20%) respectively). In addition in the HRT group only, FGF21 was approximately doubled post exercise (at 0 and 3h,  $P < 0.05$ ), Erythropoietin levels also increased by ~20% at 3h ( $P = 0.023$ ), whereas SPARC was elevated by ~50% immediately after exercise ( $P = 0.016$ ). No myokines demonstrated a significant interaction in the two-way ANOVA, except Osteocrin ( $P = 0.004$ ), where LRT animals exhibited a greater increase than HRT.

Conclusion: Our results revealed some anticipated “exercise responses” for established myokines e.g. IL-6/LIF increasing and Myostatin decreasing, while IL-15/Irisin paradoxically dropped. Across models, there were only subtle differences including moderate temporal increases in FGF21, Erythropoietin and SPARC specific to HRT animals. Osteocrin was higher at baseline, increased significantly immediately post exercise and remained higher throughout in the LRT. We conclude that while differences exist in myokine secretion, in LRT vs. HRT, these differences are subtle and seem unlikely to drive the extreme phenotypic metabolic adaptations to exercise in LRT vs. HRT animals.

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## C073

**Expression of TASK3\_G236R channels, associated with KCNK9 Imprinting Syndrome, is reduced at the plasma membrane but this does not account for all of the functional consequences of this mutation.**

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KCNK9 Imprinting Syndrome is a severe neurological disorder caused by a mutation (G236R) of the two-pore domain potassium (K2P) channel, TASK3. The TASK3\_G236R mutant channel displays considerably reduced current density compared to wildtype (WT) TASK3 channels (1, 2). In this study, we have investigated whether this difference arises from altered expression of the mutant channel at the membrane and whether the altered functional properties of the channel can be explained by this.

Recombinant human TASK3 and TASK3\_G236R mutant channels were tagged with green fluorescent protein and transiently expressed in tsA201 cells. The plasma membrane was

stained with deep red cell mask and cells were viewed using confocal microscopy. Correlation data between the channel and the membrane were expressed as mean ( $\pm$  SEM) of the Pearson's Correlation Coefficient (PCC) and the Mander's Overlap Coefficient (MOC) and  $n$  = cells. Statistical analyses were with unpaired  $t$  tests.

For TASK3 channels, PCC values were  $0.55 \pm 0.02$  ( $n = 25$ ) compared to  $0.41 \pm 0.04$  ( $n = 24$ ) for TASK3\_G236R channels ( $p = 0.003$ ). Similarly, MCC values were  $0.84 \pm 0.01$  ( $n = 25$ ) for TASK3 compared to  $0.75 \pm 0.02$  ( $n = 24$ ) for TASK3\_G236R ( $p < 0.0001$ ). Therefore, although the mutant channel was expressed at the membrane, expression levels were significantly less than for WT channels.

The functional properties of TASK3\_G236R mutant channels expressed at the membrane are altered for mutant channels compared to WT. Activation of  $G_{\alpha_q}$ -coupled, H1 receptors by the wake-active neurotransmitter histamine, has been shown to inhibit TASK3 channels and this underlies activation of cholinergic neurons by histamine in the basal forebrain (3). Whole-cell patch clamp recordings were obtained from recombinant TASK3 and TASK3\_G236R channels both co-expressed with histamine (H1) receptors. Current through TASK3 channels co-expressed with H1 receptors was inhibited by  $74 \pm 3\%$  ( $n = 4$ ) following activation of the receptors by 20 mM histamine. Conversely, inhibition of TASK3\_G236R channels by 20 mM histamine, was significantly attenuated ( $p < 0.001$ ) and, indeed, a small activation of current of  $11 \pm 6\%$  ( $n = 5$ ) by histamine was observed.

Thus, whilst less TASK3\_G236R channels are expressed at the plasma membrane, this cannot explain all of the effects of the mutation on the channel, since inhibition of these TASK3\_G236R channels by histamine receptor activation is significantly attenuated. Attenuation of histamine inhibition may contribute to the low arousal state seen in patients with KCNK9 Imprinting Syndrome (4). Furthermore, therapeutic strategies to increase TASK3\_G236R channel expression at the membrane in KCNK9 Imprinting Syndrome will not overcome all of the functional consequences of the mutation.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## C074

**Activation of mGluR5 on parvalbumin-positive interneurons contributes to oscillation memory in mouse hippocampal CA3 region**

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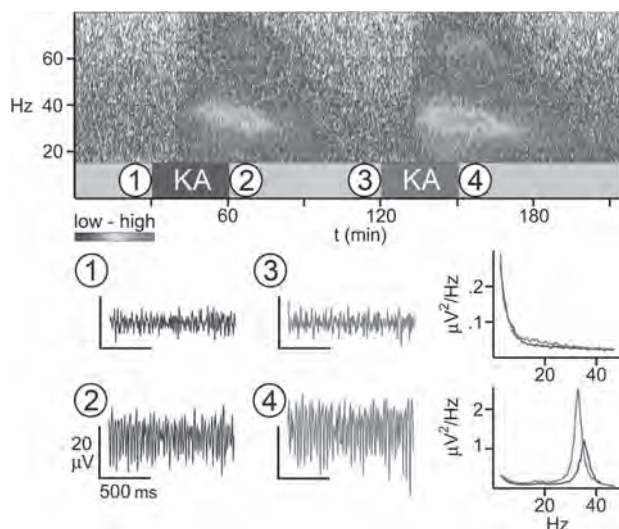
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Deficiencies of network activity in the gamma frequency band ( $\gamma$ , 30 – 80 Hz) and of parvalbumin-positive interneurons (PVI) are frequently observed in models of neuropsychiatric disorders. However, little is known of how they interact in disease. Alterations of activity-induced, mGluR5-dependent synaptic plasticity of excitatory inputs onto PVIs (Zarnadze et al., 2016) may link these phenomena and serve as a mechanism of action in neuropsychiatric pathophysiology.

We prepared acute hippocampal slices of wild-type C57Bl6/J mice (p45 – p70) and performed local field potential recordings in the CA3 region. Following 30 minutes of recording baseline activity, we induced a first, transient period of network activity by brief bath-application of kainate (KA, 150 nM). After one hour of washout, which allowed for a return to baseline activity and an additional resting period, an identical second induction of network activity resulted in a roughly doubled amplitude of  $\gamma$ -power (Mean  $\pm$  SEM: 209.38  $\pm$  15.31 % of the first induction period,  $n = 32$ ). Reminiscent of synaptic plasticity, we call this “oscillation memory” (Fig. 1).

To elucidate how mediators of synaptic plasticity contribute to oscillation memory, we continuously bath-applied D-AP5 (50  $\mu$ M) or MPEP (10  $\mu$ M), pharmacological antagonists of NMDA-R and mGluR5, respectively. Whereas D-AP5 had no significant effect (248.51  $\pm$  19.22 %,  $n = 42$ ; Mann-Whitney U-test vs Control:  $p > 0.05$ ), application of MPEP attenuated oscillation memory by approximately 50%, as did co-application of both substances (MPEP: 155.90  $\pm$  12.72 %,  $n = 36$ ;  $p < 0.01$ , D-AP5 + MPEP: 159.22  $\pm$  12.77 %,  $n = 37$ ;  $p < 0.01$ ). In mice undergoing conditional postnatal ablation of mGluR5 in PVIs (PV-mGluR5<sup>-/-</sup>), oscillation memory was impaired with no additional reduction by MPEP (PV-mGluR5<sup>-/-</sup>: 158.32  $\pm$  7.96 %,  $n = 46$ ; PV-mGluR5<sup>-/-</sup> + MPEP: 153.26  $\pm$  14.51 %,  $n = 25$ ; Mann-Whitney U-test vs PV-mGluR5<sup>-/-</sup>:  $p > 0.05$ ), indicating that mGluR5 activation on PVIs contributes to enhancing subsequent  $\gamma$ -activity in wild-type animals.

Our data suggest potentiation of  $\gamma$ -activity as a novel function of synaptic plasticity specific to PVIs. Furthermore, our finding in the conditional mGluR5 knockout, an animal model reported to exhibit behavioral hallmarks of neuropsychiatric disease (Barnes et al., 2015), hints at a functional link between PVI plasticity, oscillation memory and neuropsychiatric pathophysiology.



**Oscillation Memory in Mouse Hippocampal CA3 Region.** Transient network activity was evoked by bath-application of 150 nM KA. During washout, activity returned to baseline levels. A second induction period produced a marked increase in  $\gamma$ -power. *Left* Time-frequency plot of an exemplary trace (Band Pass filtered, 15 – 80 Hz). Insets mark exposure to KA (“KA”) and time window of analysis (1-4). *Right* Close-Up traces of baseline activity preceding KA application (1, 3) and subsequent  $\gamma$ -activity (2, 4) with corresponding Fast Fourier transforms.

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C075

## Analysis of motor learning in head fixed mice during voltage imaging

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Acquisition of complex movement sequences is termed motor skill learning<sup>1</sup>. The motor cortex provides a substrate for adaptive alterations during the achievement of these skills<sup>2</sup>. Voltage imaging techniques enable monitoring of electrical activity from a large population of Layer 2/3 motor cortex neurons in awake behaving animals<sup>3</sup> and correlates neural activity with behaviour during learning<sup>4</sup>. Here we aim to standardize a motor learning behavioural test (lever pulling task) for head fixed conditions in order to determine changes in Layer 2/3 connectivity during motor learning. We developed a novel self-initiated lever-pull task and monitored the mouse cortex in vivo using wide field voltage imaging. Twelve Rasgrf 2-2AdCre;Camk2a-tTA;Ai78 (TITL-VSFP-B) mice were used, aged 3 to 4 months. Mice were anaesthetised with ketamine (150mg/Kg) and domitor (1.5mg/Kg), the skull was thinned and a head holder frame was attached to the skull using dental cement. After 15 days of recovery, mice were water restricted (body weight was maintained >85% of the initial) for 5 days prior to training. Head-restrained mice were trained to perform a lever pull task, a four  $\mu$ l water drop was provided as a reward when the lever was pulled beyond 3° positional threshold. Mice performed 16 trials (10 seconds each) per day over 3 days. Different parameters were recorded using a custom made interface, variables are reported as means  $\pm$  S.E.M., compared by Friedman test and Dunn’s multiple comparison test. Four mice obtained less than one reward per trial on day 3 of training and were excluded. Mice obtained more water rewards on day 3 of training (294 $\pm$ 28) compared with day 2 (177 $\pm$ 39) and day 1 (189 $\pm$ 43) ( $p < 0.05$ ). Out of the 16 trials mice performed, water rewards were obtained in 69.53 $\pm$ 9.98%, 70.31 $\pm$ 9.13% and 80.47 $\pm$ 5.83% of the trials for days 1, 2 and 3, respectively; significant differences were not detected. Mice learned to associate pulling the lever with a water reward, as the total pulling time per trial on day 3 (5.42 $\pm$ 0.73s) was higher than day 2 (3.82 $\pm$ 1.01s) ( $p < 0.05$ ) and day 1 (2.93 $\pm$ 0.63s) ( $p < 0.01$ ). Conversely, the total push time (no reward) per trial decreased on day 3 (1.46 $\pm$ 0.56s) compared with day 2 (2.45 $\pm$ 1.009s) ( $p \geq 0.05$ ) and day 1 (2.93 $\pm$ 0.63s) ( $p < 0.05$ ). Mice also increased the amount of time the lever crossed the reward threshold per trial on day 3 (3.03 $\pm$ 0.73s) compared with day 2 (1.85 $\pm$ 0.81s) ( $p \geq 0.05$ ) and day 1 (1.01 $\pm$ 0.35s) ( $p < 0.01$ ). Mice improved their movement accuracy by decreasing the pulling range and keeping the lever close to the reward threshold position. Thus, mice successfully learned to pull a lever and refine motor movements to obtain greater amounts of water over 3 days of training. Our results indicate that this lever pulling test measures motor skill learning in mice that can be integrated with mesoscopic voltage imaging to generate connectivity maps during motor learning.



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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## C076

### Regulation of voltage-gated sodium channel $\beta 1$ subunit function by secretase cleavage

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Voltage-gated  $\text{Na}^+$  channels (VGSCs), composed of a pore-forming,  $\text{Na}^+$ -conducting  $\alpha$ -subunit and auxiliary  $\beta$ -subunits ( $\beta 1$ - $\beta 4$ ), are responsible for the inward  $\text{Na}^+$  current ( $I_{\text{Na}}$ ) underlying neuronal action potentials.  $\beta$ -subunits regulate  $\alpha$ -subunit function by promoting plasma membrane localisation, thereby increasing  $I_{\text{Na}}$ , and through alteration of  $\alpha$ -subunit activation and inactivation kinetics. Perturbations in  $\beta$ -subunit function contribute to various channelopathies. For example, mutations in  $\beta 1$  have been reported in generalised epilepsy with febrile seizures (GEFS+) and Brugada syndrome. Also, VGSC subunits, including  $\beta 1$ , promote breast cancer invasion and metastasis. However, the interaction between  $\beta$ -subunit domains and  $\alpha$ -subunit function is incompletely understood. Several  $\alpha$ -subunit interaction sites are within the  $\beta 1$  extracellular domain (ECD) and intracellular domain (ICD).  $\beta 1$  is sequentially processed by  $\beta$ - and  $\gamma$ -secretase, releasing the ECD and ICD respectively, yet the impact of this proteolysis on  $\beta 1$ -mediated regulation of  $\alpha$ -subunit function has yet to be delineated. Here, we investigated how secretases influence  $\beta 1$ -mediated regulation of  $I_{\text{Na}}$  in MDAMB231 (231) cells using whole-cell patch clamp recording. Results are expressed as mean  $\pm$  SEM, statistical significance determined using ANOVA with Tukey's post hoc tests. Treatment of 231 cells expressing  $\beta 1$ -GFP with a  $\gamma$ -secretase inhibitor significantly decreased  $I_{\text{Na}}$  from  $-15.2 \pm 1.3$  pA/pF to  $-8.0 \pm 1.4$  pA/pF ( $n=12-16$ ;  $P<0.01$ ) without affecting 231 cells expressing GFP alone ( $-6.9 \pm 1.1$  pA/pF vs.  $-5.9 \pm 1.1$  pA/pF;  $n=14-16$ ;  $P=0.996$ ). This result suggests that  $\gamma$ -secretase is involved in regulating  $\beta 1$ -mediated modulation of  $\alpha$ -subunit activity. To determine whether  $\beta 1$ ICD is directly involved in regulating  $\alpha$ -subunit function,  $\beta 1$ ICD-GFP was expressed in 231 cells and  $I_{\text{Na}}$  compared to 231-GFP and 231- $\beta 1$ GFP cells. 231- $\beta 1$ ICDGFP cells displayed a significantly larger  $I_{\text{Na}}$  than 231-GFP cells ( $-9.3 \pm 1.0$  pA/pF vs.  $-4.4 \pm 0.6$  pA/pF;  $P<0.01$ ;  $n=14-16$ ). Furthermore, the  $I_{\text{Na}}$  in 231- $\beta 1$ ICD-GFP cells was not significantly different from that of 231- $\beta 1$ GFP cells ( $-11.7 \pm 0.9$  pA/pF;  $n=15$ ;  $P=0.2$ ). These data support the notion that cleaved  $\beta 1$ ICD is involved in regulating  $I_{\text{Na}}$ . To further investigate the importance of secretase cleavage, a cleavage-resistant  $\beta 1$  (SR $\beta 1$ ), lacking the  $\beta$ -secretase site (amino acids 142-147), was expressed in 231 cells. We anticipated that if  $\beta 1$ ICD is necessary for increasing  $I_{\text{Na}}$ , then SR $\beta 1$  should not increase  $I_{\text{Na}}$  compared to 231-GFP control cells. Surprisingly, SR $\beta 1$  expression significantly increased  $I_{\text{Na}}$  compared to 231-GFP cells ( $-12.9 \pm 1.2$  pA/pF vs.

$-5.9 \pm 0.8$  pA/pF;  $n=15$ ;  $P<0.05$ ). The  $I_{\text{Na}}$  of 231-SR $\beta 1$ -GFP cells was also significantly smaller than the  $I_{\text{Na}}$  in 231- $\beta 1$ GFP cells ( $-21.2 \pm 2.8$  pA/pF;  $n=15$ ;  $P<0.01$ ). In conclusion,  $\beta 1$  regulates  $I_{\text{Na}}$  by both secretase-dependent and secretase-independent mechanisms. Further work is required to elucidate the molecular pathways involved.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## C077

### Neural mechanisms of numerical processing in typically developing children and children with dyscalculia

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Dyscalculia is a learning disability affecting the acquisition of arithmetical skills in children with normal intelligence and age-appropriate education. There are two main hypotheses about dyscalculia: The deficit in quantity processing and the deficit in matching quantities with symbols. It is still unclear which neural mechanisms are related with dyscalculia. In this study, number processing in children with dyscalculia and typically developing children were examined via functional magnetic resonance imaging (fMRI).

The first stage of the study, we tested 1944 third-grade students (7-9,5 years) in elementary schools with Mathematics-and-Arithmetic-Performance-Tests, and Raven-Progressive-Matrices-Test for fluid intelligence. Secondly, determined children were tested two-years later with mathematics tests and Weschler-Intelligence-Scale-for-Children, and evaluated by child psychiatrists. After co-morbidities were eliminated, the dyscalculia ( $n=12$ , mean age: 11.25) and control ( $n=15$ , mean age: 11.26) groups were precisely identified. Participants were asked to perform a numerosity comparison paradigm while undergoing fMRI scan. Paradigm consists of two types of number sense condition and two difficulty levels. Imaging data is analyzed with repeated measures ANOVA with group (dyscalculia and control), number sense (symbol and dot) and difficulty (0.5 and 0.7 ratios) as factors via a MATLAB toolbox SPM12. Intelligence quotient and age of participants were added as covariates.

The main effect of number activations was bilateral intraparietal sulcus (IPS), dorsolateral prefrontal cortex (DLPFC). The main effect of difficulty activations was bilateral insular cortex,

anterior cingulate cortex. For the main effect of group orbitofrontal cortex and ventromedial prefrontal cortex was activated. For group and number interaction hippocampus was activated. A region of interest (ROI) analysis which performed on hippocampal region showed that hippocampus only activated during symbol comparison condition at dyscalculia group.

In the number sense literature, it is suggested that IPS is related to perceiving numerosity and DLPFC is responsible for the cognitive control (attention, memory) which related to numerosity. The frontoparietal network which activated in number task is in line with the literature. The neural network which consists of insular cortex and anterior cingulate cortex activated in the main effect of difficulty. This network is called salience network and it is related to orienting attention to the task. Frontal activation in dyscalculia could be related to the requirement of more cognitive control as a result of a deficiency of parietal mechanism which related to basic number sense. Hippocampal activation in the symbol condition showed that patients with dyscalculia needed to access memory during the task. This might be caused by the deficit in matching symbols and quantities.

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C078

# Functional role of chloride/proton exchangers CIC-3 and CIC-5 in exocytosis of large dense core vesicles in chromaffin cells

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The CLC family is a large family with nine members which fulfill diverse functions, at the plasma membrane or in intracellular compartments.

CIC-3 and CIC-5 belong to a sub-branch of intracellular Cl<sup>-</sup>/H<sup>+</sup> antiporters. The physiological relevance of CIC-3 in the central nervous system (CNS) is demonstrated by the *Clcn3*<sup>-/-</sup> knock-out animal model, which exhibits a pronounced hippocampal and retinal degeneration<sup>[1]</sup> as well as alterations in the excitatory/inhibitory neurotransmission<sup>[1],[2],[3]</sup>.

Beside its role in neuronal transmission, CIC-3 was more recently proposed as regulator in the neuroendocrinal system<sup>[4],[5]</sup>. CIC-5 is required for endocytosis in the proximal tubule, and so far no role has been assigned in neuronal or neuroendocrinal cells.

The aim of this project is to characterize the role of CIC-3 in regulated exocytosis of large dense core vesicles (LDCVs) in neuroendocrinal cells. We characterized exocytosis in adrenal chromaffin cells through a combined technique of cell membrane capacitance and amperometric recordings. In contrast to previous results<sup>[4],[5]</sup> where exocytosis in adult (P60) *Clcn3*<sup>-/-</sup> chromaffin cells was severely affected, we could not observe any dramatic effect in exocytosis measured in new born (P0) *Clcn3*<sup>-/-</sup> mice. Therefore, we investigated whether another chloride/proton exchanger might compensate for the absence of CIC-3 in early developmental stages.

Quantitative real-time PCR experiments showed that CIC-5 is less abundant (down-regulated) in adrenal glands extracted

from adult mice. CIC-5 was up-regulated in absence of CIC-3 in new born mice, suggesting a possible compensating role. To test the effect of CIC-5 in exocytosis, we reduced the expression of CIC-5 (via shRNA Knockdown strategy, using the lentiviral expression system) in chromaffin cells isolated from *Clcn3*<sup>-/-</sup> P0 mice. The exocytotic response in such double mutant (*Clcn3*<sup>-/-</sup>/*Clcn5*<sup>KD</sup>) cells was significantly decreased, similarly to the impairment observed in exocytosis measured in *Clcn3*<sup>-/-</sup> P60 cells. Furthermore, analysis of single amperometric events did not reveal any impairment in the amount of released catecholamines, indicating that most probably CIC-3 and CIC-5 do not regulate neurotransmitter loading in LDCVs. The reduced spike frequency observed in *Clcn3*<sup>-/-</sup> P60 cells and DKO P0 cells reveals a decreased LDCVs fusion frequency, reflecting a reduced number of primed vesicles. We therefore monitored cell membrane capacitance in response to depolarizing stimuli in order to evaluate the vesicle priming process. (*Clcn3*<sup>-/-</sup>/*Clcn5*<sup>KD</sup>) cells exhibit a significant reduction in the total cell membrane capacitance as well as in the first 4<sup>th</sup> depolarizing step, suggesting that Cl<sup>-</sup>/H<sup>+</sup> exchangers regulate LDCVs priming.

We conclude that CIC-3 and CIC-5 are crucial regulators in the priming process of LDCVs in adrenal chromaffin cells.

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C079

# Astrocytic glutamate clearance is regulated by neuron-dependent Notch signalling as well as cAMP

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Glutamate clearance is an important function of astrocytes, preventing excitotoxic neurotransmitter build-up. The two astrocytic transporters responsible for this glutamate clearance are EAAT1 (*Slc1a3*) and EAAT2 (*Slc1a2*). Neurons have been shown to induce astrocytic expression of *Slc1a2* and *Slc1a3* genes; however, the pathways mediating this induction are unclear. As altered astrocytic glutamate uptake is implicated in numerous neurodevelopmental and neurodegenerative diseases, such as Alzheimer's disease, elucidating the mechanisms that regulate these transporters is of clinical importance<sup>1,2</sup>. Using a novel mixed-species RNAseq approach, our lab has recently shown

that numerous astrocytic genes are regulated by neurons, including glutamate transporter and Notch pathway genes<sup>3</sup>. We used these findings to investigate astrocytic EAAT function and the mechanism behind their regulation. Primary cultures of cortical mouse astrocytes (from E19.5 pups) were grown either alone as monoculture, or cocultured with cortical rat neurons (from E21.5 pups)<sup>3</sup>. Glutamate transporter function was measured electrophysiologically in astrocytes by transporter currents induced by application of the EAAT agonist L-aspartate (200  $\mu$ M) in the presence of AP-5 (100  $\mu$ M). Current identity was confirmed by the EAAT inhibitor TFB-TBOA (20  $\mu$ M). Results are given as mean  $\pm$  S.E.M. assessed by unpaired t-test. There was a significant increase in EAAT currents in cocultured compared to monocultured astrocytes ( $I = 3.2 \pm 0.9$  vs  $34.2 \pm 5.4$  pA,  $p < 0.01$ ,  $n = 19$  (mono),  $n = 12$  (co)). To investigate the involvement of Notch signalling in this induction, the  $\gamma$ -secretase inhibitor DAPT was applied to cocultured astrocytes to inhibit Notch signalling. DAPT treated astrocytes had significantly lower EAAT currents compared to untreated cells ( $I = 9.9 \pm 1.8$  vs  $34.2 \pm 5.4$  pA,  $p < 0.01$ ,  $n = 10$  (DAPT),  $n = 12$  (ctrl)). To confirm the role of Notch signalling, monocultured astrocytes were transfected with the constitutively active Notch effector CBF1-VP16 or a globin control. Astrocytes expressing CBF1-VP16 had greater EAAT currents than controls ( $I = 15.8 \pm 2.8$  vs  $4.2 \pm 1.2$  pA,  $p < 0.01$ ,  $n = 10$  (CBF1)  $n = 8$  (globin)). Finally, cyclic AMP (cAMP) analogues have been shown to increase astrocytic EAAT expression. To determine the ability of cAMP to increase EAAT function, monoculture astrocytes were treated with 8-Br-cAMP (0.5 mM). After 1wk of treatment there was an increase in astrocytic EAAT currents ( $I = 59 \pm 12$  vs  $13 \pm 4$  pA,  $p < 0.01$ ,  $n = 25$  (8-Br),  $n = 20$  (ctrl)). This enhancing effect was also observed in coculture astrocytes, and separately in astrocytes treated with a combination of forskolin and IBMX to boost cellular cAMP levels. These data show that both neuron-dependent Notch signalling and cAMP can regulate astrocytic EAAT function, offering new clinically relevant targets to explore to alter astrocytic glutamate uptake capacity. Beart PM, O'Shea RD. Transporters for L-glutamate: An update on their molecular pharmacology and pathological involvement. *British Journal of Pharmacology*. 2007;150(1):5-17.

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C080

# **ASICs in mechanosensation: ASIC2a deletion enhances stretch-evoked responses in mouse muscle spindle primary afferents.**

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Amiloride-sensitive, or acid-sensing, ion channels (ASICs) are highly expressed in all primary mechanosensory nerve endings examined. However, their role there is unclear, since an involvement in neurosensory mechanotransduction is

disputed<sup>1-3</sup>. Nevertheless, ASICs are one of the few putative mechanosensory channel families with a selective conductance for Na<sup>+</sup>, the predominant current carrier of the receptor potential in muscle spindles<sup>4</sup>. We have shown high expression of ASIC2a in muscle spindle primary sensory terminals<sup>3</sup>. We therefore generated knockout (*Asic2a*<sup>-/-</sup>) mice and examined the effect on mechanosensory responses of parvalbumin-expressing sensory neurones innervating muscle spindles. Soleus nerve-muscle preparations were removed from *Asic2a*<sup>-/-</sup> mice or their wild-type littermates ( $n = 5$  each genotype), after humane killing. Their afferent firing was then recorded in response to stretch-hold-release (ramp) and saw-tooth length changes of 0.2, 1 and 5 Hz. The number of action potentials (APs) fired, or the firing rate (APs/sec) in the whole muscle nerve during particular phases of the stretch profile were quantified and compared between genotypes. The sensitivity of firing to amiloride, a broad-spectrum blocker of ASICs, was also examined. Data presented as mean  $\pm$  SEM, and WT vs KO. The significance of differences were ascertained by ANOVA, threshold  $P = 0.05$ .

ASIC2a knock out enhanced both hold-phase spindle afferent firing rate ( $178.9 \pm 8.4$  vs  $219.1 \pm 10.0$  APs/sec;  $P < 0.01$ ), and inhibition of firing during release (release deficit,  $-34.1 \pm 2.3$  vs  $-47.6 \pm 3.0$  APs;  $P < 0.001$ ). Dynamic firing was enhanced at low stretch rates (dynamic excess;  $0.2\text{Hz} - 195.0 \pm 1.0$  vs  $255.8 \pm 1.2$  APs,  $P < 0.0001$ .  $1\text{Hz} - 72.8 \pm 0.3$  vs  $76.1 \pm 0.3$  APs,  $P < 0.0001$ ; data normalised to means) and inhibited at low release rates (release deficit;  $0.2\text{Hz} - 156.8 \pm 1.2$  vs  $148.6 \pm 1.2$  APs;  $P < 0.0001$ ;  $1\text{Hz} - 25.6 \pm 0.3$  vs  $-21.9 \pm 0.3$  APs;  $P < 0.0001$ ; data normalised to means). Similar trends were found at 5Hz. Interestingly, amiloride (1mM) was more effective in inhibiting spindle responses in *Asic2a*<sup>-/-</sup> soleus both during hold phase-firing ( $91.3 \pm 3.07$  vs  $62.1 \pm 5.1$  APs/sec;  $P < 0.0001$ ; data normalised to final control value) and dynamic sawtooth stretches (e.g.  $1\text{Hz}$  dynamic excess –  $85.9 \pm 2.4$  vs  $68.2 \pm 3.4$  APs;  $P < 0.0001$ ; data normalised to final control value). In behavioural tasks, *Asic2a*<sup>-/-</sup> mice had significant deficits in balance beam walking.

We conclude that ASIC2a is a molecular determinant regulating mechanosensitive firing rates in mouse proprioceptors.

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C081

### Loss-of-function mutation causes gain-of-pain syndrome: neuropathy patient harbors a trafficking deficient sodium channel mutation.

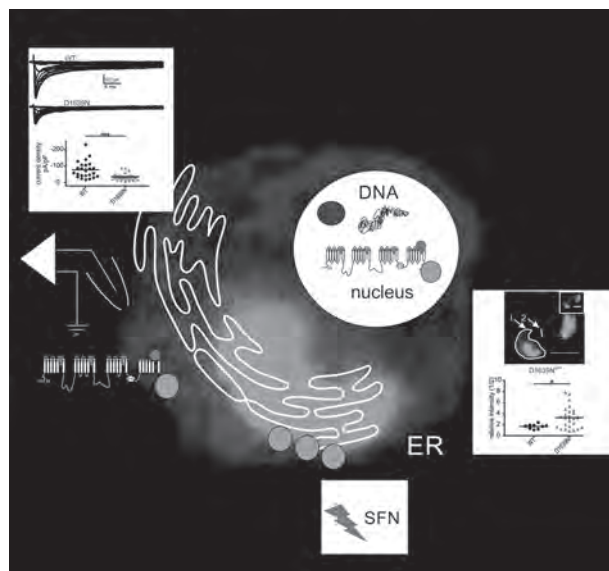
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Mutations in the voltage-gated sodium channels (Nav) 1.7, 1.8, and 1.9 are linked to human pain syndromes. Most of the mutations studied to date present with an intuitive pathomechanism, a direct link between the function of the channel and the phenotype of the patient. However, recent studies reported mutations that cause a gain of channel function but loss-of-pain in the patient, or the opposite: gain-of-pain in the patient but impaired channel function. For these mutations the underlying pathomechanism needs to be elucidated.

In this study, we investigate the D1639N mutation of the SCN10A gene encoding Nav1.8, that has previously been described in a patient suffering from the chronic pain syndrome small fiber neuropathy (SFN). We used the neuroblastoma cell lines (ND7/23 and N1E115 cells) for whole cell patch-clamp experiments and immunofluorescence microscopy. We show that the amino acid substitution within in the channel leads to a significant reduction in current density, but does not alter any other tested biophysical gating properties of the channel (activation, fast and slow inactivation and recovery from fast inactivation). Thus, the D1639N mutation causes loss-of-function of the channel. Neither co-expression of  $\beta$ -subunit 1 or 3, nor overnight incubation at lower temperature (27°C) rescued the current density. However, incubation with 1 mM lidocaine, but not 100  $\mu$ M phenytoin, overnight restored the current density of Nav1.8/D1639N transfected cells. Ion channel blockers have been reported to support channel trafficking to the membrane by stabilizing trafficking deficient proteins, and thus the rescuing effect of lidocaine suggests a trafficking deficit of the D1639N mutation. Accordingly, we show enhanced protein levels in Nav1.8/D1639N injected oocytes (SDS Page). Nav1.8/D1639N transfected neuroblastoma cells harbor enhanced fluorescence intensity ratios when we compare the main cytoplasm with a small cytoplasmic hem near the nucleus. Thus, these enhanced amounts of protein likely accumulate in cell organelles in the cytoplasm, such as the endoplasmatic reticulum (ER). This relative fluorescence intensity is reduced after incubation with 1 mM lidocaine overnight, suggesting that lidocaine dissolves the accumulation of Nav1.8/D1639N and enhances trafficking to the membrane.

According to our results, Nav1.8/D1639N is trafficking deficient. The accumulation of misfolded or trafficking deficient proteins in cell organelles in the cytoplasm, e.g. the ER, is known to possibly result in ER stress. ER stress is a relevant pathogenic factor in systemic diseases. Thus, the accumulation of Nav1.8/D1639N may result in ER stress causing the systemic disease SFN. Lidocaine could potentially be a suitable therapeutic option for this SFN patient carrying the D1639N mutation, and further clinical testing is needed.



Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

C082

### Assesing Empathy in Valproic Acid Induced Autism Model of Rats

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Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder with genetic, neurobiological and environmental roots. This complex disorder has a variety of symptoms from social interaction, communication and empathy deficits to repetitive and stereotyped behavior (1). Animal models play a key role to understand symptoms and mechanism of ASD. Prenatal exposure to valproic acid (VPA) increases the risk of ASD in humans and autism-like symptoms in rodents (2). VPA model of ASD is widely used, however empathy deficits has not been studied with this method yet.

Wistar Albino pregnant females used in this study were grouped to receive either single 400 mg/kg VPA dissolved in saline (n=3) or same volume of saline (control, n=2) intraperitoneally on embryonic day 12.5. On postnatal day 21 (P21) rats were separated from mother and caged in with same sex siblings. Rats were tested for empathy on P30 and P60 in a 25x60x30 cm Plexiglas box. The set up includes a familiar rat from the same cage of subject rat where familiar rat is in a restrainer at the centre of the box. Subject rat, then, was placed in the box and its behavior was observed for 10 minutes. To measure acute empathy and avoid effects of learning, pups were not trained before the test and were not given any reinforcements or punishments. Besides testing for empathy, rats were tested for olfactory discrimination, social recognition, social preference, pre-pulse inhibition, locomotor activity and marble burying for validation of the VPA model. To assess empathy, the socially attentive time of the subject rat with the familiar was measured and subject's tendency to free the restrained rat was calculated by the frequency of attempt to remove the restraining block. Both values were

analyzed with one-way ANOVA and Tukey-Kramer post hoc test. For attentive behavior, control females (CF) (n=15) showed higher values than VPA females (VF) (n=9) on both P30 and P60 ( $p<0.01$ ;  $p<0.01$ ). On the other hand, control males (CM) (n=7) showed significantly higher values than VPA males (VM) (n=17) on P60 only ( $p<0.01$ ). Total control and VPA values differed on P30 and P60 ( $p<0.01$ ;  $p<0.01$ ). Escape attempt frequencies of CF-VF and CM-VM on P30 showed no differences where as on P60 values of CF over VF and CM over VM were higher ( $p<0.01$ ;  $p<0.05$ ). Total control values were higher than VPA on P30 and P60 ( $p<0.05$ ;  $p<0.01$ ). Our results suggest that empathetic behavior in VPA group pups is disrupted compared to controls. Elevation of values on P60 implies that measured behavior improves over time and present in females from earlier days. As a conclusion, VPA-induced model of ASD shows promising data to model empathy defects, thus, gives us an insight on biopsychosocial aspects of ASD.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

C083

## Multi-site phosphorylation of bHLH transcription factors regulates neural differentiation in *Xenopus*

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Throughout embryogenesis there is a critical balance between progenitor cell proliferation and subsequent differentiation; this balance is also pivotal when deranged in diseases like cancer, and to promote differentiation in cellular reprogramming for regenerative medicine (1). During neurogenesis, basic Helix Loop Helix (bHLH) proneural transcription factors act in cascades to drive and coordinate the various stages of neuronal differentiation, yet the same master regulators at the top of the cascade can also critically promote progenitor maintenance to ensure formation of a nervous system of the correct size, shape and integrity. How are these opposing roles coordinated? Proneural proteins Neurogenin2, Ascl1 and NeuroD4 contain conserved Serine/Threonine-Proline (S/T-P) sites that can be targeted by proline directed kinases such as cell-cycle-associated cyclin-dependent kinases (cdks); thus coordinating the activity of the cell cycle and differentiation machinery. Using neurogenesis assays in developing *Xenopus* embryos, the Philpott Lab has established a multi-site phospho-regulation model whereby phosphorylation on S/T-P sites by cdks or other proline-directed kinases, directly inhibits proneural activity to drive differentiation (2-4). Using NeuroD4 as an example, the wild-type (WT) gene was cloned into a

plasmid vector and the serine or threonine residues mutated to alanine (S/T-A) by site-directed mutagenesis to prevent phosphorylation, creating a range of single and sequential site mutant constructs; the latter shown in Figure 1A. mRNA was transcribed *in vitro* and injected into two cell stage *Xenopus* embryos and neurogenesis was assayed in neurula stage embryos by qPCR and *in situ* hybridisation for expression of neural- $\beta$ -tubulin as described in (4). For proneural proteins, this multi-site phosphorylation acts like a rheostat, with proneural activity sensitively regulated by the number of phosphorylation events (Figure 1B). Mechanistically, western blot analysis of over-expressed WT and phospho-mutant NeuroD4 demonstrates that the dephosphorylated protein has enhanced protein stability (Figure 2A). Additionally, by cross-linking the embryos prior to fractionation to extract cytoplasmic and nuclear fractions, even correcting for total protein level the dephosphorylated protein displays a greater association for chromatin (Figure 2B). Together these properties enable the dephosphorylated protein to drive the expression of differentiation genes that require epigenetic remodelling prior to activation (5). Multi-site phosphorylation enables coordination of differentiation activity within the cellular protein kinase environment during development. Additionally, manipulation of proneural protein phospho-status can dramatically enhance neurogenesis for cellular reprogramming (3).

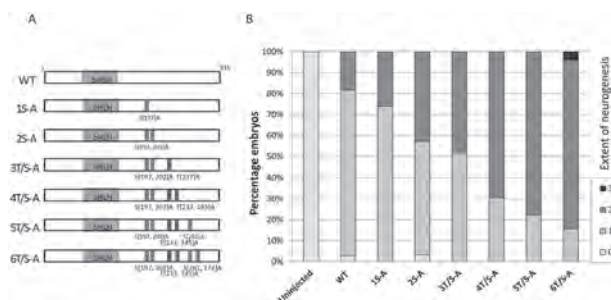


Figure 1: (A) Schematics of WT NeuroD4 and a sequential phospho-mutant series indicating the SP or TP sites that are mutated to AP. (B) Semi-quantitative scoring of neural- $\beta$ -tubulin as described in (4); embryos injected with mRNA encoding constructs; (N=59-77 embryos per category from 3 independent experiments).

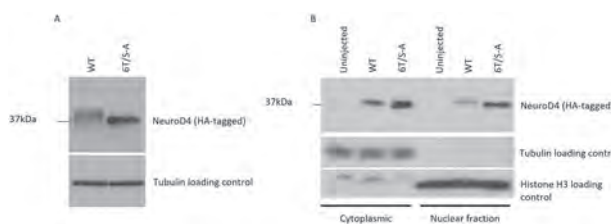


Figure 2: (A) Western blot analysis from whole embryo lysates over-expressing equivalent amounts of WT and phospho-mutant NeuroD4 mRNA; N=3. (B) Western blot of embryos over-expressing WT and phospho-mutant NeuroD4 with cross-linking prior to fractionation as described in (4); N=2.

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Special thanks to Professor Anna Philpott and all members of the Philpott Lab.

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## C084

### Hypoxia-inducible Factor 2 alpha is Essential for Proper Brain Development

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Sufficient tissue oxygenation is required for regular function and survival of cells. Especially in the brain, oxygen supply must be tightly regulated to avoid hypoxia (shortage of oxygen) and irreversible cell damage. The transcription factor complex hypoxia-inducible factor (HIF) plays a key role in the adaptation of cells to hypoxia. Particularly, neural stem cells are sensitive to fluctuating oxygen concentrations. To examine the role of the HIF isoform HIF-2 $\alpha$  during brain development, mice with a neuronal-specific knockout of HIF-2 $\alpha$  were bred. When brains of adult mice were evaluated, a reduction of neurons, especially pyramidal cells, of synapses and of oligodendrocytes in the cortex in HIF-2 $\alpha$  knockout mice was observed compared to wild type animals. Loss of pyramidal neurons occurred mainly in the retrosplenial cortex, a brain region that underpins a range of cognitive functions, including memory and navigation. Accordingly, our behavioral studies showed disturbed cognitive abilities in HIF-2 $\alpha$  knockout mice, mainly concerning learning aptitude and ability of retrospection.

For mechanistic studies, neural stem cells were extracted from postnatal brains and cultivated as neurospheres (3-dimensional cell clusters) in various oxygen conditions (21%, 1% and 0.2% O<sub>2</sub>). Here, we found that loss of HIF-2 $\alpha$  impaired differentiation into neurons and oligodendrocytes, synapse formation and migration as well as prohibiting neural stem cells from death. Genetic analyses confirm that loss in oligodendrocytes is accompanied by diminished *Olig2* expression. Furthermore, we hypothesize that up-regulation of *NeuroD1* in HIF-2 $\alpha$  deficient stem cells leads to a migration deficit to the retrosplenial cortex due to its role in building topographical hierarchies in the developing cortex. This needs to be further evaluated in future.

In conclusion, HIF-2 $\alpha$  plays a significant role in adequate development of the brain and in adaptation of cells to hypoxia.

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## C085

### Effect of insulin on GABAergic signalling in hippocampal dentate gyrus granule cells in a mouse model of Alzheimer's disease

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Epidemiological data suggest that altered insulin signalling can increase the propensity for the development of cognitive decline & progression of Alzheimer's disease (AD) in humans. Also, peripheral glucose & insulin resistance was reported in several mouse models of AD after 9 months (1). The dentate gyrus (DG) is the principal gateway of cortical input to the hippocampus (2) & plays an important role for the formation & recall of memory. It has been shown that the DG region is one of the first & where most plaques are deposited both in AD mouse models & in the brains of humans with AD (3). Also, patients with AD show elevated inhibitory neurotransmitter GABA levels in the cerebrospinal fluid. It has been hypothesized that an abnormal increase in GABAergic tonic conductance in the hippocampus may, in part, underlie the memory impairment in AD (4). As insulin & insulin sensitizers can improve cognitive function in AD (1, 4), we examined the effect of physiological concentrations of insulin on GABAergic signalling in DG granule cells (GCs) in aged wild type (WT) & transgenic (tgSwe) mice overexpressing betaAPP with Swedish mutation (5).

Aged (10–12 months old) male & female tgSwe & C57BL6 WT mice were euthanized by cervical dislocation procedure. Whole-cell patch-clamp recordings were performed on DG GCs from dorsal hippocampal slices (350 $\mu$ m thick). Patched neurons were filled with neurobiotin for further 3D reconstruction. Neuritic plaques were detected by Thioflavin S stain. Values are means $\pm$ S.E.M., compared by two-tailed Student's *t*-test.

The phasic & tonic GABAergic currents were recorded in GCs of dorsal hippocampus in aged WT & tgSwe mice. The frequency of spontaneous inhibitory post-synaptic currents (sIPSCs) recorded from GCs were similar in WT & tgSwe mice. However, the GABA-evoked tonic current amplitude was significantly higher in GCs of DG of tgSwe mice compared to the WT mice (9.2 $\pm$ 1.3pA n=12 vs 4.2 $\pm$ 1.3pA, n=6, p<0.01, respectively) & was partially suppressed by selective inverse agonist for the GABA<sub>A</sub> receptors containing  $\alpha$ 5 subunit L-655,708 (100nM) by 36 $\pm$ 16%, n=3. Further, we pre-incubated slices with insulin (1 nM) & then measured the GABA-activated tonic current amplitude in DG neurons in WT control (4.2 $\pm$ 0.5pA, n=6) & compared with the amplitude obtained in tgSwe mice (7.2 $\pm$ 2.2pA, n=10) but the results were, on the average, similar (p>0.05). sIPSCs of GCs were not modulated by insulin in old WT mice. The frequency of sIPSCs in tgSwe mice was significantly higher in the presence of insulin compared to control condition (0.9 $\pm$ 0.1Hz, n=10 vs 0.6 $\pm$ 0.1Hz, n=9, p<0.05, respectively).

Thus, the data suggest that GABA-activated tonic inhibitory current in DG neurons is increased in tgSwe mice. Insulin at physiological concentrations modulates GABAergic signalling in AD mouse model.

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## C086

### Calcium homeostasis is significantly disrupted in dentate gyrus neurons of acute, ex vivo brain slices of a mouse model of Alzheimer's disease.

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Recent work suggests that disruption of neuronal calcium homeostasis may be one of the early initiators of Alzheimer's disease (AD)<sup>1,2</sup>. However many of these findings have been generated using primary neuronal cultures<sup>3</sup> which are a relatively poor representation of intact brain tissue. To this end, the aim of the present study was to determine if previous work in our lab, which has demonstrated that intracellular calcium regulation was disrupted in primary hippocampal neurons from a transgenic mouse model of AD (3xTgAD mouse), relative to wildtype (non-Tg) controls could be replicated using more physiological ex vivo acute hippocampal brain slices.

Acute hippocampal brain slices (250µm) were prepared from age-matched (18.7±10.3 days, n=61) 3xTgAD and non-Tg mice using a McIlwain tissue chopper and stored in a slice holder containing aCSF for 1 hour prior to experimentation. Slices were then loaded with the calcium-sensitive dye, fluo 2AM (8µM) in a microfluidic oxygenator<sup>4</sup>, before being placed into a perfusion chamber and continuously superfused with aCSF (2ml/min) containing TTX (1µM). Intracellular calcium (Ca<sup>2+</sup>) responses of neurons in the dentate gyrus (DG) to the group 1 metabotropic glutamate receptor agonist (S)-dihydroxyphenylglycine (DHPG), in the absence and presence of elevated K<sup>+</sup> (7.5mM) extracellular aCSF (control K<sup>+</sup>=2.5mM), and/or absence and presence of the cyclic ADP ribose inhibitor, nicotinamide (5mM), were monitored and recorded using WinFluor software. Data were analysed by unpaired Student's t-test and are expressed as mean±S.E.M.

Under control conditions (i.e. K<sup>+</sup>=2.5mM), DHPG (50µM)-evoked Ca<sup>2+</sup> signals were significantly (P<0.05) larger in 3xTgAD neurons relative to those in control neurons (123±29%, n=20-53). However, in the presence of elevated (7.5mM) K<sup>+</sup> aCSF although the magnitude of DHPG-evoked Ca<sup>2+</sup> signals in the non-Tg neurons significantly increased (20±12%; n=53, 5 animals, P<0.05) relative to control, those evoked in the 3xTgAD neurons significantly decreased (32±7%; n=20, 4 animals, P<0.05) relative to controls. Further, we also found that there was no significant difference between mouse genotypes in the degree of attenuation of mGluR – mediated Ca<sup>2+</sup> release by nicotinamide (non-Tg by 11±5.2%; n=30 cells, 4 animals, P<0.05; 3xTgAD by 23±6.2%; n=22 cells, 3 animals, P<0.01).

In conclusion, we have shown that, as with primary hippocampal neuronal cultures, significant differences in calcium handling exist between 3xTg and non-Tg neurons at an early age. This supports the suggestion that AD pathogenesis may occur well before more overt, neurohistopathological

alterations. Additionally, we have also demonstrated that calcium imaging using acute hippocampal slices is a versatile, less time consuming and, arguably, more physiological methodology than primary neuronal culture.

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## C087

### Neuronal coding of food-seeking and food intake in hypothalamic networks

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Feeding is largely regulated by hypothalamic neuronal circuits, yet little is known about neuronal coding of different feeding-related behaviours by the lateral hypothalamus (LH) and its extrahypothalamic inputs. Combining optogenetics with electrophysiological recordings in freely behaving mice, we have identified a novel top-down pathway from medial prefrontal cortex via lateral septum to lateral hypothalamus, which utilizes gamma synchronization (30-90 Hz) to regulate food-seeking by dynamic reorganization of functional cell groups in the LH (1). During viral injections and electrode implantations mice were anaesthetized using carprofen (s.c., 5 mg/kg), lidocain (local) and isoflurane (inhalation, 1.5–3% in oxygen). All animal experimental procedures accorded with current national legislation/guidelines.

The gamma-rhythmic input enabled fine-time scale separation of LH cells according to their feeding-related activity: a subset of LH neurons, active at the food location ("food-match" cells), prominently reduced their firing during the gamma oscillation trough. In contrast, LH cells, which were preferentially active distantly from the food zone ("food-mismatch" cells), showed high excitability at the gamma trough. The selective induction of food seeking (reduced latency: control: n = 8 mice, opsin: n = 10 mice; P = 0.0004, t-test; preference of the food zone: control: n = 8 mice, opsin: n = 12 mice, P = 0.0047, binomial test), without changes in food intake (control: n = 8 mice, opsin: n = 9 mice, P = 0.8, t-test) by gamma-rhythmic stimulation contrasts with an increased food intake during somatic optogenetic stimulation of LH GABA (Vgat-positive)

cells (optogenetic stimulation (control:  $n = 7$  mice, opsin:  $n = 5$  mice,  $P = 0.0078$ ), a subgroup of LH cells, which also mediates arousal (2). To investigate intrahypothalamic network responses which underlie differential functions of LH Vgat cells vs. lateral septum-LH projections in feeding behaviours, we studied effects of these manipulations on firing of LH neurons. Optogenetic stimulation of LH Vgat cells evoked a prominent increase in firing of “food-match” but not “food-mismatch” cells (mismatch:  $n = 31$  cells, match:  $n = 15$  cells;  $P = 0.007$ , t-test). In contrast, gamma-frequency optogenetic stimulation of LS-LH projections increased firing of “food-mismatch” rather than “food-match” neurons (mismatch:  $n = 12$  cells, match:  $n = 20$  cells;  $P = 0.019$ , Mann–Whitney test). Thus, neuronal populations in the lateral hypothalamus, as well as top-down gamma-rhythmic signaling, differentially code food-seeking and food intake.

Carus-Cadavieco M, Gorbati M, Ye L, Bender F, van der Veldt S, Kosse C, Börgers C, Lee SY, Ramakrishnan C, Hu Y, Denisova N, Ramm F, Volitaki E, Burdakov D, Deisseroth K, Ponomarenko A, Korotkova T (2017). Gamma oscillations organize top-down signaling to hypothalamus and enable food seeking. *Nature*, 542(7640):232-236.

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## C088

### A human TRPA1-specific pain model

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The cation channel TRPA1 plays an important role in the perception of pain. One of its major functions is to act as a sensor for irritating and potentially hazardous electrophilic substances. TRPA1 involvement has been demonstrated for several diseases. However, for TRPA1 in particular, species differences are pronounced and limit translational research. Therefore, a human TRPA1-specific pain model was established. Our own previous study used carvacrol as TRPA1-agonist. While there is no doubt about TRPA1 activation, the required concentration was high, and a specific antagonist only allowed for a partial (31%) inhibition (Schwarz et al., 2017). JT010 is the most potent published and commercially available agonist, a half-maximal activation in the nanomolar range was reported (Takaya et al., 2015). Using this substance and the so far most potent available antagonist A-967079 we established the first TRPA1-specific pain model. With approval of the ethics committee, 16 subjects were tested in two sessions in a double-blind manner. The first session introduced to the experimental paradigm and contained an injection of phosphate buffered synthetic interstitial fluid, without addition as control and with capsaicin as a painful reference. Intracutaneous injections into the volar forearm were rated every five seconds. In a second session, multiple concentrations of JT010 with and without antagonist were applied in a double-blind manner with a pre-randomized sequence. JT010 caused a higher pain rating than control injections

( $p < 0.001$ , t-test). Pain ratings peaked after 30 s and fully subsided in less than five minutes. The JT010-induced pain was concentration-dependently inhibited by the TRPA1 antagonist, almost complete inhibition (98%) could be achieved. This specific model provides a new tool to validate new drug candidates with respect to their efficacy in humans.

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## C089

### Transient impairment of *in vivo* burst firing in surviving dopamine neurons is associated with a temporary motor deficit after partial lesion

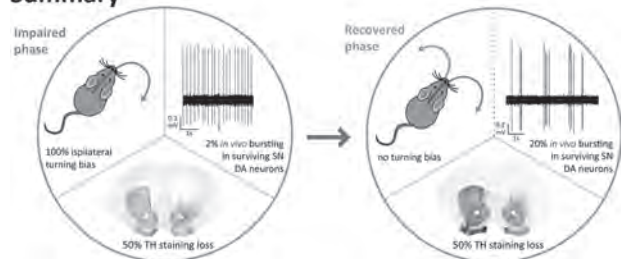
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Parkinson disease (PD) is the second most prevalent neurodegenerative disorder and characterized the selective degeneration of vulnerable neuron populations, prominently among them, dopamine (DA) neurons in the substantia nigra (SN). It is currently unclear to what degree surviving DA SN neurons either homeostatically adapt their activity during PD to compensate for reduced population size and dopamine depletion, or enter a functionally impaired state even before degeneration. To study the long-term temporal profile of post-lesional adaptations of the nigrostriatal DA system, we utilized a unilateral partial 6-hydroxydopamine (6-OHDA) model. By monitoring spontaneous motor behavior of the 6-OHDA and ACSF-infused control mice for 3 month post-surgery, we identified two distinct phases. As expected, the 6-OHDA infused mice displayed a strong contralateral turning deficit compared to ACSF-infused mice during the first few weeks (impaired phase). However, over the course of 2 months this turning deficit completely disappeared (recovered phase). To compare the functional activities of surviving DA SN neurons during these two behavioral states, we carried out *in vivo* single unit extracellular recordings combined with juxtacellular labelling for immunohistochemical und morphological characterization. By focusing on the medial SN, we found that the firing frequencies and patterns of surviving DA neurons recorded during the recovered phase were not significantly different from DA SN neurons from ACSF-infused controls. In contrast, the activity of DA SN neurons from 6-OHDA infused mice recorded during the earlier impaired phase, displayed a significant 7-fold reduction in burst rate (control:  $0.31 \pm 0.14$ ,  $n=9$ ; 6-OHDA:  $0.04 \text{ Hz} \pm 0.02$ ,  $n=9$ ), while their mean frequency of firing was not affected (control:  $5.4 \text{ Hz} \pm 0.51$ ,  $n=9$ ; 6-OHDA:  $4.13 \text{ Hz} \pm 0.59$ ,  $n=9$ ). We are currently investigating the possibility of a causal role of the temporary reduction of burst rate in surviving DA SN neurons for the impaired turning behavior.

## Summary



In our hemiparkinsonian mouse model the spontaneous behavior and *in vivo* firing pattern of the surviving DA neurons changed over time, while the TH staining loss in the striatum remained at 50%. In the early impaired phase (21<sup>st</sup> lesion day) the mice presented with a strong ipsilateral turning bias and the surviving DA neurons displayed reduced bursting in comparison to controls. In the later recovered phase (>3 months) - the 6-OHDA mice showed no turning deficits and the bursting of DA neurons was not significantly different from the controls.

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## C090

### Effects of crocins on intracerebroventricular streptozotocin induced neuroinflammation and peripheral immune changes in experimental rats of Alzheimer's disease

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Different animal models are used to investigate the mechanism of neurodegeneration in AD. Intracerebroventricular streptozotocin injected experimental rats of Alzheimer disease (ICV STZ-AD) develop insulin resistant brain state associated with memory impairment and neurodegenerative changes that share many features in common with sporadic Alzheimer disease (AD) in humans. A possible neuroinflammatory mechanism has been implicated in the memory impairment and neurodegeneration in ICV STZ-induced experimental AD rats which might influence the peripheral immune compartment through the blood brain barrier. The antioxidative and anti-inflammatory effects of crocins have been studied in different experimental animal models of cerebral ischemia, acute and chronic inflammation. The anti-inflammatory and antioxidative potentials of crocins have not been sufficiently explored in the ICV STZ-AD rats. Crocin was applied to find any protective role on the ICV STZ induced neuroinflammation (proinflammatory markers namely TNF- $\alpha$  and IL-1 $\beta$ , ROS and nitrite), histopathological (Cresyl violet staining for Nissl granules) and peripheral immune changes. Brain levels of TNF- $\alpha$  and IL-1 $\beta$ , ROS and nitrite levels were significantly increased in hippocampus and serum of ICV STZ AD rats. The appreciable changes in most of the inflammatory parameters and chromatolysis of Nissl granules of STZ-AD rats were protected after i.p. administration of crocin in these rats. Thus it can be concluded that Crocins by its antioxidative and anti-inflammatory processes can inhibit the STZ induced neuroinflammation and peripheral immune changes in AD rats.

DST, Govt. Of India for INSPIRE FELLOWSHIP to Rupsa Ghosh

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## C091

### Junctophilin-2 Supports Functional Coupling Between Type 2 Ryanodine Receptors and BK Channels in Vascular Smooth Muscle Cells

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Junctophilin-2 (JPH2) is a structural protein that tethers the sarcoplasmic reticulum (SR) to the plasma membrane (PM) within the cardiac dyad. JPH2 is vital for efficient excitation-contraction coupling in cardiomyocytes and is critically important for maintaining the structure of Ca<sup>2+</sup> microdomains that enable crosstalk between ion channels on the PM and SR. Contractile regulation of vascular smooth muscle cells (SMCs) also relies on close interactions between the PM and SR, but little is known about the molecular architecture of these peripheral coupling sites. This study tested the hypothesis that JPH2 is critical for maintaining interactions between the SR and PM and associated signaling pathways in SMCs. C57/BL6 mice were euthanized according to approved protocols by the Institutional Animal Care and Use Committee of the University of Nevada, Reno, and the UK Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act 1986. We found that mRNA encoding JPH2 was present in native, contractile SMCs purified from enzymatically dispersed cerebral pial arteries and enriched using fluorescence-activated cell sorting. JPH2 protein expression was confirmed using the Wes capillary electrophoresis-based immunoassay. Super-resolution microscopy showed significant co-localization of JPH2 and type 2 ryanodine receptors (RyR2) in immunolabeled contractile SMCs. In cerebral arteries SMC, RyR2 on the SR are functionally coupled with Ca<sup>2+</sup>-activated K<sup>+</sup> (BK) channels on the PM, a negative feedback system that buffers vasoconstriction. We employed a molecular interference approach using selective morpholinos to knockdown JPH2 expression in isolated cerebral pial arteries, due to the lack of selective pharmacology. JPH2 protein levels were reduced by ~50% compared with scrambled sequence controls. Perforated patch-clamp electrophysiology was performed to record spontaneous transient outward currents (STOCs), which represent the activation of clusters of BK channels by Ca<sup>2+</sup> released from the SR through RyR2. These studies found that STOCs were essentially absent from SMCs isolated from vessels treated with JPH2-targeted morpholinos, but STOC frequency and amplitude in SMCs treated with scrambled control morpholinos did not differ from untreated controls. To complement our knockdown strategy, we designed a novel inhibitory peptide that is homologous to the Membrane Occupation and Recognition Nexus (MORN) domain of JPH2, necessary for JPH2 to interact with the PM. Conventional whole cell patch clamp method was employed. These experiments showed that STOC frequency was significantly reduced in SMCs loaded with the MORN domain peptide compared to a scrambled control. This study is the first to show the presence of JPH2 in contractile arterial SMCs and its role in maintaining functional coupling between RyR2 and BK channels.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.



C092

# **Nitrate-independent components of beetroot juice reduce blood pressure and improve vascular function in pregnant eNOS<sup>-/-</sup> mice**

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Hypertension and uteroplacental insufficiency remain unsolved problems of pregnancy pathologies, such as fetal growth restriction (FGR). Nitric oxide (NO) is the main mediator of vascular adaptation to pregnancy, maintaining a high flow-low resistance system that favours oxygen and nutrient delivery to the fetus. Beetroot and green leafy vegetables, rich in nitrate, can provide an alternative source of NO. Dietary nitrate supplementation has been shown to lower blood pressure and enhance endothelial function in the non-pregnant state.

We hypothesized that maternal dietary nitrate administration, via beetroot juice (BR), reduces blood pressure (BP) and improves vascular function and fetal growth in the endothelial nitric oxide synthase knockout mouse (eNOS<sup>-/-</sup>), an established model of fetal growth restriction, associated with chronic hypertension and uteroplacental vascular dysfunction.

Pregnant C57Bl/6J (WT) and eNOS<sup>-/-</sup> mice were supplemented with nitrate-containing beetroot juice (BR+, ~1mmol/kg/day nitrate) in the drinking water from embryonic day (E)12.5. Control mice received either an equivalent dose of nitrate-depleted beetroot juice (BR-) or normal drinking water. Systolic BP was measured at E12.5 and E17.5 by tail cuff. Placentas and fetuses were weighed following delivery at E18.5. Endothelium-dependent relaxation to acetylcholine (ACh) was assessed in isolated maternal uterine (UtA) and mesenteric arteries (MesA) at E18.5 using wire myography.

Supplementation with either BR+ or BR- decreased systolic BP (P<0.05) in eNOS<sup>-/-</sup> mice and enhanced UtA relaxation to ACh (P<0.01) in both strains, compared to water controls. Endothelial function was unaffected by treatments in MesA of eNOS<sup>-/-</sup> or WT mice. Fetuses from eNOS<sup>-/-</sup> mice were significantly smaller compared with WT dams (P<0.001) and this difference was not affected by BR+ or BR- supplementation.

These data suggest that nitrate-independent components of beetroot juice lower BP and improve endothelial relaxation within the reproductive vascular system in pregnancies associated with hypertension and reduced NO bioavailability, however, these beneficial outcomes were not associated with an increase in fetal weight.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

C093

# **Mutations of the pore and calcium-binding regions of TMEM16A differentially affect the blocking actions of CaCC<sub>inh</sub>-A01 and niflumic acid.**

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The Ca<sup>2+</sup>-activated chloride channel, TMEM16A, plays an important role in smooth muscle contraction, sensory neuron excitability and fluid secretion. Blockers of the channel like niflumic acid (NFA), A9C and CaCC<sub>inh</sub>-A01 have been characterized previously [1] but the exact binding mechanism of these drugs has not been studied. In the present study, mutations of different regions of the TMEM16A channel were investigated and the effect of niflumic acid and CaCC<sub>inh</sub>-A01 observed. Resultant currents were studied using the whole-cell patch clamp technique from HEK293 cells expressing the wild-type (WT) or mutant channels. For comparison, currents evoked at +100 mV are reported as mean values ± S.E.M.

TMEM16A assembles as a dimer with each monomer unit containing an individual pore and ten transmembrane alpha helices [2]. Point mutation of the Ca<sup>2+</sup>-binding residue E702A on the alpha 7 transmembrane resulted in Ca<sup>2+</sup>-insensitive channels. Application of 30 μM NFA to the WT currents reduced the current amplitude from 2.06 ± 0.24 nA to 0.73 ± 0.13 nA (n=6, p<0.05). However, in the E702A mutant, the effect of NFA was reversed, with it acting as an activator, increasing the current amplitude from 0.58 ± 0.08 to 1.36 ± 0.32 nA (n=6, p<0.05). The effect of CaCC<sub>inh</sub>-A01 (10 μM) did not change, with mutant E702A currents being blocked from 1.06 ± 0.19 to 0.29 ± 0.06 nA (n=6, p<0.05).

CaCC<sub>inh</sub>-A01 (10 μM) reduced current amplitude in the WT from 3.15 ± 0.58 to 0.74 ± 0.17 nA (n=6, p<0.05). Three pore mutations R535A, R515A and K588A [3] situated in α3-α4 linker, α3 and α5 respectively were constructed and the effect of 10 μM CaCC<sub>inh</sub>-A01 compared. The blocking effect of CaCC<sub>inh</sub>-A01 was markedly reduced in the R535A mutant (from 3.08 ± 0.47 to 1.62 ± 0.22 nA, n=6, p>0.05), suggesting it might possibly be a binding site residue for the drug. However, 30 μM NFA blocked R535A mutant currents similar to WT (from 1.69 ± 0.18 to 0.66 ± 0.09 nA, n=6, p<0.05). The R515A mutant resulted in channels with altered kinetics and neither NFA, nor CaCC<sub>inh</sub>-A01, were able to effectively block these currents (e.g. 10 μM CaCC<sub>inh</sub>-A01 reduced them only from 0.85 ± 0.14 to 0.72 ± 0.13 nA, n=6, p>0.05; 30 μM NFA reduced them from 1.08 ± 0.24 to 0.79 ± 0.18 nA, n=6, p>0.05). The effect of CaCC<sub>inh</sub>-A01 was not altered on the K588A mutant, with the channels being blocked as in the WT. These results reflect two distinct regions in the TMEM16A channel being possible binding pockets for NFA and CaCC<sub>inh</sub>-A01. The Ca<sup>2+</sup>-binding region may be contributing to the functionality of NFA, as mutations in that region reversed its action, while the pore might be the binding region for CaCC<sub>inh</sub>-A01.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

C094

### Soluble adenylyl cyclase links store-operated $\text{Ca}^{2+}$ entry (SOCE) to $\text{Ca}^{2+}$ /cyclic AMP-response element binding protein (CREB) activation in human coronary artery smooth muscle cells

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In vascular smooth muscle,  $\text{Ca}^{2+}$  influx through store-operated channels activates  $\text{Ca}^{2+}$ /cyclic AMP-response element binding protein (CREB) and induces transcription of a distinct set of genes (1). CREB activation by store-operated  $\text{Ca}^{2+}$  entry (SOCE) is associated with early response/ proliferative gene transcription, although the physiological outcome and role of CREB activation in vascular repair or disease states is controversial (2). Here we show that the  $\text{Ca}^{2+}$  and  $\text{HCO}_3^-$ -sensitive enzyme, soluble adenylyl cyclase (sAC), links SOCE with CREB activation in human coronary artery smooth muscle cells (hCASMCs) and may therefore represent an important regulatory point in the  $\text{Ca}^{2+}$ -transcription pathway.

In fura-2-loaded hCASMCs, internal  $\text{Ca}^{2+}$  stores were depleted by incubation with the sarcoplasmic reticulum  $\text{Ca}^{2+}$  ATPase inhibitor, thapsigargin (2  $\mu\text{M}$ ) in  $\text{Ca}^{2+}$ -free solutions. This induced a transient increase in cytosolic  $\text{Ca}^{2+}$  (Fig 1A;  $n=18$  cells). SOCE was then induced by the addition of  $\text{Ca}^{2+}$  (1.8 mM) to the extracellular solution. In hCASMCs expressing the FRET-based cyclic AMP biosensor H187, the same protocol produced changes in cyclic AMP that mirrored cytosolic  $[\text{Ca}^{2+}]$  (Fig 1B;  $n=19$ ). These cyclic AMP changes were blocked by the Orai1 inhibitor, GSK7975A (30  $\mu\text{M}$ ;  $n=25$ ). SOCE also activated the cyclic AMP effector protein kinase A (PKA). PKA activity was measured in hCASMCs expressing the PKA reporter, AKAR4-NES ( $n=19$ ), and by immunoblot analysis to assess phosphorylation of the PKA substrate, vasodilator-stimulated phosphoprotein ( $n=3$ ). Immunoblot analysis also revealed CREB phosphorylation (serine 133) in response to SOCE ( $n=3$ ). Cell-permeant transmembrane adenylyl cyclase inhibitors, SQ22,536 (100  $\mu\text{M}$ ;  $n=18$ ) or dideoxyadenosine (DDA; 100  $\mu\text{M}$ ; Fig 1C;  $n=28$ ), had no effect on the SOCE-induced rise in cAMP. In contrast, sAC inhibitors 2-hydroxyestradiol (2-CE, 20  $\mu\text{M}$ ;  $n=29$ ) or 4-hydroxyestradiol (4-CE, 20  $\mu\text{M}$ ; Fig 1D;  $n=18$ ) completely abolished the generation of cAMP in response to SOCE. In immunoblots, another sAC inhibitor, KH7 (20  $\mu\text{M}$ ), inhibited SOCE-induced phosphorylation of both VASP and CREB ( $n=3$ ). 48-hour incubation with individual sAC inhibitors also significantly reduced viable hCASMC numbers compared to vehicle controls (2-CE, 39.2  $\pm$  4.7% reduction; 4-CE, 44.7  $\pm$  7.2%; KH7, 36.1  $\pm$  1.8%;  $n=3$ ; mean  $\pm$  SEM;  $P<0.05$  one-way ANOVA; Tukey post-hoc).

Our data suggest that in hCASMCs SOCE activates sAC, which in turn activates the cAMP/PKA/CREB axis. sAC is directly activated by  $\text{Ca}^{2+}$  and  $\text{HCO}_3^-$  and responds to ATP (3). Since  $\text{CO}_2$  is in almost instantaneous equilibrium with  $\text{HCO}_3^-$ , sAC functions as a  $\text{CO}_2/\text{HCO}_3^-$  (and indirect pH) sensor. This ability to respond to several physiological signals suggests sAC acts as an integrator within signalling pathways and maybe an overlooked regulatory node in  $\text{Ca}^{2+}$ -transcription coupling.

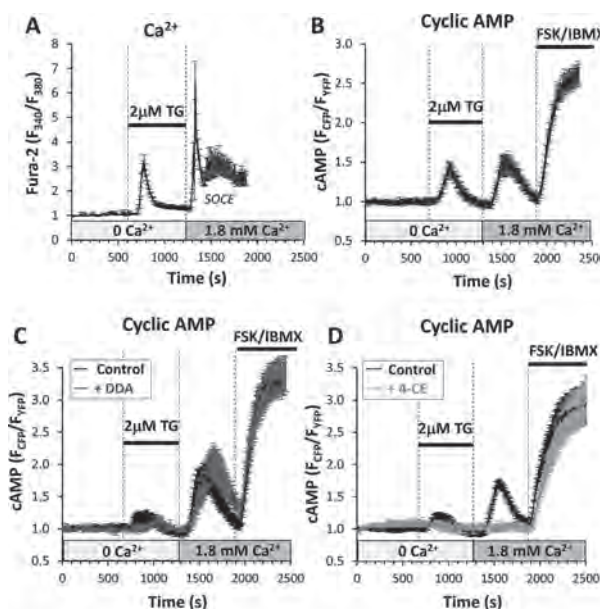


**Figure 1.** Inhibition of soluble adenylyl cyclase (sAC) abolishes changes in intracellular cyclic AMP induced by store-operated  $\text{Ca}^{2+}$  entry (SOCE). **A.** Changes in intracellular  $[\text{Ca}^{2+}]$  measured in fura-2-loaded human coronary smooth muscle cells (hCASMCs) in response to SOCE ( $n=18$  cells; mean  $\pm$  SEM). Internal  $\text{Ca}^{2+}$  stores were depleted by incubation in nominally  $\text{Ca}^{2+}$ -free solution followed by addition of the sarcoplasmic reticulum  $\text{Ca}^{2+}$  ATPase inhibitor, thapsigargin (TG; 2  $\mu\text{M}$ ). SOCE was then induced by addition of  $\text{Ca}^{2+}$  (1.8 mM) to the extracellular solution. **B-D** Real-time cyclic AMP changes measured in response to SOCE in hCASMCs transduced with adenoviruses encoding the FRET-based cyclic AMP biosensor H187. At the end of these experiments saturating concentrations of the transmembrane adenylyl cyclase activator, forskolin (FSK; 20  $\mu\text{M}$ ) and the pan-phosphodiesterase inhibitor, IBMX (500  $\mu\text{M}$ ) were added to generate maximal cyclic AMP responses. The cell-permeant inhibitor of transmembrane adenylyl cyclases, dideoxyadenosine (DDA; 100  $\mu\text{M}$ ), had no effect on the SOCE-induced rise in cAMP (**C**;  $n=28$ ). In contrast, the sAC inhibitor 4-hydroxyestradiol (4-CE, 20  $\mu\text{M}$ ) completely abolished generation of cAMP in response to SOCE (**D**;  $n=18$ ).

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

C095

### Role of TRPA1 and TRPM8 channels in intrinsic vascular responses to cold

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It is well known that cold induces vasoconstriction in skin blood vessels as a protective response against heat loss. This phenomenon is thought to be mediated by an efferent reflex to the activation of cold-sensitive afferent nerves in the skin. In contrast to this view, we found in *ex vivo* pressure myography experiments that cooling to 10 degrees induced a 28 percentage contraction in endothelium-denuded plantar arteries dissected from wild type (WT) mice. Cold produced a significantly smaller vasoconstriction in the presence of the TRPA1 inhibitor HC030031 and in arteries dissected from *Trpa1* or *Trpm8* knockout (KO) animals. Application of HC030031 virtually abolished the responses to cold in arteries from *Trpm8* KO. Neither TRPA1 nor TRPM8 channels could be detected in vascular smooth muscle cells, suggesting that the effects of cold are mediated by activation of these

channels in perivascular sensory and/or sympathetic nerves. Cold-induced vasoconstriction was potentiated in the presence of the CGRP receptor inhibitor BIBN 4096 (40 %) and reduced after depletion of catecholamines from the sympathetic nerve terminals with guanethidine. Cold had no effect in arteries were incubated with both BIBN 4096 and guanethidine. We detected mRNA of both TRPA1 and TRPM8 channels in sympathetic ganglia isolated from WT mice and we could confirm the presence of perivascular sympathetic nerves in confocal images of plantar arteries labelled with an anti-TH antibody. Taken together, our data demonstrates that cold has a dual action, mediated by both vasodilatory and a vasoconstrictor responses via the activation of TRPA1 and TRPM8 channels. To our knowledge, our results represent the first evidence for an intrinsic response to cold in cutaneous arteries and for the presence and the functional role of sensory TRP channels in efferent nerve fibers.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### C096

##### **Deficiency of prolyl-4-hydroxylase domain 2 (PHD2) in neurons enhances microvessel formation in the postnatal mouse brain through HIF-dependent and -independent mechanisms**

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In contrast to embryonic vascularization of the CNS, mechanisms underlying postnatal angiogenesis and remodeling of quiescent adult vasculature are still poorly understood. Regarding forebrain vascularization, the roles of neurons and neuronal oxygen-dependent mechanisms (PHD-HIF axis) remain completely elusive. The Cre-loxP technique was used in mice to induce deficiency of PHD2 (P2), HIF-1 $\alpha$ /HIF-2 $\alpha$  (H1/H2) and PHD2/HIF-1 $\alpha$ /HIF-2 $\alpha$  (P2/H1/H2) in postnatal neurons. Brains were harvested at 0, 1 (n=5) and 12 weeks (n=6), respectively. Co-detection of CD31 and intravenously applied EvansBlue by fluorescence microscopy was used to determine the density of functional vessels. Endothelial cell proliferation was evaluated by Ki67/CD31 staining. Immunoblotting, real-time RT-PCR, and RNAscope® *in situ* hybridization were performed to analyze gene expression. Values are means  $\pm$  SD, compared by two-way ANOVA. The density of functional vessels in 12 week-old P2 knockout (KO) mice was increased by 55% (255 $\pm$ 7 vs 396 $\pm$ 7 vessels/ $\mu$ m<sup>2</sup>, p<0.001) as compared to wildtype control (WT). Ablation of H1/H2 resulted in 20% less perfused vessels (261 $\pm$ 17 vs 210 $\pm$ 9, p<0.001). Interestingly, P2/H1/H2 KO showed no significant difference as compared to WT, but elicited 17% more functional vessels in comparison to H1/H2 KO (246 $\pm$ 8 vs 210 $\pm$ 9, p<0.01). At day of birth, no significant difference in vascular density was found in any KO. Earliest alterations occurred at 1 week: +50% (p<0.05) in P2 KO, -20% (p<0.05) in H1/H2 KO, and no difference in P2/H1/H2 KO. Accordingly, endothelial cell proliferation was elevated at 1 week in P2 KO (38 $\pm$ 6 vs 60 $\pm$ 1 Ki67<sup>+</sup> endothelial cells/ $\mu$ m<sup>2</sup>, p<0.001) and P2/H1/H2 KO (25 $\pm$ 5 vs 39 $\pm$ 3, p<0.05). Gene expression analysis revealed an up-regulation of the key angiogenic factor *Vegf* (1.5-fold, p<0.05) in P2 KO, and enhanced transcript levels of angiogenic *Spp* (1.9-fold, p<0.05) and *Adm* (1.5-fold, p<0.01). HIF-1 $\alpha$  protein levels were elevated in P2 KO by 2.9-fold (p<0.001). Despite the loss of HIF- $\alpha$  in neurons, *Vegf* transcript levels

were increased by 1.7-fold (p<0.05) in juvenile P2/H1/H2 KO as compared to WT. Although HIF-1 $\alpha$  protein abundance in P2/H1/H2 KO was reduced to 0.7-fold (p<0.05) of WT levels, it was still elevated as compared to H1/H2 KO (0.7 vs 0.3, p<0.001). These findings suggest that lack of PHD2 in neurons leads to HIF-dependent up-regulation of *Vegf* in non-neuronal cells. *In situ* hybridization confirmed astrocytic *Vegf* up-regulation in P2 KO (18 $\pm$ 0.4 vs 24 $\pm$ 0.5 transcripts/astrocyte, p<0.01) and in P2/H1/H2 KO (18 $\pm$ 0.4 vs 21 $\pm$ 0.1, p<0.01), while neurons only minorly contributed to *Vegf* expression in P2 KO, but not in P2/H1/H2 KO. Collectively, our findings clearly indicate that the PHD-HIF axis in neurons controls microvasculature formation during early postnatal CNS development.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### C097

##### **A novel role for cyclooxygenase, NO-synthase and soluble guanylyl cyclase in resistance arteries from patients with cardiovascular disease.**

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We previously reported large relaxing responses (>75%) to the endothelium-dependent vasodilators acetylcholine and bradykinin in pericardial resistance arteries isolated from patients requiring coronary artery bypass or cardiac valve replacement surgery [1-2]. In the present study we determined, in arterial segments in parallel from the same patient in a multi wire-myograph setup, the involvement of prostaglandins and of the NO/cGMP pathway. The use of human materials was approved by the Medical Ethical Committee of the Region of Southern Denmark (S-2010044 and S-20140202) and were performed according to the principles expressed in the Declaration of Helsinki. Informed written consent was obtained from all patients prior to surgery. Relaxing effects of 1  $\mu$ M Na-nitroprusside (NO-donor) were not reduced by 100  $\mu$ M L-NAME (NOS-inhibitor) but abolished by 300  $\mu$ M cPTIO (NO-scavenger) or 10  $\mu$ M ODQ (inhibitor of guanylyl cyclase). In vessels contracted with 32 mM K<sup>+</sup>, the relaxing effect of 1  $\mu$ M bradykinin was, on average, only moderately reduced by L-NAME or ODQ and not by cPTIO. In vessels contracted with 32 mM K<sup>+</sup> in the presence of 10  $\mu$ M indomethacin (non-selective COX-inhibitor), bradykinin-induced relaxation was markedly reduced by L-NAME or ODQ but not significantly modified by cPTIO. In vessels contracted with 0.25-0.5 nM endothelin-1, the strong relaxing effect of bradykinin was not reduced by either L-NAME, cPTIO or ODQ in absence and presence of indomethacin. These findings are in line with earlier observations of a prostaglandin- and NO/cGMP-independent vasodilator pathway in resistance arteries from patients with residual cardiovascular disease. They also indicate for the first time, presence of an additional pathway that is i) sensitive to inhibition of NO-synthase or guanylyl cyclase but not scavenging of NO, ii) modulated by endogenous prostaglandins and iii) unlikely to cause hyperpolarization.



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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

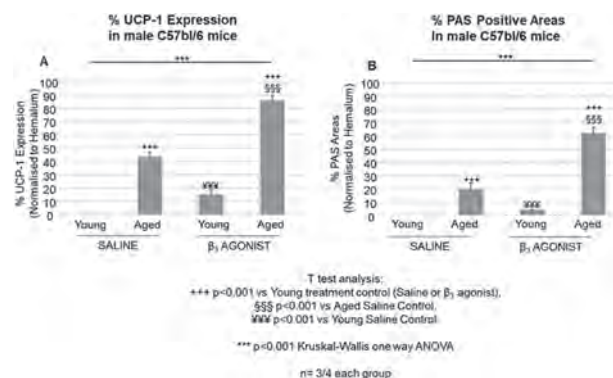
C098

### Age, perivascular adipose tissue and browning: the effect of chemically induced browning differs from endogenous browning in aged mice.

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Perivascular adipose tissue (PVAT) regulates vascular tone through release of anti-contractile factors and studies report dysfunctional PVAT in conditions of cardiovascular disease. In pathologies such as hypertension, where dysfunctional PVAT is present, browning i.e. the shift from white to brown adipocyte phenotype may be a protective mechanism to prevent cardiovascular disease. The prevalence of cardiovascular disease is increasing with age and we therefore aimed to study the role of browning of PVAT in aging. Browning was induced by  $\beta_3$  agonist in aged male C57bl/6 mice (32-34 weeks old) through stimulation with  $\beta_3$  agonist (CL-316.243 1 mg/kg/day n=4) or saline (n=4) for one week. We interrogated our model through histology and wire myography to evaluate levels of browning, inflammation and vascular function, comparing aged animals with young (12-14 weeks old). Histological analysis confirmed increased uncoupling protein-1 (UCP-1, marker of browning) in  $\beta_3$  agonist treated animals. Saline treated old animals displayed increased levels of UCP-1 compared to young animals. Old animals treated with  $\beta_3$  agonist displayed further increased levels of UCP-1 (Figure 1A). Periodic Acid-Schiff Reagent Staining (PAS) showed increased expression of positive areas in the aged animals exposed to browning compared to young animals exposed to browning and compared to old saline controls (Figure 1B). Wire myography was employed to evaluate the contractile function, endothelium-dependent and independent relaxation in the presence and absence of PVAT and the data is currently under analysis. Taken together, our data suggest that aging in male C57bl/6 mice increases the endogenous level of browning as well as the susceptibility to browning from  $\beta_3$  adrenergic agonist. However, this chemically induced browning in aged animals is also associated with increased presence of glycogen and glycoproteins typically associated with inflammation and fibrosis. These data imply that while endogenous browning is enhanced by aging, chemically induced browning in the context of aging may be detrimental. Further analysis of data from wire myography will clarify the impact of the observed results on vascular function.



Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

C099

### Dynamics and heterogeneity of renal lymphatic vessel development

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Renal lymphatic vessels have roles in fluid homeostasis and immunomodulation, and are thought to be key players in chronic kidney disease and transplant rejection. However, there is a paucity of information regarding how the lymphatic system of the kidney develops. Historically, identifying lymphatics has been challenging and requires multiple markers together with appropriate imaging techniques. We have overcome these difficulties by combining wholemount immunofluorescence for early lymphatic markers, Prox1 and Lyve-1, with optical clearing and three-dimensional imaging of the mouse embryonic metanephros, the direct precursor to the mature kidney. All animal experiments were approved by the UK Home Office. Our data shows that the majority of renal lymphatic vessels arise at embryonic day 14.5, four days after the kidney rudiment initiates, as an endothelial cell plexus restricted to the renal hilum. These endothelial cells extend towards the renal cortex, express classic markers of lymphatic endothelium, and form lumina later in nephrogenesis. We also identified a novel population of Prox1<sup>+</sup>/Lyve-1<sup>+</sup> cell clusters in the developing kidney, anatomically distinct from the main lymphatic tree, which co-express FoxD1, a marker of the renal interstitial stroma. Cre recombinase based lineage tracing using the pan-endothelial *Tie2* promoter labelled the majority of the renal lymphatic tree, but not Prox1<sup>+</sup>/Lyve-1<sup>+</sup> cell clusters. These findings suggest that renal lymphatic vessels arise heterogeneously, by both lymphangiogenesis and lymphvasculogenesis, from endothelial and non-endothelial contributions. Elucidating novel mechanisms for renal-specific lymphatic development may serve as a foundation for lymphatic-based therapies for kidney disease.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## C100

### Wall stress-mediated RGS5 expression in vascular smooth muscle cells controls arterial remodeling during hypertension

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During the onset of hypertension, vascular smooth muscle cells (VSMCs) are exposed to an increase in wall stress which translates to biomechanical stretch that induces a compensatory thickening of the vascular wall. Eventually, this leads to arterial stiffening which poses as a main risk factor for cardiovascular diseases including myocardial infarction and stroke. In this context, G-protein signaling has been reported to control blood pressure and VSMC activation. So far, it remains unclear how G-protein activity is controlled in VSMCs under these conditions. To this end, we investigated the impact of the regulator of G-protein signaling 5 (RGS5), an endogenous inhibitor of  $G_{\alpha_{q/11}}$  and  $G_{\alpha_{i/o}}$  activity, on blood pressure and the VSMC phenotype during experimental hypertension in mice. To analyze the onset of hypertension, deoxycorticosterone acetate pellets (50 mg/pellet) were implanted subcutaneously for 10 days and the mice were fed 1 % sodium chloride in the drinking water. All results are expressed as mean  $\pm$  S.D. and analyzed by one-way ANOVA. Loss of RGS5 in mice (RGS5<sup>-/-</sup>) did not affect baseline mean arterial pressure or the development of hypertension (WT 102 $\pm$ 7.5 vs. RGS5<sup>-/-</sup> 106 $\pm$ 3.8 mmHg, hypertension WT 117.7 $\pm$ 5.5 vs. RGS5<sup>-/-</sup> 114.6 $\pm$ 2.1 mmHg, n=5-6, not significant) but prevented hypertension-induced arterial remodeling, i.e. thickening of the vascular wall due to VSMC proliferation (PCNA-positive nuclei hypertension WT 53 $\pm$ 11.2 vs. RGS5<sup>-/-</sup> 2 $\pm$ 3.7, n=5, p<0.01). Under these conditions, RGS5<sup>-/-</sup> VSMCs maintained a contractile phenotype as evidenced by the presence of  $\alpha$ -smooth muscle actin (grey intensity hypertension WT 1292.03 $\pm$ 143.1 vs. RGS5<sup>-/-</sup> 1605.5 $\pm$ 473.1, n=5, p<0.05) and smooth muscle-myosin heavy chain (relative grey intensity hypertension WT 0.5 $\pm$ 0.4

vs. RGS5<sup>-/-</sup> 1.3 $\pm$ 0.4, n=8-11, p<0.05). Mechanistically, exposing isolated arteries to hypertensive pressure levels or VSMCs to biomechanical stretch was sufficient to increase RGS5 expression (arteries 1.9 fold $\pm$ 1.3, n=13, VSMCs 3.2 fold $\pm$ 1.1, n=3, p<0.05). Further, the absence of RGS5 severely impaired RhoA activity - a key protein that controls arterial remodeling in mice and humans - in arteries of hypertensive mice (WT 1.5 $\pm$ 0.3 vs. RGS5<sup>-/-</sup> 1.1 $\pm$ 0.2, n=4, p<0.05) as well as in stretch-exposed VSMCs (WT 1.1 $\pm$ 0.6 vs. RGS5<sup>-/-</sup> 0.6 $\pm$ 0.08, n=3, p<0.05). However, stretch-induced RhoA activity could be rescued by PKC inhibition that mimics RGS5-mediated termination of  $G_{\alpha_{q/11}}$  signaling (solvent 1.2 $\pm$ 0.3 vs. PKC inhibitor 2.2 $\pm$ 0.7, n=5, p<0.05).

Collectively, our findings identify RGS5 as a mechanoresponsive protein and demonstrate that RhoA activation depends on the restriction of G-protein activity. This mechanism seems to be a prerequisite for (stretch-dependent) vascular remodeling processes and might serve as a therapeutic target to combat hypertension-induced detrimental vascular changes.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## C101

### Specific G $\beta$ subunits differentially regulate Kv7.4 channels

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The Kv7.4 channel, from the Kv7 family of voltage gated potassium channels, is important in the regulation of vascular smooth muscle tone. Recently it was shown that Kv7.4 channels and native vascular Kv7 channels are positively regulated by G protein  $\beta\gamma$  subunits, and that these subunits are essential for the basal activity of these channels (1). However, there are 5 different G $\beta$  and 12 G $\gamma$  subunits and it is increasingly clear that in the regulation of other G $\beta\gamma$  effectors individual isoform combinations can perform specific functions. Which subunits are important in the regulation of Kv7.4 channels is unclear, so here we investigate the effect of G $\beta$ 1-5 subunits on Kv7.4 whole cell currents

Chinese Hamster Ovary cells were transfected with the relevant plasmids using Lipofectamine 2000/ optiMEM for 24 hours. A total of 3 $\mu$ g was transfected in a 1:1 ratio of Kv7.4:EV (Empty Vector) or Kv7.4:G $\beta$ 1-5. Whole cell K<sup>+</sup> currents were studied by ruptured patch electrophysiology. Proximity Ligation Assay was performed to detect protein-protein interactions between Kv7.4 and G $\beta$  subunits. Rat renal artery myocytes were isolated from freshly dissected arteries of male wistar rats (175-225g). Animals were culled by cervical dislocation, in accordance with the Animals (Scientific Procedures) Act 1986. Currents recorded from cells expressing Kv7.4 and either G $\beta$ 1, G $\beta$ 3 or G $\beta$ 5 were significantly greater than those expressing Kv7.4 alone (Kv7.4 alone = 13.5 $\pm$ 2.1 pA/pF (n=24), with G $\beta$ 1 = 35.8 $\pm$ 12.5 pA/pF (n=7), with G $\beta$ 3 = 22.7 $\pm$ 5.9 pA/pF (n=5), or with G $\beta$ 5 = 23.4 $\pm$ 5.4 pA/pF (n=6), as measured at +40 mV). Conversely, co-expression of G $\beta$ 2 or G $\beta$ 4 with Kv7.4 resulted in significantly decreased currents than in control (with G $\beta$ 2 = 5.8 $\pm$ 1.4 pA/pF (n=7) and with G $\beta$ 4 = 5.9 $\pm$ 2.1 (n=8)). In rat renal artery myocytes proximity ligation assay detected interactions between Kv7.4 and G $\beta$ 1 (12.7 $\pm$ 1.4 puncta/cell (N=1, n=7)) and G $\beta$ 3 (27.6 $\pm$ 5.7 puncta/cell (N=1, n=6)) which were reduced when cells were treated with the general G protein  $\beta\gamma$  subunit inhibitor, gallein (G $\beta$ 1 - 3.2 $\pm$ 1.4 puncta/cell (N=1, n=5) and G $\beta$ 3 - 3.3 $\pm$ 1.4 puncta/cell (N=1, n=5)).

These results show that specific G $\beta$  subunits have divergent role in positively (G $\beta$  1, 3 and 5) or negatively (G $\beta$  2 and 4) regulating Kv7.4 channels, which could have important implications in the regulation of vascular tone.

Stott JB et al. (2015) *Proc Natl Acad Sci USA*. 112:6497-6502

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

C102

### Mitochondrial ATP regulates spontaneous calcium signalling in native endothelial cells

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It is generally accepted that endothelial cells are highly glycolytic and do not rely on mitochondria for ATP generation. Rather than providing energy, mitochondria may act as signalling organelles that control cytosolic Ca<sup>2+</sup> signalling or modify reactive oxygen species. The majority of studies suggesting endothelial cells are glycolytic have been performed in cell culture systems where ATP production may be significantly altered from the native cell type. This study was designed to investigate the regulation of endothelial function by mitochondria in intact, native arteries. Our results suggest that mitochondrial ATP production is critical in regulating local Ca<sup>2+</sup> signalling in intact endothelial cells.

Endothelial Ca<sup>2+</sup> signalling (Cal-520/AM), endothelial mitochondria (TMRE), and the internal elastic lamina (IEL; auto fluorescence) were visualised by fluorescence microscopy of the vascular wall of small mesenteric arteries from Sprague Dawley rats (1). Under basal conditions, intact endothelial cells exhibited extensive subcellular Ca<sup>2+</sup> signalling activity. Spontaneous Ca<sup>2+</sup> events occurred in 12.5  $\pm$  1.5% of endothelial cells (N = 27 fields, n = 9). Ca<sup>2+</sup> events arose via IP<sub>3</sub>-mediated release from the endoplasmic reticulum. In support, Ca<sup>2+</sup> events were not significantly altered by a Ca<sup>2+</sup>-free bathing solution, but were abolished by the SERCA inhibitor, CPA, or the IP<sub>3</sub>R inhibitor, 2-APB (n = 5 for each treatment, paired data, significance assessed at p<0.05 using Student's paired t-test). Spatial maps of the location and distribution of local Ca<sup>2+</sup> sites showed the majority occurred close to junctions with smooth muscle cells (holes in the IEL). The distance between Ca<sup>2+</sup> events and IEL holes was smaller than would be expected if Ca<sup>2+</sup> events occurred randomly throughout the cytosol (p < 0.05, paired t-test of real vs randomly permuted data). Mitochondria, are morphologically heterogeneous in the native endothelial cells and occupied a significant proportion (8.7  $\pm$  0.2%) of cell area. However, there was no correlation between sites of Ca<sup>2+</sup> event initiation and mitochondrial position (p = 0.86, paired t-test of real vs randomly permuted data). However, endothelial Ca<sup>2+</sup> signalling was dependent on a polarised mitochondrial membrane potential. The uncoupler, CCCP, or the complex 1 inhibitor, rotenone, each depolarised the mitochondrial membrane potential and nearly eliminated endothelial Ca<sup>2+</sup> events (n = 5 for each, paired data, p < 0.05 using Student's paired t-test). Each drug reduced Ca<sup>2+</sup> activity whether applied alone or in combination with the mitochondrial ATP synthase inhibitor, oligomycin, to reversal of the ATP synthase. Oligomycin alone also significantly inhibited endothelial Ca<sup>2+</sup> activity.

In contrast to studies in cultured endothelial cells, these results suggest that mitochondrial ATP production is critical to the function of the native endothelium and exerts a long-range control of IP<sub>3</sub>R-mediated intracellular Ca<sup>2+</sup> signalling.

Wilson C, Lee M & McCarron J (2016). Acetylcholine released by endothelial cells facilitates flow-mediated dilatation. *The Journal of Physiology* 594, 7267-7307.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

C103

### Smooth muscle cell iron homeostasis is autonomously controlled by the hepcidin/ferroportin axis and its disruption leads to pulmonary arterial hypertension

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Iron deficiency (ID) is the most common nutritional disorder in the world, leading to anaemia in ~25% of cases. ID is a co-morbidity in chronic heart failure and pulmonary arterial hypertension (PAH). However, the mechanistic link between ID and cardiovascular disease, and the importance, if any, of anaemia in this link remain unclear. Ferroportin (FPN), the only known mammalian iron exporter, releases iron from duodenal enterocytes, splenic macrophages and hepatocytes; the sites of iron absorption, recycling and storage respectively. The liver-derived peptide hepcidin controls systemic iron homeostasis by antagonizing FPN at those sites. Previously, our work in the heart has shown that hepcidin produced locally by cardiomyocytes is required for cell-autonomous control of intracellular iron levels, through action on cardiomyocyte FPN. Furthermore, we demonstrated that disruption of the cardiomyocyte hepcidin/FPN axis, through knock-in of the FPN C326Y isoform (which retains iron export function but is resistant to hepcidin), leads to cardiomyocyte iron depletion and fatal heart failure. Heart failure occurred against a background of normal systemic iron levels and no anaemia<sup>1,2</sup>. FPN is also present in smooth muscle cells, where its function remains unknown.

Aim- To define the function of FPN and of its regulation by hepcidin in smooth muscle cells.

Methods- We generated mice with a tamoxifen-inducible smooth muscle-specific knock-in of FPN C326Y (iSM-FPNC326Y). We characterised cardiac function by Cine-MRI and assessed pulmonary vascular function by right heart catheterisation. We also carried out lung histology and assessment of iron indices. Finally, we investigated the effect of intravenous iron administration in this mouse model.

Results- iSM-FPNC326Y mice did not differ from controls in terms of blood iron indices. Nonetheless, they showed elevated right ventricle systolic pressure (RVSP) at 6 weeks and 3 months post tamoxifen-injection. This was associated with increased muscularisation of the pulmonary arterioles. Cine-MRI showed increased RV end systolic volume, and reduced RV ejection fraction at both timepoints. These observations are consistent with the development of PAH. Intravenous iron, given at weeks 1, 3 and 5 post-tamoxifen injection prevented the development of PAH at 6 weeks.

Conclusion- These data demonstrate, for the first time, that normal pulmonary vascular function requires control of intracellular iron levels in smooth muscle cells, and that this



control is dependent on a local FPN/hepcidin axis. Disruption of this local axis leads to PAH against a background of normal systemic iron homeostasis and no anaemia. These data provide a mechanistic explanation for the known link between ID and PAH and for the reported benefits of iron supplementation in this disease setting.

Lakhal-Littleton S, Wolna M, Christian H, Santos A, Diaz R, Biggs D, Davies B, Robbins P. (2016) An essential cell-autonomous role for hepcidin in cardiac iron homeostasis. *Elife*. Nov 29;5.

Lakhal-Littleton S, Wolna M, Carr C, Miller J, Christian H, Ball V, Santos A, Diaz R, Biggs D, Stillion R, Holdship P, Larner F, Tyler D, Clarke K, Davies, Robbins P. (2015) Cardiac ferroportin regulates cellular iron homeostasis and is important for cardiac function. *Proc Natl Acad Sci USA* 10;112(10):3164-9.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## C104

### Anatomic distribution of Bradykinin 2 receptors in the upper rat ureteric smooth muscle corresponded to regions of high sensitivity to Bradykinin

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Bradykinin (BK) is present in the urine and induces inflammatory and contractile responses in the ureter of different animals (Catacutan-Labay 1966; Ribero et al., 2016) by stimulating B<sub>2</sub> receptors (Eggerickx et al., 1992), however there are no data existed illustrating the anatomical and functional distribution of B<sub>2</sub> receptors in the rat ureter. Frozen sections from the upper, middle and lower ureters of male wistar rats (6-8weeks) that were humanely killed using CO<sub>2</sub> anaesthesia followed by cervical dislocation in accordance with the UK legislation were immunostained with anti-B<sub>2</sub> receptor antibodies and examined under fluorescent microscope and their level of the expression was calculated using confocal microscope. The ureteric segments were also exposed to cumulative doses of BK (0.1, 0.2, 0.5, 1, 2.5, and 5 µM) for 5 minutes and force was measured using a special apparatus designed by Professor Burdyga (university of Liverpool). Immunohistochemical studies revealed homogenous distribution of B<sub>2</sub> receptors in the urothelium. While, their expression was significantly higher in the smooth muscles (SMs) of the proximal upper (93%±0.6, n=7), lowest in the middle (41%±0.8, n=9), and modest in the lower part, (51%±0.7, n=11) relative to the urothelium. Force measurement revealed that the upper SMs was much more sensitive than other parts. The threshold concentration, which evoked spontaneous activity was 0.5 µM, while in the middle and the lower segments were 2 to 5 µM. In the upper ureter, BK at 0.2 µM induced small elevation of the baseline force (7.5±0.5%) and at 0.5 µM to 16.4±0.3% which was superimposed by spontaneous phasic contractions, with an average frequency of 1.06±0.6 cycles/min (n=9). At 5 µM, BK further increased the baseline force to 38.4±0.6% superimposed by spontaneous contractions, which appeared either as single phasic contractions or long lasting groups of fused phasic contractions. The middle ureter were significantly less sensitive to BK. In 50-60% of the samples, only high concentration (5 µM) produced elevation of the base line force to 6.8 ±0.3% (n=7), which was superimposed by the phasic activity, with an average frequency of 0.9 ±0.1 cycles/min, and in the rest of the samples, without phasic activity. Similarly, the SMs of the lower ureter were also significantly

less sensitive to BK. at 0.2 µM, BK had little or no effect on the baseline force. In the presence of 0.5 µM, the base line force was increased to 3.7±0.2%. At 5 µM, BK increased both the tonic contraction to 11.9±0.3% and the phasic contractions to 1.6±0.4 cycles/min (n=6). These data demonstrate a higher level of expression of B<sub>2</sub> receptors in the proximal ureter corresponded to areas of increased sensitivity of the rat ureteric smooth muscle to BK.

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## C105

### Role of mineralocorticoid receptors in mediating endothelial dysfunction in experimental diabetes

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Introduction: Elevations in circulating levels of aldosterone correlates with increased cardiovascular deaths in diabetic patients. Endothelial dysfunction is a key player in the development of vascular complications seen in diabetic patients. Increased levels of aldosterone and activation of the mineralocorticoid receptor are associated with endothelial dysfunction and antagonism of the mineralocorticoid receptor is suggested to improve endothelial function. The mineralocorticoid receptor in the endothelium (ECMR) has been found to be harmful in pathophysiological conditions.

Hypothesis: We hypothesize, that the detrimental vascular effect of aldosterone in diabetic vascular complications occurs through the mineralocorticoid receptor in the endothelium. Methods: Transgenic mice (MR<sup>fllox/fllox</sup>;Tie2-Cre) with a specific deletion of the MR in endothelium and their wildtype littermates (MR<sup>fllox/fllox</sup>) were given streptozotocin (STZ) for five consecutive days (55mg/kg/day) to induce diabetes or vehicle. Diabetic mice were re-injected with STZ (single bolus) six weeks after initial injection to maintain diabetes. A 10 % sucrose solution was given during the five injection days to avoid hypoglycemia. Bodyweight and blood glucose levels were measured weekly. After 10 weeks, mesenteric second order resistance arteries were isolated and perfused and both constriction and dilation was assessed in presence of aldosterone and an MR antagonist.

Results: STZ injections significantly increased blood glucose levels compared to vehicle controls in both ECMR-KO (18.11±1.11mM vs 6.42±0.12mM) and WT (19.24±1.06mM vs 6.50±0.20mM) with no difference between sexes. There

were no difference in weight gain between STZ and VEH. Preliminary data suggests that STZ-treatment only affects dilatation in WT animals where maximal dilatation to acetylcholine was decreased compared to controls (WT:  $10.13 \pm 4.109\%$  vs.  $32.75 \pm 6.540\%$  and KO:  $37.39 \pm 0.3814$  vs.  $27.19 \pm 2.481\%$ ,  $n=3-4$ , mean $\pm$ SEM). After incubation with aldosterone ( $10^{-9}$ M, one hour), dilatation was further decreased in WT ( $7.219 \pm 5.167\%$  and  $20.31 \pm 1.682$ ,  $n=3-4$ , mean $\pm$ SEM), but not in KO ( $36.59 \pm 2.554\%$  and  $29.66 \pm 4.992\%$ ,  $n=3-4$ , mean $\pm$ SEM) no matter of STZ-treatment or vehicle. Incubation with the MR antagonist spironolactone ( $10^{-7}$ M, 30min) improved maximal dilatation in both STZ-treated WT and KO ( $15.87 \pm 6.49\%$  and  $41.84 \pm 5.35\%$ ,  $n=3$ , mean $\pm$ SEM).

Conclusion: These data suggests that the endothelial cell specific mineralocorticoid receptor is involved in decreasing dilatation to acetylcholine in STZ-treated mice, but more mice needs to be included before solid conclusions can be made.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## C106

### Skin lymphatic vasculature participates in sodium and blood pressure homeostasis

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Salt accumulation in the skin leads to lymphangiogenesis. Whether such increase in lymphatic vessels results in enhanced lymph vessel function is unknown. Oppositely, how inability for lymphangiogenesis in skin influence blood pressure regulation is not established. We therefore studied, after salt ingestion, lymphatic function in a rat model shown to induce lymphangiogenesis in skin and blood pressure development in the Chy mouse model that has a mutation resulting in missing lymphatics in skin and lymphoedema. Male Sprague-Dawley rats were given either a high salt diet (chow with 8% salt and 0.9% saline, HSD) for 2 weeks or deoxycorticosterone (100 mg pellet subcutaneously, chow with <0.1% salt and 0.9% saline, DOCA) for 3 weeks. Control rats received low salt diet (chow with <0.1% salt and tap water, LSD) for 2 weeks. Blood pressure was measured with the tail-cuff method. Immunohistochemistry was used to determine the number of lymph vessels in the skin. Na<sup>+</sup> accumulation was measured with flame photometry in dry ashed skin. Lymph flow was estimated from tissue clearance of intradermally injected fluorescently labelled albumin, measured with optical imaging. In a separate series of experiments, Chy and wild type (WT) mice were given high salt diet (chow with 4% and 0.9% saline, HSD) for 2 weeks. Blood pressure was measured with 24-hours telemetric recording before the onset and at the end of the high salt diet period. DOCA and HSD rats had a mean blood pressure that was  $162 \pm 24$  mmHg ( $n=18$ ) and  $129 \pm 12$  mmHg ( $n=24$ ), respectively, whereas the LSD group had a mean blood pressure of  $110 \pm 13$  mmHg ( $n=24$ ). The salt content in skin of DOCA and HSD rats was  $0.145 \pm 0.009$  mmol/ml ( $n=5$ ) and  $0.113 \pm 0.003$  mmol/ml ( $n=5$ ), respectively, both higher than the  $0.093 \pm 0.001$  in the LSD group ( $n=6$ ) ( $p<0.005$  for both comparisons). Lymph vessels per mm<sup>2</sup> in skin were  $167 \pm 43$  in DOCA ( $n=3$ ) and  $96 \pm 23$  in HSD ( $n=3$ ) compared with  $69 \pm 30$  in the LSD group ( $n=3$ ). The removal rate of albumin was higher after salt ingestion, suggesting

an increased lymph flow. Thus, the percent of remaining labelled albumin 24-h after injection in skin was estimated to  $14.6 \pm 1.34\%$  in HSD ( $n=20$ ) and  $20 \pm 1.00\%$  in LSD ( $n=19$ ) groups. Chy mice ( $n=3$ ) tended to have a higher systolic blood pressure than WT ( $n=3$ ) before start of the HSD ( $125 \pm 5$  mmHg vs.  $113 \pm 13$  mmHg,  $p>0.05$ ). The HSD resulted in a significant increase in systolic blood pressure during night-time in Chy mice ( $141 \pm 3$  mmHg,  $p<0.05$ ), whereas the pressure in WT was unchanged ( $119 \pm 11$  mmHg). The heart rate was the same in the two groups after the diet ( $594 \pm 11$  BPM vs.  $612 \pm 34$  BPM). Collectively, our data showing that newly formed lymph vessels after salt ingestion are functional and that mice incapable of lymphangiogenesis get salt-induced increase in blood pressure suggest that lymphatics in skin contribute in salt and blood pressure homeostasis.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## C107

### Multiple Stimuli are Detected by Specialised Cells in the Vascular Endothelium

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The endothelium is the innermost layer of blood vessels and controls virtually all cardiovascular activities. The endothelium uses a multitude of receptors to detect various extracellular agonists. Endless streams of information arrive simultaneously at the endothelium to modulate vascular function. However, it is not yet fully understood how the endothelium can detect multiple simultaneously arriving signals and separately process the information held within each activator. Here, we show the endothelium manages the multitude of extracellular signals by using spatially-distinct endothelial cells that are primed to detect specific extracellular signals. Furthermore, each activator elicits a Ca<sup>2+</sup> signal with unique characteristics. When multiple activators are present simultaneously communication occurs between endothelial cells and computations are carried out to generate a new unique Ca<sup>2+</sup> signals that are more complex than a simple summation of each signal.

To determine how signals evoked by multiple agonists (ACh, ATP, ADP & histamine) were transduced in the endothelium we studied Ca<sup>2+</sup> signalling evoked by the agonists in intact arteries. Male Sprague-Dawley rats (150-250g,  $n=5$ ) were euthanized by CO<sub>2</sub> overdose (Schedule 1; Animals (Scientific Procedures) Act 1986) and second order mesenteric arteries were extracted, cut open and pinned flat. The endothelium was loaded with a Ca<sup>2+</sup> indicator, Cal-520/AM and imaged using fluorescence microscopy. Changes in intracellular Ca<sup>2+</sup> in thousands of endothelial cells were measured individually. For each agonist, concentration response curves were carried out to obtain the concentration at which 25% (EC<sub>25</sub>) of the entire population of endothelial cells were activated. The EC<sub>25</sub> concentration of each of the four agonists was applied to the same artery to determine which cells were activated. The Ca<sup>2+</sup> signal for each agonist began in spatially-distinct clusters and propagated among cells as Ca<sup>2+</sup> waves. There was no significant overlap in the cells that responded to each agonist ( $P<0.05$ ). Interestingly, the calcium signal for each agonist was unique and the Ca<sup>2+</sup> signal was altered when agonists were applied in combination. For example, the Ca<sup>2+</sup> signal evoked by ACh had a slow steady increase to a maximum peak were

it remained elevated. However, the  $\text{Ca}^{2+}$  signal evoked by ATP increased to maximum sharply before falling to baseline level. When both agonists were applied simultaneously the  $\text{Ca}^{2+}$  signal was a complex computation of both individual signals. The new  $\text{Ca}^{2+}$  signal had a much sharper increase to maximum than ACh alone, but shallower than for ATP. The new  $\text{Ca}^{2+}$  signal remained elevated at a much higher level than for ACh alone.

These findings show that the endothelium comprises of spatially-distinct populations of cells that are primed to detect specific agonists. These spatially-distinct populations of cells may process the endless streams of information in parallel to control specific cardiovascular functions.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## C108

### Uremic serum affects endothelial nanomechanics and expression of ion channels

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Patients with chronic kidney disease (CKD) have a markedly increased incidence of cardiovascular events and cardiovascular disease (CVD) mortality compared with the age-matched general population. The high concentration of circulating uremic toxins in CDK patients leads to vascular inflammatory responses, thereby inducing endothelial dysfunction, which is associated with CVD development and progression.

It is postulated that serum, derived from CKD patients (uremic serum), changes the nanomechanical properties of endothelial cells leading to endothelial dysfunction and vascular inflammation. In addition, the influence of CKD on the expression of endothelial ion channels and its potential modification by mineralocorticoid receptor (MR) inhibition was studied which would consequently also affect endothelial cell function.

To test this we employed primary human endothelial cells (HUVEC and HAEC) cultured in medium supplemented with 10% pooled sera from either healthy or uremic patients undergoing dialysis. The mechanical stiffness of the outer layer of the cells was monitored using Atomic Force Microscopy (AFM)-based nanoindentation measurements. To obtain a complete profile of ion channel subunit expression, we performed high-throughput qPCR of 92 ion channel genes using a custom-designed Taqman low-density array card. In addition, we characterized the influence of uremic serum on nitric oxide (NO) production.

Our data show that uremic serum potently increased the mechanical stiffness of endothelial cells which was abolished by application of the MR-antagonist spironolactone. In addition, uremic serum augments MR expression and induced remodeling of ion channel expression patterns, including increased expression of  $\alpha\text{ENaC}$ , a known transcriptional target of MR that promotes endothelial stiffness and dysfunction. Exposure of HAEC to uremic serum significantly decreased NO production. Our data identify the vascular endothelium as important target of uremic toxins with the MR as possible mediator of uremia-induced endothelial dysfunction. This supports the proposed beneficial effects of MR inhibition during CKD.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## C109

### Patterns of medical school e-learning: Effects of social media and introduction of a Smartphone mobile platform on learning

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#### Introduction

Hull York Medical School (HYMS) teaches 700 students. Year 1 & 2 (Phase I), students are campus-based – at Hull & York Univ. Years 3-4 (Phase II) & 5 (Phase III), students are taught at sites spread over 8600 sq Km of N.Yorkshire by 1000 staff. A smartphone e-learning app, Blackboard mobile (BB) was introduced in July 2011 to aid learning and communication between widely dispersed staff & students.

#### Aims

To determine extent of:

Students accessing mobile e-learning and to variation by year of study and mobile platform.

Use of e-learning by year of study

Use of social media as an adjunct to BB

#### Methods

Questionnaires were administered to 65, 55 & 50 students respectively in Phase I, II and III respectively in 2012. Responses were analysed with multivariate analysis (SPSS v.20). Log in data from York Univ was analysed according to Smartphone platform (Apple, Android, Blackberry), time of day & day of week.

#### Results

86% Phase I students reported owning a Smartphone, with 79 % overall HYMS student & 78% staff ownership, compared with 50% general UK population. 50% students had unlimited data usage contracts. 55% of BB users used Apple iOS & 39% Android; 90% logins were via Apple iOS & 9% via android.

44% students and 10 % staff downloaded BB in the first 9 months available. 398 unique visitors went to Blackboard between Oct 2011 – March 2012, with 179 unique visitors in March 2012. Peak usage was on Tuesday & Friday with nadir on Wednesday, Thursday, & weekend. Peak usage by hour of day was at 9am & 1pm (Fig. 1).

Mean + SD (range) time spent on Blackboard (BB) and Facebook (FB) respectively were 20.8 + 25.8 (0-180) & 60.3 + 78.2 (0-480) min/ day respectively ( $p = 0.001$ , ANOVA). Time spent on both BB and FB were lower in Phase III than Phase I ( $p = 0.001$ , 0.006, Fig. 2) Only 30% students used the electronic forum on BB to interact, students preferred talking face to face, by telephone, email or FB.

35% students admitted skipping lectures because of content available on BB. Students skipped a median + SD (range) of 3 + 11.6 (0-50) lectures/ year.

BB problems cited by students were poor or absent search facilities, the disorganized filing of resources, “clunkiness” and availability of resources elsewhere e.g. books.

43%, 27% & 14 % respectively of Phase 1,2 & 3 students respectively admitted to using FB to aid learning, particularly Phase I students, to aid problem-based learning (PBL). Methods included direct messaging, posting articles to groups/ individuals, asking where information was located, arranging meetings to prepare presentations, asking questions, checking deadlines.



## Conclusions

1. FB usage was higher than BB usage
2. Both BB and FB usage declined as students progressed through the medical school, despite them being more widely dispersed;
3. FB usage in early years engendered a learning community enhancing PBL

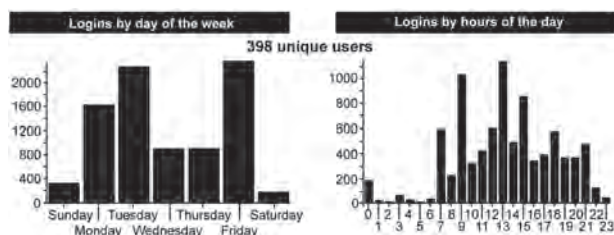


Fig.1 Log-ins by day of week and by time of day (398 unique visitors per half year) post introduction of Blackboard mobile app.

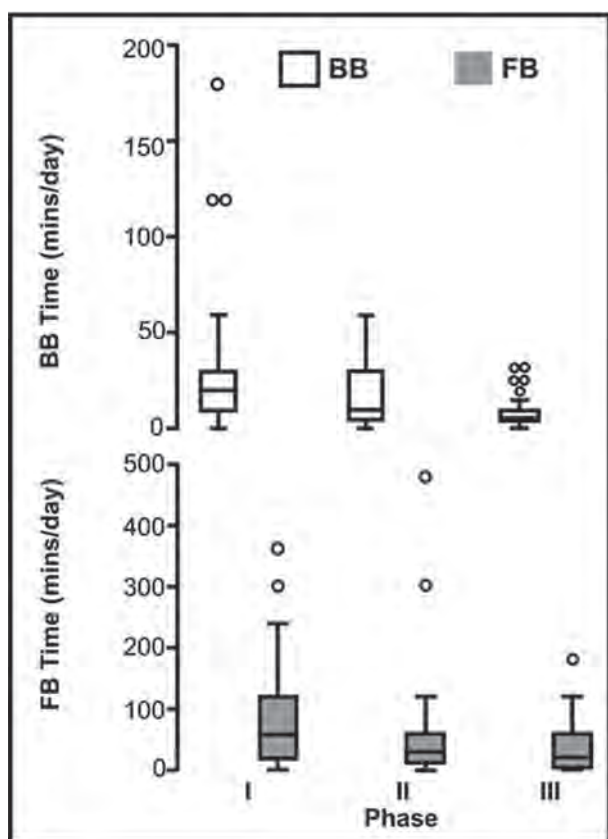


Fig.2 Box and whisker plots for time spent per day on Blackboard (upper panel, BB) and Facebook (lower panel, FB) by Phase of study (abscissa). Horizontal line represents median, box shows first – third interquartile range (IQR), whisker shows range ie bottom value (1st quartile -1.5 IQR) to highest value (3rd quartile +1.5 IQR). Outliers shown by open circles

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C110

## Developing an undergraduate course to address the skills gap in the communication of research to a diverse audience beyond academia

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Students in their future careers, will have to demonstrate their ability to communicate with many different audiences, skills that are often overlooked within undergraduate programmes. These skills are required by many graduate employers and are associated with further research degrees. The aim of this project was to develop a taught course for undergraduate students studying any discipline across the University of Leeds. Beneficial for those students who wish to effectively communicate research findings and engage with wider audiences. This course aimed to emphasise the link between research and student education.

A Level 5, undergraduate course was created and delivered; its content encompassing the planning, delivery and evaluation of successful public engagement/outreach events based on current research at Leeds. The course consisted of 12 x 2 hour seminars delivered by experts in the field, with appropriate supplementary resources made available on the virtual learning environment. Seminars were delivered in a stimulating and interactive way, by both internal colleagues and external experts in the field. The topics included discovering key research areas at Leeds, event planning and organisation, understanding audiences, effective communication skills, working with the media, working with partner organisations, evaluation, funding and impact.

The assessment was a written piece of coursework communicating, in a creative way, a key research finding published by academics at Leeds. This involved planning an event or resource by which to communicate this research to a chosen audience outside of academia. Students also engaged in a mid-way presentation of their suggested communication event or resource and a reflective blog.

The course has been delivered to 2 cohorts of students (n=39). 82% of students were studying a programme within the biosciences. From the end of course survey (n=25) 96% of respondents rated the course as one of the most interesting courses they had studied so far. All respondents stated that they were satisfied or extremely satisfied with the course. 80% indicated they had developed new skills and all respondents stated they had enhanced existing skills, in particular communication, presenting, creativity, planning, organisation, research interpretation & engagement skills.

This programme of work has demonstrated that undergraduate public engagement courses enable students to gain valuable work experience and develop key skills and attributes required by graduate employers, thereby significantly enhancing their employability.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

C111

# Designing authentic assessment strategies to evaluate multiple domains of competency in Physiology

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Assessment of student learning is a multi-pronged approach that mainly focuses on meeting the criteria for certification, continuous improvement, as well as on preparing the student for the workplace [1,2]. Authentic assessment in particular requires students to apply competencies to settings that mimic real work place settings [3,4]. There is a growing realisation of the benefits of incorporating authentic assessment to improve the learning of Physiology in medical education. The purpose of the study was to employ technology supported strategies to develop authentic assessments for Physiology students. The technologies employed included online, network and stand-alone device based platforms. Authentic assessment strategies using these platforms were used in the systematic collection of evidence on the student's demonstration of acquired knowledge, skills, attitudes and values. To validate the results, students' performance on authentic assessments was compared with performance on standardised assessment. A cohort of students in the Clinical Associate programme was used. Authentic assessment enabled students to demonstrate their knowledge and understanding of both Systems Physiology and Applied Physiology in a manner that was consistent with workplace competencies stipulated in the curriculum. The crucial finding from the study was that no single assessment strategy was superior, but that multiple strategies were more reliable in measuring student performance and progress. There was no statistically significant difference between platforms that supported flexibility in terms of the time and the place of taking the assessment and those that were not flexible. Authentic assessment may be used as an alternative or complimentary form of assessment in Physiology education. Furthermore, programs that enable the collection of data on student performance from multiple types of assessment platforms and provide analysis will be valuable not only for determining effectiveness of authentic assessment strategies but for also informing decisions on how to improve pedagogy, curriculum and instructional strategies. Bennett RE. Formative assessment: A critical review. *Assessment in Education: Principles, Policy & Practice*. 2011, 18:5-25.

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C112

# Drowning and asphyxia in the time of Edmund Goodwyn

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This paper addresses the visionary work of a virtually unknown English medical student by the name of Edmund Goodwyn (1756-1829), whose graduation thesis (1) represents one of the most progressive scientific contributions of his time. While miscellaneous aspects of his remarkable monograph, titled *The Connexion of Life with Respiration*, have been occasionally mentioned in a few history books and other medical publications, the unusually lucid and innovative nature of his work has yet to be recognized.

Goodwyn's thesis contains several important discoveries: the first experimental demonstration that drowning is caused by the exclusion of atmospheric air from the lungs; the mechanism by which artificial ventilation treats asphyxia; and the first description of an oxygen-conserving reflex commonly known as diving bradycardia, which until recently had been attributed to Paul Bert (1833-1886). (2) It also holds one of the earliest (if not the earliest) experimentally-supported arguments that pulmonary circulation is continuous in all phases of respiration, and not intermittent, as it was widely believed at the time (3).

In addition, Goodwyn's thesis includes one of the earliest repudiations of suspended animation, a mystical concept that confused physicians into the early part of the twentieth century, and which sparked an era in the history of medicine known as the "premature burial panic." (4)

Thus, this paper intends to show the innovative nature of Goodwyn's thesis in its historical context, and to provide a first biographic glimpse of this remarkable physician.

Goodwyn E. *The Connexion of Life with Respiration; or, an Experimental Inquiry into the Effects of Submersion, Strangulation, and Several Kinds of Noxious Airs, on Living Animals: with an Account of the Nature of the Disease They Produce; Its Distinction from Death Itself; and the Most Effectual Means of Cure*. London: T. Spilsbury; 1788.

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C113

### Explorative vs classical practical course – how to inspire scientific thinking in medical students

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**Introduction:** Recently, the skills of medical students in scientific thinking have been identified as an important issue in medical education. Scientific thinking cannot be imparted by classic lectures, but require active involvement of students. We modified a practical course in physiology. A study was designed to test whether the new course facilitates scientific thinking without impairing physiological knowledge transfer. **Methods:** The study group consisted of 226 first year medical students of the Medical Faculty Mannheim of Heidelberg University. Written consent for participation in the study was obtained from all participants. The group was randomly divided into 2 groups (traditional vs. modified course). Subject of both courses was a laboratory experiment in skeletal muscle physiology. In the traditional course the students addressed topics already presented in lectures. In the modified course students dealt with the same topics as in the traditional course, but the experiment was extended to include one issue not taught before. When working on this issue, the students were instructed in scientific thinking. All participants filled a questionnaire with 15 multiple choice questions, addressing the physiological background, and 4 open questions, addressing criteria of scientific methodology.

**Results:** Physiological knowledge in both groups did not differ ( $F(1)=2.08$ ,  $p=0.15$ ). Scores in scientific thinking in the modified course were higher ( $M=4.17$ ,  $SD=1.91$ ) than in the traditional course ( $M=2.04$ ,  $SD=1.91$ ) with  $F(1)=70.69$ ,  $p<0.01$ ,  $\eta^2=0.24$  (large effect).

**Conclusions:** Our study demonstrates that small adjustments of courses in medical education can facilitate scientific thinking without impairing knowledge transfer. However, the level of scientific thinking was still quite moderate. Thus, the teaching of these principles should be improved.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

C114

### How much do pre-clinical medical students utilise the internet to study physiology?

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Physiology remains a central focal point of pre-clinical medical teaching<sup>1,2</sup> with didactic lectures still the core instructional methodology utilised by most institutions<sup>3,4</sup>. However, universities are now faced with ever increasing numbers of 'Generation Connected' (Gen C) students who are reliant upon the internet and social media platforms for both their intellectual and personal needs. This development is liable to challenge traditional physiology teaching as students seek ways to integrate their online life with their educational experience.

Anecdotal evidence suggests that medical students utilise both online resources and social media relatively extensively to facilitate and propagate their understanding of Physiology but little actual research has been conducted to determine to what extent this occurs.

The initial aim of the current study was therefore to determine how first year medical students from both direct entry (DEM; 72 students) and graduate entry (GEM; 67 students), medical streams utilise internet resources and social media to facilitate or supplement their Physiology learning. For the study, students completed an anonymised 24 item survey which collected information on both student demographics and on their use of online learning Physiology learning resources.

We determined that 97.8% of all students surveyed had used internet platforms to source Physiology information, with 89.2% doing so at least once per week. The vast majority (76.3%) used YouTube as their primary source of learning. 69.8% of students stated that they interacted with social media (e.g. Twitter, Snapchat, Facebook, etc.) in excess of 12 times per week, with 84.7% of students indicating that they had used social media platforms to discuss course related issues with their peers. Significantly, 94.2% of all students indicated that they would first search for an answer online if they did not understand something in Physiology, with 70.8% and 50.4% of respondents signifying that they would not contact the instructor either by e-mail or in person, respectively, if they were still unsure. Somewhat worryingly however, only 31.4% of students ever "fact-checked" Physiology information obtained from online sources (e.g. by using textbooks, papers and/or instructors).

Overall, our findings have revealed that Gen C pre-clinical medical students utilise both social media and a variety of internet resources extensively to facilitate their study of Physiology. As such, it suggests that the blending of elements such as YouTube videos into Physiology teaching would provide both a means of enhancing student engagement with, and consequently, the understanding of, the subject as well as providing academic and ethical oversight of such online content.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

C116

### Teaching physiology using a Gamified Flipped Classroom model.

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**Background:** Evidence suggests that millennial students favour active teaching methodologies<sup>1</sup>. In response to the need for more active learning modalities, flipped classroom and gaming have increasingly become part of health professions education<sup>2-3</sup>.



**Activity:** Flipped classroom sessions presented to undergraduate and medical students were augmented with a review activity conducted using gaming. Students were provided with learning materials, in the format of assigned reading and recorded lecture, for review prior to the activity-driven classroom session. At the session, students were consigned to randomly allocated small groups. Each group was given a separate clinical vignette to solve and to present their findings to the other groups. Based on the pre-session and in-classroom activities, student groups then participated in a gamified review quiz. Teams were awarded points for correctly answered quiz questions. At the end of the sessions, students were asked to complete an anonymous survey on the effectiveness of the activity to their learning and to rank other learning activities they found beneficial.

**Results:** A total of 31/ 48 undergraduate and 28/44 medical students responded to a feedback survey- 90% of respondents found the quiz gamification highly effective. A Mann-Whitney test (*U*) indicated that the delivery of review content was highly effective for both student groups. However, the undergraduate-students found gamification more engaging than the medical-students (*U* = 326, *p* = .04). Interestingly, among seven flipped classroom activities (FCA), *Problem based learning* was ranked 1<sup>st</sup> by both student-groups, and *Games* was ranked 2<sup>nd</sup> by the medical-students. Nevertheless, statistical test suggested the symmetry of FCA-ranking between both student groups (*p*>0.05).

**Discussion:** Participant feedback, reported that students found the gaming activity as an effective complement to flipped classroom pedagogy. Students reported high levels of engagement while participating in both activities. Suggested improvements to the quiz format included: A quiz league for all flipped classroom activities; greater review of quiz answers, and improved mechanisms for identification of each team during the quiz. Insights from this pilot are being shared within the institution as a discussion vehicle for incorporating more active learning strategies into the curriculum.

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C117

### **A simple assessment instrument for physiology coursework; does it drive learning?**

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It is well established that assessment drives learning behavior in students (Biggs & Tang, 2007, Ramsden, 1992). Rowntree (1987) cited six purposes of assessment including maintenance of standards, motivating students, motivating teachers, and preparing students for professional life. Indeed, Davies (1994) has posited that a curriculum designed to promote

deep learning is pointless if the assessment encourages students to adopt a surface approach.

With the aim of using assessment to promote learning, I have developed a coursework feedback instrument for use within the Centre for Biomedical Science Education in QUB. Using this instrument, several criteria (alterable depending on the modality of assessment; essay, poster, oral presentation, or experimental write-up) are graded on a conceptual scale from first class honours to pass. These different grades are then combined and a final mark given with comments. I have had staff give a commitment to have this detailed, personalised and signed coursework returned within two weeks of the submission date.

To evaluate the effectiveness of the feedback instrument in promoting learning, a questionnaire was distributed to a Scientific Methods class on the day that two pieces of coursework (a referencing exercise and a critical review of a scientific article) were returned. Questions were posed on the effectiveness of the feedback in driving student learning, whether it would change learning behavior and whether it initiated a dialogue between students and staff. Anecdotal evidence suggested that increased feedback made student marks a starting point for a negotiation on grades. To address this, a further 2 questions were asked on the perceived fairness of the process. A 5 point Likert scale was used to evaluate the response to each of the questions with 5 indicating strong agreement with a statement and 1 strong disagreement. Ratings are given as mean marks out of 5 ± S.E.M, *n* = 47. In addition to the Likert questions, there were open ended questions asking students about the positives and negatives of the process and how it could be improved.

Students responded with Likert scores of 4.4 ± 0.8 agreeing that the assessment and feedback tools used here had driven their learning and 4.2 ± 0.8 agreeing that the feedback had altered how they would approach assessment in the future. The assessment was considered fair (4.7 ± 0.1), and was considered to have initiated a dialogue between students and staff (4.1 ± 0.1). There was a reassuringly low score in response to the question asking whether the feedback had made students disappointed with the grade achieved (1.9 ± 0.2).

These findings suggest positive engagement with the assessment process and are encouraging about students maturity and ability to take direction from academics, once given appropriately detailed and timely feedback.

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C118

### Integration of student-led lectures as an innovative interactivity in teaching

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Interactivity in teaching is prevalent in lectures. As student number and teaching information increase in Higher Education, the variety and quantity of interactivity employed seems decreasing, and is mostly reduced to a simple Q&A. This can have negative impacts on teaching, such as reduced interests in subjects and understanding for lectures, consequently affecting grades. Developing effective interactivity in teaching is therefore important.

In this presentation, we report student-led lectures as a new form of interactivity in teaching, which we employed in level 4 Human Physiology module within Biomedical Sciences course. A case study, and its advantages, feedback, and future improvement are explained.

The content of student-led lectures can be summaries or extra information to the module. There are several benefits to have student-led lecture: 1. When students deliver a lecture, they need to fully understand the module content and beyond. This improves their self-study ability and independency, and subsequently improve the directed learning, which is crucial as it can improve student's marks and framework of knowledge. This also requires an ability to present in their own words. 2. Module Leader will know to what extent student has understood the module, providing useful firsthand feedback on his lectures. 3. Other students will benefit from it with (a) increased confidence and intellectual stimulation by peer lecture delivery, increased understanding of content and studying ability. (b) Exposure to different perspective in the same module prompts them to think, so they form their own views on the module and not simply "absorb" a monotonous view by the module leader. This promotes a full understanding of the module and connection of knowledge between different modules. (c) Learning from someone with a similar level of knowledge may be better than from someone with more knowledge of the subject, as patterns of understanding can be learned.

This idea was executed by a student delivering a 30 min lecture containing an original case study and a summary of the Semester 2 Human Physiology module to the entire class (n=141). The student delivered the lecture with the help from the module leader.

As a result, the student achieved improved exam mark (83% to 95%). Most of students in the class gave positive feedback commenting on its benefits and how it aided their understanding of the module as a whole. There was also an increase in the mean grade of the class (5% above previous year).

By integrating student-led lectures as further interactivity in Physiology and other modules, students will benefit from sharing their learning experiences, increasing their understanding and involving them in discussions. More improvement could be done in the future. Hopefully, this new form interactivity of student-led lectures will be incorporated in more teaching.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

C119

### Preparing physiology students for group assessed work

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Stage 2 Physiological Sciences students at Newcastle University report that they enjoy working in groups during practical sessions. However, personal communication and module evaluation comments indicated the presence of well-documented problems with group dynamics and student dissatisfaction with related group assessment (Gibbs, 2009). As practical leaders spent substantial time informing students on the value of group work and providing guidelines on appropriate group behaviour this outcome was disappointing. In 2015-16, a peer-assessment component, using WebPA software (Loughborough University, 2006) to moderate the group mark, was introduced allowing students to evaluate self and peer attendance at group meetings, adherence to deadlines and provision of feedback. Module evaluation indicated that students appreciated use of this tool to moderate marks when problems were encountered but that students were unsure how to manage issues as they arose. To address this we created a Context Café workshop, aiming to prepare these students for group assessed work.

During this workshop, held in the first week of the semester, students worked in small groups to discuss group characteristics and dynamics and to analyse case studies based on previously reported problems. Refreshments were provided and group members rotated to ensure students had the opportunity to meet all of their Physiological Sciences course-mates, having previously only completed common biomedical sciences modules in a cohort of ~400 students.

Following the workshop, held in each of the last two years, students were asked to complete a brief questionnaire relating to how beneficial they felt the workshop had been and how it would influence their approach to group assessment. Two thirds of students (37/56) reported meeting the other people on the course and feeling better equipped to handle new and difficult situations encountered during group assessment as the most beneficial outcomes. Nearly half of respondents said that when approaching group assessment they would be more aware of effective group organisation (including setting deadlines, planning, making frequent contact) and how to improve group dynamics (including avoiding and solving problems early).

The first Context Café workshop appeared successful as no problems with group assessment tasks were reported to practical leaders and only minor issues were indicated and accounted for in peer assessment using WebPA. In the second year some groups did encounter significant issues which they raised with practical leaders. However, in contrast to previous years, students felt confident managing these problems, ensuring they completed the assessment on time and were satisfied that peer assessment would result in fair allocation of marks. The benefits of these workshops were further highlighted in end of semester module evaluation comments indicating that students felt more confident in approaching group assessments.

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Loughborough University. 2006. WebPA [Online]. Available: <http://webpaproject.lboro.ac.uk/>

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## C120

### **Women in Physiology: Development of an educational resource to improve awareness of the contributions of historical female physiologists.**

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The aim of this study was to investigate the perceptions of students and academic staff regarding key historical female researchers in physiology. It was hoped that this study would raise the profile of women in medical science, help students learn about the history of physiology, and assess whether infographics are a useful method of communicating with a broad audience.

Ten historical female physiologists were selected from a variety of physiological specialities and infographic pull-up posters developed that provided information about the life and achievements of these individuals. These posters formed the core of a public exhibition entitled 'Women in Physiology', staged at the University of Aberdeen, UK.

Paper-based feedback surveys were available so visitors could provide their views in relation to the use of infographics to communicate the information, and their perceptions of key female medical scientists. The survey included a series of 5 point Likert scale questions to assess level of agreement with various statements, and a free text area for additional responses.

Thirty-seven anonymous completed surveys were left at the exhibit. Students made up 73% of the respondents, with 24% being university staff, and 3% from the general public. Only 24% of respondents had any knowledge of the female physiologists in the exhibit, with the majority of this knowledge (66%) coming from lectures. All respondents disagreed that women were treated fairly in the history of medical science. Ninety-two per cent of respondents agreed they knew more about male researchers in medical sciences, and 95% agreed that infographic posters were an effective way to learn about the history of physiology. No respondents disagreed with these last two statements. In relation to infographic posters, 100% agreed they were an effective way of representing these women and their contributions, and for engaging a broad audience with scientific concepts/information. Eighty-nine per cent felt that university courses focused more on the contributions/discoveries of one gender than another (male or female), and 97% felt teachers should give equal attention to the contribution of males and females in the subject matter they teach. None disagreed with the last two statements. 97% wanted to learn more about women in physiology, and 100% wanted more of such exhibitions around university locations. This study has shown that universities may have to do more to raise the profile of the contributions of female medical scientists, but that exhibits such as this may be an effective method of engaging a broad audience of students and staff with such topics, as well as improving how we teach the history of physiology. Future exhibits will take place in more accessible areas of university campus and during public engagement events to increase the visibility of women in physiology.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## C121

### **Enhancing engagement with physiology using infographic presentations.**

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Poster presentations to communicate scientific knowledge and understanding are a commonly used assessment tool within undergraduate programmes. However, it is a challenge for students to deliver clear and detailed information whilst minimising volume of text on the poster. Infographics are progressively being used to demonstrate key scientific concepts in simple graphical form and developing students to effectively communicate with both a scientific audience and the general public is increasingly important within an academic curriculum. To enhance communication skills and also student engagement in this process we adapted an existing physiology research project assessment and increased the emphasis on public communication skills with the use of Infographics.

Students were asked to undertake a 11 week project on a physiological topic of their choice and present it in an infographic format online on a dedicated Twitter feed and also present the same work in a more traditional printed poster session. Staff and peers graded the infographics at the poster session. In addition, students had to submit an abstract reporting their findings, and the abstract had to be formatted as if they were submitting it to a Physiological Society meeting. Students could explore any area of physiology they wanted to as long as they were excited about the topic, and they could communicate the important messages or concepts involved in their project clearly and concisely. A range of free software/websites that could be used to produce infographics were demonstrated, and students could choose whichever one they felt was most suitable for them. Examples of infographics that summarised and communicated complex biomedical concepts or information were also made available to the class.

Students were invited to complete an anonymous questionnaire on the delivery and outcomes of the exercise. This considered how easy it was to simplify the scientific material and how infographic presentation compared with a standard scientific poster. Comparison between online and printed delivery was also evaluated along with the impact on the overall learning experience. Students rated various aspects of the project on Likert scales from 0-10.

Forty-six student completed the questionnaire, with the vast majority preferring online infographic posters rather than printed ones. Other findings included that students felt that infographics could communicate complex scientific concepts more effectively than traditional scientific posters. Students found the science easier than trying to pitch the content at the correct level for the audience. They appeared to give more thought as to how information should be presented and delivered to different audiences, and became more critical of their own work. This pilot initiative has been a success in revitalising a physiology project assignment, whilst teaching participants new skills and helping them further develop a range of graduate attributes.

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C122

### A New Elective Course at the Faculty of Medicine: Robophysiology

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**Introduction:** Increasing the impact of technology, especially electronics and software, on the medicine, requires the education of medical doctors using these technologies to be revised. It is necessary to make some changes in training for the students to understand and use the technology that they use in medicine and to develop new technologies. While it was not possible for medical doctors to intervene in hardware and software before, developments in software and hardware technology have made it possible in recent years. The development in the software is the spread of the "Python" programming language. The development in hardware is the widespread use of the "Arduino" microcontroller (which can be thought of as a very small and inexpensive computer fitted to a chip). The course, which we started as Hacettepe Faculty of Medicine for the first time in 2017-2018 education year under the name of "Mathematical Physiology" covers these topics. Next year this 16 week elective course (2 hours/week) will be expanded and given in two terms under the names of "Robophysiology I: Arduino" and "Robophysiology II: Python". **Objective:** Through these lessons, we aim to make medical school students understand the working principle of the instruments used for medical diagnosis and treatment. It is also the goal of this course to ensure that students who complete this course will be able to develop a new medical tool as well as gain the ability to perform both physical and mathematical models of specific physiological systems.

**Method:** The reason for choosing Python language in software is very easy to learn because it is very similar to English grammar and spoken language. Despite being easy, it is pretty powerful. According to the list published by the Institute of Electrical and Electronics Engineers in 2017, Python is the best programming language. Arduino is the best tool to learn the hardware. Advanced mathematical knowledge is not a requirement for Arduino although it is beneficial. However, it is still a must to use the conventional microcontrollers.

**Findings and Conclusion:** The lesson we started with mathematical physiology in the fall semester 2017-2018, hardware with Arduino and software with Python, will be given in the fall and spring semesters next year by extending the feedback from our students twofold. The technological change we are experiencing is a sign that these courses will be included as compulsory courses in the Faculty of Medicine curriculum in the near future. In order to prepare for the future, I think that this initiative we started in Hacettepe is necessary to be expanded as an elective course in all medical faculties. Medicine, inevitably, will be a space where robots and people will collaborate in the future. Hacettepe University has initiated its first initiative as an institution that not only teaches today but also educates future doctors.

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C123

### Teaching physiology to medical students in an integrated computer-assisted problem-based context

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Teaching basic sciences, including physiology, to medical students require innovative strategies that stress the relevance to their future profession. In response to this, many medical schools now have adopted integrated curricula in order to contextualize the teaching of basic sciences. However, at least some of these strategies do not achieve the desired actual integration. In this study, we evaluated the use of computer-based clinical cases in motivating the students to learn basic sciences, including physiology, in problem-based classes. The Faculty of Medicine in Suez Canal University adopt problem-based learning (PBL) as one of its main strategies. Starting in November 2010, the authors led a group of 10 students in the 3<sup>rd</sup>, 4<sup>th</sup> & 5<sup>th</sup> years who have strong computer skills to design 6 interactive problems in Internal Medicine and General Surgery. Scientific support was provided by subject experts in both departments. Each problem basically starts with a short video clip of a patient presenting his/her complaint with one of the students taking the relevant findings in the history. This is followed by a summary of the findings of the clinical examination and the relevant investigations. Throughout the case presentation, questions related to the basic and clinical sciences relevant to the case are inserted and the students have to discuss it and select an answer before proceeding to the next step. Following each question, explanation of the answers is complemented by audiovisual material that re-enforce the acquisition of basic science knowledge. After the classes, assessment of students' satisfaction was done using a 10-item anonymous questionnaire, with a 5 Likert scale for each item. Questions addressed their perceived effect in general, and the effect on brain storming, knowledge acquisition. The analysis showed that students believe that e-problems helped them to understand basic sciences better (79 %), learn deeper and remember longer (61%), participate more in discussions (70%), render PBL sessions more interesting (79%), focus their discussion (63%). In conclusion, computer-based cases can be used to stimulate the students to acquire relevant basic science knowledge and achieve a high level of integration. Also, this project presents a good example of how students can be motivated to help in improving their learning environment with a relatively low cost as compared to the professional case analysis computer programs.

This project was jointly funded by the Suez Canal University and the Project Management Unit in the Ministry of Higher Education

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C124

### **Institutional Openness in Animal Research Under EU Directive 2010/63/eu**

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This submission uses empirical work into the self-reporting performed by institutions – government, private, charity sector – of their use of biomedical research animals. Under EU Directive 2010/63/eu such entities are obliged to publicly acknowledge their experimental work with all vertebrates. Under national law, other key details for disclosure can include the quantity, species and care arrangements of such animals. Some organisations see this as a response to animal rights groups which allege a culture of secrecy; others more fully embrace the ‘transparency’ agenda.

The paper aims to build on a current/unpublished EARA project, supported by the European Commission, which evaluates and rates the online profiles of a representative sample of organisations using animals in biomedical research – including in basic and veterinary research. The authors have measured the extent to which public-facing websites accurately report on their publishers’ use of animals in a clear/accessible fashion. In the process, we consider whether biomedical researchers are addressing the concerns raised in the November 2017 review of EU Directive 2010/63/eu. In its review the European Commission stated that the Directive has introduced elements aimed at improving transparency and openness. It also reported that there has been some progress on transparency but suggested that more was needed. By rigorously evaluating institutions’ websites, in terms of, for example, their use of original photography, non-technical case studies and simple online navigation, the presentation would aim to identify the extent to which EU transparency guidelines on animal research transparency are being adhered to across EU Member States.

The methodology involves data entry based on six criteria: does the website feature a statement on the use of laboratory animals; can species used or types of research be identified (‘more information’); is there an attempt at, say, community engagement or answering FAQs (‘extensive information’); are images of animals used, especially original ones; are case studies available; could the information provided be considered prominent? When compiled, such data allows for national openness scores. The paper can then consider the overall state of institutional openness in EU animal research, which can be displayed using pie charts and illustrated with ‘good practice’ screenshots from various websites.

Whereas empirically the paper considers the actual content and presentation of the sites involved, the materials assembled permit discussion of a wider question: namely, to what extent is the desire for openness expressed in the revised EU Directive 2010/63/eu turning transparency into a normative principle linked to biomedical research?

Thanks to colleagues at the European Animal Research Association for their assistance with this project.

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C125

### **How can we help undergraduate students in large cohorts identify as Physiologists?**

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Many higher education institutions (including ours) no longer offer Physiology as an ‘entry’ degree, and first year undergraduate students (UG) are often taught Physiology in large mixed cohorts (e.g. Biomedicine or Bioscience). This situation poses significant challenges for new undergraduates who want to identify and network with peer- and near-peers who have a shared interest in Physiology. Student-led collaborative learning communities can enhance student engagement and improve module outcomes (Rutherford et al. 2015; Amici-Dargan et al. in press). We are currently using outputs of student-led focus groups to empower students to establish their own ‘Communities of Practice’ (Wenger 1998; Webber 2016), to enable them to identify as Physiologists whilst studying in large mixed cohorts.

Constructivist grounded theory (Charmaz, 2014) was the chosen methodological approach for this study, because it focuses on developing theory from rich qualitative data rather than aiming to prove or disprove a hypothesis. This study involved 8 participants: 2 students with an interest in Physiology from each UG year group, and 2 Physiology graduates (both did a placement year). Focus groups were conducted, transcribed and anonymised by a student researcher, and transcripts coded by 2 independent researchers. Emerging themes were identified and are currently being used to inform the establishment of a student-led ‘Community of Practice’ for UG Physiology students.

Four dominant themes emerged from student-led focus groups:

- (1) Any UG Physiology community should have shared ‘cross-year group’ leadership by a small group of students representing different stages of study
- (2) The community should hold regular informal Physiology-focused activities, run by near-peer students with occasional input from invited academics
- (3) A virtual community should be developed in parallel; online platforms should be chosen by students and must be intuitive to use with good functionality on different electronic devices
- (4) Academics should provide background support, validate the initiative and encourage student engagement

Student-led focus groups are an excellent way to facilitate the development of novel initiatives that engage and meet the actual needs of existing cohorts. Our findings reveal that the level of academic input in any UG community of practice needs to be very carefully considered and agreed by the students, and will depend on the main aims of the scheme. Expectations of staff and students need to be clearly set and new initiatives work best if launched at the beginning of an academic year. Any online platforms should be pre-populated with information and, in our experience, sign up should be voluntary to get a good level of engagement. These findings provide a foundation for developing effective collaborative learning communities to support students with an interest in Physiology.

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Rutherford, S. M., Mistry, S. and Scott, J.L. (2015) Assessing the potential of Web 2.0 technologies for supporting collaborative learning in Higher Education, in formal and informal learning environments. In: Gillies, R. (Ed.) *Collaborative Learning: Developments in Research and Practice*. Nova, New York. pp 265- 294.

Webber, E. (2016). *Building successful communities of practice: discover how connecting people makes better organisations*. 1st ed. TACIT.

Wenger, E. (1998). *Communities of practice: Learning, meaning and identity*. Cambridge: Cambridge University Press.

David Jordan Teaching Award from the Physiological Society

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

C127

### **Tumor microenvironmental acidosis and hypoxia differentially regulate the expression of tumor-related microRNAs**

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Small non-coding RNAs (microRNAs) have emerged as important regulators of stress response and thus play a critical role in the development and progression of cancer. They regulate the expression of their target genes on a post-transcriptional level and can act either as tumor suppressors or oncogenes (oncomiRs). MicroRNA expression is regulated by the tumor microenvironment, as for instance shown for hypoxia. However, besides hypoxia also a decrease in extracellular pH (acidosis) is often found in tumors. At present, the impact of acidosis on microRNA expression is widely unknown. Thus, the aim of this study was to analyze the role of tumor acidosis on microRNA expression.

The expression was analyzed in two experimental tumor lines, subline AT-1 of the Dunning prostate carcinoma and Walker-256 mammary carcinoma, *in vitro* and *in vivo* (subcutaneously implanted in male Copenhagen and Wistar rats, respectively). By Next Generation Sequencing 33 microRNAs were identified in AT-1 and 38 in Walker-256 cells which were regulated by acidosis (pH=6.6 for 24 h). 11 microRNAs were regulated in both tumor cell lines consistently in the same direction. By TaqMan qPCR we could confirm 3 down-regulated (miR-183, miR-203, miR-215) and 1 up-regulated microRNA (miR-7). These results were compared to expression changes under hypoxic condition (pO<sub>2</sub>=1.5 mmHg, pH=7.4) and combined hypoxia+acidosis (pO<sub>2</sub>=1.5 mmHg, pH=6.6). Acidosis-sensitive microRNAs were either contrariwise or not regulated by hypoxia. The effect of hypoxia+acidosis resembled an additive effect of acidosis and of hypoxia alone. *In vivo* most of the microRNAs were downregulated when the tumor pH was artificially lowered by inspiratory hypoxia and metaiodobenzylguanidine (MIBG) administration. In AT-1 tumors microRNAs were mostly regulated in the same direction as in isolated cells whereas in Walker-256 tumors several microRNAs showed an opposite regulation as the cells *in vitro*.

To elucidate the impact of microenvironmental regulation of microRNA expression on cellular behavior, putative microRNA targets were studied. The expression of Brip1, Ercc6l, Ikbke, Per3 and Tlr5 was affected by acidosis. Further studies are needed to clarify whether these changes depended on the altered microRNA expression and which of the regulated microRNAs were critical for target regulation.

In conclusion, the regulation of microRNAs differs profoundly depending on whether acidosis, hypoxia or acidosis+hypoxia are present in the tumor. Acidosis regulates microRNAs miR-7, miR-183, miR-203 and miR-215 in prostate and breast cancer cells *in vitro* and *in vivo* and could by this influence tumor cell transcriptional program thereby tumor cell behavior.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

C128

### **Contribution of cyclooxygenase (COX) products to exercise hyperaemia in young Black Africans (BAs) and White Europeans (WEs): are there gender differences?**

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The contribution of prostaglandins (PGs) to exercise hyperaemia is controversial. However, most studies were performed on mixed groups of men and women and few mention ethnicity. BAs are at higher risk of developing hypertension relative to WEs; reduced endothelium-dependent dilatation is associated with hypertension. We hypothesised that young BAs show a blunted contribution of PGs to exercise hyperaemia compared to WEs, but the contribution of PGs is relatively preserved in young BA women by the facilitatory influences of oestrogen on endothelium.

In 16 WEs and 19 BAs (10/6,11/8: M/F respectively, aged 18-29 years), we recorded changes in arterial pressure (ABP) by finger photoplethysmography and forearm blood flow (FBF) by venous occlusion plethysmography evoked by 2 min rhythmic handgrip exercise at 60% of maximum voluntary contraction before and after the COX inhibition with aspirin (600mg p.o). Values were compared by ANOVA. All subjects were recreationally active and normotensive (SP/DP <140/90mmHg). Resting FBF was similar in WEs and BAs (5.5±0.7 vs 5.6±0.4 ml/100ml tissue/min). Following rhythmic handgrip, FBF increased to 49.5±6.6\* in WEs and 39.1±3.9\* ml/100ml tissue/min in BAs (\*: p<0.001 vs rest). The evoked increases in FBF were greater in WEs than BAs (P<0.05 WE vs BA). Further, within both ethnic groups, the increases in FBF were smaller in women: to 60.0±8.9 and 32.0±2.7<sup>§</sup> ml/100ml tissue/min in WE men vs women (<sup>§</sup>: p<0.05 men vs women) and to 45.6±4.6 vs 28.9±5.4<sup>§</sup> ml/100ml tissue/min in BA men and women.

COX inhibition did not affect resting ABP, or FBF in WE or BA. However, the increases in FBF were attenuated in all WEs (49.5±6.6 vs 39.5±4.5<sup>†</sup> ml/100ml tissue/min: <sup>†</sup>: control vs aspirin) and all BAs (39.1±3.9 vs 33.9±3.8<sup>†</sup> ml/100ml tissue/min). Further COX inhibition attenuated the increases in forearm vascular conductance (FVC: FBF/ABP) in all WEs (to 0.57±0.05 vs 0.39±0.05<sup>†</sup> conductance units (CU)) and all



BAs ( $0.40 \pm 0.04$  vs  $0.34 \pm 0.04^{\dagger}$  CU). However, whereas COX inhibition attenuated the increases in FVC in men in both ethnic groups ( $0.49 \pm 0.07$  vs  $0.35 \pm 0.06^{\dagger}$  in WEs and  $0.42 \pm 0.07$  vs  $0.25 \pm 0.06^{\dagger}$  CU in BAs), it had no effect in women (WEs:  $0.28 \pm 0.03$  vs  $0.20 \pm 0.02$ ,  $P=0.09$ ; BAs:  $0.26 \pm 0.04$  vs  $0.29 \pm 0.04$  CU,  $P=0.9$ )

Thus, our results indicate that considered as mixed gender, young adult groups, BAs show attenuated exercise hyperaemia relative to WEs. Further, within both WEs and BAs, young women show blunted exercise hyperaemia relative to men. However, in contrast to our hypotheses, although the analysis of the full groups of BAs and WEs indicated PGs contribute to exercise hyperaemia in both ethnicities, this largely reflects a substantial contribution of PGs to exercise hyperaemia in WE and BA men; any contribution of PGs to exercise hyperaemia is weak in both WE and BA women.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

C126

### Wnt peptides control mammalian cell membrane potential

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Wnt ligands are a diverse family of secreted polypeptides, implicated in multiple pathways during development and in disease. Nineteen Wnt ligands are known in humans and activation of Wnt binding receptors leads nuclear translocation of  $\beta$ -catenin downstream. Application of subset of Wnts activate a  $\text{Ca}^{2+}$  signal in prostate, breast and bowel cancer cells lines (Thrasivoulou et al, 2013). To investigate early events in this signalling pathway we have used high throughput techniques (Qpatch) as well as single cell tight seal patch recording with  $[\text{Ca}^{2+}]_i$  imaging to show that an early step in the Wnt signal cascade is the control of membrane potential.

Using PC3 prostate cancer cell lines, a local application via a puff pipette of either Wnts 5A, 9B or 10B produced an small initial depolarisation, followed by a hyperpolarization of up to 30 mV. Simultaneous recording of  $\text{Ca}^{2+}$  with OGB5N ( $K_d = 20 \mu\text{M}$ ) showed a parallel biphasic response, with an early  $\text{Ca}^{2+}$  decrease. Determination of the Wnt concentration using a dye in the delivery pipette indicated a threshold response occurs for Wnt concentrations in the low nM range with a response latency less than 200 ms. High-throughput measurements, using controlled microfluidics, also consistently showed an early (<3s) current response at levels down to 2 nM.

As in earlier  $\text{Ca}^{2+}$  imaging experiments, a bolus delivering higher sustained concentrations to the bath produced a larger but delayed (100-200s)  $\text{Ca}^{2+}$  rise. Associated with this rise was a large cationic current reversing around +10mV, which sometimes though not always was reversible. This current was often accompanied by damped  $\text{Ca}^{2+}$  and membrane current oscillations (rate 0.07-0.05 Hz). The phenomena are simply explained by activation of a store operated calcium release. The net effect ensures Wnt activation leads to  $\text{Ca}^{2+}$  entry by a voltage-independent non-selective pathway and a release of  $[\text{Ca}^{2+}]_i$ . We suggest that Wnt control of membrane potential is a signal amplifying mechanism for low nanomolar levels of Wnt, and further suggests that modulation of this critical cell signal transduction pathway may be a means to control cell proliferation for some cancers.

C. Thrasivoulou, M. Millar, A. Ahmed, Activation of intracellular calcium by multiple Wnt ligands and translocation of beta-catenin into the nucleus: a convergent model of Wnt/ $\text{Ca}^{2+}$  and Wnt/beta-catenin pathways. *J Biol Chem* 288, 35651-35659 (2013).

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## PCA001

**Sodium nitroprusside dilates cerebral vessels in young men**N.D. Olesen<sup>1,2</sup>, M. Fischer<sup>1,3</sup> and N.H. Secher<sup>1</sup>

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**Introduction**

Cerebral autoregulation maintains cerebral blood flow (CBF) despite marked changes in mean arterial pressure (MAP) (1). Sodium nitroprusside (SNP) reduces MAP by vasodilatation, but is reported to lower CBF suggesting a critical role for its perfusion pressure when MAP is lowered (2, 3). We evaluated the influence of SNP on CBF and aimed for a 20% and 40% reduction in MAP, while keeping MAP  $\geq 50$  mmHg, to challenge cerebral autoregulation.

**Methods**

In 19 healthy men (age  $24 \pm 4$  years; mean  $\pm$  SD) right internal carotid (ICA) and vertebral artery (VA) blood flow was evaluated by duplex ultrasound and transcranial Doppler measured middle cerebral artery mean blood velocity (MCA  $V_{\text{mean}}$ ). Cerebral  $\text{CO}_2$  reactivity was determined by hyperventilation at rest and CBF ((ICA + VA flow)  $\times 2$ ) also 'corrected' according to reduction in arterial  $\text{CO}_2$  tension ( $\text{PaCO}_2$ ) with the reservation that  $\text{CO}_2$  reactivity may be affected by SNP. Brachial artery catheterisation determined MAP and total peripheral resistance was derived by pulse contour analysis (Modelflow). Analysis was by a repeated measure mixed model. Values are presented as mean  $\pm$  SD.

**Results**

Three subjects developed presyncopal symptoms during the large reduction in MAP and data obtained at that time was not included in the analysis. Infusion of SNP ( $2.0$  ( $1.5$ - $2.5$ ) and  $8.6$  ( $6.0$ - $9.4$ )  $\mu\text{g kg}^{-1} \text{min}^{-1}$ ) reduced MAP (from  $83 \pm 8$  to  $69 \pm 8$  and  $58 \pm 4$  mmHg; both  $P < 0.0001$ ), total peripheral resistance, and  $\text{PaCO}_2$  ( $41 \pm 3$  vs.  $39 \pm 3$  and  $37 \pm 4$  mmHg; both  $P < 0.01$ ). Yet, ICA flow increased at the moderate reduction in MAP (by  $27 \text{ ml min}^{-1}$ ; 95% CI:  $6$ - $48$ ;  $P = 0.013$ ) but returned to the baseline value by the large reduction in MAP while VA flow and CBF were maintained with increased ICA and VA conductance (Fig. 1). In contrast, SNP decreased MCA  $V_{\text{mean}}$  from  $69 \pm 16$  to  $59 \pm 14$  and  $52 \pm 14 \text{ cm s}^{-1}$  (both  $P < 0.001$ ). With 'correction' for the reduction in  $\text{PaCO}_2$  ( $2.8 \pm 1.0\% \text{ mmHg}^{-1}$  for CBF) SNP increased ICA flow while VA flow was unaffected and thus CBF increased whereas MCA  $V_{\text{mean}}$  remained reduced.

**Discussion**

Similar to the present results CBF increases in response to vasodilatation by, e.g. L-arginine (4) and adenosine (5). Yet, evaluation of CBF by  $^{133}\text{Xe}$  clearance indicates that CBF is reduced by even a minor SNP-induced reduction in MAP (2, 3). The discrepancy between results using duplex ultrasound and  $^{133}\text{Xe}$  clearance may relate to cerebral vasodilatation that may increase transient time for blood whereby clearance of  $^{133}\text{Xe}$  would be slowed and interpreted as a decrease in CBF. We took changes in  $\text{PaCO}_2$  into account but cerebral  $\text{CO}_2$  reactivity may be affected by SNP.

**Conclusion**

Sodium nitroprusside induces systemic and cerebral vasodilation and increased cerebrovascular conductance. The results indicate different regional regulation of CBF as SNP increased ICA but not VA flow.

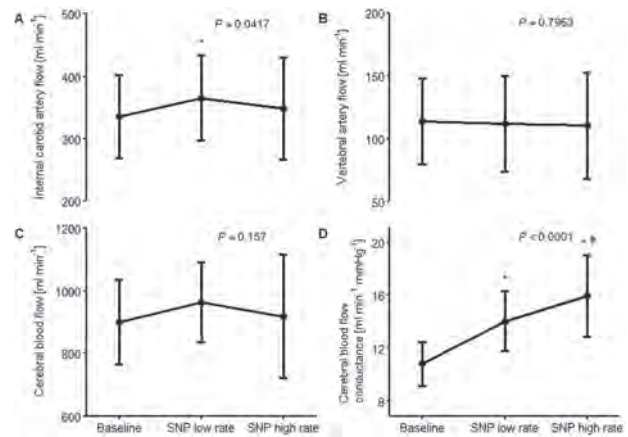


Fig. 1. A: internal carotid artery blood flow, B: vertebral artery blood flow, C: cerebral blood flow, and D: cerebral blood flow conductance at baseline and during infusion of sodium nitroprusside (SNP). Variables are without 'correction' for change in  $\text{PaCO}_2$ .  $P$ -values represent the overall effect as estimated by a repeated measure mixed model. \* $P < 0.05$  vs. baseline. † $P < 0.05$  for low vs. high SNP infusion rate.

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This study was supported by the University of Copenhagen.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA002

**Effect of Valsalva Maneuver on Ventricular Repolarization**O. Bhagat<sup>1</sup>, A. Singhal<sup>1</sup> and S. Deora<sup>2</sup>

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**Introduction:** Though Valsalva manoeuvre is well documented for its cardiovascular and autonomic responses (1) but little has been studied about its effect on ventricular repolarisation. Prolongation of ventricular repolarisation predisposes to arrhythmias (2). It causes the increase in intrathoracic pressure thereby compressing blood vessels and heart, which may lead to modulation of ventricular repolarisation. Therefore, we attempted to measure the effect of Valsalva manoeuvre on ventricular repolarisation.

**Method:** We conducted the study after ethical approval from institutional ethics committee on ten male volunteers of 17-21 years of age. ECG and respiratory movements were recorded at baseline, during strain and after release, in Valsalva manoeuvre at maximal effort for 7 seconds. BIOPAC MP150 with BN-TX RSPEC 3.0 was used for acquisition and LabChart 5 software for analysis. We measured RR interval, QT interval, QTc, JT interval, Tpeak-end duration and Tpeak-end / QT interval ratio for baseline, phase II and phase IV of Valsalva manoeuvre.

**Results:** Tpeak-end duration and Tpeak-end QT interval ratio, which is the index for transmural dispersion of ventricular repolarization (3), were increased more than double from  $40 \pm 0.01 \text{ ms}$  and  $0.14 \pm 0.01$  at baseline to  $83 \pm 0.04 \text{ ms}$  and  $0.29 \pm 0.11$  respectively in phase II (strain phase) of Valsalva

manoeuvre. These returned to near baseline values in phase IV. RR interval decreased ( $840 \pm 0.08$  to  $643 \pm 0.06$ ) whereas QTc interval increased ( $314 \pm 0.02$  to  $342 \pm 0.05$ ) in phase II of Valsalva manoeuvre (Table1).

Conclusion: Ventricular repolarisation duration increases during phase II (continued strain) thus it may trigger arrhythmic episodes in susceptible individuals on performing Valsalva manoeuvre.

Table 1: Measures of ventricular repolarization and RR interval duration during baseline, phase II and phase IV of Valsalva maneuver

|                        | Baseline (ms)   | Phase II VM (ms)  | Phase IV VM (ms)  | % change between Phase II & Baseline (%) | % change between Phase IV & Baseline (%) |
|------------------------|-----------------|-------------------|-------------------|------------------------------------------|------------------------------------------|
| RR Interval            | $840 \pm 0.08$  | $643 \pm 0.06^*$  | $1081 \pm 0.10^*$ | $-23 \pm 7.02$                           | $29 \pm 15.09$                           |
| QT Interval            | $286 \pm 0.02$  | $272 \pm 0.04$    | $279 \pm 0.02$    | $-5 \pm 12.99$                           | $-2 \pm 7.49$                            |
| QTc                    | $314 \pm 0.02$  | $342 \pm 0.05$    | $269 \pm 0.02^*$  | $9 \pm 17.47$                            | $-14 \pm 9.25$                           |
| JT Interval            | $209 \pm 0.02$  | $187 \pm 0.03$    | $204 \pm 0.02$    | $-10 \pm 15.06$                          | $-2 \pm 9.44$                            |
| T peak-end             | $40 \pm 0.01$   | $83 \pm 0.04^*$   | $42 \pm 0.01$     | $108 \pm 101.81$                         | $5 \pm 18.65$                            |
| T peak-end/QT Interval | $0.14 \pm 0.01$ | $0.29 \pm 0.11^*$ | $0.15 \pm 0.03$   | $110.16 \pm 58.25$                       | $7.42 \pm 10.36$                         |

Values are presented in Mean $\pm$ SD ; \* P<0.05, Repeated measures ANOVA with post-hoc analysis

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We acknowledge all the participants of the study and faculty and staff members of Autonomic Function test Laboratory of AIIMS, Jodhpur, India

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA003

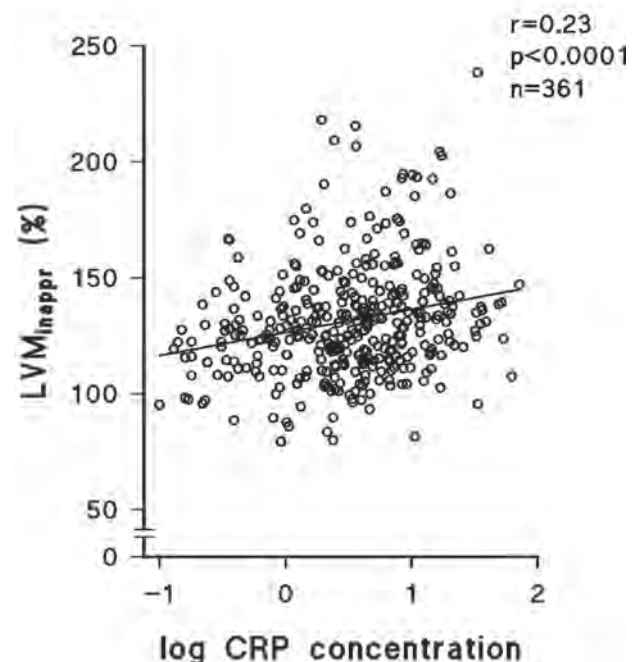
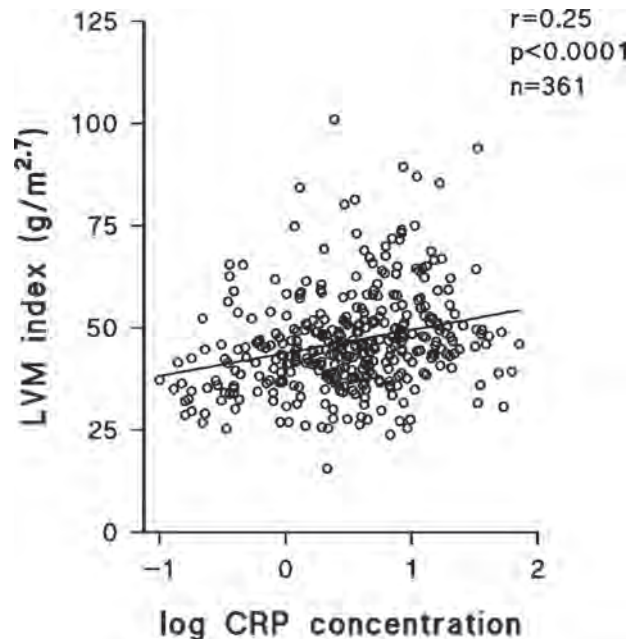
### C-Reactive Protein is Related to Left Ventricular Mass Independent of Co-Morbidities in a Community Sample with a High Prevalence of Risk-Related CRP.

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To determine whether there is a relationship between circulating C-reactive protein (CRP) concentrations and left ventricular mass (LVM) independent of co-morbidities. In 361 randomly selected participants from a community with a high prevalence of CRP concentrations considered to be high-risk (54.0%), but without cardiovascular or renal disease, the relationship between CRP and echocardiographically-determined LVM indexed to height<sup>2.7</sup> (LVMI) and inappropriate LVM (LVM<sub>inappr</sub>) was evaluated. LVM<sub>inappr</sub> was determined from the % of actual/predicted LVM. Serum CRP concentrations were correlated with both LVMI and LVM<sub>inappr</sub> (p<0.0001). With adjustments for a number of potential confounders including age, systolic blood pressure, waist circumference (or body mass index), and glucose control (glycated haemoglobin), the relationships between serum CRP concentrations and both LVMI and LVM<sub>inappr</sub> (partial r=0.11, p<0.05 for both) persisted.

The independent relationship between CRP and LVMI or LVM<sub>inappr</sub> translated into a markedly higher multivariate-adjusted LVMI and LVM<sub>inappr</sub> values in the highest as compared to the lowest quartile of CRP (LVMI; highest quartile CRP=48.8 $\pm$ 10.7, lowest quartile CRP=45.0 $\pm$ 11.0, p<0.05; LVM<sub>inappr</sub>; highest quartile CRP=137 $\pm$ 28, lowest quartile CRP=127 $\pm$ 24, p<0.02). Previous studies found relationships between CRP and LVM or LVH, but those studies were conducted in select clinical populations or populations with a low prevalence of risk-related CRP concentrations and the relationships were not demonstrated to be independent of co-morbidities. However, in randomly selected participants from a population with a high prevalence of CRP, the CRP concentrations were associated with LVMI or LVM<sub>inappr</sub> independent of co-morbidities.



Bivariate relationships between log C-reactive protein (CRP) concentration and left ventricular mass (LVM) index or inappropriate LVM (LVM<sub>inappr</sub>) in a community sample.



I would like to extend my deepest gratitude and respect to my supervisors, Professor Angela J. Woodiwiss and Professor Gavin R. Norton for their guidance and support without whom my efforts would have been futile. I thank them for allowing me to use some of the ECG's and echocardiograms in their records. I would also like to thank the Cardiovascular Pathophysiology and Genomics Research Unit (CPGRU) members for their assistance in data collection and participant recruitment notably Dr Carlos Libhaber, Mr Joseph Maseko, Mr Mthuthuzeli Kiviet, Dr Harold Majane, Ms Nomonde Molebatsi and Mrs Nkele Maseko.

I am also greatly indebted to Oppenheimer Memorial Trust for their valued sponsorship which made my studies possible. I thank the manager of Oppenheimer Memorial Trust, Ms Clare Digby for her confidence in me which enabled me to start my programme against all odds. I want to acknowledge the great contribution made by all those people in SOWETO, South Africa who availed some of their time to participate in the research.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

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PCA004

**In situ evaluation of tonic and reflex cardiorespiratory changes induced by chlorpyrifos-oxon in rats.**

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Chlorpyrifos-oxon (CPO) is the active metabolite of chlorpyrifos (CPF), an organophosphorus (OP) compound highly associated with poisoning and death worldwide (Eddleston et al. 2005; Munõz-Quezada et al. 2017). With the recent increase in the frequency of nerve agent (acetylcholinesterase inhibitors) attacks on humans it becomes crucial to understand the effects on vital visceral function and to test for effective antidotes. Previous data from our group showed that *in vivo* exposure to CPF impaired cardiovascular reflexes in rats (Cunha et al. 2018). Recent experiments using an *in situ* arterially perfused decerebrate rodent preparation, showed that 24 hours after CPF exposure, a marked reduction in peripheral chemoreflex function and basal respiratory discharge was observed (*unpublished data*). However, the temporal profile of immediate changes exerted by this compound on cardiorespiratory modulation remains unexplored. The aim of the present study was to evaluate the baseline and reflex cardiorespiratory changes induced by CPO within the first hours after poisoning. An *in situ* preparation was set up using juvenile rats ( $92.3 \pm 12.7$ g; n=17) perfused with a modified Ringers solution and neuromuscularly blocked (rocuronium bromide, 10 mg/mL), as previously described (Paton 1996). Basal records of phrenic nerve (PNA), recurrent laryngeal nerve (RLN), sympathetic nerve activities (tSNA), heart rate (HR), ECG and perfusion pressure (PP) were compared before and after CPO (15 mg/kg in DMSO 55%) or DMSO (control vehicle), which were added to perfusate. Before and 15 minutes after CPO administration, KCN (0.03%; 50, 75, 100  $\mu$ L) or phenylephrine (30 mg/mL; 100  $\mu$ L) were injected through the ascending aorta to stimulate the peripheral chemo- and baro- reflexes, respectively. Data were analysed by generalized estimating equations (GEE)

and presented as means  $\pm$  S.E.M. Within the first minutes of poisoning, CPO increased basal HR and tSNA ( $p < 0.0001$ ) and decreased respiratory frequency ( $p < 0.0001$ ). PP was transiently increased ( $p < 0.0001$ ) after CPO administration. CPO decreased the time to reach inspiratory peak of phrenic nerve discharge ( $p < 0.0001$ ) and increased duration of expiratory phase ( $p < 0.0001$ ). Duration of RLN post-inspiratory activity was increased ( $p < 0.001$ ) while amplitude was decreased ( $p < 0.0001$ ) by CPO. Chemoreflex induced sympatho-excitation ( $p < 0.05$ ) and pressor response ( $p < 0.05$ ) were decreased, while bradycardia ( $p < 0.0001$ ) was increased after CPO administration. No change in chemoreflex tachypneic response was observed. CPO exposure reduced baroreflex mediated sympatho-inhibition ( $p < 0.05$ ). CPO induced marked immediate changes in tonic and reflex cardiorespiratory function, which may have important implications in the treatment of poisoned patients.

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Fapes/CONFAP, Capes, Newton Fund/Research Councils UK, NIH (1R01AT008632-01).

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

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PCA005

**Effects of levosimendan in rats with lipopolysaccharide-induced disseminated intravascular coagulation**

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Disseminated intravascular coagulation (DIC) is a common complication of sepsis and leads to multiple organ failure and death. In patients with severe sepsis, pro-inflammatory cytokines and activation of tissue factors trigger a cascade of events leading to coagulation dysfunction and multiple organ failure (MOF). Levosimendan (LS) has been demonstrated that it has protective effects against tissue injury caused by endotoxin. The aim of this study was to examine effects of levosimendan on consumptive coagulopathy and organ dysfunction in an endotoxaemic animal model induced by lipopolysaccharide (LPS). Here, we tried to investigate effects of levosimendan on haemostatic changes in LPS-induced DIC by using Thromboelastography (TEG). Male adult Wistar rats were randomly divided into four groups: Control group, an intravenous infusion of 5% dextrose 1.2 mL/kg for 20 minutes (24  $\mu$ g/kg for 20 minutes plus 0.6  $\mu$ g/kg/min for 4 hours); LPS group, an intravenous LPS (4 mg/kg) infusion followed by dextrose administration; and LS-treated LPS group, an intravenous LPS infusion followed by LS treatment. Haemodynamics, biochemistry, haemostasis and inflammatory response were examined during the experimental period. The administration of LS significantly attenuated (i) consumptive coagulopathy displayed by TEG, (ii) the decreases of platelet count

and plasma interleukin-6 in rats treated with LPS. Thus, the treatment of LPS rats with LS reduced organ injury and coagulopathy and this protective effect may be attributed to its anti-inflammatory effects.

This work was supported by the grant MOST 105-2320-B-016-010.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA006

## Investigation of the osmopressor response in young healthy adults

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### Introduction

The osmopressor response was reported in 1999 in patients with orthostatic hypotension secondary to autonomic nervous failure. Ingestion of 480 ml water increased systolic blood pressure by 33-37 mm Hg and diastolic blood pressure by 14 mm Hg (1, 2). However, the nature and size of the osmopressor response in healthy people is unclear (3, 4, 5). The aim of this study was to determine if water ingestion increased blood pressure in young healthy volunteers.

### Methods

Thirty-seven young undergraduates (median age 22 years, range 20-25; 13 male: 24 female) were enrolled on the study. All subjects self-declared as healthy and not on medication. Institutional ethics approval for the study was granted. Subjects sat at a table in a well-lit, air-conditioned room (22-24 °C) for 80 minutes. Blood pressure was measured using automated brachial cuff monitors every 5 minutes. After 20 minutes subjects ingested 500 ml water within five minutes, or otherwise received no drink. Test runs were performed on three separate occasions for each subject. Data was analysed by two-way ANOVA with Dunnett's post-hoc tests and correction for multiple testing (Graph Pad Prism). Mean values of parameters at 25-80 minutes were compared to the control mean at 20 minutes. Differences in means with  $p < 0.05$  were considered significant. A sub-group of 13 subjects also ingested 300 ml orange juice to compare with any effects of water.

### Results

Systolic blood pressure was increased transiently 10 minutes after onset of water ingestion: 20 minutes (baseline) = 109.2 (1.7) mmHg (mean, SEM,  $n=37$ ); 30 minutes = 112.6 (1.7) mmHg ( $p < 0.05$ ). Diastolic blood pressure was also increased at this time point: 78.4 (1.4) mmHg at 30 minutes compared to baseline: 73 (1.1) mmHg ( $p < 0.05$ ). The major response to water ingestion was a long-lasting decrease in heart rate (Figure 1). Heart rate decreased from a baseline of 79.8 (1.4 bpm) to 76.2 (1.4) bpm at 30 minutes ( $p < 0.05$ ), with a maximum decrease of nearly 6 bpm at 40 minutes: 73.8 (1.3) bpm ( $p < 0.05$ ). The decrease in HR lasted until 65 minutes (45 minutes post water ingestion). Heart rate naturally decreased in the sitting position with no drink and was significantly different to baseline after 70-80 minutes. Ingestion of 300 ml orange juice had no sustained effects on any parameter.

### Conclusion

The major response to water ingestion in these young healthy volunteers was a long lasting negative chronotropic response or bradycardia. A transient increase in systolic and diastolic blood pressure was recorded 10 minutes after onset of water

ingestion but was not sustained. The bradycardia may be as a result of the baroreceptor reflex and/or increased vagal drive to the heart. Further research into the mechanism and utility of the response is planned.

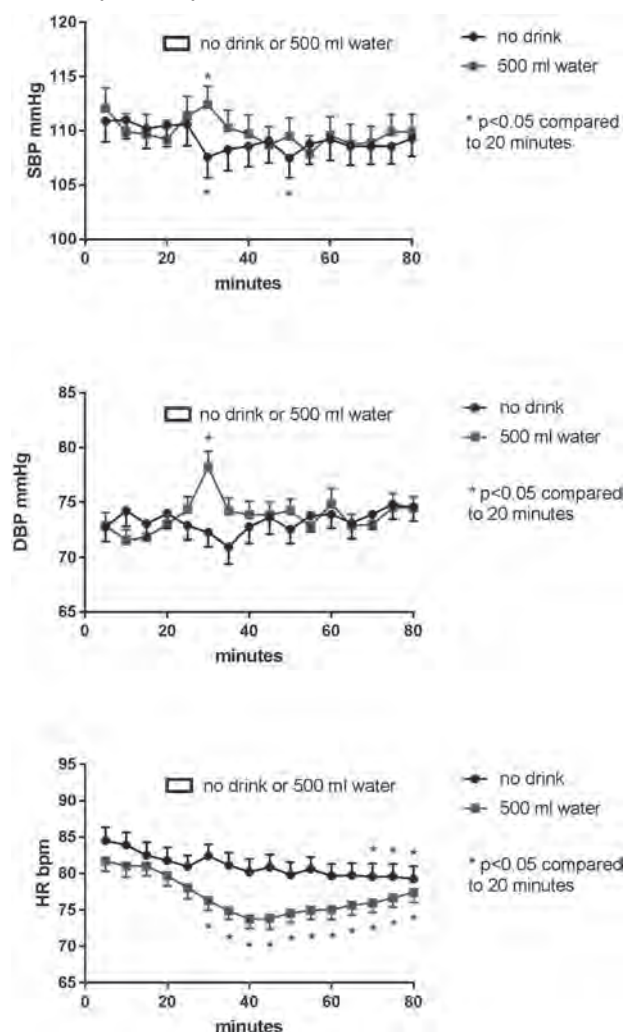


Figure 1. Effect of water ingestion (500 ml over 5 minutes) on systolic blood pressure, diastolic pressure, and heart rate in young healthy adults tested in the seated position. Controls had no drink. Mean (SEM),  $n=37$ . \* $p < 0.05$  by ANOVA.

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I would like to thank the MPharm project students and their volunteers who contributed to this study.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA007

**Do fasting healthy subjects show alterations in heart rate variability?**

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Most religions encourage a form of fasting. Fasting has been shown to have multiple health benefits. Two studies have investigated heart rate variability (HRV) during Ramadan fasting, one in healthy subjects [1] and the other in hypertensives [2]. Both showed contradicting results but more importantly, lacked a pre-Ramadan baseline. A change in HRV by Ramadan fasting would interest both clinicians and researchers. The aim of this study was to investigate the effects of Ramadan fasting on heart rate variability (HRV) in healthy subjects.

16 healthy volunteers (all male) underwent three consecutive five-minute HRV measurements over two months. Subjects were all staff and students of the University of Plymouth (UoP). The study was given ethical approval by the Research Ethics committee, UoP. All participating subjects gave informed and signed consent. Anthropometry, blood pressure and HRV were measured. To measure HRV, an ECG lead was attached to each limb. LabChart software and a PowerLab were used for data acquisition. The order of the experiments was: 1 week pre-Ramadan, 3 weeks into the month of Ramadan and 3 weeks after Ramadan ended. Data was also normalised by taking into account the heart rate [3]. The results were analysed by repeated measures ANOVA.  $p < 0.017$  was considered as significant.

The subjects' mean ( $\pm$  S.D.) age was  $31.88 \pm 8.08$  years and mean BMI was  $24.77 \pm 2.89$  kg m<sup>-2</sup>. Except BMI, all normalized and non-normalized time and frequency domain HRV variables showed no significant difference over all three occasions. HF did show a non-significant 30% increase from pre-Ramadan to Ramadan. Heart rate and blood pressure also showed no significant changes over this period. Although BMI significantly decreased in Ramadan relative to pre-Ramadan ( $p=0.0007$ ), it increased post-Ramadan ( $p=0.004$ ) similar to pre-Ramadan baseline levels.

To our knowledge, this is the first study which has investigated the effects of Ramadan fasting on HRV before, during and after fasting. Our data showed HRV in our subjects was not affected by Ramadan fasting. Despite there being no significant HRV changes, the data showed some consistent trends, such as HF, HFnu, SD1 and normalized HF all increasing during Ramadan fasting. This indicated a possible increase in parasympathetic activity during this time. The reason for such autonomic modulation is unclear. All the HRV data had a high standard deviation, indicating a large inter-subject variation. This along with the small number of subjects would have decreased the statistical power of the study. Although our findings did not show any significant HRV changes, further research is required with a larger sample population to determine if Ramadan fasting affects HRV.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA008

**Actions of aqueous extract of roots of *Carissa edulis* (Natal Plum) on cardiac properties of isolated rabbit's heart.**M. YUSUF<sup>1</sup>, M.A. Mabrouk<sup>2</sup> and K.A. MUHAMMED<sup>1</sup><sup>1</sup>Human Physiology, Kaduna State University., Kaduna, Nigeria and <sup>2</sup>Human Physiology, Bayero University, Kano, Nigeria

Hypertension is one of the major risk factors in both coronary heart disease and cerebrovascular accidents; it often remains asymptomatic until late in its course. *Carissa edulis* Vahl is a shrubby plant called Natal Plum in English. It is predominant in the northern region of Nigeria. The plant have been claimed to treat numerous ailments including hypertension. This study was designed to investigate the effects of the aqueous extract of the root of *Carissa edulis* on cardiac properties of a rabbit's heart ex vivo, using the Langendorff apparatus and technique. The apparently healthy and non-treated research animal was sacrificed by cervical dislocation, the heart was accessed after dissection of the thoracic cavity and mounted on the apparatus, upon which the test extract and other standard drugs were applied for their various effects. Especially for their chronotropic and inotropic effects, which were recorded by a Physiograph on a tracing paper. Data obtained were statistical analyzed using the paired student's t-test and the results indicated that aqueous extract of root of *Carissa edulis* showed a dose dependent effects in causing a negative chronotropic and inotropic effects on the isolated rabbit's heart. The maximum effect was observed on administration of 3.0mg of the extract, which caused a decrease in the heart rate from 120 beats/min to 90 beats/min ( $p < 0.05$ ) and a decrease in strength of contraction from  $7.92 \pm 0.154$  mV to  $1.50 \pm 0.129$  mV ( $p < 0.05$ ). The extract was seen to exhibit a similar effect as Acetylcholine on the organ, and was antagonized by Atropine. We therefore conclude that aqueous root extract of *Carissa edulis* has a negative chronotropic and inotropic effects on an isolated heart of a rabbit and may possess the antihypertensive properties.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.



PCA009

### The physiological effects of Nasal High Flow: a Theoretical Study

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Nasal High Flow (NHF) is a therapy in which humidified, warm air is delivered through the nose to spontaneously breathing patients. It has been shown that NHF affects the amplitude and frequency of breathing and that this response varies between patients and between wakefulness and disease states (1). To help us understand these physiological effects of NHF, we have developed a mathematical model of the respiratory system that couples the upper airway with lung mechanics, gas exchange and gas transport. We conduct our study in open loop (i.e. without including the respiratory feedback mechanisms) and change the breathing pattern manually to maintain physiological levels of arterial partial pressures of oxygen (O<sub>2</sub>) and carbon dioxide (CO<sub>2</sub>). We simulate the model numerically under control conditions (no NHF) and under conditions that mimic NHF. When NHF is administered, the nostrils are partially blocked while airflow is delivered; hence, the resistance to spontaneous breathing through the nose is increased. We study these two effects (pure flow and increased nasal resistance) independently as well as dependently. Our simulations show that when pure flow is administered without taking into account the increased nasal resistance, it has the same effect as reducing the lung dead space, that is, it improves the delivery of O<sub>2</sub> and the removal of CO<sub>2</sub> from the blood. However, when changes to the nasal resistance are included, the effects on the blood levels of O<sub>2</sub> and CO<sub>2</sub> could be reversed, leading to either increase or decrease in blood O<sub>2</sub> and CO<sub>2</sub> levels. We hypothesize that these changes in blood gas levels trigger the respiratory control system to change the breathing pattern and we show that changing the breathing pattern under normal conditions can bring the blood levels of O<sub>2</sub> and CO<sub>2</sub> back to normal (however, we show that a variety of breathing patterns can achieve this). Our simulations also show that administering NHF leads to an increase in baseline of airway pressure as well as an increase in pressure swings during breathing. These results are aligned with trends seen in previous experimental studies (1, 2, 3). Our theoretical study provides several new insights about the physiological effects of NHF and although it is still unclear why a particular combination of frequency and amplitude of breathing is selected by the neural system, our study demonstrates the potential of using NHF for studies of neural control of breathing.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCA010

### Can atropine and pralidoxime restore neuroreflex cardiorespiratory function after acute exposure to chlorpyrifos in rats?

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Previous work from our group showed that acute exposure to chlorpyrifos (CPF), an organophosphorus (OP) compound, impairs chemoreflex and baroreflex function in rats (Cunha et al. 2018). However, whether atropine (ATR) or/and pralidoxime (2-PAM), antidotes frequently used in OP poisoning, are capable of restoring cardiorespiratory function, remains unknown. The study abides by the Brazilian National Council for Animal Experimentation; being approved (number 058/2014) by the University Committee for Animals Ethical Use. Rats (350-400g; n=65) were catheterized under anesthesia (tribromoethanol, 250 mg/kg, i.p.) and, on the next day, injected with CPF (30 mg/Kg, i.p.) or saline (SAL, 0.9%, i.p.). One hour later, animals were split in groups receiving either ATR (10 mg/Kg, i.p.) or 2-PAM (40 mg/Kg, i.p.) separately and in combination. Mean arterial pressure, systolic arterial pressure (SAP), pulse interval (PI) and heart rate (HR) were derived from pulsatile arterial pressure while respiratory parameters were obtained through plethysmography. Recordings were performed in awake rats 24 hours after injections. Time-domain [variance, standard deviation (SD), root mean square of successive inter-beat intervals (RMSSD)] and frequency-domain parameters [normalized low (LF) and high frequency (HF) bands of spectral analysis] were used to estimate heart rate variability derived from undisturbed recordings (software CardioSeries; version 1.2). Spontaneous baroreflex gain (sBRG) was estimated for upward and downward ramps from regular beat-to-beat arterial pulses. Chemoreflex was activated by potassium cyanide injections (KCN; 10, 20, 40, 80 µg/rat, i.v.). Data were analyzed by Generalized mixed model and Generalized Estimating Equations. CPF exposure increased SD (P=0.035) and variance (P=0.028) of SAP compared with SAL animals. 2-PAM treatment restored these parameters (P= 0.023). CPF decreased RMSSD (P=0.051), which was corrected by ATR (P=0.033). CPF exposure decreased HF band (P= 0.042) compared with SAL animals, which was not restored by either ATR or 2-PAM treatment. CPF animals exhibited a marginally reduced up-ramps sBRG (P=0.061) when compared with SAL, which was restored by ATR (P=0.014). Reduced hypertensive (P=0.015) and bradycardic (P=0.001) chemoreflex responses were also observed in CPF group. While 2-PAM restored the hypertensive response (P=0.005), ATR treatment recovered the bradycardic responses (P=0.038). CPF impaired the chemoreflex tachypneic response (P=0.02), being restored by ATR (P=0.023). ATR and 2-PAM given together resulted in a summation of the responses when drugs were administered individually. These data indicate that ATR and 2-PAM treatment separately or in combination, are capable of restoring CPF induced cardiorespiratory neuroreflex impairment, having important implications for poisoned patients treatment.

Cunha AF, Felipe ISA, Ferreira-Junior NC, et al (2018) Neuroreflex control of cardiovascular function is impaired after acute poisoning with chlorpyrifos, an organophosphorus insecticide: Possible short and long-term clinical implications. *Toxicology* 398–399:13–22. doi: 10.1016/j.tox.2018.02.005

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA011

### INFLUENCE OF CHANGING GRAVITY AND EXERTED EXHALATION - LINKAGE BETWEEN OXYGEN UPTAKE AND CARDIAC OUTPUT

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**Purpose:** The study was designed to show that gravity changes comparing human centrifuge (HCF) runs to parabolic flight (PFC) maneuvers induce similar changes in pulmonary oxygen uptake in the resting and exercise condition, without metabolic processes.

**Methods:** 12 subjects having flight medical certificate and signed written informed consent (age:  $30 \pm 4$  y, weight:  $79 \pm 8$  kg, height:  $179 \pm 8$  cm, BMI:  $24 \pm 2$  kg/m<sup>2</sup>) were tested on a long-arm human centrifuge applying a posGz-protocol. The cycle of a real parabola was transcribed as follows: 1.7 g for baseline (equiv. 0 g), 25 s with 2.1 g (hyper-g, pull up), 22 s of 1.2 g (transition,  $\mu$ g), and 25 s of 2.1 g (hyper-g, pull down); this sequence was repeated 16 times. The 1.2g intervals of the protocol were performed in relaxed rest or with exhalation on exertion (forced pressure ventilation, lower extremity muscle contraction, and a combination). Cardiac output ( $\dot{Q}$ ) was measured beat to beat, and oxygen uptake ( $\dot{V}O_{2pulm}$ ) as well as carbon dioxide output ( $\dot{V}CO_{2pulm}$ ) were analyzed breath by breath. The same setting was flown in two parabolic flight campaigns.

**Results:** At rest, after gravity change '2.1 g – 1.2 g' an increase in  $\dot{Q}$  from 7.2 L/min ( $\pm 1.8$  L/min) up to 9.1 ( $\pm 2.3$  L/min) with transients for  $\dot{V}O_{2pulm}$  from 0.50 L/min ( $\pm 0.15$  L/min) to 0.72 L/min to ( $\pm 0.15$  L/min) were found and for  $\dot{V}CO_{2pulm}$  from 0.42 L/min to ( $\pm 0.33$  L/min) to 0.60 L/min to ( $\pm 0.28$  L/min), respectively. Six seconds after the onset of 1.2 g both,  $\dot{V}O_{2pulm}$  and  $\dot{V}CO_{2pulm}$  were decreased until the change '1.2 g – 2.1 g'. In contrast, with exhalation on exertion only a slight, unsystematic  $\dot{Q}$  increase was observed.  $\dot{V}O_{2pulm}$  and  $\dot{V}CO_{2pulm}$  were not increased above resting baseline. After exhalation on exertion  $\dot{V}O_{2pulm}$  and  $\dot{V}CO_{2pulm}$  showed a low significant ( $P < .05$ ) increase of approximately 0.4 L/min. The characteristic of the curve progression comparing HCF and PFC are comparable.

**Conclusion:** The results demonstrate a dependency between  $\dot{Q}$  and pulmonary  $\dot{V}O_2$  as well as  $\dot{V}CO_2$ . Exhaling on exertion seem to empty the lung tissue and leads to a significant increase in gas exchange which only partially is accompanied by  $\dot{Q}$  changes in recovery. These data allow further refining of modeling of cardiovascular influences on pulmonary  $\dot{V}O_2$  to estimate muscular  $\dot{V}O_2$  kinetics. We assume based on these findings that it could be possible to have a model of

cardiovascular and pulmonary research using a long-arm human centrifuge.

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## PCA012

### Acute cardiovascular responses to device-guided slow breathing in healthy human beings

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**Background:** Despite some studies reporting impressive improvement in blood pressure following the daily practice of so-called 'device guided breathing' (DGB) (1), the effectiveness of the Resperate® device remains controversial (2). Scepticism is exacerbated by a lack of understanding of the mechanisms underpinning claimed improvements in blood pressure. This study characterised for the first time the acute cardiorespiratory responses to DGB using the Resperate®, and explored the potential mechanisms by which DGB might elicit long term improvements in blood pressure. **Methods:** Ten healthy, normotensive participants (six male) undertook a 10-minute slow breathing intervention (Resperate®), whilst arterial blood pressure, heart rate, respiratory airflow and end-tidal carbon dioxide concentration were monitored continuously and non-invasively. Data are mean $\pm$ SD. **Results:** There was no significant changes in mean arterial blood pressure (MABP) across the intervention ( $93.70 \pm 9.52$  vs.  $90.39 \pm 9.91$ ). However, the intra-breath difference (inhalation vs. exhalation) in a number of haemodynamic variables increased significantly during the final five minutes of the intervention, compared to baseline, such that the amplitude of intra-breath perturbations of stroke volume (SV), systolic blood pressure (SBP) and heart rate maximised at a breathing frequency of  $\sim 0.1$  Hz. At this frequency, SBP variability doubled to 7.4% of SBP (from  $4.20 \pm 4.21$  to  $8.84 \pm 5.53$  mmHg;  $P = 0.067$ ), being underpinned by a tripling of SV variability to 14% of SV (from  $4.05 \pm 6.85$  to  $12.0 \pm 6.05$  ml;

$P=0.027$ ), and the two variables were highly correlated ( $R^2=0.90$ ;  $P=0.0001$ ). In an unexpected finding, participants with the greatest capacity to increase heart rate variability during DGB had the lowest baseline MABP ( $R^2=0.62$ ;  $P=0.0067$ ). **Conclusions:** Intra-breath haemodynamic perturbations during DGB were sufficient to generate error signal(s) for chronic regulation of blood pressure, providing a potential mechanism of action. Further research is required to understand the 'downstream' regulatory responses to this perturbation.

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### PCA013

#### High-fat diet causes arrhythmogenic remodelling of the atria and sinus node

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Obesity is a significant global health concern. It increases the risk of developing a variety of cardiovascular diseases, particularly atrial fibrillation (AF). Population studies have shown that increases in body mass index are associated with a parallel increase in AF risk (1). However, the mechanisms which link obesity to arrhythmias are poorly defined.

Male Sprague-Dawley rats were fed either a control diet of 10% fat ( $n=24$ ) or a high fat diet (HFD) of 45% fat ( $n=24$ ) for 24 weeks. Animals fed a HFD weighed 24% more than control animals at the end of the study period ( $631 \pm 11$  vs  $780 \pm 16$  g;  $n=24$ ,  $P<0.0001$ , ANOVA). Langendorff-perfused isolated heart experiments showed that effective refractory periods in the atrioventricular node ( $107.4 \pm 8$  ms,  $n=8$  vs.  $60.7 \pm 11$  ms,  $n=9$ ;  $P<0.01$ , Student's t-test) and ventricle ( $52.3 \pm 4.5$  ms,  $n=6$  vs.  $37.3 \pm 2$ ,  $n=6$ ;  $P<0.05$ , Student's t-test) were shorter in HFD fed animals. HFD fed animals had a slower intrinsic heart rate ( $209 \pm 8$  beats/min,  $n=10$  vs.  $177 \pm 8$  beats/min,  $n=10$ ;  $P<0.05$ , Student's t-test), which was significantly correlated to body weight ( $P=0.002$ ;  $R^2=0.4$ , Pearson's correlation coefficient). Most importantly, animals fed a HFD were more susceptible to atrial S1/S2 pacing-induced arrhythmias (3/8 control animals vs. 8/9 HFD animals;  $P<0.05$ , Chi-squared test) and showed a similar trend towards more ventricular S1/S2 pacing-induced arrhythmias (0/8 control animals vs. 3/8 HFD animals;  $P=0.06$ , Chi-squared-test). qPCR using Taqman microfluidic cards showed that of 44 genes investigated (including ion channel,  $Ca^{2+}$ -handling and connexin genes), there were significant alterations of 41%, 46% and 32% in the left and right atria and sinus node, respectively, in HFD animals. Data were analysed using RealTime Statminer software; statistical significance was tested using a non-parametric limma test and the Benjamini-Hochberg FDR with FDR-corrected  $P<0.2$ .

Pro-arrhythmic changes included significant downregulation of; calsequestrin and phospholamban in the right atrium ( $3.7 \pm 0.25$ ;  $n=9$  vs.  $1.1 \pm 0.05$ ;  $n=8$  and  $4 \pm 0.29$ ;  $n=9$  vs.  $3 \pm 0.13$ ;  $n=8$ ), connexin 40 in the left and right atria ( $0.009 \pm 0.002$ ;  $n=7$  vs.  $0.003 \pm 0.0005$ ;  $n=6$  and  $0.0038 \pm 0.0004$ ;  $n=9$  vs.  $0.0027 \pm 0.0003$ ;  $n=8$ ), and  $K_{v4.2}$

( $0.036 \pm 0.005$ ;  $n=7$  vs.  $0.024 \pm 0.002$ ,  $n=6$ ) in the left atrium. Further, there was upregulation of  $K_{vLQT1}$  ( $0.057 \pm 0.0027$ ;  $n=11$  vs.  $0.067 \pm 0.0025$ ;  $n=12$ ), TASK1 ( $0.17 \pm 0.014$ ;  $n=11$  vs.  $0.23 \pm 0.019$ ;  $n=12$ ) and  $K_{ir2.1}$  ( $0.011 \pm 0.00058$ ;  $n=11$  vs.  $0.014 \pm 0.00056$ ;  $n=12$ ) in the sinus node which may contribute to the intrinsic bradycardia observed in HFD animals.

These data show that a HFD causes a variety of electrophysiological changes, including significant remodelling of key ion channel, connexin and  $Ca^{2+}$ -handling genes, which may underlie the increased susceptibility to atrial arrhythmias.

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### PCA014

#### Discrepant findings to angiotensin II-induced hypertension in Rag-1 knockout mice cannot be explained by dose effects and salt intake alone

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**Motivation:** Several laboratories have found a resistance of Rag-1 knockout mice, lacking functional B- and T-cells, to angiotensin II mediated hypertension. Recently, however, Ji et al. have reported that their Rag-1 knockout mice strain, purchased in 2015/2016, lost the pressure resistant phenotype. Interestingly, we never observed any angiotensin II resistance of Rag-1 knockout mice, purchased in 2009. Here, we investigated whether dose effects or differences in salt intake might be responsible for these discrepant findings.

**Methods:** Rag-1 knockout mice, directly imported from the Jackson Laboratory, and corresponding C57Bl/6J wildtype mice were analysed for blood pressure via radiotelemetry under different doses of angiotensin II ( $170 \text{ ng} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ,  $490 \text{ ng} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  or  $1500 \text{ ng} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ) and under conditions of low- and high salt intake (modified special diet containing either  $0.2 \text{ g Na}^+/\text{kg}$  or  $30 \text{ g Na}^+/\text{kg}$ ). Flow cytometry measurements of lymphocytes were performed in renal and aortic tissue.

**Results:** We did not observe any difference in blood pressure response between Rag-1 knockout mice and C57Bl/6J wildtype mice to angiotensin II, neither under different doses of angiotensin II nor under different levels of salt intake (2-way ANOVA, all n.s.). Blood pressure increases in both mouse strains are very similar to those described for C57Bl/6J wildtype mice by other groups.

As expected for Rag-1 knockout mice, no T-cells were detectable in aortic or renal tissue. In C57Bl/6J wildtype mice, levels of CD3-positive-, CD4-positive- or  $\gamma\delta$ -T-cells measured as percentage of infiltrating CD45<sup>+</sup> cells, were not affected by angiotensin II treatment.

**Conclusions:** Our findings indicate that Rag-1 knockout mice are not protected from angiotensin II-mediated hypertension by lack of functional T-cells alone, since Rag-1 knockout mice and C57Bl/6J wildtype mice showed identical blood pressure increases in response to angiotensin II treatment,



independent of dose and sodium intake. Full development of the blood pressure resistant phenotype depends on the action of a yet unidentified modifier, which might be found in housing conditions or microbiome. The lack of a resistance of our Rag-1 knockout mice to angiotensin II induced hypertension comes along with an absence of an increased T cell infiltration in typical hypertensive end organs. Accordingly, the role of the adaptive immune system examined in Rag-1 knockout/C57Bl/6J mice has to be reviewed critical for investigation of angiotensin II-mediated immunological effects.

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PCA015

### Loss of cross-talk between atrial A<sub>1</sub> and A<sub>2A</sub> Adenosine receptors upon continuous atrial pacing and autonomic stimulation

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Adenosine is a natural occurring nucleoside throughout most of the body systems, resulting from the biological process of consuming ATP (energy). In the cardiovascular system, more specifically in the myocardium, adenosine exerts a well established protective action during myocardial ischemia and reperfusion injury, as well as, in several other conditions of stress, such as the ageing process and infection. These protective actions are mediated via activation of specific receptor subtypes (adenosine receptors A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub> and A<sub>3</sub>). In addition, there is also increasing evidence that optimal adenosine cardioprotection requires the activation of more than one receptor subtype. Hence, the purpose of the current study was to determine whether the cross-talk between adenosine A<sub>2A</sub> and A<sub>1</sub> receptors modulate receptor-mediated protection in conditions of cardiac rhythm changes, induced by strong autonomic activation. We have employed real-time PCR to rapidly and sensitively determine specific adenosine receptor transcript levels, resulting from continuous atrial high frequency pacing and autonomic activation (Sympathetic and Vagal stimulation isolated or combined), in both atria and ventricles from a total of 20 Wistar rats (11 to 14 wks). Briefly, the animals were anaesthetized with sodium pentobarbitone (60 mg/Kg; ip), and supplemented with 20% solution (iv), followed by neuromuscular blockade (Norcuron, 0.05 mg/kg/h, iv). Blood pressure, ECG, and body temperature were continuously monitored. Animals were randomized in five experimental groups. Control: no stimulation; Group 2: 2h high frequency atrial pacing at the right side, with a concentric bipolar microelectrode; Group 3: 2h continuous right cervical vagus nerve stimulation; Group 4: 2h continuous thoracic sympathetic trunk stimulation; Group 5: 2h Vagal and sympathetic stimulation.

Relative quantification using the housekeeping gene 18S rRNA as internal control, revealed no significant changes in the levels of A<sub>1</sub> mRNA and a very significant increase of A<sub>2A</sub> expression levels for all types of stimulation, in both atria and ventricles (p≤0.0065 vagal and sympathetic stimulation; p<0.0001 for dual stimulation and atrial pacing). As expected, the present findings emphasize the already important adenosinergic cardioprotective action of the adenosine receptors studied herein, suggesting a marked activation of the adenosine

receptor A<sub>2A</sub> as a primary response against both physical and physiological strain and sinus rhythm modulations due to ANS stimulation, which may contribute to the recovery of the myocardium cells, preventing further inflammation downstream the remodeling mechanisms.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA016

### Potassium intake reduces the elevated blood pressure response to oral salt loading as well as to voluntary hand grip in normotensive adult Nigerians.

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Oral salt loading has been shown to result in elevated blood pressure especially in salt sensitive normotensive subjects (Elias et al, 2011), while voluntary hand grip contraction increases blood pressure via activation of sympathetic nervous system (Fadel et al, 2003). However dietary potassium supplement can reduce arterial blood pressure (Gijssbers et al, 2015). The present study was designed to determine if potassium intake can ameliorate the pressor response to high salt ingestion as well as to hand grip contraction. Experiments were carried out on 30 normotensive (NT) students of Babcock University, aged 25.3±0.92 yrs (Mean±SEM), that were divided into two groups of 15 subjects each : a) salt loaded with 200 mmol sodium chloride solution for 5 days (Elias et al, 2011) and b) salt loaded for 5 days with added intake of 600 mg of potassium chloride tablets (Slow K®, Novartis). Further tests were also carried out in both groups, before and during Hand Grip (HG) at 30% of Maximum Voluntary Contraction (MVC) for one minute with the use of a Dynamometer (Camry, Model EH101). Ethical Approval was obtained from Babcock University Research and Ethics committee and the subjects gave written consent. Blood pressure (BP, mmHg) was measured with an Accoson sphygmomanometer and also serum Na<sup>+</sup> and K<sup>+</sup> concentrations (mmol/l) before and after administration of salt or salt + potassium (salt+K). The effects of 30% MVC hand grip were also determined in the two groups of subjects. Data are Mean±SEM and statistical analysis was by Students paired t test. The results show that salt intake resulted in significant elevation of Systolic Blood Pressure (SBP), from baseline of 118.3±1.8 to 123.5±1.9 (P<0.05) but not of diastolic Blood Pressure (DBP)- 65.3±1.3 vs 67.2±1.4 (P=NS). However, after salt+K, the BP change was reversed and SBP reduced significantly from 122.6±1.5 to 117.2±1.2 (P<0.05) and DBP from 70.0±1.9 to 62.1±1.5 (P<0.05). Salt intake increased serum Na<sup>+</sup> concentration significantly from 133.9±0.7 to 138.6±0.9 (P<0.05) but Salt+K however reduced it from 139.1±0.5 to 136.6±0.3 (P<0.05). However serum K<sup>+</sup> was not significantly altered either by salt or salt+K. HG significantly increased (P<0.05) SBP from 127.6±2.9 to 157.2±2.6 in controls, less so from 126.9±1.7 to 149.9±3.8 in salt loaded subjects but had no significant effect in subjects on salt+K ie 124.0±3.0 vs 127.9±2.1. During HG, DBP increased in controls from 76.9±2.5 to 95.6±2.9 (P<0.05) and after salt loading,

from  $75.4 \pm 2.8$  to  $84.7 \pm 2.2$  ( $P < 0.05$ ) but in salt+K, there was no significant change –  $82.8 \pm 2.0$  vs  $79.5 \pm 1.7$ . The results indicate that potassium intake blunts the BP elevation following salt loading or during voluntary HG. Potassium intake may therefore be effective in reducing induced changes in blood pressure.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA017

### Gender differences in the systolic blood pressure in young healthy adults

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Gender and aging influence systolic blood pressure, component of arterial hemodynamic function. Hypertension is more prevalent in male than in female. Systolic blood pressure (SBP) is an indicator of cardiovascular risk. High SBP indicates risk of heart disease and stroke. This study was designed to determine the gender differences in SBP in young healthy adults. 120 subjects (male - 60 and female- 60) within the age group of 19 -21 years were included in the study. Subjects with a habit of smoking, history of diabetes, hypertension and who were under medication were excluded from the study. The study was performed with the written informed consent of participants. Anthropometric parameters like Height (cm), weight (kg), Body Mass Index (BMI  $\text{kg/m}^2$ ) were calculated. The blood pressure was recorded in supine position using standardized mercury sphygmomanometer in the left arm by auscultatory method. Two blood pressure readings were taken at an interval of 10 minutes. The average of the two recorded systolic values and two diastolic values were taken as SBP and Diastolic blood pressure (DBP) respectively. Data was expressed as mean  $\pm$  SD, compared by Independent t test. BMI was not significantly different between males and females (male:  $22.59 \pm 2.94 \text{ kg/m}^2$  and female:  $21.74 \pm 3.66 \text{ kg/m}^2$  respectively). There was significant difference in SBP between males and females (male:  $119.95 \pm 11.46 \text{ mmHg}$ , female:  $112.62 \pm 8.84 \text{ mmHg}$ ,  $p < 0.01$ ). DBP was not significantly different between males and females (male:  $75.67 \pm 9.51 \text{ mmHg}$ , female:  $72.38 \pm 9.63 \text{ mmHg}$  respectively). SBP was significantly higher in males than in females. In conclusion, gender differences exist in SBP in young healthy adults.

Wiinberg N et al. (1995). *Am J Hypertens* 978P, 86P

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Joyner et al. (2016). *Exp Physiol* 349P, 55P

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We would like to express our sincere gratitude to the participants who volunteered in our study.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA018

### Arousal from hibernation: patterns of heart rate variability in golden hamsters.

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Hibernation-like strategies are considered promising for medical practice and space exploration. Heart rate (HR), an indicator of functional state of the body, greatly fluctuates with cardiac arrhythmias presence throughout hibernation bout. The significance of arrhythmias is still unknown and less studied especially upon arousal from hibernation, the very stage when the blood flow renormalizes in reperfusion-like manner. To clarify the peculiarities of HR dynamics upon arousal from hibernation, male golden hamsters (85–95 g,  $n=10$ ) towards to the hibernation bout end (average bout duration was  $3 \pm 0.5$  days) were transferred into a warm illuminated room ( $21 \pm 2^\circ\text{C}$ ) and sterile needle electrodes for ECG recording (placed under the paws skin), piezoelectric sensors for breathing pattern evaluation (placed at the both side between the animal's body and nest walls) and a thermocouple (placed in the cheek pouch) for body temperature (Tb) measurement were attached. Heart and respiratory signals were recorded and preprocessed with computer-based electrocardiograph Poly-Spectrum (Neurosoft) and then RR intervals data were imported into Kubios 2.2 software (Kubios Oy) to calculate time, frequency and non-linear heart rate variability measures. The recording processes were terminated at  $Tb=23.5 \pm 5.1^\circ\text{C}$ . Data were means  $\pm$  SD, compared by ANOVA. Two different patterns of RR dynamics were found. The first one was characterized by smooth progressive shortenings in mean RR distance (increase in HR) ( $1785.48 \pm 794.85$ ,  $921.43 \pm 409.31$  and  $301.73 \pm 56.88 \text{ ms}$ ,  $n=11$ ) and low standard deviation of RR intervals (STD RR) ( $14.75 \pm 15.72$ ,  $8.46 \pm 6.04$ ,  $3.75 \pm 1.42 \text{ ms}$ ,  $n=11$ ) at the start, middle and end stages of arousal from hibernation respectively. The second one was characterized by period ( $1364.3 \pm 655.6 \text{ s}$ ,  $n=17$ ) of regular alternating "long" and "short" RR intervals (bigeminy) appearance and, as the result, by increased STD RR ( $165.58 \pm 63.33$ , mean  $RR=852.15 \pm 130.28 \text{ ms}$ ,  $n=17$ ). The presence of two separate peaks in RR distribution was confirmed by time-domain and non-linear (Poincare plot) analysis. No breathing arrhythmia was revealed at simultaneous ECG and breathing recordings at this stage. Noteworthy, a period characterized by switching HR back to lower level along with total or partial ceasing of arrhythmia wedged into the arrhythmia period. All animals but one kept to a certain type of RR distribution pattern. That one alternated between both patterns in the course of 9 successive bouts. Parasympathetic activation re/appearance was revealed by spectral analysis of heart rate variability only after abrupt arrhythmia termination. Arrhythmia-free and arrhythmia dynamics are possible upon hamsters' arousal from hibernation. The same animal may "produce" both patterns. Arrhythmia episode and switching back into lower HR during that episode are probably to slowing HR and/or metabolic rate increased upon arousal.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA019

**The *in vitro* effect of polyvinylpyrrolidone and citrate coated silver nanoparticles on erythrocytic oxidative damage and eryptosis**Z. Ferdous<sup>1</sup>, S. Beegam<sup>1</sup>, S. Tariq<sup>2</sup>, B.H. Ali<sup>3</sup> and A. Nemmar<sup>1</sup><sup>1</sup>Physiology, United Arab Emirates University, College of Medicine and Health Sciences, Al-Ain, United Arab Emirates, <sup>2</sup>Anatomy, United Arab Emirates University, College of Medicine and Health Sciences, Al-Ain, United Arab Emirates and <sup>3</sup>Pharmacology and Clinical Pharmacy, Sultan Qaboos University, College of Medicine and Health Sciences, Al-Khod, UK

Silver nanoparticles (AgNPs) are increasingly used as antimicrobial agents and drug carriers in various biomedical fields. AgNPs can encounter erythrocytes either directly following intravenous injection, or indirectly via translocation from the site of administration. However, information regarding the pathophysiological effects and possible mechanism of action of AgNPs on the erythrocytes are still inadequately studied. Thus, the aim of our study was to investigate the mechanism underlying the effect of coating and concentration of AgNPs on mouse erythrocytes *in vitro*. We studied the interaction of polyvinylpyrrolidone (PVP) and citrate (CT) coated AgNPs (10 nm) at various concentrations (2.5, 10, 40 µg/ml) with mouse erythrocytes *in vitro*. Following incubation of the erythrocytes with AgNPs, both PVP- and CT- AgNPs induced significant and dose- dependent increase in hemolysis. Transmission electron microscopy revealed that both PVP- and CT- AgNPs were taken up by erythrocytes. The erythrocyte susceptibility to lipid peroxidation measured by malondialdehyde was significantly increased in both PVP- and CT- AgNPs. The concentration of reduced glutathione and catalase activity were significantly decreased by both types of AgNPs. Additionally, PVP- and CT- AgNPs significantly increased intracellular calcium in a dose -dependent manner. Likewise, the concentration of the cellular protein annexin V was significantly and dose - dependently enhanced by both types of AgNPs. Furthermore, PVP- and CT- AgNPs induced significant increase in calpain activity in incubated erythrocytes. We conclude that both PVP- and CT- AgNPs causes hemolysis, and are taken up by erythrocytes. Moreover, we demonstrated that AgNPs induces oxidative stress and eryptosis. These findings provide evidence for the potential pathophysiological effect of PVP- and CT- AgNPs on erythrocyte physiology.

This work was supported by funds of the College of Medicine and Health Sciences, United Arab Emirates University (UAEU) grant and UAEU-SQU grant.

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We investigated ATP release in cardiomyocytes isolated by collagenase digestion from adult SD rat hearts. Cells were treated with lactic acid (LA, 10mM) which mimics increased work; ATP released into the bathing medium was measured with the luciferase assay and expressed as mean±SEM % of release from control cells. All changes were  $P<0.05$  in ANOVA. LA treatment increased extracellular ATP to  $215\pm19\%$  ( $n=79$ ), which was blocked by inhibiting LA entry to cells with  $\alpha$ -cyano hydroxycinnamic acid. Total internal reflection microscopy (TIRF;  $n=4$ ) showed that LA addition increased Ca in the zone  $<150\text{nm}$  below the cell membrane; the intracellular Ca chelator, BAPTA, or the Na/Ca exchanger inhibitor, SN6, blocked both Ca elevation and ATP release ( $47\pm3\%$  with BAPTA or  $12\pm2\%$  with SN6 ( $n=16$ )), suggesting that Ca signaling in the near-membrane area is involved in ATP release.

LA increased intracellular cAMP from  $0.40\pm0.03$  to  $0.58\pm0.03\text{nM}$  ( $n=14$ ); the LA-induced increase in ATP release was blocked by inhibition of CFTR with CFTR<sub>inh</sub>172 ( $75\pm3\%$  ( $n=24$ ) without or  $70\pm11\%$  ( $n=16$ ) with LA) or by CFTR siRNA ( $57\pm7\%$  without or  $61\pm3\%$  with LA ( $n=12$ )), suggesting that CFTR activation is a necessary step in the signaling pathway. In  $\text{HCO}_3^-$ -free medium, extracellular ATP decreased to  $39\pm4\%$  ( $n=10$ ) in the presence of LA, suggesting that CFTR-mediated or CFTR-regulated  $\text{HCO}_3^-$  entry to the cell is required for ATP release.

Cardiomyocytes were homogenised and mitochondria isolated by differential centrifugation. Increasing  $\text{HCO}_3^-$  in the mitochondrial incubation medium from 0 to 40 mM doubled cytochrome C expression ( $n=6$ ) and increased mitochondrial ATP release from  $32\pm8$  to  $237\pm59\%$  ( $n=16$ ), which was reduced to  $0.5\pm0.04\%$  ( $n=8$ ) by DIDS, suggesting that increased ATP release from mitochondria in the near-membrane area may drive the increase in cellular ATP release.

The LA-induced increase in ATP release from cardiomyocytes was blocked by inhibition of pannexins with Fast Green-FCF ( $64\pm4\%$  ( $n=19$ ) without or  $56\pm7\%$  ( $n=16$ ) with LA) or by Panx1 siRNA ( $54\pm3\%$  without or  $51\pm4\%$  with LA ( $n=12$ )) suggesting that Panx1 may be the ATP release channel. Immunofluorescent staining ( $n=25$ ) showed that CFTR and Panx1 did not colocalise in cardiomyocytes, which rules out direct interaction between them. In a live-cell caspase assay, intracellular caspase activity increased within 10s of adding LA to the medium, and medium ATP in the presence of LA was reduced from  $174\pm6\%$  ( $n=56$ ) to  $94\pm6\%$  ( $n=32$ ) by caspase 3 inhibitor, suggesting that Panx1 may be activated by caspase cleavage during LA treatment.

These data suggest that LA stimulates cardiomyocytes to release ATP via Panx1 through a pathway that involves entry of Ca and  $\text{HCO}_3^-$ , CFTR activation and mitochondrial signaling.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA021

**HIF2a mediates hypoxia-induced expression of the Wilms tumor protein WT1 in Kelly neuroblastoma cells**L. Catanese<sup>1</sup>, L.K. Sciesielski<sup>2</sup>, K.M. Kirschner<sup>1</sup>, H. Scholz<sup>1</sup> and K. Krueger<sup>1</sup><sup>1</sup>Inst. of Vegetative Physiology, Charité - Universitätsmedizin Berlin, Berlin, Germany and <sup>2</sup>Department of Neonatology, Charité - Universitätsmedizin Berlin, Berlin, Germany

Neuroblastoma is a malignant childhood tumor arising from the ganglia of the peripheral sympathetic nervous system.

## PCA020

**ATP release from cardiomyocytes involves cystic fibrosis transmembrane conductance regulator (CFTR), pannexin1 (Panx1), Ca signaling and mitochondria**

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Cardiac interstitial ATP increases with work, and ATP breakdown products contribute to blood flow regulation.



A striking feature of this cancer is its biological and clinical heterogeneity. Tissue hypoxia favors an undifferentiated tumor phenotype with poor prognosis. Recent findings indicate that the clinical outcome of a subgroup of neuroblastomas, which lack amplification of the *MYCN* oncogene negatively correlates with the expression level of the Wilms tumor gene *WT1*. To obtain novel insights into the molecular mechanisms underlying neuroblastoma growth, we analyzed the expression of *WT1* in human neuroblastoma cell lines with the *MYCN* amplification (IMR-32, SK-N-BE(2), Kelly) and in non-*MYCN* amplified (SK-N-AS, SH-EP1, SH-SY-5Y) neuroblastoma lines. In addition to normoxic conditions (21% O<sub>2</sub>), experiments were also performed at 1% O<sub>2</sub> to mimic tumor hypoxia.

In normoxia (21% O<sub>2</sub>), robust *WT1* protein levels were detected in 4 out of the 6 investigated neuroblastoma cell lines. *WT1* expression did not correlate with the status of *MYCN*-amplification. Exposure to 1% O<sub>2</sub> (24 h) increased *WT1* mRNA and protein more than 10-fold in Kelly cells ( $p < 0.01$ ,  $n = 6$ ). Hypoxia stimulated *WT1* expression also in SK-N-BE(2) cells but not in the other cell lines tested. *WT1* mRNA and protein levels in Kelly cells at 21% O<sub>2</sub> were enhanced approximately 9-fold by stabilization of hypoxia-inducible factors (HIFs) with dimethyloxallylglycine (DMOG) and 2,2'-Dipyridyl (2,2'-DP, 100  $\mu$ M each). Silencing of HIF2a, but not HIF1a, by RNA interference significantly reduced *WT1* protein and mRNA levels at 1% O<sub>2</sub>. Since transfection with siRNAs did not completely abolish HIF expression in hypoxia, we generated Kelly cell lines with inactivation of either HIF1a or HIF2a using CRISPR/Cas9. Mutations in the coding regions of both genes were identified by sequencing of PCR-amplified genomic DNA, and gene inactivation was confirmed by the lack of HIF1a and HIF2a proteins in the hypoxic knockout cell lines. HIF2a deficiency caused a loss of *WT1* expression in Kelly cells exposed to either 1% O<sub>2</sub> or 2,2'-DP (21% O<sub>2</sub>). By contrast, hypoxia-induced expression of *WT1* was preserved in Kelly cells with HIF1a knockout. Finally, silencing of *WT1* by RNA interference significantly reduced the migration of SKNAS neuroblastoma cells in transwell filter assays.

These findings demonstrate that hypoxia stimulates *WT1* expression in Kelly cells via HIF2a. In conclusion, up-regulation of *WT1* expression in hypoxia may contribute to the malignant growth phenotype by stimulating tumor cell migration in a subgroup of neuroblastomas.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA022

### The evolution and function of atrial smooth muscle in turtles

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By the end of the 19<sup>th</sup> century, unusual undulations in resting tension ('tonus waves') had been repeatedly reported in atrial preparations from freshwater turtles (Emyidae). By the 1920s, these waves were married with the histological demonstration that, unlike in other animals, smooth muscle coats the luminal side of turtle atria. Since then, precious little attention has been placed on this peculiar smooth

muscle, and its evolution and physiological function remain elusive. We used fluorescent immunohistochemistry to identify smooth muscle (smooth muscle actin) and cardiac muscle (cardiac troponin I) in fixed hearts from 8 distantly related species of turtles (*Chelodina mccordi* (N=3), *Pelodiscus sinensis* (N=2), *Chelydra serpentina* (N=3), *Pelomedusa subrufa* (N=3), *Cyclanorbis senegalensis* (N=2), *Chelonoidis carbonaria* (N=3), *Testudo hermanni* (N=3) and *Trachemys scripta* (N=8)) along with reptile outgroups (two species of crocodilian (*Caiman crocodilus* (N=1) and *Alligator mississippiensis* (N=2)) and one lizard (*Hemisphaeriodon gerrardii* (N=1))). All animals were euthanised with an overdose of pentobarbital (200 mg kg<sup>-1</sup>) and all procedures were completed in accordance with Danish animal care legislation. Atrial smooth muscle was not detected in reptiles other than turtles (although smooth muscle was readily detected in outflow tract vasculature). Sparse atrial smooth muscle was present in atria from turtles across the phylogeny, but extensive coverage (i.e. >10% of the total tissue area on sections) was only identified in the emydid species (*T. scripta*). The closely related land tortoises (*T. hermanni* and *C. carbonaria*) had little atrial smooth muscle. In *in situ* perfused hearts (e.g. Joyce et al., 2016) of the emydid red-eared slider (*T. scripta*; N=6), the addition of wortmannin, which inhibits smooth muscle contraction, increased cardiac output from  $24.3 \pm 7.4$  (mean  $\pm$  S.E.M.) to  $42.1 \pm 5.3$  ml min kg<sup>-1</sup> ( $P < 0.01$ ; paired t-test). Histamine, which elicits smooth muscle contraction, stimulated a prolonged diastolic contracted state of the atria during which cardiac output was reduced from  $32.6 \pm 7.8$  to  $13.36 \pm 5.8$  ml min kg<sup>-1</sup> ( $P < 0.01$ ; paired t-test). Extensive smooth muscle, we conclude, has evolved within turtles, where it provides a novel mechanism for the control of cardiac output. This mechanism may contribute to the tremendous diving capacity of emydid turtles.

Joyce W et al. (2016). *J Exp Biol* 219, 2220–2227.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA023

### Autonomic regulation of the pulmonary vasculature in freshwater turtle

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In reptiles, the vagal innervation of the pulmonary vasculature causes constriction by stimulation of muscarinic ACh receptors, whereas sympathetic stimulation appears to cause dilatation through  $\beta$ -adrenoceptors. The specific anatomical site(s) of constriction, however, remains debated and may differ among species. Thus, some studies point to the extrinsic pulmonary arteries as the main site of vasoconstriction, while more indirect measures point to a weak vasoconstriction of the intrapulmonary arteries. Using myography, we demonstrate that the intrapulmonary arteries (100-900  $\mu$ m) constricts as strongly as the truncus as well as the proximal and distal segments of the extrapulmonary artery in the freshwater turtle (*Trachemys scripta*). Thus, the effects of ACh appear similar throughout the pulmonary vasculature and given their small diameter, the intrinsic vessels probably represent the major site to alter pulmonary vascular resistance during vagal

stimulation. We also show widespread relaxation in response to  $\beta$ -adrenoceptor stimulation, but not to  $\alpha$ -adrenoceptor stimulation. The nitric oxide (NO) donor, sodium nitroprusside and adenosine only induced small vasodilatory responses. ACh and  $\beta$ -adrenoceptor agonists also exerted pronounced effects on the pulmonary veins. In conclusion, our study shows that the intrapulmonary arteries appear to be under autonomic regulation, which could implicate that the intrinsic vasculature is involved in changes of pulmonary vascular resistance during parasympathetic stimulation during apnea.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

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PCA024

### The NADPH Oxidase Nox4 controls polarization of macrophages in a NFKB-dependent manner

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The family of NADPH oxidases represents a source of reactive oxygen species (ROS). Within this family, Nox4 is special, as it constitutively produces  $H_2O_2$ . The loss of Nox4 promotes inflammation. A major cellular component of inflammation is the macrophage population, which divides in several subpopulations with pro-inflammatory M1 and wound-healing M2 macrophages being extremes of this functional spectrum of cells. As controversial findings on Nox4 expression in macrophages exist, we hypothesize that Nox4 expression is only present in a subpopulation of macrophages, where it may determine the phenotype. In a murine inflammation-driven fibrosarcoma model Nox4-deficiency results an increase in the expression of pro-inflammatory genes and cytokines and higher amounts of pro-inflammatory Ly6C<sup>+</sup> macrophages in the tumors. Ex vivo, bone marrow-derived macrophages deficient in Nox4 displayed a reduced M2 polarization whereas M1 markers were elevated. ROS levels were augmented in Nox4<sup>-/-</sup> cells polarized to M1 accompanied by a higher expression of Nox2. Mechanistically, Nox4-deficiency reduces STAT6 activation and promotes NFKB activity, with the latter being responsible for the higher level of Nox2 in Nox4-deficient M1-polarized macrophages. Collectively the data obtained in this study propose an anti-inflammatory role of Nox4 in macrophages. This is achieved by an enhanced M2 polarization and a suppression of NFKB activity.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

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PCA025

### The effects of tumour necrosis factor-alpha and interleukin-1-beta on cardiac intracellular calcium handling

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Cytokines including tumour necrosis factor-alpha (TNF- $\alpha$ ) and interleukin-1 beta (IL-1 $\beta$ ) are known to mediate systolic and

diastolic myocardial dysfunction in inflammatory disease such as sepsis. In severe cases, this myocardial depression leads to death. To provide a cellular basis, we separately studied the effects of 50 ng/ml TNF- $\alpha$  and IL-1 $\beta$  on intracellular Ca handling and contractility in sheep ventricular myocytes.

All procedures used accord with the Animals (Scientific Procedures) Act, UK, 1986 and Directive 2010/63/EU of the European Parliament. Ventricular myocytes were isolated from young (aged 18 months) sheep, and loaded with the ratio-metric Ca indicator Fura-2. Cells were field stimulated at 0.5 Hz and intracellular Ca and contractility dynamics measured by epi-fluorescent photometry and video sarcomere detection respectively. Relative changes in sarcoplasmic reticulum (SR) Ca content were estimated from the amplitude of Ca transients evoked by application of 10 mM caffeine.

50 ng/ml TNF- $\alpha$  and IL-1 $\beta$  reduced SR Ca content by 27 % and 41 % respectively, accounting for a 17 % and 24 % reduction each in the amplitude of systolic Ca. With TNF- $\alpha$ , the reduction of systolic Ca was associated with a 20 % reduction in sarcomere shortening, however IL-1 $\beta$  increased sarcomere shortening. The rate constant of systolic Ca decay was unaffected by both TNF- $\alpha$  and IL-1 $\beta$ . In response to both TNF- $\alpha$  and IL-1 $\beta$  the onset of systolic Ca decrease was rapid (<10 s), however, in 71 % of TNF- $\alpha$  treated cells and 52 % IL-1 $\beta$  treated cells, this was preceded by an immediate increase (58 and 52 % respectively) in systolic Ca, lasting for only 1-3 beats. TNF- $\alpha$  decreased diastolic Ca by 4 %, which was unchanged by IL-1 $\beta$  whilst diastolic sarcomere length was decreased by both cytokines.

Both TNF- $\alpha$  and IL-1 $\beta$  reduced SR Ca content accounting for reduced systolic Ca. In the case of TNF- $\alpha$ , this was associated with reduced contractility whereas with IL-1 $\beta$ , paradoxically, contractility was increased. This and the fact that resting sarcomere length was decreased despite no increase in diastolic Ca suggest both cytokines may increase myofilament sensitivity. The mechanism by which SR Ca content is reduced remains unclear. Whilst SERCA impairment does not appear to play a role, the initial and short-lived increase of systolic Ca may suggest a role for ryanodine receptor potentiation; a phenomenon we are currently investigating. While the changes to cell function produced by both cytokines can account for certain aspects of myocardial depression in sepsis, their individual contribution to the clinical phenotype may be more complex than previously thought.

Thank you to Professor Andrew Trafford's research group for provision of myocytes.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

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PCA026

### Sevoflurane increases plasma levels and angiogenic properties of human endothelial progenitor cells

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Plasma levels and functional properties of endothelial progenitor cells (EPCs) correlate to the efficacy of vessel expansion and repair after ischemia, whereas a consistent body of basic

research suggest stimulating effects of anesthetic preconditioning (APC) on stem cells behavior. Therefore, we tested the ability of sevoflurane to increase the mobilization and to potentiate the angiogenic properties of these cells. EPCs levels were evaluated by flow-cytometry and *in vitro* culture assay before exposure to sevoflurane (baseline) and 24 hrs afterwards, in blood samples collected from CAD patients. Sevoflurane (1 MAC in 50% O<sub>2</sub>) was administered on a facial mask in a discontinuous manner (2 cycles of 15 min interspersed by 10 min washout; n = 16), while control subjects received only oxygen (n = 10). The levels of stromal derived factor 1 alpha (SDF1alpha) and vascular endothelial growth factor (VEGF), two chemokines involved in EPCs mobilization and homing, were assessed by ELISA in plasma samples at baseline, 4 and 24 hrs after sevoflurane administration. 7-days old cultured EPCs derived from human cord blood mononuclear cells were exposed *in vitro* to sevoflurane 2% or 4% in air/5% CO<sub>2</sub>, or only to air/5% CO<sub>2</sub> (control) for 2 hrs in a modular chamber. The incorporation of preconditioned and control DilAcLDL-EPCs into tube-like structures of human umbilical vein endothelial cells (HUVEC) grown on Matrigel was evaluated 24 hrs later. Values are expressed as means  $\pm$  SEM, compared by ANOVA. Plasma CD45dim/CD34+/KDR+, CD45dim/CD34+/KDR+/CD133+ and CD45dim/CD34+/CD309+/CXCR4+ mononuclear cell populations were increased in sevoflurane treated versus sham group at 24 hrs post-exposure (172.95  $\pm$  73.74 vs. 153.84  $\pm$  30.28, p = 0.006; 248.06  $\pm$  37.4 vs. 198.62  $\pm$  29.15, p < 0.001 and 153.87  $\pm$  49.31 vs. 134.66  $\pm$  37.95, p = 0.005 respectively, expressed as % at 24 hrs vs. baseline). Although not statistically significant, the number of adherent DilAcLDL+/FITC-UEAI+ cells in 7 days old EPCs cultures from preconditioned patients was slightly higher when compared to those of sham control subjects. SDF1alpha and VEGF levels were not significantly modified by sevoflurane. In the *in vitro* preconditioning setting, sevoflurane raised the number of incorporated DilAcLDL-EPCs into tube-like HUVEC structures (n=3, p<0.01 for 4% sevoflurane) when compared to control. Augmented EPCs mobilization and ability of integrating themselves into vascular structures are candidate mechanisms underlying the regenerative and anti-ischemic processes of APC with sevoflurane. These results indicate a promising potential of preconditioning protocols for improving the endothelial and tissue repair following percutaneous coronary intervention or cardiovascular and non-cardiovascular surgery.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA027

### Calcium signaling in epicardial and endocardial ventricular myocytes from streptozotocin - induced diabetic rat

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Diabetes mellitus (DM) is a serious global health problem and cardiovascular complications are the major cause of morbidity and mortality in diabetic patients. Electrical and mechanical

function is frequently compromised in the diabetic heart. Whilst much is known about the effects of DM on contraction and Ca<sup>2+</sup> signaling in myocytes from whole ventricle much less is known about the transmural effects of DM in the ventricle. The aim of this project was to investigate the regional effects of DM on Ca<sup>2+</sup> signaling in epicardial (EPI) and endocardial (ENDO) myocytes from the left ventricle of streptozotocin (STZ) - induced diabetic rat. Ethical approval was obtained from the UAE University Animal Research Ethics Committee. Diabetes was induced in adult male rats with a single intraperitoneal injection of STZ/citrate buffer (60 mg/kg body weight). Age-matched controls received citrate buffer alone. Experiments were performed after 5 months of treatment. EPI and ENDO myocytes were isolated by enzyme and mechanical dispersal techniques. L-type Ca<sup>2+</sup> current (I<sub>Ca,L</sub>) and intracellular Ca<sup>2+</sup> were simultaneously measured in fura-2 loaded myocytes by whole-cell patch clamp and fluorescence photometry. Na<sup>+</sup>/Ca<sup>2+</sup> (NCX) current was measured in separate experiments by whole-cell patch clamp. Experiments were performed at 35-36 °C. Data are means  $\pm$  S.E.M., compared by ANOVA followed by Bonferroni-corrected t tests for multiple comparisons. Diabetes was characterized by a 5-fold increase in blood glucose. Cell capacitance was significantly (p<0.05) lower in ENDO-STZ (159.4 $\pm$ 10.8 pF, n=17) compared to ENDO-CON (203.5 $\pm$ 12.9 pF, n=17) myocytes. Amplitude of I<sub>Ca,L</sub> was not significantly (p>0.05) altered in EPI-STZ compared to EPI-CON and in ENDO-STZ compared to ENDO-CON myocytes. However, the amplitude of the Ca<sup>2+</sup> transient was significantly increased in EPI-STZ (0.419 $\pm$ 0.013 RU, (fura-2 ratio units), n=13) compared to EPI-CON (0.383 $\pm$ 0.015 RU, n=16) and in ENDO-STZ (0.459 $\pm$ 0.014 RU, n=11) compared to ENDO-CON (0.384 $\pm$ 0.010 RU, n=16) myocytes. Currents mediated by NCX was significantly reduced at +60 mV in EPI-STZ (1.93 $\pm$ 0.27 pA/pF, n=17) compared to EPI-CON (4.36 $\pm$ 0.76 pA/pF, n=17) and in ENDO-STZ (1.61 $\pm$ 0.35 pA/pF, n=14) compared to ENDO-CON (2.74 $\pm$ 0.22 pA/pF, n=16) myocytes. NCX was also reduced at -100 mV in EPI-STZ (0.97 $\pm$ 0.32 pA/pF, n=14) compared to EPI-CON (3.57 $\pm$ 0.68 pA/pF, n=17) and in ENDO-STZ (0.97 $\pm$ 0.32 pA/pF, n=14) compared to ENDO-CON (2.40 $\pm$ 0.35 pA/pF, n=16) myocytes. The data suggest that increased amplitude of Ca<sup>2+</sup> transients may be associated with reduced NCX current in EPI and ENDO myocytes from STZ-induced diabetic rat.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA028

### Amphiphysin II (Bin 1) driven transverse tubule formation in cardiac muscle.

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Regular invaginations in the surface membrane of cardiac myocytes, known as transverse tubules (t-tubules), are vital for maintaining normal contractility of the heart through the regulation of excitation contraction coupling. In cardiac diseases, such as heart failure, t-tubule loss is associated with decreased synchrony of calcium release from the sarcoplasmic reticulum, resulting in impaired contractility. Thus,



determining the mechanisms that control t-tubule formation is essential for understanding this disease. Evidence suggests that the protein Amphiphysin II controls cardiac t-tubule formation and may play a vital role in calcium regulation (Lee *et al*, 2002; Caldwell *et al*, 2014). We therefore aimed to extend these observations and determine if Amphiphysin II is sufficient to drive t-tubule formation in the heart.

Neonatal rat ventricular myocytes (NRVMs) were isolated from 2 day old Wistar rats (n=14 litters) and maintained in culture. Vectors encoding isoforms 5, 8 and 9 of the Amphiphysin II gene (*Bin1*) with a C-terminal mKate2 fluorescent protein tag were transiently expressed in NRVMs using FuGENE 6 lipofection. A vector containing the mKate2 fluorescent tag only was used as control. After 48hrs, over-expression of *Bin1* was confirmed at the mRNA and protein level. Tubule formation was assessed using the membrane dye FM-464 and confocal microscopy. Of cells successfully transfected with *Bin1*, 95% had developed tubule structures (n=6 litters). Conversely, tubules were absent in cells only expressing the fluorescent tag control. To determine if *Bin1* driven tubules are functional, transfected cells were loaded with the calcium indicator Fluo-8 AM and field stimulated.

The systolic calcium transient amplitude was larger in cells transfected with *Bin1* isoforms 5 ( $18.5 \pm 11.5\%$ , n=5 litters), 8 ( $29.8 \pm 11.8\%$ , n=7 litters) and 9 ( $29.73 \pm 11.9\%$ , n=4 litters), when compared with untransfected myocytes ( $p < 0.05$ , SPSS Statistics). Furthermore, transfection with *Bin1* isoforms 5 and 9 led to faster rise ( $63 \pm 42.6\%$ ,  $81 \pm 49.2\%$  respectively) and decay ( $83 \pm 45.7\%$ ,  $122 \pm 94.5\%$  respectively) of the systolic calcium transient ( $p < 0.05$ , SPSS Statistics, n= 4-5 litters). Transfection with the control vector only had no effect on calcium handling when compared with untransfected cells. Additionally, the calcium handling proteins NCX & RyR co-localised with *Bin1* induced t-tubules when assessed using immunocytochemistry (Manders = 0.58 (NCX); 0.54 (RyR), n=2 litters). These data suggest that *Bin1* plays a vital role in tubule formation in cardiac myocytes and specific *Bin1* isoforms may enhance calcium kinetics. Given the importance of t-tubules to normal excitation contraction coupling and their perturbation in heart failure we therefore suggest that *Bin1* might be a novel therapeutic target to restore t-tubules in disease.

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Caldwell JL, Smith CE, Taylor RF, Kitmitto A, Eisner DA, Dibb KM, Trafford AW. Dependence of cardiac transverse tubules on the bar domain protein amphiphysin ii (*bin1*). *Circ Res*. 2014;115:986-996

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCA029

##### Matrix metalloproteinase 2 acts as repressor hypertrophic growth in ventricular cardiomyocytes of rat

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Matrix metalloproteinases (MMPs) are upregulated during the process of cardiac remodeling and were originally identified as modulators of the extracellular matrix during heart failure progression. However, intracellular functions of MMPs came

in the focus of current research. They are found at nearly all intracellular locations, i.e. at the sarcomer, mitochondria, nuclei, and in the cytosol. This suggests multiple, not yet defined actions of MMPs in cardiomyocytes. Therefore, we conducted this study to determine expression levels of MMPs in isolated ventricular cardiomyocytes of rat and analysed the impact of MMPs on hypertrophic growth.

Results: In ventricular cardiomyocytes from adult rats expression of MMP1, 2, 3, 9 and 14 mRNA was detected under baseline conditions. In western blots protein expression of MMP 2, 9 and 14 could be verified. Interestingly, stimulation of cardiomyocytes with hypertrophy inducing agents (10  $\mu$ M PE, 1 ng/ml TGF $\beta$ 1 or 100 nM AngII) decreased protein expression of MMPs. This indicates that MMP down regulation may be involved in hypertrophic growth. Indeed, inhibition of MMPs with TAP-0, MMP-inhibitor III or with ARP-100, an inhibitor specific for MMP2, enhanced hypertrophic growth, determined by an increase in the rate of protein synthesis and in cross sectional area. Also in the cardiomyogenic cell line H9c2 the MMP2-inhibitor ARP-100 provoked an increase in cross sectional area and in the rate of protein synthesis. Specific downregulation of MMP2 by siRNA in H9c2 cells as well as in adult cardiomyocytes enhanced the cross sectional area of cells within 48 h to  $260 \pm 129\%$  (n=6,) and  $140 \pm 10\%$  (n=4), respectively (each with  $p \leq 0.05$  vs. untreated and scrambled siRNA controls). Hypertrophic growth effects of MMP inhibitors could be blocked by the ERK inhibitor, PD98059, and by the PI3K inhibitors, LY290042 or wortmannin, in adult cardiomyocytes.

In conclusion, MMP2 acts as repressors of hypertrophic growth in cardiomyocytes via ERK and PI3K signaling. These findings define a new intracellular action of MMP2 in cardiomyocytes.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCA030

##### Hydrogen sulfide regulates mitochondrial $\text{Ca}^{2+}$ accumulation and mitochondrial function in adult and old rat hearts

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Hydrogen sulfide is a novel gasotransmitter and is known to have a regulatory role in the mammal's organism. In cardiovascular system it performs vasodilator, antiinflammatory, antiapoptotic and proangiogenic functions. In mitochondria  $\text{H}_2\text{S}$  acts as an electrons donor for the electron transport chain (ETC) and protects organelles from ischemic-reperfusion injury (Fu M, et al, 2012). But little is known about its influence on calcium homeostasis of mitochondria, while  $\text{Ca}^{2+}$  is important for excitation-contraction coupling, ATP synthesis and apoptosis. Mitochondria have been suggested to be linked to age-related alterations through formation of ROS, respiratory chain dysfunction and by increased sensitivity of mitochondrial permeability transition pore (MPTP) opening to  $\text{Ca}^{2+}$ . Thus, the aim of our work was to investigate the influence of  $\text{H}_2\text{S}$  on  $\text{Ca}^{2+}$  accumulation in mitochondria,  $\text{Ca}^{2+}$ -induced MPTP opening and mitochondrial functions in adult and old rat hearts. In our research we used adult (5-7 months, 250-350 g) and old (22-24 months, 350-420 g) male rats. Mitochondria were isolated using differential centrifugation method after rat decapitation with guillotine. Calcium accumulation in

isolated organelles was studied by flow cytometry analysis, under conditions when mitochondria were loaded with Fluo-4 AM in concentration 2.5  $\mu\text{mol/l}$  for 30 minutes at 26 °C. The effect of  $\text{H}_2\text{S}$  on  $\text{Ca}^{2+}$  accumulation in isolated mitochondria was studied using dissolved in distilled water NaHS in the concentration range of  $10^{-7}$  to  $10^{-4}$  mol/l. MPTP opening was registered spectrophotometrically as mitochondrial swelling during 15 min. The functions of ETC were estimated using oxygraph high-resolution respirometry. In each experiment, at least 5 repetitions were used. It was shown that isolated mitochondria from old rat heart had increased ability to accumulate  $\text{Ca}^{2+}$  on first three minutes by 27% max compared to mitochondria, isolated from adult cardiac tissue (F/F0 1,77 to 1,29 respectively). The influence of exogenous NaHS on calcium accumulation was dose dependent. NaHS in concentration  $10^{-4}$  –  $10^{-5}$  mol/l decreased  $\text{Ca}^{2+}$  influx into organelles. While,  $10^{-6}$  –  $10^{-7}$  mol/l NaHS elevates mitochondria calcium capacity both in adult and old rats in 2 times for  $10^{-7}$  mol/l in adult and 1,6 times in old animals. However, exogenous  $\text{H}_2\text{S}$  also prevents MPTP opening in concentration-dependent manner. The most effective was NaHS in concentration  $10^{-5}$  mol/l, which cause the decrease of mitochondrial swelling by 77% in adult and 60% in old rats. Also inhibition of mitochondrial  $\text{H}_2\text{S}$ -synthesis enzyme 3-MST *in vivo* increases  $\text{Ca}^{2+}$ -induced MPTP opening, decreases the rate of oxygen consumption in states 3 by Chance by 26 % (from 87,6 to 64,5 nmol  $\text{O}_2/\text{min}/\text{mg}$  protein) and respiratory control ratio from 2,4 to 1,84 in adult animals. Thus,  $\text{H}_2\text{S}$  regulates mitochondrial function in adult and old cardiac tissue trough the effect on ETC, increase of  $\text{Ca}^{2+}$  accumulation and inhibition of MPTP opening.

Fu M, Zhang W, Wu L, Yang G, Li H, Wang R. Hydrogen sulfide ( $\text{H}_2\text{S}$ ) metabolism in mitochondria and its regulatory role in energy production. *PNAS*. 2012; 109(8):2943–8.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA031

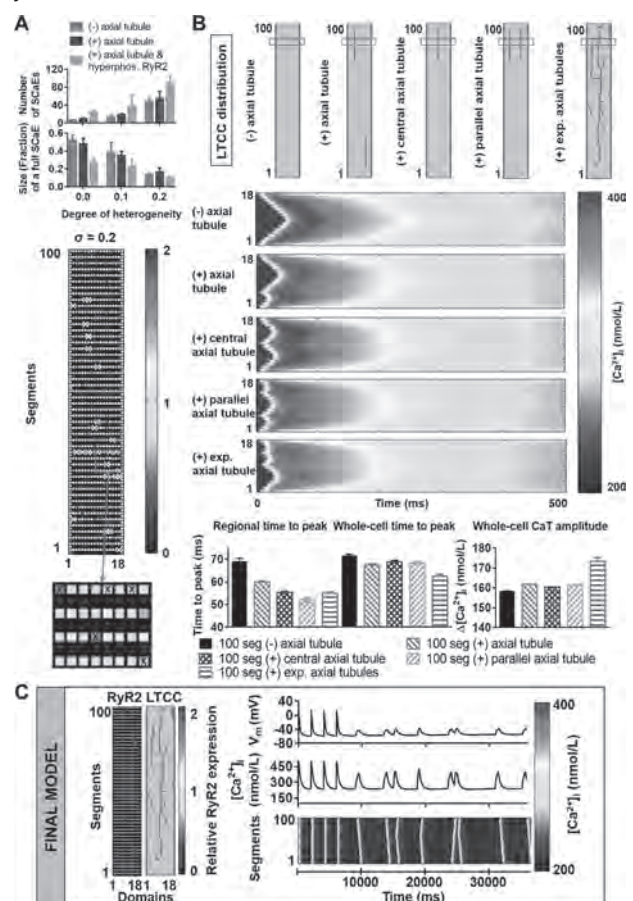
## Subcellular organization of ryanodine receptors and $\text{Ca}^{2+}$ channels modulates the propensity of spontaneous $\text{Ca}^{2+}$ waves and cardiac arrhythmias

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$\text{Ca}^{2+}$  handling is a major factor influencing cardiac electrophysiology and its dysregulation plays a crucial role in cardiac arrhythmias. Spontaneous  $\text{Ca}^{2+}$ -release events (SCaEs) from the sarcoplasmic reticulum play crucial roles in the initiation of cardiac arrhythmias by promoting triggered activity. However, the subcellular determinants of these SCaEs remain incompletely understood. Structural differences between atrial and ventricular cardiomyocytes, e.g., regarding the density of T-tubular membrane invaginations, may influence cardiomyocyte  $\text{Ca}^{2+}$ -handling and the distribution of cardiac ryanodine receptors (RyR2) has recently been shown to undergo remodeling in atrial fibrillation. Recent publications (Brandenburg et al., 2016; Macquaide et al., 2015) have suggested that the subcellular distribution of RyR2, L-type  $\text{Ca}^{2+}$  channels (LTCC) and T-tubules in cardiomyocytes modulates  $\text{Ca}^{2+}$  handling, but it is experimentally challenging to directly study the

impact of  $\text{Ca}^{2+}$ -handling protein distributions (which require fixation and antibody staining) and  $\text{Ca}^{2+}$  handling in the same cell. Here, we employ computational modeling to provide an in-depth analysis of the impact of variations in subcellular RyR2 and LTCC distributions on  $\text{Ca}^{2+}$ -transient properties and SCaEs in a human atrial cardiomyocyte model. We incorporate experimentally observed RyR2 expression patterns and various configurations of axial tubules, including previously published experimental axial tubules (Brandenburg et al., 2016) in a previously published model of the human atrial cardiomyocyte (Heijman, Erfanian Abdoust, Voigt, Nattel, & Dobrev, 2016; Voigt et al., 2014). We identify an increased SCaE incidence for larger heterogeneity in RyR2 expression (Fig. A, top), in which SCaEs preferentially arise from regions of high local RyR2 expression (Fig. A, bottom). We show that incorporation of axial tubules in various amounts and locations reduces  $\text{Ca}^{2+}$ -transient time to peak (Fig. B). Although the LTCC distribution itself does not affect SCaEs, selective hyperphosphorylation of RyR2 around axial tubules increases the number of spontaneous waves. Finally, we present a novel model of the human atrial cardiomyocyte with physiological RyR2 and LTCC distributions that reproduces experimentally observed  $\text{Ca}^{2+}$ -handling properties (Fig. C). Taken together, these results significantly enhance our understanding of the structure-function relationship in cardiomyocytes, identifying that RyR2 and LTCC distributions have a major impact on systolic  $\text{Ca}^{2+}$ -transients and SCaEs.



Brandenburg, S., Kohl, T., Williams, G. S., Gusev, K., Wagner, E., Rog-Zielinska, E. A., ... Lehnart, S. E. (2016). Axial tubule junctions control rapid calcium signaling in atria. *J Clin Invest*, 126(10), 3999–4015. doi:10.1172/JCI88241

Heijman, J., Erfanian Abdoust, P., Voigt, N., Nattel, S., & Dobrev, D. (2016). Computational models of atrial cellular electrophysiology and calcium handling, and their role in atrial fibrillation. *J Physiol*, 594(3), 537–553. doi:10.1113/jp271404

Macquaide, N., Tuan, H. T., Hotta, J., Sempels, W., Lenaerts, I., Holemans, P., ... Sipido, K. R. (2015). Ryanodine receptor cluster fragmentation and redistribution in persistent atrial fibrillation enhance calcium release. *Cardiovasc Res*, 108(3), 387-398. doi:10.1093/cvr/cvv231

Voigt, N., Heijman, J., Wang, Q., Chiang, D. Y., Li, N., Karck, M., ... Dobrev, D. (2014). Cellular and molecular mechanisms of atrial arrhythmogenesis in patients with paroxysmal atrial fibrillation. *Circulation*, 129(2), 145-156. doi:10.1161/CIRCULATIONAHA.113.006641

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCA032

### **A phospholipase A2 homologue increases the intracellular calcium concentration and induces spontaneous intracellular calcium transients and cell collapse in adult rat cardiomyocytes**

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The group of phospholipase A2 and phospholipase A2 homologue muscle toxins (PLA2-Myotoxins) from the venom of the viperid snake *Bothrops asper* targets skeletal muscle cells. Muscular effects of PLA2-Myotoxins include triggering spontaneous intracellular calcium transients, cell membrane disruption, hypercontraction of the myofilaments and cell collapse. Through those and other effects PLA2-Myotoxins lead to irreversible skeletal muscle damage. Whether and how PLA2-Myotoxins additionally target cardiac muscle cells has remained unknown.

In order to test for the first time the effects of PLA2-myotoxins in the excitation-contraction coupling of adult cardiac muscle cells, we exposed vital rat cardiomyocytes to the PLA2 homologue myotoxin II (PLA2-MtII). Anesthesia of rats was induced by inhalation of 5% isoflurane for 3 min in a box and kept by continuous inhalation of 2% isoflurane through a mask. After confirming the anesthetized state by the absence of reaction to several nociceptive stimuli, the heart was rapidly excised and a primary culture of vital ventricular cardiomyocytes was isolated. Thereafter, single cardiomyocytes were exposed to 50 µg/mL PLA2-MtII while monitoring intracellular calcium concentration (as reported by the calcium indicator FURA-2) and isotonic unloaded cell shortening by means of changes of the sarcomere length, during cyclic electrical stimulation (1 Hz).

Electrical stimulation triggered calcium transients and cell shortening in cardiac cells. Compared with the baseline before toxin application, few seconds of exposition to PLA2-MtII induced in the cardiomyocytes: a) increased diastolic and systolic calcium concentration, b) increased amplitude of the calcium transient, c) increased rate constant of calcium concentration decay during the descending phase of the calcium transient and d) increased amplitude of cell shortening. These changes reached statistical significance

levels ( $p < 0.05$ , T-test). Additionally, if kept in presence of PLA2-MtII for few minutes, cardiomyocytes progressively lost the calcium transient and cell shortening triggered by the electrical stimulus, showed spontaneous intracellular calcium transients and finally collapsed. Cell collapse was characterized by membrane damage, irreversible hypercontraction and abnormally increased intracellular calcium concentration. Thus, our results suggest that PLA2-MtII induces alterations of the intracellular calcium handling and structural damage in adult cardiac muscle cells *in vitro*. Understanding the membrane and intracellular mechanisms triggered by PLA2-Myotoxins in cardiac muscle could contribute to understand snakebite envenoming as well as to develop new molecular tools for manipulating the intracellular calcium concentration of the heart.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCA033

### **The Impact of Intentional Distortion of Image Quality on Left Ventricular Deformation Indices by Three-dimensional Speckle-Tracking Echocardiography**

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Background: Three-dimensional speckle-tracking echocardiography (3D-STE) is a novel technique that provides a comprehensive quantification of regional and global left ventricular (LV) myocardial deformation. It is increasingly used in clinical practice. 3D-STE is believed to be influenced by image quality, although quantitative evidence on this is limited.

Purpose: To assess the impact of intentionally distorted image quality on LV deformation indices measured by 3D-STE.

Methods: 18 healthy volunteers (age  $28 \pm 6$  years, 55.5% men) underwent three-dimensional echocardiography (3DE). Participants with poor images were excluded. One optimal and one intentionally impaired 3DE full-volume datasets of the LV were obtained and analysed per participant. Quality was impaired by including images with echo drop out, shadow artefacts, or poor visualization of the endocardium. Both acquisitions were free of stitching artefacts and the frame rate was maintained similar. Obtained 3D-STE parameters were LV volumetric, and global and averaged segmental LV deformation measures. The acquisition protocol was repeated on the same day to assess the test-retest repeatability. Mixed linear modelling was used to estimate the extent of bias and the intraclass correlation coefficient (ICC) was used as a measure of reliability.

Results: Sub-optimal image quality resulted in a systematic bias in all global and averaged segmental LV strain measures and LV rotational measures (Table 1). LV ejection fraction and most of LV volumes by 3D-STE were also consistently underestimated. Reproducibility was fair to good for all global and averaged segmental LV strain measures (ICC 0.47 – 0.72) and poor for twist and torsion (ICC 0.39 and 0.33 respectively). LV volumes and ejection fraction showed excellent reproducibility irrespective of image quality (ICC 0.78 – 0.92). Reproducibility was improved for all measures when restricting the analysis to undistorted quality images [i.e. good image quality] (Table 1).



**Conclusions:** Sub-optimal image quality adversely affects the reproducibility of 3D-STE derived measures and introduces a moderate systematic bias (<10%). Image quality should be assessed and accounted for in 3DSTE studies.

|                                                    | Good                 | Sub-optimal          | Mean $\Delta$ | 95% CI      | P      | ICC <sup>†</sup> | Good1                | Good2                | ICC <sup>‡</sup> |
|----------------------------------------------------|----------------------|----------------------|---------------|-------------|--------|------------------|----------------------|----------------------|------------------|
| <b>Global LV deformation measures</b>              |                      |                      |               |             |        |                  |                      |                      |                  |
| GLS, %                                             | -21.4 (-22.2, -20.5) | -20.2 (-21.1, -19.3) | -1.1          | -1.6, -0.47 | 0.001  | 0.50             | -21.6 (-22.5, -20.7) | -21.2 (-22.0, -20.3) | 0.62             |
| GCS, %                                             | -28.0 (-28.0, -27.0) | -25.7 (-26.7, -24.7) | -2.3          | -2.9, -1.7  | <0.001 | 0.72             | -28.3 (-29.5, -27.1) | -27.7 (-28.5, -26.6) | 0.88             |
| Twist, °                                           | 13.7 (11.2, 16.1)    | 10.8 (8.3, 13.3)     | 2.9           | 0.64, 5.16  | 0.012  | 0.39             | 13.7 (11.0, 16.4)    | 13.6 (11.0, 16.3)    | 0.40             |
| Twist, °/cm                                        | 1.5 (1.2, 1.8)       | 1.2 (0.96, 1.5)      | 0.32          | 0.046, 0.59 | 0.018  | 0.33             | 1.5 (1.2, 1.8)       | 1.5 (1.2, 1.8)       | 0.37             |
| <b>Averaged* segmental LV deformation measures</b> |                      |                      |               |             |        |                  |                      |                      |                  |
| LS, %                                              | -20.4 (-21.2, -19.5) | -19.6 (-20.5, -18.8) | -0.78         | -1.4, -0.02 | 0.043  | 0.47             | -20.6 (-21.5, -19.7) | -20.1 (-21.0, -19.3) | 0.66             |
| CS, %                                              | -28.2 (-29.3, -27.2) | -25.7 (-26.7, -24.6) | -2.5          | -3.1, -1.9  | <0.001 | 0.72             | -28.5 (-29.6, -27.3) | -28.0 (-29.2, -26.8) | 0.86             |
| PTS, %                                             | -13.0 (-14.1, -11.9) | -11.4 (-12.5, -10.3) | -1.6          | -2.3, -0.96 | <0.001 | 0.62             | -13.4 (-14.6, -12.2) | -12.8 (-13.8, -11.4) | 0.42             |
| RS, %                                              | -40.9 (-41.6, -40.2) | -38.2 (-38.9, -37.5) | -2.6          | -3.06, -2.1 | <0.001 | 0.72             | -41.1 (-41.9, -40.3) | -40.5 (-41.2, -39.8) | 0.80             |
| <b>Global LV volumetric measures</b>               |                      |                      |               |             |        |                  |                      |                      |                  |
| EDV, ml                                            | 123.9 (115.4, 132.4) | 117.7 (109.2, 126.3) | 6.1           | -1.7, 8.6   | <0.001 | 0.91             | 123.2 (114.2, 132.2) | 124.6 (115.6, 133.6) | 0.97             |
| ESV, ml                                            | 53.6 (49.0, 58.2)    | 53.6 (49.2, 58.4)    | -0.22         | -1.5, 1.0   | 0.743  | 0.92             | 52.8 (47.6, 57.8)    | 54.4 (49.4, 59.4)    | 0.96             |
| EF, %                                              | 56.9 (55.6, 58.2)    | 54.3 (53.0, 55.5)    | 2.6           | 2.0, 3.3    | <0.001 | 0.98             | 57.3 (55.8, 58.7)    | 56.3 (55.1, 58.0)    | 0.94             |
| SV, ml                                             | 70.2 (65.8, 74.7)    | 63.8 (58.4, 68.3)    | 6.4           | 4.8, 7.9    | <0.001 | 0.98             | 70.3 (65.8, 74.8)    | 70.2 (65.6, 74.7)    | 0.97             |

CI, confidence interval; CS, circumferential strain; EDV, end-diastolic volume; EF, ejection fraction; ESV, end-systolic volume; GCS, global circumferential strain; GLS, global longitudinal strain; ICC, intra-class correlation coefficient; LS, longitudinal strain; LV, left ventricular; mean  $\Delta$ , mean difference; PTS, principle tangential strain; RS, radial strain; SV, stroke volume. \*Averaged based on 16 segments model. †ICC < 0.4 = poor reproducibility, 0.4  $\geq$  ICC < 0.75 = fair to good reproducibility, and ICC  $\geq$  0.75 = excellent reproducibility. ‡ICC represents data from all 4 scans per participant (i.e. good and sub-optimal quality 3DE data sets of the first and second scans). ††ICC after restricting the analysis to undistorted image quality (i.e. good quality images; Good1 = the first scan and Good2 = the second scan).  $p < 0.05$  was considered statistically significant.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCA034

### Role of Nitric Oxide on Cardiac Regeneration through Fibrotic Elimination in Zebrafish (*Danio Rerio*) Model of Myocardial Infarction

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Fibrosis is one of the main hallmarks in post myocardial heart and leading to cardiac failure. Surprisingly, the teleost fish and urodele amphibian having high capacity of regeneration and complete elimination of fibrosis in cryo-injury model of myocardial infarction. However, the mechanism behind this elimination in post MI heart of zebrafish is still unclear. The objective of the present study was to evaluate the role of reactive nitrogenous species on regenerating heart with special reference to fibrosis elimination. After getting approval from the institutional ethics committee the adult zebrafish of both sexes were used in the present study. Animals were subjected induction of myocardial infarction model using cryoinjury under surgical anesthesia (0.2 % of Tricaine in tank water) (González-Rosa & Mercader, Nat Protoc. 2012, 29;7(4):782-8). During postoperative care, the fish were subjected to ECG analysis and sacrificed at various time points (0, 3, 7, 15, 30, 45 and 60 days POP (n=8)) by overdose of anesthesia (Tricaine 5 % tank water). The heart was dissected out and subjected to various analyses. The level of reactive nitrogenous species (n=3) (RNS) (0 –  $p < 0.001$ , 3-  $p < 0.001$ , 7- $p < 0.01$ , 15  $p < 0.01$ ) and Nitric oxide synthase (NOS) (n=10) (nNOS  $p < 0.01$ , eNOS

$p < 0.001$ , iNOS  $p < 0.001$ ) were significantly increased in initial time points and they were reduced or reversed towards normal in the rest of the periods. BrdU staining (n=5) showed marked increase in the dividing cell population in cryoinjured heart in time dependent manner when compared to control. Accumulation of fibrin (0 –  $p < 0.001$ , 3 –  $p < 0.001$ , 7 –  $p < 0.01$ , and 15 –  $p < 0.001$  days) and collagen (30 –  $p < 0.001$  and 45 –  $p < 0.001$  days of POP) in the injured area (n=5) was increased in time-dependent manner. Subsequently, the collagen also completely replaced by newly formed cardiomyocytes at 60<sup>th</sup> days POP. However, the administration of NOS inhibitor significantly altered all the above parameters in post injured heart when compared to control. From the present study, it was obvious that nitric oxide plays an essential role in fibrotic elimination and cardiac regeneration in cryoinjured zebrafish heart.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCA035

### Hearts subjected to ischemia-reperfusion benefit from adenine nucleotide translocase 1 overexpression

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The occlusion of coronary vessels blocks the supply of cells with oxygen, substrates and ATP and finally results in myocardial infarction. Oxygen deficiency during ischemia induces mitochondrial membrane depolarization and reperfusion provokes opening of the mitochondrial permeability transition pore (MPTP). The adenine nucleotide translocator 1 (ANT1) is a protein in the inner membrane of mitochondria that regulates the opening of the MPTP and has a physiologic key function in cellular energy metabolism. It was shown that I/R reduces the activity of ANT and thus inhibits oxidative phosphorylation. Overexpression of ANT1 should therefore have a protective effect. Therefore, we now analyzed whether ANT1-overexpressing hearts are protected against ischemia reperfusion (I/R) injury.

Hearts of 5 – 8 month old wildtype (WT) and ANT1-TG rats were perfused retrogradely in Langendorff Apparatus with constant pressure of 50 mmHg and submitted to 45-minute global ischemia followed by 2-hour reperfusion. Hemodynamic parameters were measured continuously. Mitochondria were isolated and mitochondrial respiration, membrane potential and calcium retention capacity were measured. After I/R, ANT1-TG hearts showed a significant decrease in diastolic pressure (WT:  $98.7 \pm 4.3$  mmHg; ANT1:  $80.7 \pm 3.4$  mmHg,  $p < 0.01$ ) and aortic pressure (WT:  $98.7 \pm 4.3$  mmHg; ANT1:  $80.7 \pm 3.4$  mmHg,  $p < 0.01$ ). In accordance with better hemodynamics, ANT1-overexpressing mitochondria of I/R hearts showed a higher calcium retention capacity (WT:  $50.0 \pm 6.3$  nM  $\text{CaCl}_2$ ; ANT1:  $97.5 \pm 8.5$  nM  $\text{CaCl}_2$ ;  $p < 0.01$ ) and a weaker decrease in membrane potential than WT mitochondria (WT:

37.2 ± 0.6 a.u.; ANT1: 25.5 ± 1.7 a.u.;  $p < 0.01$ ). In addition, ANT1-TG mitochondria from I/R rats are not restricted in their respiration (complex 1: WT 18.9 ± 1.4 basal vs 12.9 ± 1.7 I/R,  $p < 0.05$ ; ANT1 18.3 ± 1.2 basal vs 17.4 ± 1.6 I/R; in nmol O<sub>2</sub> \* min<sup>-1</sup> \* mg protein<sup>-1</sup>). Furthermore, mitofusin-2 (Mfn-2), a mitochondrial membrane protein contributing to the maintenance and operation of mitochondria, was downregulated in WT mitochondria after I/R, but was stable in ANT1-TG mitochondria (WT basal: 102.2 ± 1.2 %; WT I/R: 48.1 ± 3.4 %; ANT1 basal: 119.6 ± 9.4 %; ANT1 I/R: 102.9 ± 26.3 %). Optic atrophy protein 1 (OPA1) another mitochondrial fusion protein and critical regulator of mitochondrial respiration was significantly higher expressed in ANT1-TG hearts compared to WT hearts (WT: 100.0 ± 3.7 %; ANT1: 148.9 ± 1.7 %).

ANT1-overexpressing rat hearts cope better with the consequences of I/R. This is reflected in better hemodynamics and greater stability of the mitochondria. This is indicated by the improved expression of mitochondrial proteins and results in a superior energy balance and improved calcium handling of mitochondria, which ultimately leads to a lower rigor of the surviving ANT1-TG cardiomyocytes.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA036

#### Calcium/calmodulin-dependent protein kinase II is partly responsible for beta-adrenergic stimulation of slow delayed and inward rectifier potassium currents

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**Introduction and aim:** During acute  $\beta$ -adrenergic receptor ( $\beta$ -AR) stimulation the ventricular action potential (AP) shortens by a robust increase of cardiac potassium currents. Besides the well-established protein kinase A pathway, some of the downstream effects of  $\beta$ -AR stimulation are mediated by calcium/calmodulin-dependent protein kinase II (CaMKII). Our aim was to investigate the extent of CaMKII involvement in mediating the effect of  $\beta$ -AR activation on the most important potassium currents flowing during canine ventricular AP.

**Methods:** Experiments were performed on canine left ventricular cardiomyocytes produced by enzymatic digestion using the segment perfusion technique. Adult mongrel dogs were anesthetized with I.M. injections of 10 mg/kg ketamine + 1 mg/kg xylazine as approved by the local ethical committee. A "canonical" AP was used to stimulate every cell under action potential voltage clamp conditions at 37 °C without intracellular calcium buffering. Rapid delayed rectifier potassium current (IKr), slow delayed rectifier potassium current (IKs), transient outward current (Ito) and inward rectifier potassium current (IK1) were measured in this order by using their specific blockers in a cumulative manner (1  $\mu$ M E4031, 0.5  $\mu$ M HMR-1556, 100  $\mu$ M Chromanol 293-B and 50  $\mu$ M BaCl<sub>2</sub>, respectively). Data were collected in four study groups: Tyrode solution (CTRL), after beta-adrenergic stimulation with 10 nM isoproterenol (ISO), inhibition of CaMKII with 1  $\mu$ M

KN-93 (KN-93), after beta-adrenergic stimulation with inhibited CaMKII (KN-93+ISO).

**Results:** Neither Ito nor IKr differed significantly in the four groups studied. Moreover, there was no difference in either IKs or IK1 among CTRL and KN-93 group. On the contrary, IKs amplitude and total charge were larger in ISO than in CTRL or in KN-93 conditions by about 6- and 8-fold, respectively. In the KN-93+ISO group, IKs showed an about 2.5 times smaller amplitude and carried roughly half as much total charge compared to the ISO group. The total charge carried by IK1 in the ISO group was 25% and 15% larger compared to CTRL and to KN-93+ISO group, respectively. Under beta-adrenergic stimulation, IK1 starts to activate earlier during the AP plateau. IK1 density was about 3 times greater both at +20 mV and at 0 mV membrane potential under the command "canonical" AP in ISO compared to CTRL. Similarly, IK1 density was about 60% and 90% larger at +20 mV and at 0 mV, respectively, in KN-93+ISO compared to KN-93.

**Conclusion:** CaMKII activation plays an important role in beta-adrenergic stimulation of potassium currents. Part of the beta-adrenergic enhancement of IKs and IK1 is caused by CaMKII activation.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA037

#### Chronic oxidative stress-mediated remodeling of cardiac gap junctions in an in vitro model for arrhythmogenic cardiomyopathies

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Age-related arrhythmogenic cardiomyopathies (ACs), such as atrial fibrillation, are a group of heart diseases characterized by rapid and irregular beating of the heart. Ageing and systemic diseases including diabetes and hypertension are major risk factors for this disease complex. At the molecular level, ACs are associated with elevated levels of reactive oxygen species (ROS) and an altered protein expression pattern at the intercalated disk, predominantly gap junctions built from connexin-43 (Cx43). So far, a causal link between chronic oxidative stress and altered Cx43 expression has not yet been identified. Here, we focus on the characterization of the subcellular signaling pathways that link rapid pacing and enhanced ROS production with gap junction remodeling. We tested the hypothesis that prolonged oxidative stress influences the expression of Cx43 by modifying the activity of a specific microRNA, miR-1, that modulates translation of Cx43 mRNA.

Induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) were used as model to investigate the effect of tachypacing, an established experimental technique mimicking the conditions of ACs, on Cx43 expression. Confluent iPSC-CMs were tachypaced at 4 Hz for 24 and 48 hours, and compared with cells paced at 1 Hz and non-paced cells. ROS levels were monitored using CM-H<sub>2</sub>DCFDA fluorescence and confocal imaging. Levels of miR-1 were quantified and Cx43 expression was assessed by immunocytochemistry

and Western blot analyses at different pacing frequencies and after manipulation with a specific miR-1 inhibitor.

Our data revealed three effects of tachypacing: First, tachypacing iPSC-CMs for 24 h at 4 Hz significantly increased ROS compared to 1 Hz pacing (mean $\pm$ SEM: 4.7 $\pm$ 0.9 fold increase, n=5, p<0.05, Student's t-test). In addition, tachypacing significantly increased the activity of ROS-producing enzymes (slope: 0.17 $\pm$ 0.03 at 4 Hz vs. 0.04 $\pm$ 0.01  $\Delta$ F/s at 1 Hz, n=3, p<0.05). Second, tachypacing for 48 h at 4 Hz increased miR-1 expression by 20% (n=4). Third, tachypacing for 24 and 48 h at 4 Hz significantly reduced total protein expression of Cx43 (24 h: 0.4 $\pm$ 0.2, 48 h: 0.3 $\pm$ 0.1 fold decrease, n=3, p<0.05). Next, we investigated the effect of miR-1 on iPSC-CMs. Expression of the specific anti-miR-1 probe significantly increased sarcolemmal Cx43 expression compared to control scrambled miR (2.4 $\pm$ 0.2 fold increase, n=4, p<0.05). The effect of anti-miR-1 on Cx43 expression resulted in a significant increase in total Cx43 (1.3 $\pm$ 0.1 fold increase, n=3, p<0.05).

Our data suggest that chronically increased ROS levels by tachypacing lead to elevated miR-1 expression which reduces Cx43 expression in iPSC-CMs. This is the first experimental evidence that chronic oxidative stress may be causally linked to the pathophysiological remodeling of gap junctions underlying arrhythmogenic diseases.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCA038

##### Imaging of human neutrophil granulocytes by active targeting of perfluorocarbon-nanoemulsion and non-invasive $^{19}\text{F}$ MRI

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Magnetic resonance imaging (MRI) is one of the most important clinical imaging modalities since it provides excellent contrast between soft tissues and is free of radiation. However, conventional  $^1\text{H}$  MRI is unable to visualize distinct cell-types. To overcome this, contrast agents based on iron oxide or gadolinium have been applied for cell tracking. Recently,  $^{19}\text{F}$ -based contrast agents (Perfluorocarbon nanoemulsions = PFCs) emerged for non-invasive imaging of monocytes and macrophages at inflammatory hot spots.  $^{19}\text{F}$  is nearly absent from biological tissue, therefore accumulation of  $^{19}\text{F}$ -loaded cells results in unequivocal 'hot spots' without any background. Since neutrophil granulocytes are the first cells that infiltrate the infarcted myocardium and play a pivotal role for the subsequent healing process, we developed a targeting approach for the specific targeting of PFCs to human neutrophil granulocytes and their visualization by  $^{19}\text{F}$  MRI.

For targeting of neutrophils granulocytes, we utilized the neutrophil specific peptide (NG2), as well as non-specific control peptide (NGC)<sup>1</sup>. First, we tested the binding of NG2/NGC to neutrophils and found that the NG2, but not NGC bound to neutrophils and that neither of the peptides bound to monocytes or lymphocytes. In addition, we found that NG2 does not bind to neutrophils from mouse, rat or pig. Interestingly, we found a stronger binding of NG2 to neutrophils from patients with ST-elevated myocardial infarction (STEMI) (MFI: 4.27 $\pm$ 1.85; n=8) compared to healthy donors (MFI: 1.71 $\pm$ 0.59; n=6). Stimulation of healthy neutrophils

with LPS also increased the binding of NG2. For  $^{19}\text{F}$ -MRI, we coupled the NG2/NGC peptides to fluorescently labelled PFCs to generate NG2-/NGC-PFC. Upon incubation with NG2- or NGC-PFCs  $^{19}\text{F}$ -MRI revealed a strong  $^{19}\text{F}$  signal in healthy neutrophils (AUC: 1.3 $\pm$ 0.5; n=6) compared to NGC-PFC (AUC: 0.2 $\pm$ 0.1, n=9). Again, we observed a stronger  $^{19}\text{F}$  signal in neutrophils from patients after myocardial infarction (AUC: 2.8 $\pm$ 1.1; n=5). These results were confirmed by flow cytometry which revealed that NG2-PFCs but not NGC-PFCs were specifically taken up by neutrophils. Monocytes showed little and lymphocytes no uptake of NG2-PFCs. Internalization of NG2-PFCs was verified by confocal microscopy. Here, we could demonstrate that NG2 specifically binds to human granulocytes and that NG2-PFCs can be utilized to visualize neutrophils by  $^{19}\text{F}$ -MRI and flow-cytometry. Importantly, STEMI patients showed the increased binding of the NG2 and stronger labelling with PFCs. Our targeting approach holds the potential to specifically visualize the infiltration of human neutrophil granulocytes into inflamed tissue via non-invasive  $^{19}\text{F}$  MRI.

Mazzucchelli, L. *et al.* Cell-specific peptide binding by human neutrophils. *Blood* 93, 1738–1748 (1999).

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCA039

##### Cardiosphere-derived cells as a Model System for Myocardial Infarction

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Coronary heart disease is the most common cause of death in Europe<sup>1</sup>. Although tremendous achievements were made in prevention and treatment of myocardial infarction the resulting fibrotic scar remains a major threat. Ischemia and reperfusion (I/R) of the myocardium has to be thoroughly studied on a molecular basis to discover and understand new targets to treat I/R injury.

The experiments to unveil new targets were performed with cardiosphere-derived cells (CDCs). CDCs are a heterogeneous primary cell culture created from murine neonatal hearts<sup>2</sup> containing cardiomyocytes, endothelial cells, smooth muscle cells and stem cells<sup>3</sup>. In order to simulate myocardial infarction, oxygen-glucose deprivation (OGD; 0.2% O<sub>2</sub>, glucose-free medium) was performed for several periods of time (1h, 2h, 4h, 6h, 8h) followed by one hour of reperfusion with oxygen and glucose. Additionally, the response to hypoxia alone was studied by exposing the cells to hypoxia (1% O<sub>2</sub>) for 24h, 48h, and 72h with a normoxic control to investigate the impact of hypoxia-inducible factor stabilization in the cells.

Because exposure to low oxygen and lack of glucose is a tough challenge we presume that the CDCs metabolism and ultrastructure will suffer under OGD. First results showed significant changes in mRNA expression of various genes. Among these, we observed a significant down regulation of *connexin 43* (Cx43), a substantial part of the myocardial gap junction. This might impact myocardial conduction and lead to arrhythmia, a common complication after ischemic heart injury. Furthermore, the expression of *Adra1b* (*alpha-1b adrenergic receptor*) and *Adra1d* (*alpha-1d adrenergic receptor*) were significantly decreased, suggesting that the challenge has a negative impact on the mitotic capability of CDCs.



Cx43 and the alpha adrenergic receptors are only two possible examples to tackle complications after I/R injury. It is important to further investigate these and other possible targets to improve the long-term outcome of myocardial infarction.

EuroStat: [http://ec.europa.eu/eurostat/statistics-explained/index.php/Causes\\_of\\_death\\_statistics](http://ec.europa.eu/eurostat/statistics-explained/index.php/Causes_of_death_statistics) 22.05.2018

Tan et. al.; Isolation and expansion of cardiosphere-derived stem cells, Current protocols in stem cell biology 2011

Hosoyama et al., Cardiosphere-derived cell sheet primed with hypoxia improves left ventricular function of chronically infarcted heart, American Journal of Translational Research 2015

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#### PCA040

### Sixteen loci identified for T-wave morphology changes in response to exercise and recovery from UK Biobank implicate genes governing ventricular repolarization

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**Background:** The T-wave morphology restitution (TMR) index, quantifying the variations in the morphology of the T-wave in response to variations in heart rate (HR) have been shown to be strongly associated with increased arrhythmic risk leading to sudden cardiac death in chronic heart failure patients [1, 2]. Currently, the genetic basis of TMR remains to be elucidated. The aim of this study was to discover single-nucleotide polymorphisms (SNPs) associated with TMR.

**Methods:** 1-lead ECG recordings of 52,330 participants of European ancestry from the UK Biobank study who had an exercise stress test were analysed. The study has approval from the Ethics Committee and all participants provided informed consent. The test used a bicycle ergometer and included six minutes of graded cycling exercise and 1-minute recovery [3]. Two TMR phenotypes were derived, quantifying the T-wave morphology changes between resting and peak exercise, and, measured between peak exercise and 50 s post-exercise. We performed a discovery genome-wide association studies (GWASs) for each phenotype in 30,000 individuals and a validation experiment in the remaining 22,330 samples. We also conducted a GWAS in the full data set, all from UK Biobank. **Results:** Four (*RNF207*, *KCNQ1*, *SOX5* and *KCNJ2*) and three (*NOS1AP*, *KCNQ1* and *SOX5*) loci for and, respectively, formally replicated. In the full data set GWAS, four further loci for (*NOS1AP*, *SCN5A*/*SCN10A*, *PREP* and *KCNH2*) and five for (*SSBP3*, *SCN10A*, *LINC01213*, *CAMK2D* and *KLF12*) were genome-wide significant ( $P \leq 5 \times 10^{-8}$ ). In total, we discovered 16 loci, with four being common across traits. A large proportion of the identified loci map to genes functionally related to ventricular repolarization.

**Conclusion:** Our findings demonstrate the genetic contribution of the response of T-wave morphology to changes in HR, and indicate shared physiological mechanisms with QT

interval. Future studies will evaluate the contribution of TMR and the associated genes to cardiovascular risk prediction.

Ramírez et al (2017). *J Am Heart Assoc* 6(5), e005310.

Ramírez et al (2017). *PLoS One* 12(10), e0186152.

UK Biobank: Protocol for a large-scale prospective epidemiological resource. (2007) *UK Biobank Coordinating Centre*

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#### PCA041

### Molecular basis of arrhythmic substrate in ageing, Pgc-1β-deficient murine hearts modelling mitochondrial dysfunction

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**Background:** Ageing and chronic metabolic disorders are associated with mitochondrial dysfunction, in turn associated with increased incidences of arrhythmia. An ageing Pgc-1β-deficient mouse model of mitochondrial dysfunction demonstrates atrial and ventricular arrhythmias and reduced myocardial action potential (AP) conduction velocity, θ (1, 2). However, the molecular changes underlying these electrophysiological findings are currently unknown. We investigated expression levels of voltage-gated NaV1.5 channels and gap junction connexin (Cx) proteins, both known to affect AP conduction.

**Methods:** Generation, breeding and sacrifice (cervical dislocation) of mice and tissue retrieval by cardiectomy are described in (1, 2). The four experimental groups consisted of young and old, and wild-type and Pgc-1β<sup>-/-</sup> C57/B6 mice (min. n=4/group). Western blotting (WB) was performed on cardiac tissue lysates. Expression levels of NaV1.5 and the Cx isoforms Cx40 and Cx43 were obtained through densitometry. Immunofluorescence (IF) was also used to quantify Cx40 and Cx43 expression in paraffin-embedded tissue sections. Two-way ANOVA with Tukey's post-hoc test was used to investigate for independent or interacting effects of age or Pgc-1β deletion. **Results:** In the atria, WB demonstrated that increased age and genotype showed no significant independent or interacting effects on NaV1.5 expression levels. Significant independent effects of increased age (Cx40:  $P < 0.0001$ ; Cx43:  $P = 0.0018$ ) and Pgc-1β deletion (Cx40:  $P = 0.0098$ ; Cx43:  $P = 0.0286$ ) decreased

atrial Cx40 and Cx43 expression levels, without interactions between factors. IF experiments demonstrated increased age had no effect on atrial Cx40 expression, but Pgc-1 $\beta$  deletion reduced Cx40 expression ( $P=0.0027$ ). Increased age ( $P<0.0001$ ) and Pgc-1 $\beta$  deletion ( $P<0.0001$ ) independently reduced Cx43 expression.

In the ventricles, WB experiments demonstrated interacting effects of age and genotype on Nav1.5 expression levels ( $P=0.0418$ ). Young Pgc-1 $\beta$ -/- ventricles showed higher Nav1.5 expression than young WT ventricles ( $P=0.0253$ ). Both WB and IF experiments did not show either interacting or independent effects of age or Pgc-1 $\beta$  deletion on ventricular Cx43 expression.

**Discussion and conclusion:** The present results demonstrate functional rather than expressional changes in atrial and ventricular Nav1.5, and ventricular Cx43. In contrast, down-regulated Cx40/Cx43 expression can be implicated in reduced  $\theta$  following increased age or Pgc-1 $\beta$  deletion in the atria. These results suggest differing atrial and ventricular susceptibilities in protein expression patterns nevertheless culminating in proarrhythmic phenotype. Additionally, altered Ca<sup>2+</sup> homeostasis, oxidative stress or altered cellular redox potentials may contribute to the altered electrophysiological abnormalities seen in response to mitochondrial dysfunction.

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2. Valli H, Ahmad S, Chadda KR, Al-Hadithi A, Grace AA, Jeevaratnam K, et al. Age-dependent atrial arrhythmic phenotype secondary to mitochondrial dysfunction in Pgc-1 $\beta$  deficient murine hearts. *Mech Ageing Dev.* 2017;167:30-45.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

#### PCA042

##### Ultrastructure and ion channel expression of the Purkinje-ventricular junction in rabbit heart

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**Background:** For decades, the Purkinje fibre (PF)-ventricular junction has been implicated in arrhythmogenesis, but it remains an enigmatic and understudied region of the heart.

**Objective:** We aimed to unravel the ultrastructure and ion channel expression of the PF-ventricular junction.

**Methods:** The PF-RV region was excised from 3 rabbit hearts, fixed and stained for serial block face-scanning electron microscopy (SBF-SEM). Megascan 3D datasets for cardiomyocytes (a height of 0.8-mm) were collected over 1 week, with voxel sizes 30, 30, 150 nm in the X, Y, Z directions, respectively. Ion channel expression was measured in the free-running PFs, the junctional region and the ventricular wall of the right ventricle using quantitative PCR.

**Results:** There were two types of cell in the junctional area. One type of cell (J1) was long, large, and rod-shaped and had a 3.7 $\times$  larger volume and 2.1 $\times$  larger surface area than free-running PF cells. The other type of cell (J2) was short, wide and had a 2.0 $\times$  larger volume than free-running PF cells. In addition, the boundary between two junctional cells had

numerous wrinkles (like a curtain), which increased the area for the intercalated disc. Ion channel expression of the junctional area was unique, sometimes like that of the PFs, sometimes like that of the ventricular muscle and sometimes different from both tissues (e.g. KvLQT1 was significantly lower in the junctional tissue compared to the PFs and ventricular muscle). **Conclusion:** As long suspected, there is a distinct junctional tissue with unique properties at the border of the PFs and ventricular muscle. The unique properties of the junctional tissue presumably boost the incoming weak signal of the PFs to allow it to activate the large mass of surrounding ventricular muscle, as well as explaining its role in arrhythmogenesis.

Comparison of each cell type (mean  $\pm$  SEM)

|                                  | Free-running PF (n=7) | J1 (n=10)          | J2 (n=20)          | Right ventricle (n=58) |
|----------------------------------|-----------------------|--------------------|--------------------|------------------------|
| Volume ( $\mu\text{m}^3$ )       | 7,097 $\pm$ 348       | 26,394 $\pm$ 2,898 | 14,144 $\pm$ 1,012 | 10,558 $\pm$ 460       |
| Surface area ( $\mu\text{m}^2$ ) | 4,706 $\pm$ 291       | 10,034 $\pm$ 573   | 7,248 $\pm$ 438    | 4,915 $\pm$ 171        |
| Length ( $\mu\text{m}$ )         | 127 $\pm$ 11          | 145 $\pm$ 7        | 96 $\pm$ 6         | 98 $\pm$ 3             |
| Width ( $\mu\text{m}$ )          | 15.2 $\pm$ 0.9        | 31.5 $\pm$ 2.6     | 39.7 $\pm$ 2.7     | 18.5 $\pm$ 0.6         |

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

#### PCA043

##### Diabetes mellitus is associated with increased ghrelin level in the rat heart

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Diabetes mellitus (DM) is associated with increased risk of cardiovascular diseases leading to premature death. The structure and function of cardiac muscle are grossly impaired after the onset of DM. The functions of atrial and ventricular cardiomyocytes are controlled by a variety of neurotransmitters from both the sympathetic and parasympathetic pathways. In addition to neurotransmitters, peptides such as ghrelin have been shown to affect the function of the heart. The pattern of distribution of ghrelin in the diabetic rat heart is poorly understood. The cellular localization and distribution of ghrelin in the heart of a rodent model of DM was examined using immunohistochemistry. DM was induced by intraperitoneal injection of streptozotocin (60 mg kg<sup>-1</sup>). Wistar rats with blood glucose level equal or greater than 300 mg dl<sup>-1</sup> were considered diabetic. Four weeks after the induction of DM, control and diabetic rats were humanely euthanized with chloral hydrate according to the rules and regulations established by the Institutional Animal Ethics Committee. The ventricles of the heart were removed and processed for ghrelin immunohistochemistry using established method (1). Ghrelin was observed in both the sarcolemma and sarcoplasm of ventricular cardiomyocytes. The nuclei of cardiomyocytes were spared. The number of ghrelin-positive ventricular cardiomyocytes increased significantly ( $p < 0.05$ ) after the onset of DM. Since it has been reported that ghrelin protects the heart by inhibiting apoptosis in cardiomyocytes, the increased cardiac tissue level of ghrelin observed in this study, may be a result of a compensatory mechanism that is established to help in restoring cardiac structure and function in the ailing heart.

Lotfy M, Singh J, Rashed H, Tariq S, Zilahi E, Adeghate E. Mechanism of the beneficial and protective effects of exenatide in diabetic rats. *Journal of Endocrinology* 2014; 220: 291-304.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA044

### Iron deficiency reduces cardiac contraction by downregulating RyR2 channels and suppressing SERCA pump activity

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**Introduction:** Iron deficiency, irrespective of anemia, has been shown to be detrimental to cardiac function in chronic heart failure patients. Clinical trials have consistently demonstrated a beneficial effect of intravenous iron supplementation on cardiac function (1). However, the mechanisms of this effect are unknown. We investigated the link between iron deficiency (anemia), cardiac dysfunction and iron supplementation in mice fed an iron-deficient diet.

**Methods:** Mice were weaned on an iron-deficient diet for 6 weeks; some received intravenous iron as ferric carboxymaltose (FCM) after 3 weeks on the diet. *In vivo* cardiac function was assessed by CINE-MR imaging at the end of weeks 3, 4 and 5 of diet. To investigate the effect of iron deficiency (anemia) on gene expression, animals were sacrificed at the end of week 6 of diet, and their cardiac mRNA was subject to RNA-seq (Illumina HiSeq 4000) and analyzed for differential gene expression and altered pathways. The dynamics of cardiac calcium handling were interrogated using a variety of methods, including electrophysiology and live-cell fluorescence  $\text{Ca}^{2+}$ -imaging of electrically evoked  $\text{Ca}^{2+}$  transients in enzymatically isolated cardiac myocytes loaded with the dyes Fluo-3 or FuraRed.

**Results:** Following 5-weeks of an iron-deficient diet, animals became anemic (Hb (g/L): Control 147.7 vs Deficient 63.6). By week 3 of diet, ejection fraction of iron-deficient anemic animals was significantly decreased to 64% (compared to 70% Control). Whole-transcriptome sequencing (RNA-seq) suggested altered gene expression in several pathways, including cardiac  $\text{Ca}^{2+}$  signalling. Western blotting of cardiac lysates indicated a remodeling of proteins associated with the sarcoplasmic (SR)  $\text{Ca}^{2+}$  store: a two-fold downregulation of RyR2 channels and phospholamban phosphorylation at Thr17. Ventricular myocytes from iron-deficient anemic animals had reduced CaT amplitude (by 33%), with no change in diastolic SR  $\text{Ca}^{2+}$  load, L-type current or  $\text{Ca}^{2+}$  buffering. Reduced fractional  $\text{Ca}^{2+}$  release was attributable to downregulated RyR2 channel activity. The constancy of diastolic SR  $\text{Ca}^{2+}$  load was maintained by the combination of reduced RyR2 permeability and right-shifted SERCA pump activity. Intravenous injections of FCM were able to reverse the anemia and iron deficiency and restore CaT amplitude and cardiac ejection fraction.

**Conclusion:** Herein, we present a molecular mechanism underpinning cardiac remodeling in iron deficiency anemia and confirm the efficacy of intravenous iron supplementation in restoring normal function at the cellular and whole-organ level.

Anker et al (2018) "Effects of ferric carboxymaltose on hospitalisations and mortality rates in iron-deficient heart failure patients: an individual patient data meta-analysis" *Eur J Heart Fail.* 20(1):125-133.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA045

### The cardiovascular role of proprotein convertase subtilisin/kexin 9 (PCSK9)

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Hypercholesterolemia and hypertension belong to the most severe risk factors of cardiac disease. Proprotein convertase subtilisin/kexin 9 (PCSK9) regulates hepatic LDL receptor expression and furthermore participates in oxLDL-dependent effects on adult rat ventricular cardiomyocytes. The molecular mechanisms by which PCSK9 exerts its function in cardiomyocytes remained unclear. The current study was performed to clarify whether oxLDL causes the release of PCSK9 from cardiomyocytes, whether PCSK9 affects cardiac function via an autocrine mechanism, whether PCSK9 has intracellular functions in terminal differentiated myocytes, and whether PCSK9 also affects other pathways that reduce cardiac function such as that of angiotensin II (Ang-II). To address these questions, adult rat ventricular cardiomyocytes (ARVC) were cultured for 24 h and exposed to oxLDL (0-30  $\mu\text{g/ml}$ ), Ang-II (100 nM), siRNA directed against PCSK9 (xx), hPCSK9 and combinations thereof. Release of PCSK9 was quantified by ELISA, cell function by load-free cell shortening. Intracellular PCSK9 was induced independent from oxLDL by transfection of cardiomyocytes. Binding partners of PCSK9 in cardiomyocytes were identified by MALDI-TOF. oxLDL, but not Ang-II, increased the amount of extracellular PCSK9 in a concentration-dependent way (566 $\pm$ 220 to 2804 $\pm$ 305 pg/ml). Administration of extracellular PCSK9 and Ang-II but not transfection of cardiomyocytes with PCSK9 reduced load-free cell shortening (%-cell shortening: 7.21 $\pm$ 3.67; 7.01 $\pm$ 3.10; 9.31 $\pm$ 3.20; n=90 cells, 10 culture dishes, 2 preparations). siRNA directed against PCSK9 inhibited the effect on load-free cell shortening caused by oxLDL and Ang-II (%-cell shortening: 7.01 $\pm$ 3.10 vs. 10.64 $\pm$ 2.62). A receptor antagonist directed against the Ang-II receptor type 1 attenuated the effect of hPCSK9. None of these treatment regimes altered significantly the steady-state expression of PCSK9 in cardiomyocytes. Upon immunoprecipitation of PCSK9, alpha-crystallin B, myosin, and actin were identified. Finally, changes in mRNA expression of PCSK9 affected the mRNA expression of alpha-crystallin B. Silencing of crystalline-B potentiated reduced cell shortening. In conclusion, oxLDL acts via release of PCSK9 and extracellular PCSK9 seems to interact with Ang-II receptors. Intracellular mRNA levels of PCSK9 affect the steady-state of alpha-crystallin B expression and thereby reducing PCSK9 mRNA expression helps to withstand Ang-II-dependent effects on cell function.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.



PCA046

# **Cardiac specific overexpression of PGC-1 $\alpha$ isoform 4 in mice causes distinct changes in postnatal cardiomyocyte Na<sup>+</sup>/K<sup>+</sup> ATPase function and leads to early death by dilating heart failure**

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Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 $\alpha$ ), a transcriptional coactivator, is a master regulator of cellular energy metabolism. In cardiac myocytes, PGC-1 $\alpha$  contributes also to expression of many proteins involved in the processes of contractile function (1). PGC-1 $\alpha$  isoform 4 (PGC-1 $\alpha$ 4) has been shown to regulate skeletal muscle growth and its transgenic overexpression in mice induced skeletal muscle hypertrophy (2). In this study, we aimed to investigate the effects of PGC-1 $\alpha$ 4 on heart muscle by using a transgenic mouse model where PGC-1 $\alpha$ 4 expression is restricted to cardiac myocytes with Cre-Lox recombination system. PGC-1 $\alpha$ 4 overexpressing mice ( $\alpha$ 4<sup>+</sup>) died by the age of five weeks (n=9) and histological analysis showed severe cardiac dilation at the age of four weeks (Fig.1). Calcium handling and electrophysiological traits (action potential, sodium and calcium currents) were drastically changed in cardiomyocytes isolated from 4-week-old  $\alpha$ 4<sup>+</sup> mice. To pinpoint initial actions of PGC-1 $\alpha$ 4 in our model, we performed RNA sequencing from ventricular tissue of  $\alpha$ 4<sup>+</sup> mice at neonatal stage when their survival was yet unaffected by the overexpression. As expected, neonatal  $\alpha$ 4<sup>+</sup> hearts had only 202 differentially expressed genes (DEG) compared to control hearts whereas 4-week-old  $\alpha$ 4<sup>+</sup> hearts had 1554 DEGs (threshold for DEG: adjP<0.01, absolute logFC>1; n=4). Interestingly, at both time points gene enrichment analysis indicated notable changes in processes involved with plasmalemmal ion transport. One of the most prominent expressional changes was increase in Na<sup>+</sup>/K<sup>+</sup> ATPase (NKA) subunit alpha-3, which at both time points translated into increased protein expression as assessed with western blot (n=4). In addition, expression of NKA subunit alpha-2 increased in neonatal, but not in four-week old  $\alpha$ 4<sup>+</sup> hearts. Next, we examined NKA current in isolated cardiomyocytes with patch clamping under exposure to its pharmacological inhibitor ouabain at saturating concentration (1 mM) and at concentration that blocks only the more sensitive alpha-2 and -3 isoforms (10  $\mu$ M) (3). Proportion of more ouabain sensitive component of the total pump current was increased in both neonatal (26.6 $\pm$ 3.3% in control [n=7] vs 40.9 $\pm$ 4.5% in  $\alpha$ 4<sup>+</sup> [n=9], p<0.05) and 4-week-old (20.5 $\pm$ 2.5% in control [n=12] vs 40.7 $\pm$ 3.5% in  $\alpha$ 4<sup>+</sup> [n=8], p<0.001) cardiomyocytes (Fig.2). Increased NKA expression leading to its overt activity will increase the cardiomyocyte energy consumption, which might be one of the causative changes inducing heart failure in this model. In conclusion, the present study proposes role for PGC-1 $\alpha$ 4 in the regulation of cardiomyocyte ion homeostasis and its overexpression during cardiac development lethally impairs the heart growth in the immediate postnatal stage.

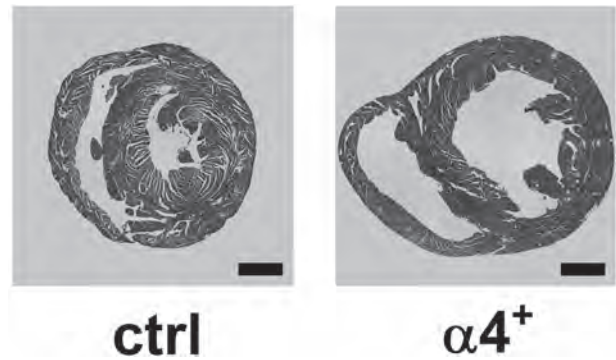


Figure 1. MTC stained cardiac cross sections from control (ctrl) and Pgc-1 $\alpha$ 4 overexpressing ( $\alpha$ 4<sup>+</sup>) mice. Scale 1 mm.

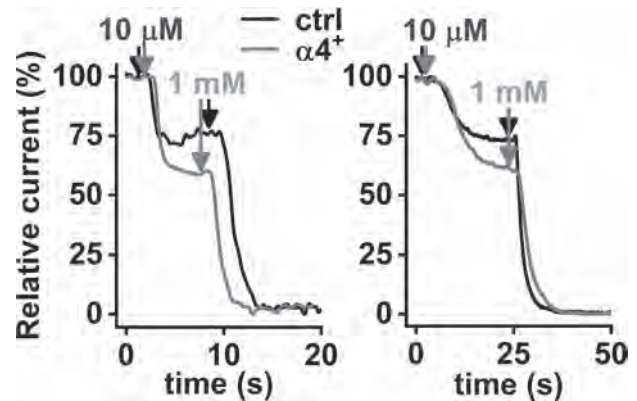


Figure 2. Representative relative Na<sup>+</sup>/K<sup>+</sup> ATPase current traces under consecutive applications of 10  $\mu$ M and 1 mM ouabain in neonatal (left) and 4-week-old (right) cardiomyocytes isolated from control (ctrl) and Pgc-1 $\alpha$ 4 overexpressing ( $\alpha$ 4<sup>+</sup>) mice.

Schilling, J. and D. P. Kelly (2011). Journal of Molecular and Cellular Cardiology 51(4): 578-583.

Ruas, J. L., et al. (2012). Cell 151(6): 1319-1331.

Hilgenberg, L. G., et al. (2009). Journal of Biological Chemistry 284(25): 16956-16965.

Authors would like to acknowledge Dr. Jennifer Estall from University of Montreal for providing the Lox-Pgc-1 $\alpha$ 4 mouse model for the study and Anne Karppinen for the technical assistance.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA047

# **The cardiovascular role of uncoupling protein 2 (Ucp2): Is it cardiac-specific and an uncoupling protein?**

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Uncoupling protein 1 (ucp1), also known as thermogenin, is an uncoupling protein in the brown fat tissue. Two other ucp isoforms, named ucp2 and ucp3, seemed to play distinct roles in various tissues including the heart although a relative high homology among ucp family members suggests a similar function. Upregulation of ucp2 in hypertrophic hearts may indicate a role for ucp2 this protein in this process, but the potential mechanism remains unclear. We performed experiments with adult ventricular cardiomyocytes from mice and rats (AMVC and ARVC) to study the role of ucp2 during hypertrophy and transition to

heart failure. In ARVC, silencing of *ucp2* significantly increased a process known as “cell spreading” that is required for cellular adaptation to stress conditions ( $15 \pm 7\%$  vs.  $27 \pm 6\%$  of all cells). Furthermore, silencing of *ucp2* by siRNA directed against the molecule as well as genipin, a pharmacological inhibitor of ucp2, attenuated an angiotensin II (Ang-II)-dependent loss of load free cell shortening (%-cell shortening:  $6.11 \pm 2.85$  vs.  $9.52 \pm 2.56$ ;  $6.39 \pm 2.82$  vs.  $8.34 \pm 2.54$ ;  $n=120$  cells; 15 culture dishes; 3 preparations). These experiments suggest that *ucp2* participates in the transition of adaptive hypertrophy to maladaptive hypertrophy. It has been suggested that *ucp2* reduces oxidative stress in mitochondria. However, neither pharmacological inhibition nor genetic depletion (*ucp2*<sup>-/-</sup> mice) affected ROS formation of isolated mitochondria. On the other hand, silencing of *ucp2* increased the amount of glucose transport proteins, such as Glut-4 and Glut-12 (x-fold:  $1.31 \pm 0.18$  and  $1.42 \pm 0.32$ ). Furthermore, silencing of *ucp2* reduced the concentration of intracellular lactate indicating an improvement of oxidative phosphorylation with glucose as the dominant substrate (by 31%). In ARVC *ucp2* is the main isoform of the ucp2s. Silencing of *ucp2* significantly improved the cell function upon exposure to Ang-II, improved cellular adaptation (spreading) and reduced glycolysis. In contrast, in AMVC *ucp2* and *ucp3* are equally expressed but silencing of *ucp2* did not improve Ang-II-dependent functional loss. Nevertheless, *ucp2* mice show increased Glut expression and improved function upon hypertrophic stimuli such as pulmonary artery banding (PAB). In conclusion, animals benefit from low *ucp2* expression by upregulation of glucose transporters and promotion of oxidative phosphorylation.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA049

### HIF-1 $\alpha$ controls the expression of Na/K-ATPase in hypoxic cardiomyocytes

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Na/K-ATPase is an integral membrane protein that functions as an ion pump by hydrolyzing ATP to pump three Na<sup>+</sup> out the cell in exchange for two K<sup>+</sup> per each pump cycle. Pump activity is important for regulating ion gradients, cell volume, resting membrane potential and electrical activity, and signal transduction in the heart. In ischemic heart and heart failure expression of the pump subunits decreases, contributing to decreased pump activity. Since in the ischemic heart cellular adaptation to hypoxia is regulated by hypoxia-inducible transcription factors (HIF), we tested whether the decreased Na/K-ATPase expression in hypoxic cardiomyocytes involves HIF signaling.

Rat ventricular cardiomyocyte H-10 cells were used for the experiments. HIF-1 $\alpha$  and HIF-2 $\alpha$  were silenced in by adenoviral introduction of shRNAs at 200MOI (multiplicity of infection). Cells were exposed to normoxia (20% O<sub>2</sub>) and hypoxia (1.5% O<sub>2</sub>) for 24h. mRNA expressions of HIF-1 $\alpha$ , HIF-2 $\alpha$ ,  $\alpha$ 1- and  $\beta$ 1-Na/K-ATPase was measured by RT qPCR and normalized to 18S rRNA. The surface expression of  $\alpha$ 1-Na/K-ATPase subunit was measured after biotinylation and separation of biotin-labelled proteins with streptavidin coated beads. Proteins in the non-biotinylated fraction were considered intracellular.

Protein expressions were measured by Western blot. Values are means  $\pm$  S.D, compared by ANOVA or t-test.

Hypoxia decreased the mRNA expression of  $\alpha$ 1-Na/K-ATPase (normoxia  $1.08 \pm 0.6$  vs. hypoxia  $0.43 \pm 0.3$ ,  $p=0.01$ ) and  $\beta$ 1-Na/K-ATPase (normoxia  $0.89 \pm 0.4$  vs. hypoxia  $0.42 \pm 0.3$ ,  $p=0.036$ ). The decrease in Na/K-ATPase mRNA subunits was partially prevented by silencing HIF-1 $\alpha$  but not HIF-2 $\alpha$  ( $\alpha$ 1-Na/K-ATPase: hypoxia  $0.43 \pm 0.3$  vs. hypoxia HIF-1 $\alpha$   $0.68 \pm 0.4$ ,  $p=0.011$ ;  $\beta$ 1-Na/K-ATPase: hypoxia  $0.42 \pm 0.3$  vs. hypoxia HIF-1 $\alpha$   $0.79 \pm 0.4$ ,  $p=0.02$ ). Hypoxia significantly decreased both the plasma membrane and intracellular expression of  $\alpha$ 1-Na/K-ATPase compared to normoxic cells ( $n=5$ ,  $p<0.05$ ); silencing HIF-1 $\alpha$  fully prevented hypoxic inhibition of  $\alpha$ 1-Na/K-ATPase in both fractions ( $n=5$ ,  $p<0.05$ ). Results indicate that hypoxia decreased Na/K-ATPase in cardiomyocytes, which is due to inhibition of transcription and translation. Decreased mRNA-expression and amounts of  $\alpha$ 1-Na/K-ATPase protein inserted in to the plasma membrane and located intracellular in hypoxia is controlled by HIF-1 $\alpha$ .

This work was supported by TUBITAK-BIDEB 2232-115C039 and by Acibadem University, ABAPKO 2016/04/03

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA050

### The Impact of Palmitoylation on Na<sup>+</sup>-Ca<sup>2+</sup> Exchanger (NCX) Function

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The sodium-calcium (Na<sup>+</sup>-Ca<sup>2+</sup>) exchanger (NCX) is an antiporter membrane protein that regulates cytoplasmic Ca<sup>2+</sup> by facilitating its electrogenic exchange for 3 Na<sup>+</sup>. Cardiac NCX (NCX1) is implicated in the pathogenesis of heart failure and a number of cardiac arrhythmias. A single cysteine in position 739 of the NCX1 large intracellular loop is palmitoylated, which regulates NCX1 inactivation (Reilly *et al.* (2015). *FASEB J.* 4532-4543). We investigated the impact of palmitoylation on NCX1 function using resin-assisted capture, and Fluorescence Resonance Energy Transfer (FRET) in HEK293 cells transfected with wild type (WT) or unpalmitoylatable (C739A) NCX1. NCX1 functions as a dimer: interactions between NCX1 dimers were visualised as FRET changes between exchanger proteins with CFP or YFP inserted at position 266 within the large cytoplasmic loop. NCX1-NCX1 FRET was greater in cells expressing WT than C739A NCX1, and the broad-spectrum palmitoylation inhibitor 2-Bromopalmitate reduced NCX1-NCX1 FRET only in cells expressing WT NCX1. Affinity purification followed by mass spectrometry identified the thioesterase APT1 as an NCX1-interacting protein: APT1 inhibition increased NCX1 palmitoylation and NCX1-NCX1 FRET. The palmitoylation of NCX1 and NCX1-NCX1 FRET were altered by the ion composition of the extracellular buffer: Ca-free extracellular solutions favoured FRET and elevated NCX1 palmitoylation, whereas Na-free extracellular solutions did the opposite. C739A-NCX1 FRET was unaffected by Ca-free conditions. We conclude that palmitoylation influences the structure of the NCX1 intracellular loop to modify FRET between NCX1 dimers. The impact of extracellular solution composition on NCX1 palmitoylation implies the existence of mechanisms to dynamically couple NCX1 palmitoylation to its function.

Reilly *et al.* (2015). *FASEB J.* 4532-4543

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA051

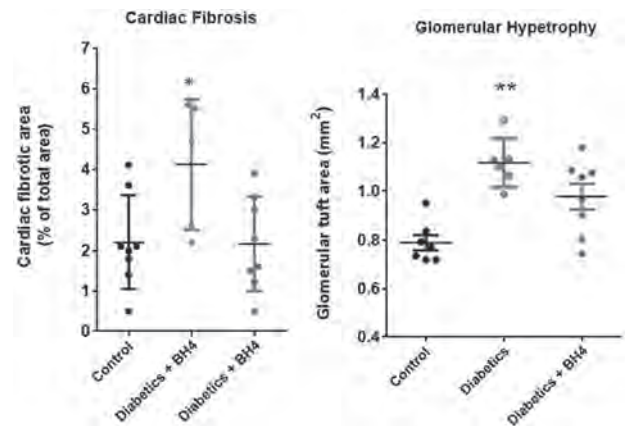
### Tetrahydrobiopterin (BH<sub>4</sub>) supplementation prevents the cardiorenal effects of diabetes in mice

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The effects of diabetes in the cardiovascular system as well as in the kidney are profound, which include hypertrophy and fibrosis (1). Diabetes also induces oxidative stress (2), at least in part due to uncoupling of nitric oxide synthase (NOS); this is a shift of NO production towards superoxide production due to reduced levels of the NOS cofactor tetrahydrobiopterin (BH<sub>4</sub>) (3). With this in mind, we tested the hypothesis that BH<sub>4</sub> supplement may prevent the development of diabetic cardiomyopathy and nephropathy.

For this purpose we induced diabetes in Balb/c mice (n= 30, male, 30-40 g) with three doses (100, 100 and 200 mg kg<sup>-1</sup>, i.p.) of streptozotocin. Then, diabetic mice were divided into two groups: one group provided with BH<sub>4</sub> (sapropterin) in drinking water (daily doses of 20 mg kg<sup>-1</sup> day<sup>-1</sup>, during eight weeks) and the other that received only water. A third group of normoglycaemic mice that received only water were used as control. Data are means ± standard deviation, compared by ANOVA or Kruskal-Wallis test. Cardiac levels of BH<sub>4</sub> were increased in mice treated with BH<sub>4</sub> (8.8 ± 2.2 control, 9.6 ± 4.7 diabetics and 209.7 ± 99.9 pmol BH<sub>4</sub> mg protein<sup>-1</sup> in diabetics + BH<sub>4</sub>, p=0.0019). Diabetes induced cardiac hypertrophy, evaluated as the ratio of heart weight /tibia length, which was prevented in the group that received BH<sub>4</sub>: (7.6 ± 1.03 g/mm control, 8.6 ± 0.63 g/mm diabetics and 7.38 ± 0.5 g/mm diabetics + BH<sub>4</sub>, p< 0.05). In addition, hypertrophy was evaluated as cardiomyocyte cross sectional area. This area was reduced in diabetic mice that received BH<sub>4</sub> (1190 ± 460 μm<sup>2</sup> control, 1194 ± 389 μm<sup>2</sup> diabetics and 1106 ± 375 μm<sup>2</sup> diabetics + BH<sub>4</sub>, p= 0.0012). In addition we evaluated cardiac fibrosis that is also a hallmark of diabetic cardiomyopathy, by Masson trichrome staining (Figure, left panel). Diabetes induced cardiac interstitial fibrosis that was reduced in mice that received BH<sub>4</sub> treatment (2.2 ± 1.1% control, 4.12 ± 1.6% in diabetics and 2.16 ± 1.2% in diabetics + BH<sub>4</sub>, p< 0.05). On the other hand, we evaluated in the kidney the impact of BH<sub>4</sub> treatment on glomerular morphology. Diabetes induced glomerular hypertrophy compared to normoglycaemic mice and was prevented by BH<sub>4</sub> treatment (Figure, right panel): 0.79 ± 0.08 mm<sup>2</sup> in control, 1.12 ± 0.1 in diabetic and 0.98 ± 0.15 mm<sup>2</sup> glomerular tuft size in diabetics + BH<sub>4</sub>, p=0.0004). In addition, diabetic mice presented increased glomerular collagen deposition, which was prevented in mice that received BH<sub>4</sub>: 1.01 ± 0.25 in control, 2.25 ± 0.29 in diabetics and 1.46 ± 0.33 score units in diabetics + BH<sub>4</sub> (p<0.0001). Overall, these results suggest that chronic treatment with BH<sub>4</sub> in mice ameliorates the cardiorenal effects of diabetes, probably by restoring the nitric oxide production. This offers a possible new alternative to explore a BH<sub>4</sub>-based treatment for the organ damage of diabetes.



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(2) Huynh K. et al. *Pharmacol Ther*. 2014;142(3):375-415

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA052

### miR-221/222 in cardiac electrical and structural remodeling

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#### Introduction

Cardiac remodeling involves structural and electrical alterations that can lead to fatal conditions like heart failure or sudden cardiac arrest. The underlying mechanisms are not fully understood. Previously we could show that epidermal growth factor receptor (EGFR) knockout (KO) mice developed a severe heart hypertrophy and changes in the electrical properties of the heart (prolonged P-duration, QRS- and QTc-intervals). NGS analysis revealed increased levels of the miR-221/222 cluster in KO mice. Additionally, we analysed two mouse models with pharmacologically induced cardiac hypertrophy (chronic angiotensin II (ANGII) or isoprenaline (ISO) treatment). miR-221/222 expression was elevated in ANGII mice but not in ISO mice. RNA-Seq experiments followed by gene enrichment analyses identified differentially regulated ion channels associated to the t-tubule cluster in EGFR KO mice which were also predicted targets of miR-221/222. This implicates a possible role of dysregulated miR-221/222 levels in cardiac electrical remodeling. Therefore, our aim was to evaluate a potential molecular mechanism underlying the miR-221/222 effects on electrical and structural cardiac remodeling in mouse models and in a cardiomyocyte cell line (HL-1).

#### Methods

We correlated miR-221/222 levels with expression (qPCR, Western blot) of cardiac ion channels (L-type Ca<sup>2+</sup> channel (LTCC) subunits: Cacna1c, Cacnb2, Cacna2d1; voltage gated K<sup>+</sup> channel Kv4.2; acetyl choline-activated K<sup>+</sup> channel subunit GIRK4) in EGFR KO, ANGII and ISO treated mice. Perivascular and interstitial fibrosis were also evaluated in these models. Dual luciferase assays were performed in HEK-293 cells to validate direct interactions between the miRs and their putative target ion channel 3'-UTRs. In HL-1 cells transfected with



mimics for miR-221/222 we analyzed cell size, cardiac Ca<sup>2+</sup> and K<sup>+</sup> channel expression as well as function (LTCC: whole cell patch clamp recording, GIRK1/4: fluorescence-based TI<sup>+</sup> flux assay). Additionally, as enhanced interstitial fibrosis was only observed in mice without elevated miR-221/222 expression, we further analysed fibrosis markers in these HL-1 cells.

#### Results

Cacna1c, Cacnb2, Cacna2d1, Kv4.2 and GIRK4 mRNA expression was decreased in mouse models with increased miR-221/222 levels. Interstitial fibrosis was only enhanced in mice without elevated miR-221/222 expression (ISO), but not in EGFR KO and ANGII mice. Luciferase assays revealed both LTCC and GIRK4 as direct targets of miR-221/222. HL-1 cells transfected with miR-221 mimics showed increased cell size, a decrease in mRNA expression of LTCC subunits, a significant reduction of Cacna1c and GIRK4 protein content as well as LTCC current density.

#### Conclusion

Increased expression of miR-221/222 influences electrical remodeling at least in part by downregulation of LTCCs and possibly also GIRK4. We hypothesize that miR-221 may also modulate structural remodeling.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCA053

### Electrical remodelling of the atrioventricular node causes heart block in athletes

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**Background:** Athletes present with atrioventricular node (AVN) dysfunction manifesting as prolonged PR intervals and heart block. This can necessitate electronic pacemaker implantation, known to be more frequent in athletes with a long training history. The underlying aetiology is unexplored but previously we reported that exercise-induced bradycardia is due to electrical remodelling of the sinus node, the heart's primary pacemaker (D'Souza et al, 2014; 2017). Here we investigated the molecular basis of training-induced AVN dysfunction.

**Methods:** 24 hour radio-telemetry ECG data were collected in sedentary and trained Standardbred racehorses (age:5-6 years), a translational, large-animal model of the athlete's heart. AVN electrophysiology and molecular profile were explored further in mice; ten-week-old C57BL/6j mice were trained by swimming for 60 min/day for 5 months following which AVN function was assessed by programmed electrical stimulation (PES) *in vivo*. mRNA levels of 96 ion channels, transporters and connexins were measured by qPCR in laser capture microdissected AVN. Protein levels of selected targets were assessed by immunohistochemistry and corresponding ionic currents measured in isolated AVN myocytes by patch clamp with suitable voltage clamp protocols. Values are given as mean±SEM, compared by ANOVA.

**Results:** Trained horses presented with a longer PR interval (sedentary, 298±7.7 ms; trained, 361±18 ms; p<0.05, n=12) and a striking increase in the incidence of second-degree

Mobitz type 1 heart block vs. sedentary horses (sedentary, 0.4±0.4; trained, 46.4±18.5 episodes/hour; p<0.0001, n=12), indicating training-induced AVN dysfunction. Similarly, trained mice showed prolonged PR intervals (sedentary, 40±1.0 ms; trained, 44.0±1.0 ms; P<0.05, n=7-8) and PES (n=8-9) revealed training-induced increases in Wenckebach cycle length (sedentary, 85.5±2.1 ms; trained, 99.8±1.4 ms; p<0.001) and AVN effective refractory period (sedentary, 62.6±2.8 ms; trained, 80.1±2.6 ms; p<0.05). These alterations were concomitant with widespread downregulation of ion channels in the trained AVN, notably the pacemaker HCN4 channel and the L-type Ca<sup>2+</sup> channel subunit Ca<sub>v</sub>1.2 (p<0.05, n=8/8). A 22% reduction in HCN4 protein levels in the trained AVN was confirmed by immunolabelling (p<0.05, n=4-5). Patch clamp recordings in isolated AVN myocytes demonstrated significantly reduced I<sub>f</sub> (~60%) and I<sub>Ca,L</sub> (~40%) current density in trained vs. sedentary mice (p<0.05, n=8-9).

**Conclusions:** Endurance exercise causes AVN dysfunction characterised by striking transcriptional remodelling that translates into reduced current density of key ionic currents involved in AVN impulse generation and conduction. We conclude that AVN electrical remodelling is a key mechanism underlying heart block in athletes.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCA054

### Novel technique for the simultaneous isolation of purified cardiac fibroblasts and epicardial cells from the infarcted murine heart

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Cardiac fibroblasts (CF) migrate and differentiate into myofibroblasts (activated cardiac fibroblasts - aCF) to mediate healing after acute myocardial infarction (MI). However, the CF population is described to be heterogeneous and difficult to capture by conventionally used markers. An additional problem, which so far has not been addressed, concerns the contamination with epicardium-derived mesenchymal cells (EPDC), formed on the epicardial surface after MI.

We have established a novel technique for the simultaneous isolation of CF and EPDCs from the infarcted murine heart. For MI induction mice were anaesthetized (1.5% isoflurane) and the left anterior descending coronary artery was ligated for 50 min followed by reperfusion. Cells were isolated from hearts 5 days post MI and compared to non-infarcted control hearts. Data are represented as mean±SD and statistically analyzed by Student t test. The elaborated methodology

involves enzymatic digestion of the isolated heart via the coronaries (Langendorff) for CF isolation and the simultaneous enzymatic removal of the EPDC layer from the outside in a single procedure. Subsequently, the cell suspension derived from the EPDC-depleted heart is separated from cardiomyocytes by centrifugation and endothelial cells as well as immune cells are removed by magnetic bead purification. Purified CF were analyzed for CF markers by flow cytometry: Pdgfr $\alpha$  as well as MEFSK4 was expressed on the majority of the isolates (72.2 $\pm$ 2.9% and 52.1 $\pm$ 4.6%, respectively), while CD90+, Sca1+ and Vim+ cells amounted to 23.3 $\pm$ 2%, 12.4 $\pm$ 0.7% and 35.5 $\pm$ 2.1%, respectively (n=4). Markers for pericytes and smooth muscle cells were expressed at minor percentages (Ng-2: 1.1 $\pm$ 0.3 %,  $\alpha$ -SMA: 12.3 $\pm$ 0.5%). Gene array analysis of cell sorted CF, aCF and EPDC revealed 736 and 336 differentially expressed genes comparing CF and aCF as well as aCF and EPDC, respectively (p $\leq$ 0.005, logFC $\geq$ 1.5; n=5). Genes of interest were further analyzed by qPCR. Analysis revealed that expression of WT-1, an established epicardial marker, was 20-fold higher in EPDC compared to aCF (n=5). Expression of activation markers in aCF and EPDC were in a similar range and higher as compared to CF. Cardiac genes and transcription factors were expressed in CF, aCF and EPDC but with large differences: expression of troponin T was 17-fold higher in CF/ aCF when compared to EPDC, while Tbx-5 was 4-fold higher in EPDC when compared to CF/ aCF. These profound differences between EPDC and aCF/ CF highlight that the removal of EPDC is an essential prerequisite for studies of the CF phenotype after MI.

In summary we report a rapid isolation technique for CF pre and post MI, which are largely free of contaminating cells. This protocol is well suited for the analysis of MI-induced phenotype changes in freshly isolated as well as in cultured CF, aCF and EPDC.

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PCA055

### Mitochondrial DAMPs in ex vivo ischemia-reperfusion injury in mouse hearts

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#### Background:

Acute myocardial infarction results in necrosis and initiation of a local sterile inflammation activated through Damage-Associated Molecular Patterns (DAMPs). Mitochondria are highly immunogenic due to their bacterial origin and the cardiomyocyte consists of ~30% mitochondria. In this study, we attenuate Toll-like receptor 4 (TLR4) activation in isolated hearts after ischemia-reperfusion and in cardiomyocytes. We also investigate cardiomyocyte viability and inflammatory responses after exposure to mitochondrial fragments.

#### Methods:

Langendorff heart perfusion of adult C57BL/6N male mice was retrogradely perfused at constant pressure (70 mmHg). Left-ventricular pressure was monitored through a fluid-filled balloon placed in the left ventricle. The experimental set-up: 20 minutes stabilization, 35 minutes global ischemia, and 60 minutes reperfusion. TLR4 signaling was investigated by adding a TLR4 inhibitor at reperfusion. Isolated adult mouse

primary cardiomyocytes were exposed to increasing concentrations of cardiac mitochondrial debris or LPS. Expression and release of cytokines were measured by qPCR and ELISA.

#### Results:

Inhibition of TLR4 at reperfusion in ex vivo Langendorff perfused mouse hearts showed reduced damage after ischemia-reperfusion. IL-6 release was significantly reduced in TLR4 inhibited hearts compared to control. Moreover, mitochondrial debris reduced viability and activated IL-6 expression in cardiomyocytes. This effect was abolished when mitochondrial debris was added in combination with TLR4 inhibitor. LPS, a known TLR4 ligand, activated the NF- $\kappa$ B pathway in cardiomyocytes.

#### Conclusions:

Mitochondria released after myocardial necrosis may reduce viability of neighboring cardiomyocytes. This in turn may initiate an inflammatory response in neighboring cardiomyocytes mediated in part through TLR4 and the NF- $\kappa$ B pathway.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCA056

### Quantitative mass spectrometry-based proteomics determines the uniqueness of the sinus node

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The sinus node is a collection of highly specialised cells that constitute the natural pacemaker of the heart. Although primarily composed of myocytes and fibroblasts like the rest of the heart, the protein expression landscape of the sinus node differs from the surrounding cardiac tissue, endowing it with its unique ability to trigger the heartbeat. Here we performed quantitative proteomics experiments to profile protein expression in the sinus node and compare it to the protein expression in the neighbouring atrial muscle. Sinus node and right atrial biopsies were collected from 30 mice sacrificed in accordance with the Animals (Scientific Procedures) Act, 1986. High-resolution mass spectrometry quantified >7,000 proteins. In the sinus node, the expression of 575 proteins was significantly different from that in the atrial muscle, showing the uniqueness of the tissue. As an example, significant differences were observed in (i) pace-making ion channels, (ii) contractile proteins linked to sinus node dysfunction, (iii) proteins involved in the storage of lipid (most important source of energy for the heart), (iv) proteins of the important regulatory natriuretic peptide system, (v) transcription factors responsible for the maintenance of the tissue and (vi) the proteins making up an elastic extracellular matrix mechanically supporting and protecting the sinus node. 59 ion channel subunits were detected, including sarcolemmal ion channels, the four major cardiac gap junction channel subunits, and intracellular ion channels (such as the mitochondrial Ca<sup>2+</sup> uniporter and the sarcoplasmic reticulum Tric-A/Tmem38a ion channel). The sarcolemmal ion channels include less described channels, such as the store-operated Ca<sup>2+</sup> channel (Orai1 and Stim1), Trpm7, purinergic receptor channels (P2rx4 and P2rx7), the two-pore K<sup>+</sup> channel, Task-1, and various Cl<sup>-</sup> channels. Of the sarcolemmal ion channels, the pacemaker channels, HCN4 and HCN1, two important Ca<sup>2+</sup> channel accessory subunits (Ca<sub>v</sub> $\alpha$ 2 $\delta$ 1 and Ca<sub>v</sub> $\alpha$ 2 $\delta$ 2) and the

T-type  $\text{Ca}^{2+}$  channel,  $\text{Ca}_v3.2$ , were significantly more abundant in the sinus node, whereas two  $\text{K}^+$  channels (Task-1 and  $\text{K}_{ir}3.1$ ) were more highly expressed in the atrial muscle. For the first time, the dataset shows the relative abundance of the ion channels, pumps and exchangers responsible for the pacemaker activity of the sinus node. By computational analysis, we show that the quantified differences in ion channel expression help explain the pacemaker ability of the sinus node. This quantitative proteomics dataset represents the most comprehensive investigation of the sinus node to date and offers a highly detailed insight into the unique composition of the pacemaker of our heart, and is an important resource for understanding sinus node disease.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA057

### Hypoxia regulates purine metabolism and adenosine- $\text{A}_{2B}\text{R}$ signalling of epicardium-derived cells formed after myocardial infarction

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Epicardium-derived mesenchymal cells (EPDC) play a pivotal role in heart development by differentiating into coronary vascular precursors, fibroblasts and cardiomyocytes. In the adult heart, EPDC are reactivated by myocardial infarction (MI) and are considered as endogenous cell source involved in cardiac remodelling. EPDC proved to secrete paracrine factors, including VEGF and FGF2, which have beneficial effects on post-MI remodelling. We have recently shown that the paracrine activity of EPDC after MI, especially the secretion of major regulatory cytokines such as IL1 $\beta$ , IL6 and VEGF, is regulated via adenosine- $\text{A}_{2B}\text{R}$  and ATP-P2X $_7\text{R}$  signalling (Hesse et al., FASEB J 31:3040-3053, 2017). However, the individual factors modulating the purinergic signalling of EPDC are presently unknown. Since hypoxia has been reported to regulate tissue remodelling processes, we explored the effects of hypoxia on the purinergic metabolism and signalling of cultured adult EPDC, isolated from Wistar rat hearts 5 d after MI.

MI was induced in mechanically ventilated rats with isoflurane (1.5% [vol/vol]) anaesthesia by ligation of the left coronary descending artery for 60 min and subsequent reperfusion. EPDC were digested from the heart surface with collagenase (1,200 U/ml) and cultured for up to 3 wk. Hypoxic conditions (1%  $\text{O}_2$ ) were established using a  $\text{CO}_2$  incubator with oxygen control. Values are means  $\pm$  SD, compared by Student's *t* test. Immunofluorescence of EPDC after 24h of cultivation under hypoxic conditions confirmed nuclear accumulation of hypoxia-induced factor (HIF)1 $\alpha$  (n=2). Quantitative Real-Time (qRT)-PCR revealed significantly increased expression of HIF1 $\alpha$  target genes *Car9* (CAIX, 2.7 $\pm$ 0.6 fold, *p*<0.01), *Vegfa* (2.4 $\pm$ 0.4 fold, *p*<0.01) and *Nt5e* (CD73, 1.2 $\pm$ 0.1 fold, *p*<0.05) after 24 h of hypoxia (n=5). Functional analysis of ATP, NAD and AMP metabolism by the purinergic enzyme cascade on the EPDC cell surface after 24 h of hypoxia via HPLC showed a decline in ATP and AMP degradation (-1.4 $\pm$ 0.3 fold and -1.3 $\pm$ 0.1 fold), while NAD degradation was enhanced (1.3 $\pm$ 0.3 fold) (4 min after substrate addition, n=3). Cultivation for 24h under hypoxic conditions significantly amplified  $\text{A}_{2B}\text{R}$  (BAY 60-6583)-dependent induction of adenosine-generating CD73

(1.7 $\pm$ 0.4 fold, *p*<0.05),  $\text{A}_{2B}\text{R}$  (1.6 $\pm$ 0.3 fold, *p*<0.05) and IL6 (3.7 $\pm$ 1.3 fold, *p*<0.05) expression as measured by qRT-PCR (n=5). Interestingly, immunofluorescence after 24 h incubation showed that  $\text{A}_{2B}\text{R}$  activation under normoxic conditions is sufficient to induce HIF1 $\alpha$  stabilization in EPDC and this at least to the same extent as hypoxia (n=2).

Our data demonstrate that hypoxia is a regulator of the purinergic metabolism and signalling in EPDC. Thus, local myocardial  $\text{pO}_2$  levels may crucially shape the potential pro-inflammatory and pro-fibrotic activities of EPDC in post-MI cardiac remodelling.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA058

### A shift in the atrial expression of voltage-gated sodium channels is seen in old age with $\text{Na}_v1.5$ declining and $\text{Na}_v1.8$ increasing – a prelude to atrial fibrillation.

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The cardiac voltage-gated sodium channel (VGSC) isoform,  $\text{Na}_v1.5$ , is primarily responsible for the fast sodium current ( $\text{I}_{\text{Na}}$ ) which underlies the upstroke of the cardiac action potential (AP) and determines cellular excitability. Recently, the 'non-cardiac' VGSC isoform  $\text{Na}_v1.8$  has been detected in the heart, where it plays a critical role in the initiation and maintenance of AF. AF is an age-associated problem with the majority of cases presenting in the elderly. Therefore, we investigated age-associated remodelling of  $\text{Na}_v1.5$  and  $\text{Na}_v1.8$  VGSC isoforms in the right atria.

We examined atrial tissue from young (6 months of age) and old (24 months of age) Wistar rats. Right atrial cryosections were triple labelled with pre-conjugated complexes of anti- $\text{Na}_v1.5$  (Alomone, Israel) to Alexa 488 (ThermoFisher Scientific, UK), anti- $\text{Na}_v1.8$  to Alexa 555 and anti-desmin to Alexa 647 viewed by confocal microscopy (LSM710 Zeiss, UK). Western blot was conducted of right atrial tissue homogenates with the same primary antibodies conjugated to HRP. Microelectrode impalements of tissue from young and old rats were used to record atrial APs. All procedures were conducted with University of Hull ethical approval and in accordance with current UK legislation. Data are mean  $\pm$  SEM, n = number of rats per group, analysed by Student's *t*-test or ANOVA with significance at *p*<0.05.

Fluorescent labelled  $\text{Na}_v1.8$  protein was observed as striated labelling, co-expressed with  $\text{Na}_v1.5$  labelled protein, predominantly at t-tubules of atrial myocytes as determined by labelled desmin protein: a novel finding of subcellular distribution.  $\text{Na}_v1.5$  immunofluorescent labelled protein was significantly reduced from 100  $\pm$  11.8 % in the young to 61  $\pm$  8.4 % in the old rat (n=5; *t*-test *p*=0.01), whereas  $\text{Na}_v1.8$  protein fluorescence significantly increased by 25% in the old rat (100  $\pm$  3.6 % young vs. 125  $\pm$  6.3 % old; *t*-test *p*=0.02). Western blot analysis showed a similar significant reduction of  $\text{Na}_v1.5$  protein expression by 50 % in the old rat (100  $\pm$  12.3 % young vs. 50  $\pm$  8.7 % old; n=5; ANOVA *p*=0.03), with a significant increase in  $\text{Na}_v1.8$  protein expression of 57 % in the old rat (100  $\pm$  6.7 % young vs. 157  $\pm$  22.2 % old; n=6; ANOVA *p*=0.02). The atrial AP amplitude significantly reduced from 105  $\pm$  12 mV (young, n=6) to 74  $\pm$  8 mV in the old rat (n=7) (*t*-test *p*=0.04).

In conclusion, we found loss of  $\text{Na}_v1.5$  protein from the right atria but an increase in  $\text{Na}_v1.8$  in old rats. These changes



associate with significant changes in the atrial action potential. Such remodelling is likely to underpin the reduction in fast  $I_{Na}$  and increased late  $I_{Na}$  observed in AF and may be a key contributing factor to the increased prevalence of AF in the elderly. Manipulation of this shift or the individual isoforms may prove useful for treatment or prevention of AF.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA059

# **A free-fluid transport pathway of fibrous connective tissues connecting the extremity end with the heart surfaces in a human gross anatomic study**

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A pathway of oriented fibrous connective tissues was revealed by fluorescein from ankle dermis, which argued against that free-fluid be entrapped within interstitium. Here, we speculate oriented fibrous tissues constitute a free-fluid transport pathway, connecting the distal end of extremities with fibrous tissues on heart, and verify this conjecture in human cadavers. 14 human cadavers were tested from 2016 to 2018. To visualize free-fluid pathway, 0.2ml fluorescein sodium was injected into distal end of right thumb of 10 subjects to observe fluorescein movements for 2.5 hrs firstly. Secondly, continuous chest compressions were performed right on heart for subsequent 2.5hrs by automatic chest compressions device with 120bpm. The fluorescent pathways in right hand, forearm, upper arm, axilla and thorax were dissected layer-by-layer. Interesting samples were studied by histological methods. Another 3 subjects were injected by a paramagnetic tracer. The other 1 subject was injected by India ink for lymphatic vessels.

There was only local confusion of fluorescein around injection point during first 2.5hrs of 10 subjects. After 2.5hrs repeated chest compressions, a longitudinally fluorescent pathway from thumb to heart surfaces was found in 7 subjects, but not in the 3 subjects with severe cachexia. By layered dissection, fluorescein pathways in hand and forearm was clearly seen in dermis, hypodermis, fascia on tendon or muscles, superficial tissues surrounding veins. In cubital fossa, upper arm, axilla and thorax, fluorescein pathways stained superficial tissues surrounding cephalic vein, axillary sheath around axillary vein, outer walls of superior vena cava, superficial tissues on right atrium. The transport course of fluorescein coincided with MRI findings. By histological analysis, fluorescent pathways were fibrous connective tissues and NOT blood or lymphatic vessels. By microCT and laser confocal microscopy, the intrinsic architecture of fluorescent pathway was longitudinally running fibers, upon which, free-fluid could flow and be visualized by fluorescein.

Together with explorations on interstitium for decades, the anatomic studies testified a systemic free-fluid transport

pathway of fibrous connective tissues, connecting superficial tissues of extremity end with a visceral organ. The intrinsic meshwork of fibrous connective tissues over the whole body probably constitute a huge interfacial transport system of body fluid between vascular conduits and cells. Both their interfacial dynamics and physiological/pathophysiological functions require extensive studies in life science.

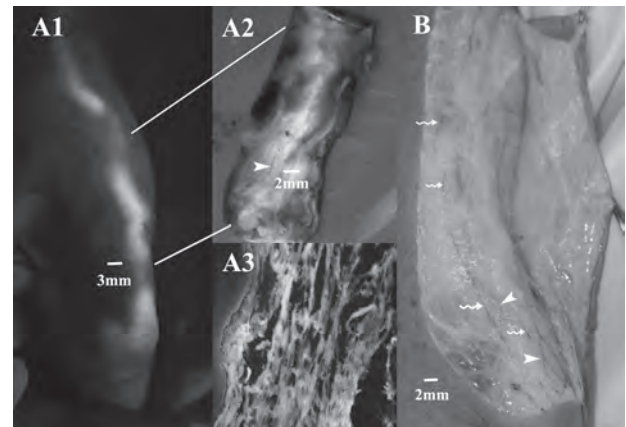


Fig. 1. The fluorescent pathway and lymphatic vessels in right hand of two cadavers (A and B). A1, a fluorescent pathway originated from the injection point and was clearly seen on the skin. A2 showed the hypodermic tissues of the inner side of A1 that were significantly stained by fluorescein. There was a venous vessel pointed by the arrow head in A2. A3 was the frozen section of A2 and displayed both the dermis and the hypodermis tissues were stained by fluorescein. B showed the collecting lymphatic vessels (pointed by arrows) near the venous vessels (pointed by arrow heads). These pictures displayed the fluorescent pathway was neither the vein nor the lymphatic vessel.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA060

### Cardiac autonomic modulation in adults with and without small abdominal aortic aneurysm

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Patients diagnosed with small abdominal aortic aneurysm (AAA), defined as the abnormal dilatation of the abdominal aorta (30-55 mm), are at increased risk of cardiovascular events and mortality compared to older adults living without the condition<sup>1</sup>. Imbalances in autonomic modulation of cardiac activity (i.e. reduced parasympathetic and/or increased sympathetic activation) have been associated with increased cardiac risk in a range of chronic conditions, but not for patients with AAA. The aim of this study was to examine the influence of AAA on cardiac autonomic modulation, as assessed by heart rate variability (HRV), in older adults with and without small AAA. This study was approved by the Human Research Ethics Committee at the Metro North Hospital and Health Service in Brisbane. Patients with AAA (n=16; age, 72±4 y) and non-AAA older adults (n=19; age, 72±5 y) volunteered for the study, and rested quietly in the supine position for 25 min with the final 5-min period assessed for HRV. Recordings were analysed via specialised HRV software to compute linear parameters within the time-domain (SDNN, the standard deviation of RR intervals; RMSSD, root mean square of successive RR intervals; pNN50, % of adjacent RR intervals that differ in duration by >50 ms), the frequency-domain using the absolute power of the low frequency (LF, 0.04-0.15 Hz) and high frequency (HF, 0.15-0.40 Hz) bands, as well as non-linear geometric indices (SD1 and SD2 obtained by Poincaré plot). Groups were compared using an independent t-test and data are presented as mean±SE. Resting heart rate was similar between patients with AAA and older adults (60±3 vs 57±4 bpm; p=0.352). Patients with AAA exhibited significantly less time-domain and non-linear measures of HRV compared with older adults, including lower SDNN (16.3±2.7 vs 29.6±4.6 ms; p=0.018), RMSSD (17.7±3.8 vs 32.0±5.8 ms; p=0.046), pNN50 (1.2±5.1 vs 9.5±2.9 %; p=0.021), SD1 (12.5±2.7 vs 22.7±4.1 ms; p=0.046), and SD2 (18.9±2.8 vs 34.5±5.2 ms; p=0.014). There was also a tendency for lower frequency domain measures of HRV (LF, 146±63 vs 609±225 ms<sup>2</sup>; p=0.061; HF, 150±73 vs 473±165 ms<sup>2</sup>; p=0.087) in patients with AAA compared with older adults. Patients with AAA

exhibited lower HRV compared with age-matched older adults, indicative of less parasympathetic and/or greater sympathetic modulations of heart rate. Reduced cardiac autonomic modulation may contribute to the elevated cardiovascular risk in AAA patients. Interventions aimed at improving cardiac autonomic modulations may reduce cardiovascular risk and improve long-term cardiovascular outcomes in this high-risk group.

Bath, M.F., Saratzis, A., Saedon, M., Sidloff, D., Sayers, R. & Brown, M. J. (2017). *Patients with Small Abdominal Aortic Aneurysm are at Significant Risk of Cardiovascular Events and this Risk is not Addressed Sufficiently.* *Eur J Vasc Endovasc Surg* 53(2): p. 255-260.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA061

### Gasotransmitters modulation of the voltage-gated K<sup>+</sup> channel Kv1.5.

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The voltage-gated K<sup>+</sup> channel Kv1.5 has key roles in the regulation of the vascular system<sup>1</sup> and in atrial excitability<sup>2</sup>, and contributes to apoptosis in various tissues. In this study we examined the regulation of recombinant and native Kv1.5 by carbon monoxide (CO); a product of the cytoprotective heme oxygenase enzymes, with recognized beneficial cardiovascular effects<sup>3</sup>. Whole cell patch clamp recordings were made from HEK293 cells expressing recombinant hKv1.5. Values are means ± S.E.M. Bath application of CO was achieved using the CO donor molecule CORM-2, which reversibly inhibited Kv1.5 in a concentration-dependent manner by 55.2 ± 3.78%, n=19 measured at 50mV (30μM; P< 0.05, Student's paired t test). Application of the inactive form (iCORM) had no significant effect. To determine the mechanism behind this CO effect we examined multiple signalling pathways; the actions of CO were significantly suppressed by the reducing agent DTT (1mM) and the superoxide dismutase (SOD) mimetics MnTMPyP (50μM) or MnTBAP (10μM) (85.8 ± 1.14%, n=5; 79.2 ± 0.85%, n=5; 81.1 ± 6.4%, n=5, P< 0.05 respectively) indicating an involvement of reactive oxygen species (ROS). Moreover, pretreatment of cells with the sGC inhibitor, Rp-8-Br-cGMPs (100nM) or the NOS inhibitor, L-NAME (1mM, 1hr at 37°C) significantly reduced the inhibitory effects of CORM-2 on Kv1.5 by 73.8 ± 1.9%, n=5 and 81.8 ± 7.45%, n=6, P< 0.05, indicating involvement of NO formation. CO also elevated intracellular peroxynitrite (ONOO<sup>-</sup>) formation, and a peroxynitrite scavenger FeTPPS markedly attenuated the ability of CO to inhibit Kv1.5 by 79.9 ± 5.7, n=5, P< 0.05, measured via the ONOO<sup>-</sup> indicator, 2-[6-(4'-amino) phenoxy-3H-xanthen-3-on-9-yl]benzoic acid (APF; n=5). CO also caused nitrosylation of Kv1.5, and two mutant forms of the channel, C331A and C346A (n=3). Hydrogen peroxide exposure induced augmentation of Kv1.5 by 15.62 ± 4.4% (n=4; P< 0.05); an effect fully reversed by CO.

In murine atrial HL-1 cells CO inhibited native Kv1.5/I<sub>Kur</sub> by 49.84 ± 4.9% (n=10 measured at 50mV), prolonged spontaneous action potentials and increased their amplitude by 7.2 ± 1.5 mV, n=6. A similar effect was observed when HL-1 cells were exposed to DPO-1, a blocker of KV1.5 channel and the I<sub>Kur</sub> current. Furthermore, in the presence of DPO-1, CO

did not increase the amplitude or duration of spontaneous action potentials further (n=6), suggesting that both CO and DPO-1 may act at the same site.

Our data indicate that Kv1.5 is a target for modulation by CO via multiple mechanisms<sup>4</sup>. This regulation has important implications for its diverse cellular functions, including excitability, contractility and apoptosis.

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Al Owais *et al.* Cell Death Dis. 2017; 8(11):e3163.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## PCA062

### Impact of novel calmodulin mutations on SK3 channels

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**Background and aims:** Calmodulin (CaM) is a ubiquitous Ca<sup>2+</sup>-sensing protein regulating many important cellular processes and playing a crucial role in Ca<sup>2+</sup>-dependent signaling in all eukaryotic cells. Over the last few years several CaM-associated variants have been identified in a small group of patients of young age with cardiac arrhythmias. CaM plays an important role in the cardiac contraction cycle, among which we find it as an interacting partner with the small-conductance Ca<sup>2+</sup>-activated K<sup>+</sup> (SK) channels. In order to understand the molecular mechanisms underlying the different phenotypes, we here aimed at studying the impact of novel CaM variants on SK3 channels.

**Methods:** For the electrophysiological evaluation, HEK293 cells stably expressing hSK3, were transiently transfected with pXOOM-rCaM<sup>WT/variant</sup>. Whole-cell patch-clamp recordings were acquired upon application of voltage ramps using a calculated free Ca<sup>2+</sup> concentration of 400 nM. For the immunocytochemistry studies MDCK cells were transiently transfected with pXOOM-rSK3<sup>WT/variants</sup> and were grown to a confluent, polarized state on glass coverslips before they were stained and imaged. Laser scanning confocal microscopy was performed using Zeiss LSM 710 confocal system.

**Results:** Out of the 9 variants evaluated in this study, the variants CaM<sup>N54I</sup>, CaM<sup>D96V</sup>, CaM<sup>A103V</sup>, CaM<sup>D130G</sup> and CaM<sup>F142L</sup> significantly suppressed I<sub>SK,Ca</sub> compared to CaM<sup>WT</sup>. Further, CaM<sup>D129G</sup> affected the membrane expression in contrast to CaM<sup>N54I</sup>, CaM<sup>D96V</sup>, CaM<sup>A103V</sup> and CaM<sup>F141L</sup> which more likely render the SK3 channel non-functional.

**Conclusion:** This study puts forward the suggestion that CaM variants have diverse effects on the SK3 channels and are giving rise to various molecular disease mechanisms.

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## PCA063

### TRPM4 knockdown modulates the sodium current in mouse cardiac myocytes.

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Transient receptor potential melastatin member 4 (TRPM4) encodes a Ca<sup>2+</sup> activated non-selective cation channel and functionally expressed in several tissues including the heart. Pathogenic mutants in TRPM4 gene have been reported in patients with inherited cardiac diseases including conduction blocks and Brugada syndrome. Heterologous expression of mutant channels in cell lines indicates that these mutations can lead to either increase or decrease in the expression and function of TRPM4 at the cell surface. While the expression studies and clinical variant studies further complement the importance of TRPM4 in cardiac function, electrophysiological characterization of cardiac phenotype in TRPM4 knockdown mice model can reveal more consolidated role of the ion channel *in situ*.

In this study we performed patch clamp studies on isolated atrial and ventricular cardiac myocytes from male C57BL/6N mice, Wild type (WT) or TRPM4 knockdown (TRPM4<sup>-/-</sup>). Action potentials (AP) were measured in current clamp using perforated patch-clamp configuration and data were presented as mean ± SEM (N - number of mice, n - number of cells studied). There significance were studied using student t-test. In ventricular myocytes we observed a decrease in the AP upstroke velocity of TRPM4<sup>-/-</sup> mice (115.75 ± 3.7 V/s (WT) vs 89.7 ± 3.7 V/s (TRPM4<sup>-/-</sup>), N=5, n=14). The decrease in the upstroke velocity in TRPM4<sup>-/-</sup> mice was concomitant to a decrease in whole cell peak sodium current density by ~ 27% (-36.0 ± 4.4 pA/pF vs -26.1 ± 1.8 pA/pF, N = 5; n=10). AP recordings from atrial myocytes did not show any change in the upstroke velocity (108.0 ± 15.5 V/s vs 107.0 ± 15.1 V/s, N=5; n=23). However, the peak sodium current density was significantly reduced in these cells by ~ 25% in TRPM4<sup>-/-</sup> (-48.0 ± 2.3 pA/pF vs -36.3 ± 2.5 pA/pF, N = 5; n=20). We further explored the effect of decrease in sodium current in cardiac myocytes recording pseudo electrocardiogram (ECGs) on explanted Langendorff perfused mice heart. Under basal condition, the (ECGs) did not show any change, however introducing mexiletine (40 µg/ml) a sodium channel inhibitor in the perfusate broadened the QRS by 18 ms (11.8 ± 1.8 ms (buffer) vs 29.1 ± 3.6 ms (mexiletine) N = 5) in WT hearts. The effect of mexiletine was much more prominent in TRPM4<sup>-/-</sup> with a broadening of QRS complex by 41 ms (10.3 ± 0.9 ms (buffer) vs 51.1 ± 10.3 ms (mexiletine) N=5). The increased sensitivity of TRPM4<sup>-/-</sup> mice ECGs towards mexiletine, further complement our observation of decreased peak sodium current in ventricular myocytes.

Our study gives the first evidence of TRPM4 expression directly impacting sodium current in mice cardiac myocytes. Future studies on patient derived cardiac iPSc (induced pluripotent stem cell) harboring TRPM4 loss-of-function variants and evaluating the impact on sodium current will be critical for developing novel therapeutic management in such patients.

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## PCA064

**Coronary microvascular perfusion in rheumatoid arthritis: the role of subtypes of obesity**A. Sandoo<sup>1,2</sup>, A. Stavropoulos-Kalinoglou<sup>3</sup>, J.P. Smith<sup>2</sup> and G. Kitas<sup>2</sup><sup>1</sup>School of Sport, Health and Exercise Sciences, Bangor University, Bangor, UK, <sup>2</sup>Clinical Research Unit, Dudley Group NHS Foundation Trust, Dudley, UK and <sup>3</sup>Carnegie School of Sports, Leeds Beckett University, Leeds, UK

Rheumatoid arthritis (RA) is a chronic inflammatory musculoskeletal disease affecting 0.8% of the UK population. RA patients also have 50% greater risk for cardiovascular disease (CVD) due, in part, to a greater prevalence of classical CVD risk factors. Some risk factors such as obesity have been reported to express phenotypes that protect against CVD. Patients who are obese but metabolically healthy (i.e. absence of insulin resistance and hyperlipidaemia) may have a lower CVD risk than patients who are normal weight but have an adverse metabolic profile. At present, the associations between subtypes of obesity and cardiovascular outcomes in RA is unclear. Thus, the present study examined different obesity subtypes on a validated surrogate measure of coronary microvascular perfusion (subendocardial viability ratio (SEVR)) in a large cohort of RA patients. 176 RA patients (age  $65 \pm 11$  years, 141 females) underwent assessments of body weight (including body mass index (BMI)) and metabolic health (fasting blood lipids, glucose, insulin, homeostasis model assessment, quantitative insulin sensitivity check index, waist circumference). Pulse wave analysis was used to derive SEVR and was calculated by dividing the area under the diastolic curve (coronary perfusion) by the area under the systolic curve (cardiac workload). RA patients were split into six categories for metabolic profile; metabolically healthy normal weight (MHNW,  $n = 42$ ), metabolically obese normal weight (MONW,  $n = 14$ ), metabolically healthy overweight (MHOW,  $n = 44$ ), metabolically obese overweight (MOOW,  $n = 16$ ), metabolically healthy obese (MHOB,  $n = 35$ ), metabolically obese, obese (MOOB,  $n = 25$ ). Univariate Analysis of Variance adjusted for age revealed that MHNW patients had better SEVR (SEVR:  $154 \pm 21\%$ ) than MOOW (SEVR:  $137 \pm 20\%$ ,  $p = .03$ ), and MHOB patients (SEVR:  $140 \pm 23\%$ ,  $p = .005$ ). There was a trend for worse SEVR in MONW patients ((SEVR:  $139 \pm 25\%$ ,  $p = .06$ ). These findings suggest that patients presenting with metabolically obese phenotypes have greater impairment in coronary microvascular function. Thus, it may be important to include measures of metabolic health alongside standard measures of BMI to help identify patients likely to develop CVD in RA.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA065

**Investigation role of Urotensin II on electrical activity of ventricular myocytes**H.S. Al Ali<sup>1</sup>, G. Rodrigo<sup>1</sup> and D. Lambert<sup>2</sup><sup>1</sup>Cardiovascular Sciences, University of Leicester, Leicester, UK and <sup>2</sup>Cardiovascular Sciences, University of Leicester, Leicester, UK

Evidence from studies on patients with heart failure show that a reduction in contractile function is associated with changes

in  $\text{Ca}^{2+}$  regulation (Houser et al. 2000). Furthermore, levels of Urotensin II (UII) are elevated in the plasma of patients with heart failure (Ng et al 2002), and it is suggested that this may lead to myocardial dysfunction. Our previous work has shown that UII reduces contraction strength of adult rat ventricular myocytes (ARVMs) and this reflects a reduction in systolic calcium (Al Ali et al. 2018). The aim of this study is to investigate the effect of UII on the action potential and L-type calcium currents (LTCC) in ventricular myocytes. ARVMs were isolated via enzymatic digestion of adult Wistar rat hearts. Electrical activity was recorded from freshly isolated ARVMs. Action potentials (AP) were recorded using the whole cell patch clamp technique with high resistance pipettes ( $12\text{--}15\text{M}\Omega$ ). LTCC was recorded using low resistance pipettes ( $2\text{--}4\text{M}\Omega$ ), filled with an electrode solution containing 10mM BAPTA. To investigate the effects of UII on electrical activity of the heart, the percentage change in action potential duration ( $\text{APD}_{30/50/90}$ ) was calculated in freshly isolated ARVMs in response to an acute application of UII (200nM for 10 mins). The data show UII reduced  $\text{APD}_{30}$  from  $9.5 \pm 1.4$  ms in normal Tyrode (NT) to  $5.3 \pm 0.9$  ms in UII ( $p < 0.01$ ) and  $\text{APD}_{50}$  from  $19.5 \pm 2.1$  ms in NT to  $12.1 \pm 1.6$  ms in UII ( $p < 0.001$ ). However, there was no significant change in  $\text{APD}_{90}$  between UII at  $57.2 \pm 5.2$  ms in NT versus  $50.9 \pm 6.0$  ms in UII ( $p > 0.05$ ,  $ns$ ;  $n = 6$  hearts; 41 cells). We set out to investigate the effect of UII on the LTCC, which is suggested by the reduction in  $\text{APD}_{50}$ . The data show that UII (200nM) decreased the LTCC recorded from ARVMs at 0mV from  $14.7 \pm 0.6$  pA/pF in NT to  $12.6 \pm 0.7$  pA/pF in UII ( $n = 4$  cells  $p < 0.001$ ). The data show a reduction in  $\text{APD}_{30/50}$  and LTCC in response to acute application of UII in ventricular myocytes, which may contribute to the reduction in contraction strength.

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Ng, L. L., Loke, I., O'Brien, R. J., Squire, I. B. and Davies, J. E. (2002), *Circulation*, 106(23), 2877–2880.

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## PCA066

**The effect of chronic inflammation on circadian rhythms in the preterm fetal sheep**V. King<sup>1</sup>, S. Dhillon<sup>1</sup>, R. Galinsky<sup>2,1</sup>, C. Lear<sup>1</sup>, A.J. Gunn<sup>1</sup> and L. Bennet<sup>1</sup><sup>1</sup>Physiology, The University of Auckland, Auckland, New Zealand and <sup>2</sup>Ritchie Centre, Hudson Research Institute, Melbourne, VIC, Australia

Circadian rhythms are important regulators of physiological activity, but little is known about fetal patterns, particularly during preterm life, and the effect of adverse events such as infection. We examined circadian patterns in preterm fetal sheep and the effect of inflammation induced by lipopolysaccharide (LPS). Experiments were approved by the animal ethics committee of Auckland University. Pregnant ewes 98–99d gestation (term=147d) were anaesthetised with propofol (5 mg/kg iv) followed by 2–3% isoflurane in  $\text{O}_2$ . Fetuses were exteriorised, instrumented for fetal heart rate (FHR), mean arterial blood pressure (MAP), and electroencephalographic (EEG) activity measurements and returned to

the uterus. Animals recovered from surgery until 104d when fetuses were randomised to receive either a 5d iv saline (n=7) or LPS (n=7) infusion (ramping dose doubled each day from 200ng), followed by 5d of recovery. Ewes were fed *ad libitum* and actiography showed ewes ate primarily during the day. The housing light period was 06.00-18.00h. Data were evaluated using ANOVA, data are mean $\pm$ SEM. In controls, FHR fell with age (189.0 $\pm$ 2.0 to 174.0 $\pm$ 1.5bpm, 1<sup>st</sup> vs. last day, P<0.001). FHR fell primarily between ~22.00-06.00h, with mixed activity from 06.00-12.00h, a rise from 12.00 to 19.00h and then plateau. LPS did not affect these patterns. MAP increased with age (34.8 $\pm$ 0.5 vs. 40.2 $\pm$ 0.7mmHg, P<0.005), primarily during the day, with a plateau ~19.00-02.00h then fall (0.8 $\pm$ 0.5mmHg) until 06.00hrs. LPS infusion blunted the rise on days 3-5 (day 5: 36.0 $\pm$ 0.8 vs. 38.0 $\pm$ 0.8mmHg, P<0.05) without changing circadian patterns. Post-infusion MAP increased continually, and groups were not different on the last day. EEG power increased with age (16.4 $\pm$ 0.4 vs. 19.8 $\pm$ 1.0dB, P<0.05), primarily during the day with a plateau or fall at night. The rise in EEG was suppressed by LPS on days 3-5 (day 5: 17.3 $\pm$ 0.6 vs. 18.8 $\pm$ 0.4 dB, P<0.05). Post-LPS, EEG increased continually and groups were not different on the last day. Spectral edge frequency (SEF) increased with age until 108d then plateaued and began to decrease. SEF increased only at night, with a daytime plateau or fall. LPS prevented the plateau/fall phase of SEF maturation, with a sustained increase observed throughout (last day 13.0 $\pm$ 0.4 vs. 11.1 $\pm$ 0.5Hz, P<0.01). Circadian patterns were sustained. Increasing MAP reflects fetal growth, thus our data suggests that fetal growth may occur primarily during the day, consistent with ewe food intake. LPS inhibits growth, and the loss of circadian patterns post-LPS infusion may reflect catch-up growth. Catch-up brain growth may also explain changes in EEG power. Increased SEF at night may reflect neural network development. Maturation of SEF reflects sleep-state development which is delayed by LPS. These data demonstrate time dependent differences in fetal physiology that can be altered by inflammation.

HRC New Zealand

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

#### PCA067

### Intrauterine growth restriction as a consequence of increased maternal salt intake in mice

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**Motivation:** Low birth weight is associated with an increased risk for cardiovascular disease in later life. Perinatal high salt intake, as typically observed in mothers consuming a "Western diet", may be a stress factor leading to intrauterine growth restriction. The aim of this study is to establish a causative linkage between high salt consumption during the perinatal period and an elevated risk for cardiovascular disease in later life.

**Methods:** Timed mating was performed between female C57Bl/6j mice at an age of 8-12 weeks and male Balb/c mice. Female mice were either fed normal-salt diet (NS, 2.2 g Na<sup>+</sup>/kg) or high-salt diet (HS, 30 g Na<sup>+</sup>/kg). The high-salt intake started with a successful mating (HS1) or 3 weeks before mating (HS2). At gestational day 13.5, the fetal and the placental

weight were taken. Minimum litter size was defined as 8 fetuses per dam.

**Results:** Litter size did not differ between treatments. Increasing the salt intake significantly reduced fetal weights in both experimental groups as compared with the weight of the offspring from normal-salt diet fed dams (NS: 151.5 $\pm$ 1.24 mg, n=79; HS1: 143.0 $\pm$ 1.29 mg, n=56; HS2: 141.8 $\pm$ 1.78 mg, n=37; p<0.0001 compared by 1-way ANOVA). Placental weights were also significantly reduced by high salt administration in both experimental groups (NS: 89.4 $\pm$ 2.0 mg, n=25; HS1: 76.4 $\pm$ 2.7 mg, n=13; HS2: 78.1 $\pm$ 1.6 mg, n=21 p<0.001 compared by 1-way ANOVA).

**Conclusion:** These data indicate that an increased maternal salt intake during pregnancy leads to growth restriction in mice on gestational day 13.5. A reduced placental weight might be an indicator of placental malfunction with consequences on nutrition of the fetus. Whether litter is small for gestational age during the whole time of pregnancy, whether we can observe a reduction in birth weight and whether we find cardiovascular consequences in later life remains to be elucidated.

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#### PCA068

### Functional adaptation of the preterm lamb diaphragm in the first week of life is not influenced by *in utero* lipopolysaccharide exposure

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*In utero* inflammation exacerbates preterm diaphragm weakness at birth (Song et al. 2013). Persistent diaphragm dysfunction, resulting from immaturity and *in utero* inflammation, may predispose preterm infants to postnatal respiratory failure. We determined the combined effects of preterm birth and *in utero* inflammation on postnatal diaphragm function in lambs. Lambs were exposed to intra-amniotic saline (n=8) or 4mg lipopolysaccharide (LPS; n=9) 48h before caesarean delivery at 129d gestational age (GA; term=150d). Before delivery, ewes were pre-medicated with Acepromazine (0.05mg/kg, IM) and Buprenorphine (0.01mg/kg, IM) prior to anesthetic induction with Thiopentone (15mg/kg, IV) and maintenance with isoflurane (1.5-2.5%). After delivery, lambs were resuscitated, commenced mechanical ventilation immediately and weaned as early as possible. Lambs were killed (pentobarbitone, 150mg/kg IV) at 7d postnatal age (PNA). Fetal control lambs were delivered at 136d GA and killed immediately (n=7). The diaphragm was excised for *in vitro* assessment of contractile function and snap-frozen in liquid nitrogen for biochemical analysis. Data are presented as mean  $\pm$  S.D. and compared by ANOVA. There were no significant differences in diaphragm contractile function, myosin heavy chain fibre expression, markers of inflammation, protein metabolism or oxidative status, between saline and LPS groups. However, contractile properties of the saline and LPS groups were significantly different to the fetal control group, indicating postnatal adaptations to extra-uterine life.

Maximum specific force ( $N/cm^2$ ) was significantly higher in the saline group ( $18.77 \pm 1.99$ ) compared to the fetal control group ( $14.25 \pm 2.08$ ;  $p=0.007$ ). Time to peak twitch force (ms) was significantly shorter in the saline ( $117.0 \pm 15.0$ ) and LPS groups ( $110.3 \pm 9.9$ ) compared with the fetal control group ( $163.8 \pm 22.2$ ;  $p<0.001$ ). Similarly, half-relaxation time (ms) was significantly shorter in the saline group ( $172.9 \pm 41.2$ ) and LPS group ( $151.6 \pm 21.9$ ) compared with the fetal control group ( $230.3 \pm 29.0$ ;  $p<0.01$ ). Importantly, LPS lambs had a greater reliance on mechanical ventilation than saline lambs (range: LPS=4-143h, saline= 3-54h). Furthermore, duration of mechanical ventilation exhibited a significant negative correlation with maximum specific force ( $R^2=0.528$ ;  $p<0.01$ ; Pearson correlation) explaining 51% of the variation observed in maximum diaphragm force at 7d PNA, independent of LPS exposure. Although LPS-induced diaphragm weakness at birth did not persist at 7d PNA, the increased reliance on mechanical ventilation after *in utero* inflammation highlights potential interactions between clinical complications associated with preterm birth that may affect diaphragm function and contribute to the development of respiratory failure during early postnatal life.

Song et al. 2013. *Am J Respir Cell Mol Biol* 49, 866–874.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCA069

##### Novel Epo regulating transcription factors

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Erythropoietin (Epo) is the key hormone promoting red blood cell production in response to hypoxia and anemia. While the kidneys are the primary site of adult Epo synthesis, the liver is the main source during development. Epo is induced by the activation of the hypoxia-inducible factor (HIF) pathway in response to hypoxia and anemia, contributing to restore the normal plasmatic oxygen levels (Wenger et al., 2010). However, under pathological conditions, such as erythrocytosis, Epo plasma levels are high, leading to a dramatic increase of the red blood cell mass and resulting in blood hyperviscosity and thrombosis. Whereas a number of mutations in genes involved in the oxygen-sensing pathway have been identified, for the majority of congenital secondary erythrocytosis patients the cause remains unknown (Keohane et al., 2013; McMullin et al., 2008).

We previously identified a functional distal hypoxia response element (5'-HRE) within the so-called kidney-inducible element, which is exploited in the current study to hunt for additional Epo regulating mechanisms (Storti et al., 2014). Apart from the hypoxia-inducible factor 2-alpha (HIF-2 $\alpha$ ), the transcription factors GATA and the hepatocyte nuclear factor 4-alpha (HNF4a) no other transcription factors have been shown to regulate Epo expression (Jelkmann et al., 2010). The tight tissue-specific and conditional control of Epo transcriptional regulation strongly suggests that other transcription factors might be involved in its expression regulation.

An improved understanding of the molecular mechanism underlying Epo regulation is of major importance in view of the current development of various therapeutic strategies to modulate endogenous Epo levels.

To identify novel factors regulating Epo production we are sequencing various regulatory regions of the Epo locus in patients with unknown cause of erythrocytosis. In a complementary approach we employ a 5'-HRE-dependent reporter gene assay screening procedure for transcription factors regulating Epo expression. Identified factors will be studied on their potential regulation of endogenous Epo expression under siRNA interference conditions and endogenous DNA binding will be validated by ChIP assays. Moreover, co-over-expression studies of candidate factors with various HIF-2 mutants found in patients with congenital erythrocytosis will also be performed.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCA070

##### Unbiased assessment of input function for positron emission tomography molecular imaging

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Positron emission tomography (PET) is commonly used for diagnosis and evaluation of treatment efficiency. Two elements are required for dynamic quantitative PET data analysis; The activity of the region of interest (ROI) as a function of time after tracer injection, and the plasma activity as a function of time (PAF). For accurate assessment of PAF, the activity measured in the blood sample collected during the PET scanning is required. However, this is an invasive and time-consuming procedure. In this study, we aimed to develop an unbiased method to determine the PAF using the PET-signal from the abdominal vasculature. We verified our PAF by calculating PET labelled Cytochrome C clearance from kidney ( $C_{Cyt}$ ) both from PET signal and ex vivo samples. Wistar male rats (350-450g, n=8) were anaesthetized with sevoflurane (3% volume and 1l/min oxygen flow). The femoral artery was cannulated for blood sampling and the tail vein for tracer injection. After i.v. injection of a bolus of PET labelled Cytochrome C (5-10 MBq), the rats were scanned dynamically for 10 minutes. During the PET-CT scanning, seven arterial blood samples (20ml) were collected at time 30 seconds, 1, 2, 4, 5, 7, 9 minutes for ex vivo verification. Immediately following the PET scanning, the rats were killed and their kidneys removed. Both blood samples and kidneys were gamma counted.  $C_{Cyt}$



was calculated as the total tracer activity in one kidney per ml volume or gram divided by the time integrated PAF. In another series, Wistar male rats (n=6) underwent contrast CT before the PET-CT dynamic scanning to measure kidney volume and abdominal vessel diameter. We found a good correlation ( $r^2=0.99$ ) between the mean intensity of the kidney and the ROI-volume calculated by the PET-software auto-segmentation tool using a range of standardized uptake values (SUV). Similarly, there was a good correlation ( $r^2=0.99$ ) between the mean intensity from abdominal blood vessels and their ROI-volumes. We used CT to obtain anatomical dimensions for kidney and abdominal blood vessels to decide an unbiased value for the time integrated plasma activity as well as the kidney activity per unit volume from the correlations described above. Contrast CT gave a kidney volume (mean  $\pm$  SEM) of  $1227 \pm 23.7 \text{ mm}^3$ , and an abdominal vessel diameter of  $2.51 \pm 0.06 \text{ mm}$  that were used in the PET-based clearance calculations. PET-CT assessed  $C_{\text{Cyt}}$  correlated well with  $C_{\text{Cyt}}$  calculated from ex vivo blood and kidney samples ( $r^2=0.90$ ). We here present a new method showing that an unbiased input signal can be used in the analysis of dynamic PET images.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCA071

### Role of renal afferent nerves in injury and inflammation of the kidney

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Renal inflammation is associated with deranged cardiovascular homeostasis leading to hypertension. Inflammatory cytokines such as  $\text{TNF-}\alpha$  play a key role in initiating responses from the diseased kidney which ultimately results in dysregulation of blood pressure control mechanisms. Male Wistar rats (275-350g, n=23) were anaesthetised with  $\alpha$ -chloralose: urethane anaesthesia ( $3 \text{ ml kg}^{-1}$ ) and the right femoral artery and vein were cannulated to allow measurement of mean arterial pressure (MAP) and heart rate (HR), and infusion of saline and supplemental anaesthetic. The right kidney was exposed via a flank incision and a cannula was inserted into renal corticomedullary border to allow infusion of saline,  $\text{TNF-}\alpha$  ( $2 \mu\text{g kg}^{-1} \text{ h}^{-1}$ ) or capsazepine (CPZ), a  $\text{TRPV}_1$  blocker at  $17 \text{ ml min}^{-1}$ . Animals were allowed to stabilise for  $\sim 45$  minutes. Experimental protocol comprised two phases during which intrarenal infusions of either saline or a drug was done. During each phase, a baroreflex gain curve was generated by I.V. phenylephrine and sodium nitroprusside ( $50 \mu\text{g kg}^{-1} \text{ min}^{-1}$ ) to increase and decrease blood pressure, respectively. In a group of rats, the renal nerve activity from the ipsilateral kidney was blocked by perfusing lidocaine (5%), a local anaesthetic on the renal artery at  $17 \text{ ml min}^{-1}$  for 10 minutes using a small diameter cannula. A renal nerve bundle running close to the renal artery of the contralateral kidney was dissected free from connective tissues, placed on bipolar electrodes and used to record renal sympathetic nerve activity (RSNA). At the end of the experiment, the animals were euthanised and the background noise was recorded. Data were expressed as mean  $\pm$  S.E.M. and compared using student's t-test and ANOVA with significance at  $P < 0.05$ . Intrarenal infusion of  $\text{TNF-}\alpha$  blunted ( $P=0.005$ ) the sensitivity of the baroreflex by almost 65% ( $0.09 \pm 0.04$  vs.  $0.25 \pm 0.01 \mu\text{V.s mmHg}^{-1}$ ). However,

when  $\text{TNF-}\alpha$  was infused intrarenally following nerve activity blockade by lidocaine there was an approximate 75% increase ( $P=0.06$ ) in the sensitivity of the baroreflex. The blunted sensitivity of the baroreflex to intrarenal  $\text{TNF-}\alpha$  was restored to near normal levels ( $P=0.025$ ) when  $\text{TNF-}\alpha$  was infused in the presence of a background infusion of CPZ ( $0.20 \pm 0.04$  vs.  $0.10 \pm 0.02 \mu\text{V.s mmHg}^{-1}$ ). The results from this study indicate that  $\text{TNF-}\alpha$ , a pro-inflammatory mediator evokes a renal nerve-mediated response that adds to a blunted baroreflex control mechanism. This suggests that in renal injury and disease the generation of inflammatory cytokines has the potential of initiating activity within the renal afferent nerves which can ultimately modulate the blood pressure control mechanism.

I would like to give my sincerest thanks to my supervisor, Dr Mohammed Abdulla, for providing me with this research opportunity. I am very grateful to him for sharing his expertise, his patience, attention and time throughout this project. I would like to thank him for his encouragement and guidance to apply for this chance to present my work.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCA072

### Screening asymptomatic school children for early asthma by determining mild airway narrowing through PEFR measurement

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Peak expiratory flow rate (PEFR), has widely been used as a predictor of treatment of asthma patients. The serial PEFR measures airway variability and is usually the most appropriate first-line investigation in workers suspected of having occupational asthma.

Measurement of peak expiratory flow (PEF) and forced expiratory volume in first second ( $\text{FEV}_1$ ) are most useful parameters for the diagnosis and exclusion of asthma. Studies even recommend measurement of predicted peak expiratory flow values, as an alternative to spirometry.

Spirometry is not often available in the primary care setting and economic factors may limit referrals for its testing.

Mild airway narrowing may be present in asymptomatic children, which can be identified by determining their PEFR. We hypothesized that children with  $<80\%$  of the predicted values of PEFR will show abnormal lung functions on spirometry which will confirm the diagnosis of obstructive disease in asymptomatic children. This will enable us to initiate early treatment.

So we planned this study to detect evidence of mild airway obstruction in asymptomatic children through PEFR and further, to evaluate pulmonary functions by digital spirometer in children showing airway narrowing.

We selected 200 asymptomatic children (123 males & 77 females) of age group 10-15 years without history of smoking, tuberculosis or any other acute or chronic respiratory illness. A detailed family history about asthmatic symptoms like

shortness of breath, wheezing, cough, heaviness of chest and night time exacerbations of symptoms, was sought. 47 children gave a positive family history of asthma.

PEFR values were then recorded in standing position, using the Mini Wright Peak Flow meter.

40 children showed PEFR values less than 80% of their predicted values. To confirm whether the low observed values were because of airway obstruction, we performed their spirometry by digital spirometer (Cosmed microquark). Out of these 40 children, only 9 had a family history of asthma. This means that 9 out of 47 (19.14%) children coming from asthmatic families and 20.26% (31 out of 153) children from non-asthmatic families, showed PEFR values below 80% of the predicted value. ( $p>0.05$ )

Considering a decrease in the  $FEV_1/FVC$  ratio to  $<80\%$  for diagnosis of obstructive disease, only 2 out of 35 children were found to have it.

The data was found to be non-parametric by Shapiro-Wilk test. Spearman test to see correlation, if any, between PEFR and  $FEV_1/FVC$  ratio was found not significant with  $r = 0.314$  and  $p = 0.065$ .

We conclude that asymptomatic children with low PEFR values may not show abnormal lung functions on spirometry. However, these children, particularly those having the risk of family history of asthma, may be followed for development of airway obstruction.

The study was funded by AIIMS, Jodhpur.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCA073

##### Oral anethole improves respiratory function in an ovalbumin-induced asthma model

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*Croton zehntneri* Pax et Hoffm. is a Euphorbiaceae species, popularly known as “canela de cunhã”, a native plant from Northeastern Brazil that produces anethole, an essential oil popularly used as an anti-inflammatory and antioxidant agent. Due to its importance and complexity, asthma has been extensively studied. However, numerous questions related to its pathophysiology have not been answered yet, and the development of new therapeutic agents has been limited. The present study investigated the therapeutic outcomes of anethole in an OVA-induced asthma model. The study was approved by our institutional animal ethics committee (Ceará State University, N° 3114549/2014). 32 BALB/c mice were used, randomly divided into four groups ( $n=8$ ). Two groups were sensitized and challenged with saline and treated with oral anethole (300 mg/kg) (SA) or Tween 80 0.1% (ST). The OT and OA groups were sensitized and challenged with ovalbumin and treated with oral anethole (300 mg/kg) (OA) or Tween 80 0.1% (OT). 24 hours after the treatment, the animals were anesthetised with sodium pentobarbital (50 mg/kg, i.p., Hypnol® 3%, Syntec, Brazil), tracheostomised, connected a mechanical ventilator, and paralysed with pancuronium bromide (0.5 mL/kg, i.p., Cristália, Brazil). Respiratory mechanics was assessed by the forced oscillation technique and quasi-static PV (pressure-volume) curve. Values are means  $\pm$  S.D., compared by ANOVA. The respiratory mechanical properties

determined by the forced oscillation technique were: Newtonian resistance ( $R_N$ ): ST =  $0.143 \pm 0.015$ , SA =  $0.134 \pm 0.022$ , OT =  $0.252 \pm 0.047$  and OA =  $0.159 \pm 0.034$  cmH<sub>2</sub>O/mL/s; tissue damping (G): ST =  $3.31 \pm 0.38$ , SA =  $3.14 \pm 0.36$ , OT =  $6.41 \pm 1.02$  and OA =  $3.37 \pm 0.47$  cmH<sub>2</sub>O/mL; and tissue elastance (H) ST =  $12.27 \pm 1.56$ , SA =  $12.13 \pm 1.53$ , OT =  $21.36 \pm 2.40$  and OA =  $13.10 \pm 1.47$  cmH<sub>2</sub>O/mL. The pressure-volume (PV) curve led to an estimated inspiratory capacity (IC) of: ST =  $1.15 \pm 0.16$ , SA =  $1.17 \pm 0.15$ , OT =  $0.79 \pm 0.13$  and OA =  $1.11 \pm 0.20$  mL; static compliance ( $C_{ST}$ ) of: ST =  $0.123 \pm 0.010$ , SA =  $0.125 \pm 0.011$ , OT =  $0.072 \pm 0.016$  and OA =  $0.115 \pm 0.022$  mL/cmH<sub>2</sub>O; and PV loop area of: ST =  $2.30 \pm 0.48$ , SA =  $2.21 \pm 0.48$ , OT =  $4.03 \pm 0.74$  and OA =  $2.41 \pm 0.55$  cmH<sub>2</sub>O.mL. We found significant impairment ( $p<0.05$ ) in all respiratory mechanics parameters in OT mice compared to the ST group, thus confirming the OVA-induced lung injury. The comparison of OA with OT ( $p<0.05$ ) demonstrated that anethole was able to improve respiratory function in the OVA-induced asthma group. In conclusion, our results demonstrated that anethole was able to improve the respiratory function in the OA group compared with OT. The present study serves as a starting point for conducting research with anethole to validate its putative use as an alternative asthma therapy.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCA074

##### Genetic ablation of the newly identified androglobin leads to primary ciliary dyskinesia

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Globins are small globular metallo-proteins, involved in different cellular functions via their reversible binding capacity to gaseous ligands (O<sub>2</sub>, CO and NO) and their storage, transport and detoxification (1). Besides the well-established hemoglobin and myoglobin, various new globin types have been discovered in vertebrates, including neuroglobin (2), cytoglobin (3), and more recently also androglobin (Adgb) (4). Adgb is a chimeric protein, consisting of a calpain-like domain and a globin-like domain, and is mainly expressed in testis tissue (4). In this study, we aimed to analyse the *in vivo* function of Adgb, by investigating Adgb knockout mice. We could observe that knockout animals develop symptoms of primary ciliary dyskinesia (PCD), a genetic disorder that causes abnormal cilia formation and function (5). All knockout male mice display infertility, as a consequence of abnormal sperm flagella formation. Consistently, FACS analysis revealed abnormal elongating spermatids and spermatozoa. Interestingly, we could also demonstrate a strong downregulation of Adgb expression levels in infertile men. Moreover, in roughly 20% of knockouts, we observed development of hydrocephalus within the two first months after birth. We could also notice increased accumulation of mucus in the sinus of these mice. Finally, we encountered few cases of polycystic kidney disease and cardiac hypertrophy. Ongoing electron microscopy analysis of trachea will enable the detection of possible abnormalities of the ciliary structure. Collectively, these results suggest that Adgb represents an essential protein for the formation and/or function of cilia, and that its genetic deletion leads to PCD. Ongoing *in vitro* approaches should

help identifying the underlying molecular mechanisms that link this globin to cilia formation.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA075

### Time-dependent acute lung and spermatotoxicity of exposure to C<sub>60</sub> fullerene in mice

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C<sub>60</sub> fullerene (C<sub>60</sub>) is released into the atmosphere by anthropogenic and natural sources. Scanty information exists regarding its toxicological effects on respiratory and reproductive systems. We aimed to study the time course of C<sub>60</sub>-triggered pulmonary and spermatotoxic impairments until 4 days after exposure. The study was approved by our institutional Ethics Committee on the Use of Animals (license: 139/16) and conducted according to ARRIVE guidelines and national legislation. 64 male BALB/c mice randomly composed 8 groups (n=8/group). They were anesthetized (sevoflurane) via a nasal cone, intratracheally instilled with 30 µL of vehicle (1.0% polysorbate 80 in 0.9% NaCl) or 30 µL of C<sub>60</sub> (1.0 mg/kg), and studied at 12, 24, 72 and 96 h (F12, F24, F72 and F96 groups and their corresponding controls) after exposure. At each time point, the respective groups underwent sedation (diazepam, 1 mg ip), anaesthesia (pentobarbital sodium, 20 mg/kg ip) and muscle paralysis (pancuronium bromide, 0.1 mg/kg iv). Since no difference was found among the 4 control groups (ANOVA), 2 animals randomly picked from each one composed one single control group (CTRL). Lung mechanics and morphological parameters were determined by a forced oscillation technique and microscopy, respectively. Antioxidant enzymes, oxidative damage and inflammatory cytokines were determined in lung homogenate. Number, motility and vitality of epididymal sperm were determined. Values are means ± S.D., and groups were compared by ANOVA, α = 5%. Morphofunctional and inflammatory data presented peak impairment at 24 h and subsequent decline until 96 h after instillation: tissue damping (CTRL: 2.8±0.5 vs. F24: 4.1±0.4 vs. F96: 2.7±0.4 cmH<sub>2</sub>O/mL), elastance (CTRL: 21.7±0.8 vs. F24: 30.8±1.1 vs. F96: 23.0±2.7 cmH<sub>2</sub>O/mL), area of collapsed alveoli (CTRL: 9.0±1.7 vs. F24: 29.8±1.5 vs. F96: 20.7±2.0 % of total area), mononuclear cells (CTRL: 5.9±0.6 vs. F24: 9.6±0.8 vs. F96: 6.6±0.3 cells/10<sup>3</sup>/mm<sup>2</sup> of tissue area), TNF-α (CTRL: 130.1±15.2 vs. F24: 549.2±58.0 vs. F96: 223.5±20.2 ng/mL) and IL-6 (CTRL: 58.9±2.1 vs. F24: 117.0±23.6 vs. F96: 91.9±9.7 ng/mL). There was a time-dependent imbalance in catalase activity (CTRL: 5.6±0.4 vs.

F96: 8.7±0.7 U/mg protein), superoxide dismutase (CTRL: 15.4±0.6 vs. F96: 10.7±1.1 U/mg protein) and thiol (CTRL: 0.12±0.01 vs. F96: 0.08±0.007 nmol reduced 5,5'-dithiobis-(2-nitrobenzoic acid)/mg protein). Sperm count (CTRL: 38.1±11.8 vs. F12: 26.8±3.3 vs. F96: 15.8±2.2 x 10<sup>5</sup> spermatozoa/mL), motility (CTRL: 58.7±4.9 vs. F96: 37.6±4.4 % of total count) and vitality (live:dead ratio, CTRL: 2.0±0.1 vs. F24: 0.4±0.1 vs. F96: 0.2±0.05) diminished progressively. In conclusion, possibly a single dose of C<sub>60</sub> can induce morphofunctional, inflammatory changes (peak at 24 h) and redox imbalance in mice lungs (highest at 96 h) and progressively increase the risk of infertility.

Supported by: CNPq, FAPERJ, FUNCAP

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## PCA076

### Effects of olprinone on airway reactivity and inflammation in ovalbumin-sensitized guinea pigs

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The effectiveness of selective phosphodiesterase (PDE) 4 inhibitors, e.g. roflumilast, has been confirmed previously in chronic obstructive pulmonary disease associated with neutrophil as well as eosinophilic inflammation. As the selective inhibition of PDE3 (present predominantly in airway smooth muscle and responsible for degradation of cAMP and cGMP), the aim of this study was to evaluate effects of olprinone (selective PDE3 inhibitor) in two different doses and dosing regimens on *in vivo* and *in vitro* airway smooth muscle reactivity to histamine, changes in blood cells count in blood and bronchoalveolar lavage (BAL) fluid, and some inflammatory mediators (interleukin (IL)-4 and IL-5) in ovalbumin sensitized guinea pigs with experimentally induced eosinophil inflammation (Mokry et al. 2017). Sensitized animals were administered 0.5 or 1.0 mg/kg b.w. of olprinone intraperitoneally as a single dose or once daily for 7 days. Sensitization with ovalbumin led to a significant increase in *in vivo* and *in vitro* airway reactivity (both p<0.01). Similarly, increased plasmatic levels of IL-4, and IL-5 in blood were observed (p<0.01), with significant increase in differential counts of eosinophils both in blood (p<0.05) and BAL fluid (p<0.01). The administration of olprinone suppressed significantly *in vivo* airway reactivity to histamine (p<0.01), and *in vitro* airway reactivity in tracheal tissue strips to cumulative doses of histamine (p<0.05). However, the IL-4 and IL-5 levels and changes in relative counts of inflammatory cells were only mild (p>0.05), suggesting that PDE3 inhibition has preferentially bronchorelaxing effects over anti-inflammatory action in guinea pigs with ovalbumin induced eosinophil inflammation. Inhibition of PDE3 could be considered as a potentially useful therapeutic tool for bronchodilation in obstructive airways diseases associated with allergic inflammation.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCA077

### Effect of hypoxia on adiponectin pathway in murine and cellular models: which involvement in COPD-associated cardiovascular risk ?

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**Introduction:** Modulation of adiponectin plasmatic level ( $Ad_{pl}$ ) was previously found to vary differently in COPD (Chronic Obstructive Pulmonary Disease) patients, probably due to the heterogeneity of the disease <sup>1,2</sup>. Hypoxaemia is a component of respiratory diseases frequently observed in severe COPD. It initiates compensatory mechanisms mainly mediated by a family of transcription factors (Hypoxia Inducible Factors HIFs) <sup>3</sup>. Hypoxaemia was suggested to modulate Ad pathway but mechanisms are unclear <sup>4,5</sup>. Due to its anti-diabetic, anti-inflammatory and anti-atherosclerotic properties <sup>4</sup>, we postulate that alteration of Ad pathway could participate to metabolic troubles and cardiovascular co-morbidities in COPD patients.

**Methods and Results:** To better understand the specific impact of hypoxaemia on Ad pathway, we used a mouse model of chronic hypoxaemia (CH) (n=13,  $FiO_2$  = 10%, 8h/day). Exposure to hypoxaemia for 35 days resulted in (i) an increased level of high MW multimers (CH :  $12,879 \pm 0.881$  % vs control :  $10,202 \pm 0.586$  % ; Mean  $\pm$  SEM, p<0.05, T-Test) without change in Ad plasmatic level (CH :  $8694 \pm 470$  ng/ml vs control :  $8520 \pm 528$  ng/ml ; Mean  $\pm$  SEM, NS, T-Test) (ii) a tissue-specific reduction of AdipoR protein level without change in mRNA expression. Given the role of macrophages in the atherogenic risk, we also evaluated AdipoR abundance in RAW murine macrophages exposed to low oxygen level (CH, n=4,  $PO_2$  = 2%  $P_{atm}$ , 24h). Exposure to hypoxia *in vitro* reduced AdipoR1/2 level (AdipoR1: fold change = 0.47; AdipoR2: fold change = 0.36, p<0.05, T-Test) as well as the abundance of p-AMPK, a metabolic sensor involved in Ad signalling pathway (fold change = 0.41, p<0.05, Mann-Whitney Rank Sum Test). To investigate the contribution of HIF-1 $\alpha$  in the effect of hypoxia on AdipoR2 abundance, macrophages were exposed to the chemical HIF-1 $\alpha$  inducer  $CoCl_2$  (n=6, 5 $\mu$ M, 24h). As expected, macrophages exposed to  $CoCl_2$  increased HIF-1 $\alpha$  abundance (fold change = 3.75 ; p<0.001, T-Test), and decreased AdipoR2 level (fold change = 0.68 ; p<0.05, T-Test). Investigations of the effect of shRNA-mediated HIF-1 $\alpha$  gene silencing on AdipoR2 abundance are ongoing.

**Conclusion:** Chronic hypoxaemia, per se, modifies Ad oligomerisation state and AdipoR protein level *in vivo* and in macrophages *in vitro*. These effects could be partly linked to HIF-1 $\alpha$  activation during adaptive response to hypoxia and could influence the cardiovascular risk in hypoxemic COPD patients.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCA078

### Knockout of caveolin-1 delays calcium signalling in human alveolar type I epithelial cells

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Secretion of pulmonary surfactant from alveolar type II epithelial cells is essential to maintain alveolar homeostasis and gas exchange within the lung. Earlier studies showed that mechanical forces are important for alveolar homeostasis and stretching of the epithelial cells during inspiration is believed to be the main stimulus for surfactant secretion. Yet the precise mechanisms how mechanical forces result in surfactant secretion are still elusive. The alveolar epithelium consists of alveolar type I (ATI) and type II (ATII) epithelial cells and both have been shown to respond to stretching. Moreover, paracrine signals from ATI cells can stimulate a rise in intracellular  $Ca^{2+}$  ( $Ca^{2+}_i$ ) in ATII cells, the main stimulus for surfactant secretion. Caveolae, small plasma membrane invaginations present in ATI but absent in ATII cells, function as membrane buffers and may play a role as mechanosensors. To investigate the impact of caveolae on cell stiffness and response to mechanical stress we established a CRISPR/Cas9-induced human caveolin-1 knockout (CAV1 KO) ATI cell line. The cells were cultured on flexible substrates (PDMS membranes) in mono- or co-culture with primary ATII cells. The cells were stretched intermittently with increasing amplitudes to a maximum of 75 % longitudinal distension. FURA-2 assays to measure changes in  $Ca^{2+}_i$  indicated that ATI WT cells respond more frequently (20.72 % responders) and at lower thresholds (20 % stretch) to mechanical stress than KO cells (4.98 % responders, 40 % stretch) suggesting that caveolae serve as a hub for downstream Ca-signalling in response to mechanical deformation of ATI cells. Moreover, using confocal microscopy, we quantitatively investigated the colocalization and amount of CAV1 and PTRF, both caveolar proteins, in the plasma membrane of stretched (15, 25, 50, 75 % stretch) versus unstretched ATI WT cells. The colocalization of both proteins was significantly reduced when membranes were stretched above 50 %. This

confirms the idea that caveolae disassemble and proteins are released from the plasma membrane when mechanical forces appear. These data further add to the idea that caveolae within ATI cells are potential mechanosensors in the alveolar epithelium.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA079

**Effects of high-frequency oscillatory ventilation vs conventional mechanical ventilation on lung function, inflammation and apoptosis in meconium-induced acute lung injury**

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Choice of appropriate ventilation mode in severe meconium aspiration syndrome (MAS) is still discussed. Therefore, this study compared short-term effects of high-frequency oscillatory ventilation (HFOV) and conventional mechanical ventilation (CMV) provided by two conventional ventilators on the respiratory parameters, inflammation and lung cell apoptosis in experimental model of MAS to find out which of these modes is superior.

Anaesthetized, meconium-instilled New Zealand young rabbits were oxygen-ventilated with HFOV (SLE5000, SLE Ltd., UK; MAS-HFOV, n=7), or with CMV supplied by ventilator SLE5000 (MAS-CMV-S, n=7) or ventilator Aura V (Chirana, Slovakia; MAS-CMV-A, n=7) for 4 hours. Other animals received saline instead of meconium and were CMV-ventilated by ventilator Aura V for 4 hours (VentC, n=7), or served as non-ventilated controls (C, n=6). Blood gases and respiratory parameters were regularly measured. At the end of experiment, left lung was saline-lavaged and protein content, and total and differential cell counts in bronchoalveolar lavage fluid were determined. Wet/dry lung weight ratio as a marker of edema formation was determined. Apoptosis of alveolar and bronchial lung cells was estimated immunohistochemically. Proinflammatory cytokines (TNF $\alpha$ , IL-1 $\beta$ , IL-8) in plasma and lung tissue were detected by enzyme-linked immunosorbent assay. Meconium instillation caused deterioration in gas exchange, neutrophil-mediated inflammation, lung edema formation, and lung cell apoptosis. Gas exchange was slightly enhanced in HFOV than in CMV, but the between-group differences were not significant. No significant differences among the meconium-instilled and HFOV- or CMV-ventilated groups were found in markers of lung edema formation or inflammation. However, higher number of caspase-3 immunoreactive cells in bronchial epithelium in MAS-HFOV group compared to non-ventilated and ventilated controls can indicate higher susceptibility of bronchial cells for apoptosis in HFOV. Both lung-protective ventilations (HFOV and CMV) supplied sufficient gas exchange with comparable impact on the lung, with no clear preference for any of the ventilation modes.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA080

**NADPH oxidase 2 is necessary for chronic intermittent hypoxia-induced respiratory muscle weakness in adult male mice**

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Obstructive sleep apnoea syndrome (OSAS), the most prevalent form of sleep-disordered breathing (SDB), is characterized by exposure to chronic intermittent hypoxia (CIH), as a consequence of repetitive occlusions of the upper airway in patients during sleep. CIH evokes redox changes culminating in impaired upper airway and diaphragm muscle function. There is a paucity of information regarding the molecular mechanisms underlying these effects. We sought to investigate the putative role of the superoxide-generating NADPH oxidase 2 enzyme in CIH-induced respiratory muscle dysfunction.

A mouse model of CIH was generated by the cycling of gas from normoxia (21% O<sub>2</sub>) for 210 seconds to hypoxia (5% O<sub>2</sub>) at the nadir over 90 seconds for 8hr/day during light hours for 2 weeks. 10-11 week old male (C57BL/6J) mice were assigned to one of 3 groups: normoxic controls (sham; n=24), CIH-exposed (n=24) and CIH+apocynin (NADPH oxidase 2 inhibitor, 2mM; n=24) given in the drinking water throughout the CIH exposure. In separate studies, 10-11 week old NOX2 null (B6.129S-Cybb<sup>tm1Din</sup>/J) male mice were assigned to a sham (n=12) or CIH (n=12) exposure. Sternohyoid and diaphragm muscle contractile function was examined *ex vivo*. Gene expression was examined by qRT-PCR. Western blot was used to measure protein expression. NOX enzyme activity was determined using a spectrophotometric assay. All values are expressed as mean  $\pm$  SD and data were statistically compared by unpaired Student t-test.

Exposure to CIH significantly decreased sternohyoid ( $9.4 \pm 1.6$  vs.  $5.2 \pm 1.4$  N/cm<sup>2</sup>;  $p < 0.001$ ) and diaphragm ( $22.6 \pm 5.6$  vs.  $12.5 \pm 2.3$  N/cm<sup>2</sup>;  $p < 0.01$ ) muscle peak specific force by ~45% compared with sham exposure. CIH significantly increased NOX enzyme activity in the sternohyoid ( $0.6 \pm 0.1$  vs.  $0.8 \pm 0.1$  slope/mg/hr;  $p < 0.01$ ), with no significant alterations in gene or protein expression of NOX subunits compared with sham. CIH decreased the expression of p22phox ( $1.1 \pm 0.4$  vs.  $0.6 \pm 0.4$  mRNA fold change relative to control;  $p < 0.05$ ) and rac ( $1.2 \pm 0.6$  vs.  $0.7 \pm 0.3$  mRNA fold change relative to control;  $p < 0.05$ ) genes and increased protein expression of NOX4 ( $0.5 \pm 0.2$  vs.  $1.0 \pm 0.2$  O.D./Ponceau S (a.u.);  $p < 0.01$ ) in the diaphragm, while NOX activity remained unchanged compared with sham. Administration of apocynin and NOX2 gene knockout completely prevented CIH-induced sternohyoid and diaphragm muscle weakness.

Mice show signs of profound respiratory muscle dysfunction following exposure to 2 weeks of CIH. The putative NOX inhibitor, apocynin, prevents CIH-induced respiratory muscle weakness. Studies in NOX2 null mice reveal that NOX2 is necessary for CIH-induced sternohyoid and diaphragm muscle weakness. Our results have implications for human OSAS.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA081

**Effects of pharmacological Gq protein inhibition on airway remodeling in chronic asthma**J.M. Dietrich<sup>1</sup>, A. Simon<sup>1</sup>, M. Matthey<sup>1</sup>, G. König<sup>2</sup>, B.K. Fleischmann<sup>1</sup> and D. Wenzel<sup>1</sup><sup>1</sup>Physiology 1, University of Bonn, Bonn, Germany and<sup>2</sup>Pharmaceutical Biology, University of Bonn, Bonn, Germany

G proteins are important modulators of (patho)physiological functions in many organs. In the lung, the Gq subtype is of particular relevance, as it is a key mediator of smooth muscle tone regulation in airways and also involved in chronic lung diseases such as allergic asthma. This disease is characterized by airway hyperresponsiveness, inflammation and remodeling comprising collagen deposition, goblet cell hyperplasia as well as smooth muscle thickening. Even though the severity of asthma appears to be linked to airway remodeling, currently available drugs cannot efficiently reduce this process. We therefore wondered, whether the pharmacological pan-Gq protein inhibitor FR900359 (FR) could modulate airway remodeling.

The effect of FR was investigated in a mouse model of chronic ovalbumin (OVA)-induced asthma: Balb/c mice were sensitized with i.p. injections of 20 µg OVA and 2 mg Alum on days 0 and 14. Then, mice were challenged with 1% OVA three days a week until day 37. FR or vehicle was applied one hour before every OVA challenge *via* intratracheal application (5 Vol% isoflurane, p.i.). For functional analysis mice were injected i.p. with fentanyl (50 µg/kg), medetomidine (0.5 mg/kg), midazolam (5 mg/kg), vecuronium (0.1 mg/kg) and carprofen (5 mg/kg s.c.) and airway resistance was determined with a FlexiVent system. For the *in vitro* assessment of FR effects on airway smooth muscle a cell growth assay using human bronchial smooth muscle cells (hBSMCs) was used.

In our chronic asthma model airway hyperresponsiveness could be observed, when airway resistance was compared to healthy mice upon methacholine (MCh) challenge. We also found that local pulmonary application of FR strongly reduced the MCh-induced increase of airway resistance in OVA mice (mean±S.E.M.: 4.2±0.5 cmH<sub>2</sub>O/s/mL at 50 mg/ml MCh, n=5) compared to vehicle treated controls (10.8±1.9 cmH<sub>2</sub>O/s/mL, n=7, two-way-ANOVA, p<0.01). Also collagen deposition around the airways was found to be enhanced in OVA mice as determined by Sirius Red staining of lung sections. Gq Inhibition by FR led to a decreased amount of collagen (2.8±0.2 collagen µm<sup>2</sup>/µm bm, n=10), when compared to vehicle controls (3.6±0.2 collagen µm<sup>2</sup>/µm bm, n=8, p<0.05). Moreover, FR also reduced goblet cell hyperplasia (0.07±0.01 PAS+ cells/µm bm, n=5) compared to vehicle treatment (0.13±0.02 PAS+ cells/µm bm, n=5, one-way-ANOVA, p<0.05), as determined by periodic acid Schiff staining (PAS). Furthermore, hBSMCs were stimulated with PDGF (20 ng/mL) and thrombin (1 U/mL) for 3 days *in vitro* and co-application of FR reduced growth of hBSMC compared to vehicle controls (Vehicle: 1.4±0.07 vs FR: 1.1±0.06 relative cell number, n=4, one-way-ANOVA, p<0.05).

These findings identify the pharmacological Gq inhibitor FR as a potentially promising candidate for reducing both, airway hyperresponsiveness and airway remodeling in chronic asthma.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA082

**The muscarinic receptor modulator gallamine induces proliferation of airway smooth muscle cells.**

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**Abstract**

Background: Muscarinic receptor antagonists are a usual treatment for chronic airway diseases, characterised by increased bronchial contraction, like asthma and chronic obstructive pulmonary disease. Furthermore, these diseases are usually accompanied by airway remodelling, involving, among others airway smooth muscle cell (ASMC) proliferation. The purpose of this study was to examine the effect of the M<sub>2</sub> muscarinic receptor modulator gallamine on rabbit tracheal ASMC proliferation and phenotype.

Methods: Airway Smooth Muscle Cells were isolated from adult New Zealand rabbit tracheas and primary cultures were established. Cell proliferation was estimated by incorporation of radioactive thymidine, by the Cell Titer AQueous One Solution method, as well as by cell count after Trypan blue staining. The mechanism mediating the effect of gallamine on ASMC proliferation was elucidated by the use of the PI3K and MAPK inhibitors LY294002 (20 µM) and PD98059 (100 µM), respectively, muscarinic agonists and/or 10% FBS. The effect of gallamine on ASMC phenotype was studied by indirect immunofluorescence and Western blot analysis of α-actin and Myosin Heavy Chain (MHC) expression. All data are expressed as means ± standard error of the mean (SEM) and N represents the number of independent experiments. Statistical analysis was performed using GraphPad Image 5 (GraphPad Software, San Diego, CA, USA). Differences between means were analyzed using one-way ANOVA followed by Bonferonni's post-test. Results were considered significantly different when p<0.05.

Results: Incubation with gallamine (1 nM-10 mM) for 24 h, increased methyl-[<sup>3</sup>H]thymidine incorporation and cell number of 24 h serum starved ASMCs, in a dose dependent way (LogEC<sub>50</sub>: -5.853, EC<sub>50</sub>: 1.4X10<sup>-6</sup>M, N= 4). The effect of gallamine involved the PI3K and MAPK pathways, since it was inhibited when cells were preincubated with LY294002 (20 µM) and PD98059 (100 µM), respectively. Specifically, LY294002 inhibited methyl-[<sup>3</sup>H]thymidine incorporation from 404.9±87.52 cpm (gallamine alone) to 201.1±39.96 cpm (p<0.05, N=6). A similar reduction was observed in the presence of PD98059 (p<0.05, N=6). Gallamine (10 mM), had a mitogenic effect (128.2±8.59 % of control, p<0.05, N=7) on ASMCs that acquired a contractile phenotype after 7 days of serum deprivation, as well. However, did not affect the proliferation induced by acetylcholine (10 µM), carbachol (300 nM) or 10% FBS. Finally, gallamine did not cause any change on the expression of the contractile phenotype markers, α-actin and MHC.

Conclusions: Gallamine, in the absence of any agonist, induces proliferation of both synthetic and contractile ASMCs. The mitogenic effect of gallamine is dependent on the activation of the PI3K and MAPK signaling pathways and does not affect the expression of ASMC contractile protein markers.

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PCA083

### Innovative Aspects of Scientific and Pedagogical Activity of I.P. Pavlov (according to "Pavlov's Wednesdays")

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Formation and development of Pavlov's scientific ideas (theory of reflexes and in particular the conditional reflex, theory of two signal systems, mechanisms of the higher nervous system) are traced in the historical objective material of Pavlov's school. One type of scientific and pedagogical activity of Pavlov's school was "Pavlov's Wednesdays".

"Pavlov's Wednesdays" were the original document of a creative process of development of the theory of the higher nervous system. It is a profound series of recommendations for the introduction of scientific activities and represents a new organizational and pedagogical method, the method of "collective thinking" encouraged by I.P. Pavlov. Pavlov suggested for the first time his co-workers of all physiological laboratories to meet each Wednesday for a collective discussion about open questions in current experimental work and about coordination of subsequent steps in studies. This method of scientific work was not applied before Pavlov. It was recorded in three volumes of protocols and shorthand reports.

Due to Pavlov there were two approaches how to present one's scientific work. The official approach was defined by publishing of articles, holding lectures and giving talks at congresses. In accordance with it, one should take into consideration each detail and wording. The unofficial approach addressed discussion of facts and thoughts between colleagues. It encouraged the expression of spontaneous thoughts and unconventional mental constructs. The unofficial approach of scientific creativity was defined as "collective thinking" and recorded in the minutes of "Pavlov's Wednesdays" at the physiological laboratory as well as in the minutes of "Pavlov's Clinical Wednesdays" at the clinic of neurology and psychiatry. The colleagues of I.P. Pavlov, students, physicians and foreign guests participated in the process of "collective thinking". Due to a systematic character of meetings the scientific team developed itself to the physiological scientific school at which Pavlov's Wednesdays played a significant role.

During the meetings in the clinic of neurology and psychiatry as part of discussions clinical cases were analyzed from the physiological point of view. Thus, I.P. Pavlov explained series of neurological and psychiatric illnesses based on physiology. Additionally, principles of the neuronal processes of the cerebral cortex were studied and questions about the development of experimental neuroses by animals as well as the study of the second signal system were elaborated. I.P. Pavlov created the guidance for the connection of physiological theories with medical practice.

"Pavlov's Wednesdays". Minutes and Shorthand Reports of Physiological Talks. Academy of Sciences of USSR, Moscow-Leningrad, Volume 1, 1949

"Pavlov's Wednesdays". Minutes and Shorthand Reports of Physiological Talks. Academy of Sciences of USSR, Moscow-Leningrad, Volume 2, 1949

"Pavlov's Wednesdays". Minutes and Shorthand Reports of Physiological Talks. Academy of Sciences of USSR, Moscow-Leningrad, Volume 3, 1949

"Pavlov's Clinical Wednesdays". Shorthand Reports. Academy of Sciences of USSR, Moscow-Leningrad, Volume 1, 1954

"Pavlov's Clinical Wednesdays". Shorthand Reports. Academy of Sciences of USSR, Moscow-Leningrad, Volume 2, 1955

I gratefully acknowledge my professor, Prof. Mag. Dr. H. Kerschbaum, for our fruitful discussions, Pavlov's museum in Ryazan (Russia) for help and contribution as well as Pavlov's library for donation of the required information.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCA084

### Designing "ion channel in health and disease" teaching in "Biology of Disease" module according to students' ability

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Four components were designed in "ion channel in health and disease" teaching of year 3 "Biology of Disease" module to help students learning according to student's level and ability. 1) Tailoring lecturing to different student levels. This design focused on different needs of students. I particularly paid attention to lecturing materials to meet students in different levels. The lecture consist basic knowledge, additional reading and further specific discussion. 2) Inspiring students' passion and thinking. This design aimed to increase students' passion for research and to form their thinking of how to start a new topic from scratch. The lecture particularly included three Nobel Winners' stories in ion channel research history to make student thinking in a logical order of ion channel research development. 3) Applying lecturing into practice. This design emphasised on students' engagement and to provide a chance for them to practice. I designed poster presentation exercise for student to choose freely an ion channel topic to give a presentation. This really stimulated students to initiate their "own research" in understanding the teaching 4) Combining research with teaching. This design forecasted to escalate students who are keen to approach their studies further to learn practical skills in real. I designed some small projects from my own research for those students to have a chance to learn skills. This provided an opportunity for them to get real sense of the skills taught and would boost their understanding of the topic. It is an appropriate way to prepare them for future studies more readily. Feedback from students were very positive and meet different needs from all levels from students. They felt stimulated and some of them have moved on to the field of ion channel studies.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCA085

### Assessing the value and impact of physiology in an integrated curriculum

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A new fully-integrated medical curriculum, designed and delivered through case-based learning, was introduced in Cardiff in

2013-14 (Hassoulas *et al.*, 2017). This approach allows scientists and clinicians to work together to ensure that students' understanding of basic science and clinical skills are developed in a cohesive manner. Core physiological concepts and principles are embedded into clinical thinking, however, there is a danger that the discipline could lose its identity within an integrated curriculum and that, as a result, students may fail to recognise its fundamental nature as a foundation of medicine. We aimed to evaluate the impact of integrating physiology into the curriculum by investigating the opinions of the first cohort of students to graduate from our new programme. Students' perceptions were collected anonymously using an online survey delivered on return from elective study towards the end of the final year. The survey contained both closed and open questions addressing students' opinions of physiology teaching, learning opportunities and the value of physiology. The study was conducted in accordance with published guidelines for educational research (BERA, 2011). 62 responses were received (20% of cohort); of these, 73% ( $n=45$ ) did not have a science degree before enrolling on the medical course. Although, in principle, the clinical and preclinical phases are fully integrated, physiology appeared to be visible to students only in the early years of the curriculum. 92% ( $n=57$ ) of students identified a strong component of physiology in Year 1, but this fell to 13% ( $n=8$ ) in Year 3 and 0% in Year 5. However, students recognised the wide range of environments in which they learned physiology and the range of staff who taught them across diverse applications of the discipline. 87% of respondents ( $n=54$ ) reported that they had also worked independently to consolidate these learning opportunities. Although only 69% ( $n=43$ ) of the respondents considered that basic science would be an important component of their future practice, 100% ( $n=62$ ) judged that knowledge of basic physiology is important in understanding the symptoms, process and treatment of disease.

These results show that our students value physiology as a keystone of their medical training and recognise its relevance to their clinical practice. However, despite the integrated nature of the course, the distinction between pre-clinical and clinical study appears to prevail. The perceived lack of physiology in the later undergraduate years suggests that this element needs to be made more explicit or enhanced. This study will be helpful in guiding future development of our own curriculum and could be used more widely to inform development of integrated medical curricula at other institutions. British Educational Research Association (BERA) (2011) Ethical guidelines for educational research. Available at: <https://www.bera.ac.uk/researchers-resources/publications/ethical-guidelines-for-educational-research-2011>

Hassoulas, A.; Forty, E.; Hoskins, M.; Walters, J. & Riley, S. (2017) A case-based medical curriculum for the 21st century: The use of innovative approaches in designing and developing a case on mental health. *Med Teach* 39 (5) 505-511

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCA086

### Microelectrode Techniques for Cell Physiology – The Plymouth Workshop 2018.

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The 'Microelectrode Techniques Workshop' 2018 is the 35<sup>th</sup> edition of the course hosted every September by the Marine

Biological Association at its Plymouth Laboratory. The course teaches hands-on technical aspects of electrophysiological and optical recording in single cells and tissues to 20 students, mainly post grad with around 4 post-docs. Several variations of voltage clamp, patch clamp and voltage recording methods are used to highlight the common technical problems encountered. A day is spent on circuit design, construction and testing, and demonstrations of signal processing and sampling. For optical recording, the microscope is reduced to optical elements and their properties explained, and fluorescent indicators and labels are used in conjunction with electrophysiological recording.

There are 25 lectures in basic and more specialised recording techniques. The hands-on experiments are taught as 3-day practicals in voltage or optical recording, including ion selective electrodes, voltage clamp, and patch clamp single cell or slice techniques. Specialised demonstrations and experiments are given for capacitance, amperometry, dynamic clamp, perforated patch and multi-electrode arrays. Optical methods taught are Ca or pH fluorescent indicator imaging with cameras, TIRF microscopy, microelectrode injection and virus fluorescent protein cell labelling techniques, and photolysis. There is a hands-on optical bench to demonstrate combining of optical components with light sources and detectors. Optogenetic methods are demonstrated and used in experiments with ChR in *C.elegans* and at the *Drosophila* neuromuscular junction.

The tuition for hands-on is one on one; this has proved to be the best way to learn the technical constraints associated with each method. There are around 45 instructors and lecturers who teach the course each year, some from instrument companies who loan much of the instrumentation.

The poster will illustrate the course in 2018.

[www.mba.ac.uk/microelectrode-techniques-cell-physiology](http://www.mba.ac.uk/microelectrode-techniques-cell-physiology),  
Twitter: @CPW\_plymouth

The Workshop is generously supported by the MBA for administration and facilities, by the Physiological Society for core support and affiliates grants for students, by the Company of Biologists for core support and student bursaries, and the BBSRC a 5 year grant for core support.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCA087

### Expression levels of some *microRNAs* in Prostate cancer cell Lines using Qiagen miScript real time PCR system

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MicroRNAs (miRNAs) are small non-coding RNAs that post-transcriptionally regulate gene expression. It has been shown that aberrant expressions of miRNAs drive the development, progression, metastasis and prognosis of many cancers including prostate cancer (Hosseini et al., 2018). The underlying mechanism for the aberrant expression is believed to be due to gene alteration, epigenetic modification and changes in Dicer abundance. The involvement of miRNAs in human prostate cancer (PCa), which is one of the most commonly

diagnosed malignant tumors, and so far the second significant cancer killer of men, has clearly demonstrated a correlation between miRNAs and their targets with prostate carcinogenesis in recent years (Karbasforooshan et al., 2018). Cell Culture of PCa cell lines (PC3, du145 and LNCaP) and normal prostate epithelial cells (PCS-440) was carried out. The cultured cells were washed once with PBS and then lysed directly in the well by addition of 1ml of TRIzol reagent; lysed cells were removed from the well by scraping with a cell scraper. This mixture was transferred to a 1.5ml Eppendorf tube, RNA was then extracted according to manufacturer's instructions. The total RNA was reverse transcribed using the miScript II RT Kit. The qPCR was performed using miScript real time PCR system. The targeted microRNAs include miR-15, miR-21, miR-34a and 34b, miR-155 and let-7a. The results show that miR-15, miR-21, miR-34a and 34b, miR-155 and let-7a are down regulated in PC3, du145 and LNCaP cell lines compared to the normal PCS-440. Strikingly, miR-21 which is a well-known oncomir that is normally up-regulated in most cancer cells was found to be down-regulated in all the three prostate cancer cell lines. The present study therefore suggests that miR-21 may be down-regulated in prostate cancer and may therefore affect therapeutic targeting of miRNAs in prostate cancer.

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KARBASFOROOSHAN, H., ROOHBAKHSH, A. & KARIMI, G. 2018. SIRT1 and microRNAs: The role in breast, lung and prostate cancers. *Exp Cell Res*, 367, 1-6.

The authors would like to acknowledge Usmanu Dan Fodiyo University Sokoto, Nigeria, Tetfund, and The Physiological Society for their financial support to attend this meeting.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

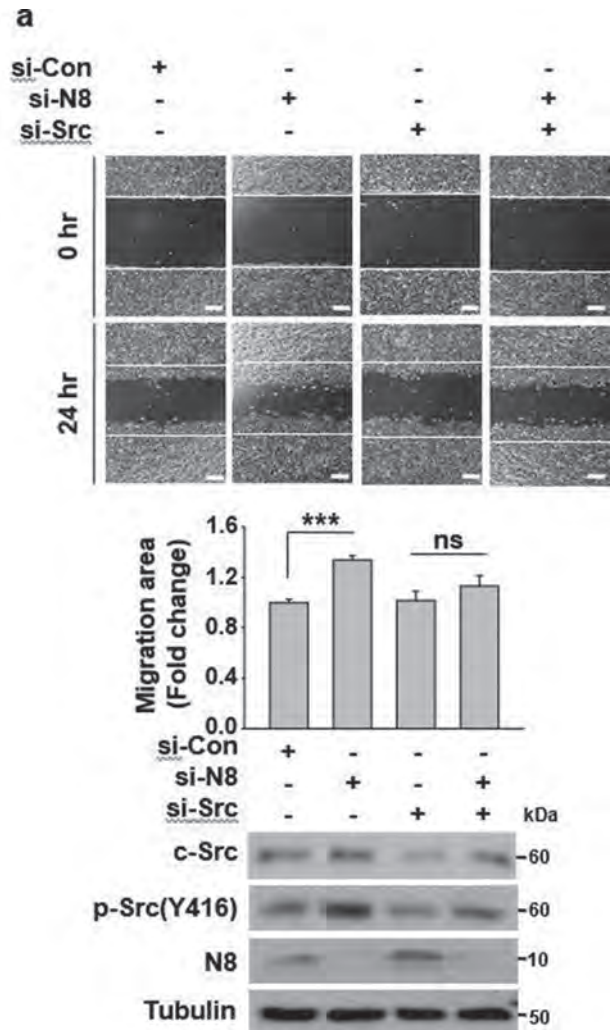
PCA088

### The E3 ligase C-CBL inhibits cancer cell migration by neddylation of the proto-oncogene c-Src.

G. Lee and J. Park

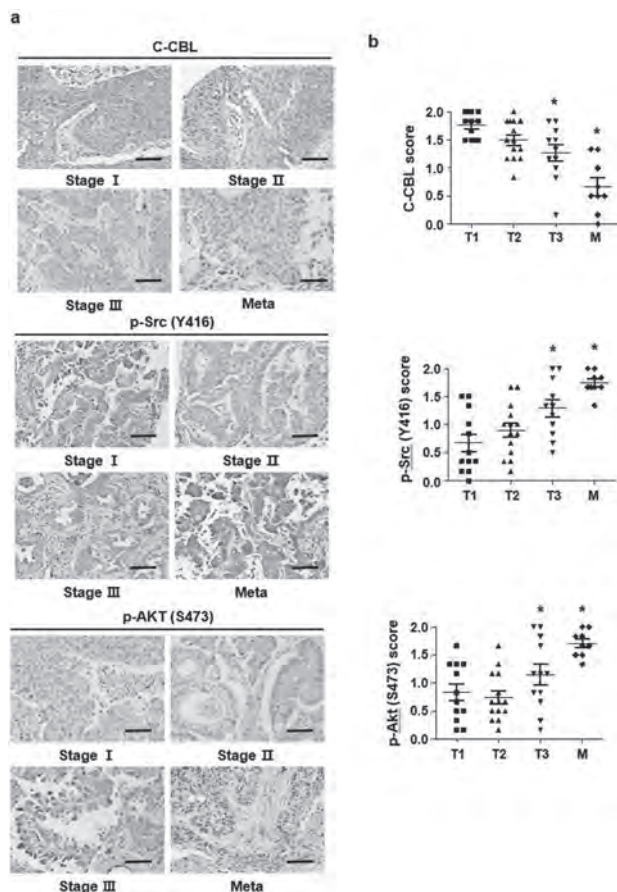
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Neddylation is a cellular process that covalently conjugates substrate proteins with the small ubiquitin-like molecule NEDD8. As neddylation is required for fast turnover of proteins in proliferating cancer cells, the neddylation process is currently regarded as a potential target for cancer therapy. However, little is known about the role of neddylation in cancer invasion and metastasis. Unexpectedly, we here found that the neddylation blockade stimulates migration of lung cancer and glioblastoma cells. Mechanistically, C-CBL acts as the E3 ligase for neddylation of the proto-oncogene c-Src. After neddylation, c-Src is poly-ubiquitinated and degraded through the proteasome, which inhibits the PI3K-AKT pathway responsible for cell migration. In human lung cancer tissues, the down-regulation of C-CBL was associated with c-Src/AKT, cancer metastasis, and poor survival in patients. Therefore, C-CBL is likely to play a tumor suppressive role by antagonizing a robust oncogenic signaling driven by c-Src. This study provides new insight about the role of neddylation in cancer metastasis. It also implies that the metastasis risk should be carefully evaluated before the clinical application of neddylation inhibitors as anticancer regimens.



c-Src knock-down attenuates the cell migration-promoting effect of NEDD8 knock-down





C-CBL and p-Src or p-AKT levels are reciprocally associated with malignancy in lung cancer.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA089

## Regulation of Gap Junction Coupling in Large Intestine Epithelial Cells by Adenosine Signalling

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The colon epithelial cell line Caco-2 was used to analyse the role of adenosine signalling in the regulation of gap junction coupling in the epithelium of the large intestine. RT qPCR experiments showed that the cells expressed Cx43, Cx32 and Cx26. In immunocytochemical experiments, the presence of Cx43 at the cell-cell borders was clearly recognized. Using gold

nanoparticle mediated laser perforation/ dye transfer assays, we found that specific stimulation of A<sub>2A</sub> or A<sub>2B</sub> adenosine receptor subtypes induced a rapid decrease in gap junction coupling. The gap junction coupling was reduced to about 50 % by 50 nM CGS 21680 (A<sub>2A</sub>) or 100 nM BAY 60-6583 (A<sub>2B</sub>) applied for 1 h. The decrease of the gap junction coupling was not related to a change in expression of Cx43 as revealed by RT-PCR analysis. Western blot experiments showed however that the stimulation of A<sub>2</sub> adenosine receptors induced a reduction of Cx43 protein in the cells. Concerning the activated signalling pathway, two different PKA inhibitors blocked the BAY 60-6583 induced decrease of gap junction coupling. Taken together the findings suggest that the A<sub>2</sub> adenosine receptors are linked to the gap junction coupling by a cAMP/ PKA-dependent pathway that affected the translation or the degradation of connexins.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

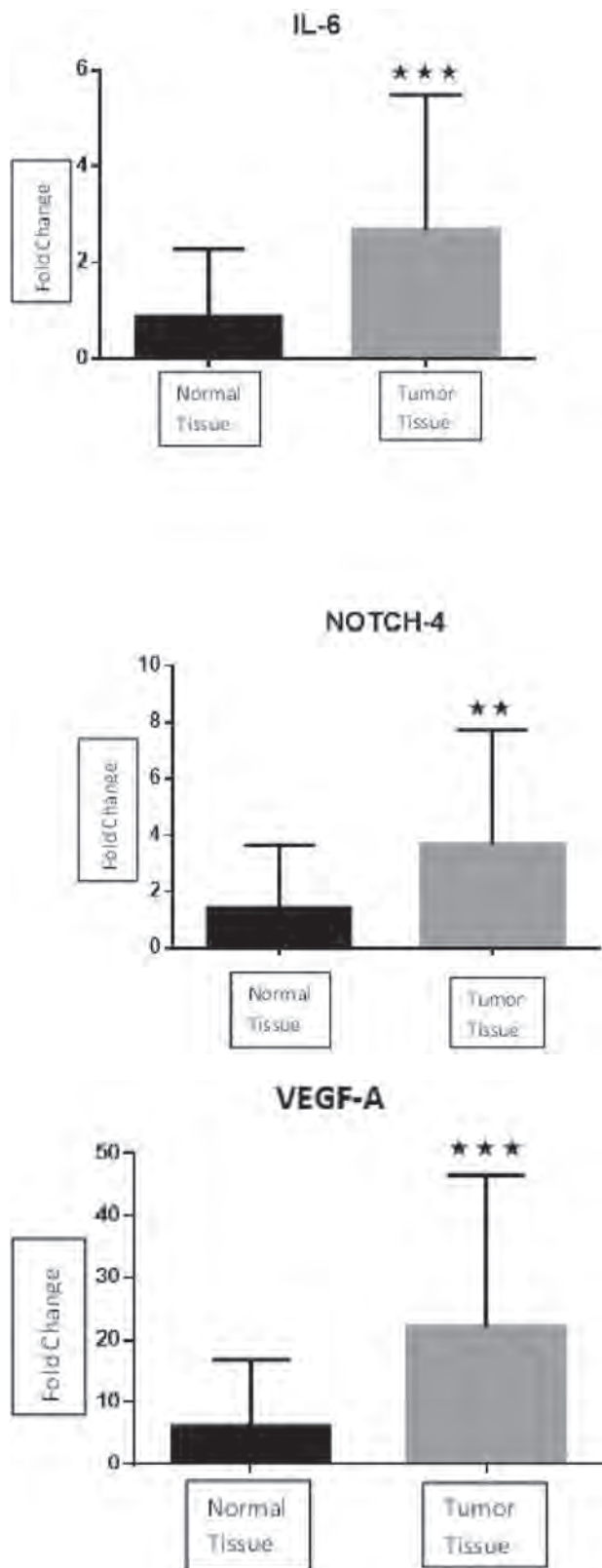
PCA090

## Is Notch -4 Associated with VEGF-A and IL-6 in the CRC Tumorigenesis?

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Colorectal cancer (CRC) is the third most common cancer in the world wide, the morbidity of patients with CRC has been increasing fastly in recent years. Recent studies have indicated that the Notch signaling plays an important role in the development and progression of cancer. The Notch signaling pathway includes Notch receptors and Notch ligands. These ligands and receptors of the Notch pathway are known to be highly oncogenic in CRC, but the effect of Notch-4 is still not clear. Angiogenic and pro-inflammatory cytokines such as VEGF and IL (interleukin)-6 are involved in CRC development mechanisms. However, other regulatory mechanisms such as Notch ligands and receptors are parallel to VEGF-A and IL-6 mediated cellular changes. The role of these three molecules has not yet been fully understood in the CRC. The aim of our study was to analyze the expression of Notch4, IL-6 and VEGF-A in malignant and neighboring normal colorectal tissues. This study was conducted with the approval of the Human Ethics Committee of Eskisehir Osmangazi University, Turkey in accordance with the Declaration of Helsinki (Approval No. 80558721/90). A total of 40 patients with CRC who underwent surgical resection at the Department of General Surgery, Eskisehir Osmangazi University Hospital were analyzed in this study. Notch4, IL-6 and VEGF-A expression were detected by using real-time polymerase chain reaction in tumorous (n:40) and neighboring normal (n:40) colorectal tissues. According to RT-PCR results, in tumorous tissue comparison with normal tissues, mRNA levels of Notch4, VEGF-A and IL-6 increased respectively (p<0.05), (p<0.001) and (p<0.001). Our study suggest that play a key role for Notch signaling in CRC, VEGF-A and IL-6. Targeting these three pathways may help to design new therapeutic strategies aimed at controlling CRC growth and angiogenesis.



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This study is supported by a grant from The Scientific And Technological Research Council Of Turkey (TUBITAK 1001, no: 116S628).

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

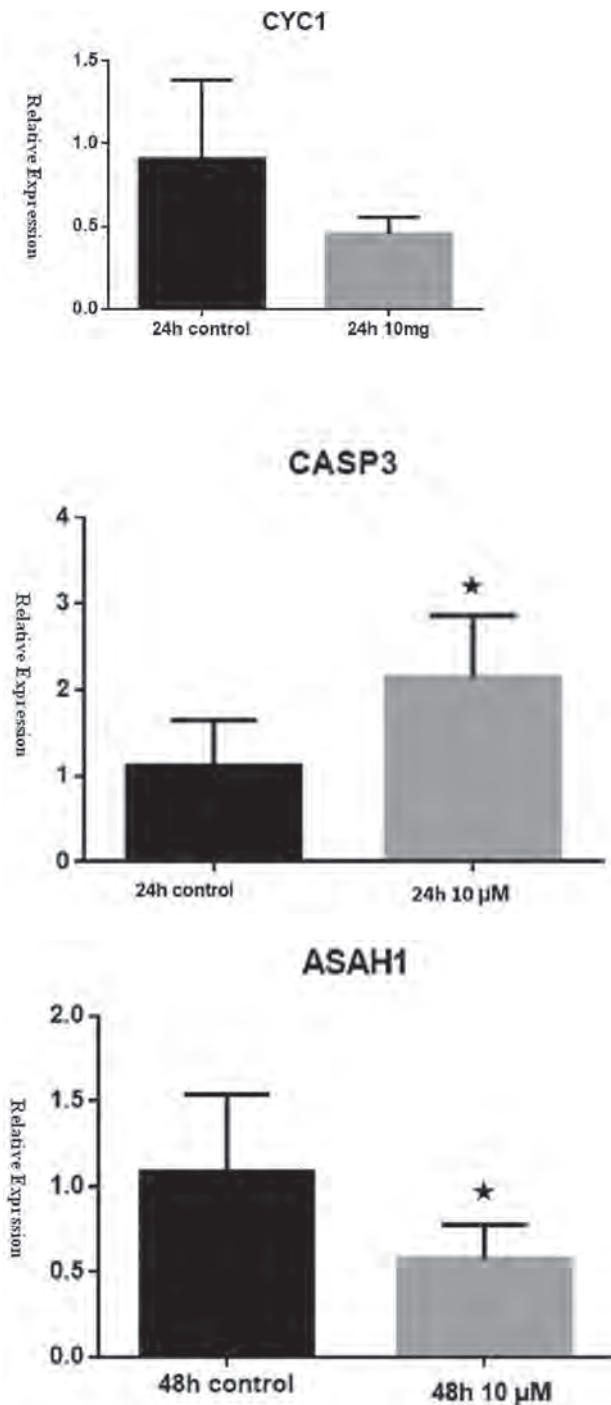
PCA091

### Targeting Inhibition of Ceramidase Induces Apoptosis in Hepatocellular Carcinoma in vitro

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Ceramide is a bioactive lipid, synthesized by hidrolization of sphingomyelin. Over-expression of ceramidases is involved in increased resistance to apoptosis which results in poor prognosis in several types of cancer including melanoma, prostate cancer and colon cancer whereas exposure to ceramides or sphingomyelinase, which generates ceramide, has been shown to induce apoptosis in cancer cells. Ceranib-2 is a novel anti-cancer drug targeting the enzyme acid ceramidase. However the possible effects of ceranib-2 on hepatocellular cancer cell line HepG-2 is not studied yet. The present study was designed to investigate the effects of ceranib-2 on the viability of human HepG-2 cells as well as Caspase-3, Cytochrome c1 (cyc1) and ASAH (acid ceramidase) expressions. The study shows that ceranib-2 increased apoptosis by decreasing ASAH expression and inducing caspase-3 expression in human hepatocellular cancer cell line in a dose and time-dependent manner. We studied the apoptotic affect of ceranib-2 in 0,1, 1, 5, 10, 25 and 50  $\mu\text{M}$  concentrations on HepG-2 cells for 24 and 48 hours by 3-(4,5-D-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide thiazolyl blue (MTT). In 10  $\mu\text{M}$  concentration of ceranib-2 we detected Caspase-3, Cyc-1 ASAH mRNA expressions by Real-Time PCR (RT-PCR). Statistical analysis was performed by using the GraphPad software program. Following the determination of Caspase-3, Cyc-1 and ASAH mRNA expression using beta-actin as a reference gene, the data obtained from RT PCR were calculated using the formula  $2^{-\Delta\Delta\text{Ct}}$ . In our study ceranib-2 decreased the viability of HepG-2 cells in 10  $\mu\text{M}$  concentration for 24 and 48 hours. This impact shows %58 difference compared to control. According to RT-PCR results, while mRNA levels of Caspase-3 increased ( $p < 0.05$ ), mRNA levels of Cyc1 were not changed in 24<sup>th</sup> hour and 10  $\mu\text{M}$  concentration compared to control group and no difference for Caspase-3 in 48<sup>th</sup> hour. mRNA levels of ASAH showed a reduction in 10  $\mu\text{M}$  concentration in 48<sup>th</sup> hour compared to control group ( $p < 0.05$ ). Our Study shows that ceranib-2 has strong anti-cancer effect as time and dose dependent. Low levels of mRNA of ASAH in treatment groups showed that 10  $\mu\text{M}$  ceranib-2 could inhibit ceramidase at 48<sup>th</sup> hour and this may suggest that ceramide levels are increasing. PCR data of the current study indicated that while cytochrome c mRNA expression level was not change, mRNA levels of Caspase-3 increased in 10  $\mu\text{M}$  concentration. Due to this contradictory results, we will focus on possible different apoptotic pathways to explain the effects of seranib-2 in our future studies.



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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## P2X4 re-sensitisation depends on recycling via acidic organelles

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P2X receptors constitute a family of seven different cation-permeable, ligand-gated ion channels present on the plasma membrane of most cells. These receptors are activated upon binding of ATP; this activation is followed by a desensitization phase and a deactivation phase upon removal of ATP. To return in its resting state the receptor needs a re-sensitization phase. Thus this cycle of de-sensitization - re-sensitization is crucial in the receptor signal transduction.

Furthermore, since some receptors of the P2X family are mainly expressed on intracellular organelles, it has been suggested that cellular trafficking might also regulate P2X receptors signaling.

P2X4 is predominately localized on the limiting membrane of late endosomes, lysosomes and lysosome-related organelles, which are all characterized by the acidic luminal pH. Since acidic pH inhibits activation of these receptors, the physiological role of their expression on acidic compartments has long remained elusive.

In this study, we investigate the trafficking of P2X4 receptor to acidic compartments and how trafficking affects re-sensitization of the receptors.

To characterize the effect of acidic pH on channel re-sensitization, we overexpressed (wt)P2X4-EGFP in HeLa cells and we performed intracellular calcium measurements ( $[Ca^{2+}]_i$ ). We compared the responses to two subsequent stimulation of ATP (5 min interval) exposing the cells to bath solution with decreasing pH and we observed a pH dependent re-sensitization of P2X4.

We subsequently increased the time interval between the two stimulations and we observed that after 30 min the recovery was almost complete. We then blocked organelles acidification, clathrin-mediated endocytosis and exocytosis. All of these perturbations significantly reduced the  $Ca^{2+}$ -peak following second ATP (< 19% for all conditions), indicating that inhibition of recycling and/or acidification inhibits P2X4 re-sensitization.

We generated P2X4 mutants where all the histidine residues on the extracellular loops were substituted by alanine, arginine or aspartate. All 3 mutants have significant effects for full recovery of P2X4 suggesting that protonation and de-protonation of critical histidine residues is crucial.

In our study, we provide evidence that targeting of P2X4 receptors to acidic organelles is essential for receptor re-sensitization. Our findings indicate that the internalization/recycling via acidic organelles facilitate protonation/de-protonation of critical histidine residues within the extracellular loop that is required for receptor recovery, in particular H286.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.



PCA093

**The Effect of Beta Adrenergic Agonist and Antagonists on Caspase 3, Caspase 8 and Caspase 9 Expression on K562 Chronic Myeloid Leukemia Cells.**

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The  $\beta$  adrenergic receptors, a family of G-protein-coupled receptors that are activated by adrenergic catecholamines, can initiate a series of signaling cascades, thereby leading to multiple cell specific responses. Beta adrenergic receptor signaling had been demonstrated to promote breast cancer growth (1), recurrence and metastasis (2) and reduce apoptosis of cancer cells (3, 4). Some researchers demonstrated that  $\beta_2$  adrenergic receptor stimulation could significantly suppress the malignant transformation of cancers, cancer cell growth and proliferation by cutting of the Raf-1/Mek-1/Erk1/2 pathway (5). Chronic myeloid leukemia is a hematopoietic stem cell malignancy characterized by myeloid cell proliferation and loss of adhesive properties of the myeloid elements. K562 cell line was established by Lozzio et al., from a patient with chronic myeloid leukemia (CML) in terminal blast crisis (1975). Previously, we found that no difference of beta 1, beta 2 adrenergic receptor expression levels on human chronic myeloid leukemia K562 cells.

In this study, we aimed to determine the expression levels of caspases 3, 8 and 9, which are effective on cell apoptosis pathway in the presence of isoprenaline and propranolol, in order to determine the role of  $\beta$  adrenoceptors in the apoptosis pathway of K562 cells.

**Methods**

K562 (ATCC No: CCL-243) chronic myeloid cells were harvested after 24 hours of treatment with agonist and / or antagonist. In these cell lysates caspase 3, 8, and 9 protein levels were determined by western immunoblotting. One-way ANOVA and Bonferroni post test statistical analyzes were used in the GraphPad Prism 5.0 program.

**Results**

Isoprenaline caused a decrease in caspase 3,8 and 9 expression compared to the control group. The propranolol has been shown to cause an increase in caspase 9 expression, leading to a decrease in caspase 3, 8 expression relative to control group. It would seem that propranolol triggers apoptosis through mitochondrial pathway in these cells as a result of the significant increase in caspase 9 expression due to propranolol.

Key Words: G protein coupled receptors, cancer, isoprenaline, propranolol, protein expression

|                | CONTROL | ATR   | CCN    | CCN+ATR | Cumene | PROP   | IPW   | PROP+CCN |
|----------------|---------|-------|--------|---------|--------|--------|-------|----------|
|                | 31.00   | 19.53 | 29.88  | 9.47    | 75.14  | 9.58   | 61.12 | 7.58     |
|                | 34.80   | 23.26 | 21.54  | 12.62   | 47.44  | 7.07   | 16.22 | 11.01    |
|                | 29.54   | 21.05 | 23.40  | 16.24   | 54.04  | 8.49   | 31.08 | 8.96     |
| Mean           | 30.27   | 22.15 | 22.50  | 11.05   | 50.74  | 7.787  | 23.66 | 8.275    |
| Std. Deviation | 1.028   | 1.552 | 1.360  | 2.229   | 4.667  | 1.008  | 10.51 | 0.9818   |
| Std. Error     | 0.7271  | 1.097 | 0.9618 | 1.576   | 3.300  | 0.7125 | 7.429 | 0.6942   |

Caspase 3 ekspresyon in K562 cells

Table 2: Caspase 8 ekspresyon in K562 cells.

|                | CONTROL | ATR   | CCN    | CCN+ATR | Cumene  | PROP   | IPW   | PROP+CCN |
|----------------|---------|-------|--------|---------|---------|--------|-------|----------|
|                | 31.59   | 32.61 | 7.73   | 17.47   | 0.71    | 2.42   | 5.90  | 1.85     |
|                | 35.75   | 31.16 | 8.41   | 15.36   | 0.91    | 2.83   | 1.37  | 2.35     |
|                | 35.62   | 23.23 | 6.34   | 14.93   | 0.83    | 3.21   | 5.57  | 1.63     |
| Mean           | 34.32   | 29.01 | 7.496  | 15.93   | 0.8235  | 2.825  | 4.284 | 1.948    |
| Std. Deviation | 2.387   | 5.050 | 1.083  | 1.362   | 0.1010  | 0.3918 | 2.526 | 0.3705   |
| Std. Error     | 1.367   | 2.916 | 0.6079 | 0.7862  | 0.05829 | 0.2262 | 1.456 | 0.2139   |

Table 3: Caspase 9 ekspresyon in K562 cells.

|                | CONTROL | ATR    | CCN    | CCN+ATR | Cumene | PROP   | IPW   | PROP+CCN |
|----------------|---------|--------|--------|---------|--------|--------|-------|----------|
|                | 16.01   | 7.77   | 32.98  | 4.60    | 1.41   | 109.01 | 5.28  | 36.81    |
|                | 17.86   | 9.80   | 35.12  | 6.36    | 1.11   | 52.74  | 0.97  | 37.08    |
|                | 19.88   | 9.84   | 34.26  | 6.02    | 1.01   | 50.62  | 2.58  | 40.35    |
| Mean           | 18.12   | 9.143  | 34.13  | 5.674   | 1.181  | 80.79  | 2.948 | 38.09    |
| Std. Deviation | 1.641   | 1.184  | 1.077  | 0.9409  | 0.2075 | 28.14  | 2.175 | 1.992    |
| Std. Error     | 0.9474  | 0.6936 | 0.6220 | 0.5432  | 0.1198 | 16.24  | 1.266 | 1.150    |

Caspase 8, 9 ekspresyon in K562 cells.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA094

**P-Coumaric Acid Ameliorates Renal Ischemia-Reperfusion-Induced Acute Lung Injury By Reducing Oxidative Stress**

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Reduction of oxygen distribution produces reactive oxygen radicals (ROS) such as hydrogen peroxide, superoxide anion and causes cellular dysfunction and necrosis. The release of massive ROS causes activation of stress-induced transcription factors and production of proinflammatory and anti-inflammatory molecules. Numerous molecules have been investigated to prevent this oxidative damage. p-Coumaric acid (p-CA), also known as 4-hydroxycinnamic acid, is one of the potential molecules which has high free radical scavenging, anti-inflammatory and antimicrobial activities. To evaluate the role of p-CA in renal ischemia/reperfusion-induced

remote lung injury, thirty-two Wistar Albino adult female rats weighing 200-250 grams (12-16 weeks) were used. These rats were randomly divided into 4 equal groups (n=8). The groups were identified as: sham (S), ischemia-reperfusion (IR), IR+ 50 mg/kg p-CA (p-CA 50), IR+100mg/kg p-CA (p-CA 100). Treatment groups were subjected to surgery in the IR group. Rats in the Sham group underwent right dorsal side nephrectomy. Other groups were clamped in the left renal artery after right dorsal side nephrectomy. p-CA was administered intraperitoneally at single dose immediately before reperfusion. Then the clamp was removed and the reperfusion period started for 3 hours. After the applications were completed, total antioxidant level (TAS), total oxidant level (TOS), superoxide dismutase (SOD), myeloperoxidase (MPO) and malondialdehyde (MDA) levels were measured spectrophotometrically. Oxidative stress index (OSI) was measured. Values are means  $\pm$  SD., compared by ANOVA. In IR, TOS, MDA, MPO and OSI increased in significant level, while TAS and SOD decreased ( $p<0.001$ ). An increase in TAS and SOD levels and a decrease in TOS, MDA and MPO values was observed in CGA applied groups ( $p<0.001$ ). Therefore, our findings suggested that the active ingredient p-CA may exert its antioxidant effects in remote lung injury induced by renal ischemia-reperfusion in rats.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCA095

#### **Ca<sup>2+</sup> tunneling as a selective intracellular signaling pathway.**

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Intracellular calcium is the most ubiquitous second messenger and is central to key events such as fertilization and cell death. The cells have only two main sources connected to the cytosol through ion channels to create intracellular Ca<sup>2+</sup> signals: the endoplasmic reticulum (ER) and the extracellular space. Because of the multitude of Ca<sup>2+</sup> targets in a cell, the signals have to be very precisely encoded in time and space. The relative localization of the Ca<sup>2+</sup> source and of the Ca<sup>2+</sup> targets are therefore of key importance. In the case of Store Operated Ca<sup>2+</sup> entry (SOCE), effectors have to be located in the immediate vicinity of the Ca<sup>2+</sup> source as SOCE cannot activate efficiently targets away from the plasma membrane (PM). One way to deliver Ca<sup>2+</sup> from SOCE to distant targets is to use the tunneling process where the ER cisterns are used as a pipeline taking Ca<sup>2+</sup> from the SOCE microdomain and releasing it through IP<sub>3</sub>Rs to distant effectors. Here we used HeLa cells to study the effect on specific intracellular targets of various Ca<sup>2+</sup> mobilizing pathways: SOCE, ER release and tunneling. In HeLa cells mitochondria localize away from the PM and constitute therefore a candidate of choice for tunneling. When Ca<sup>2+</sup> is released from the stores following the opening of stimulation IP<sub>3</sub>Rs, the mitochondrial Ca<sup>2+</sup> levels follow the rise in cytosolic Ca<sup>2+</sup>. In contrast, the mitochondria did not respond to Ca<sup>2+</sup> influx through while SOCE has little effect. Surprisingly, during tunneling, the large cytoplasmic rise observed was not associated with a significant change in mitochondrial Ca<sup>2+</sup>. Detailed analysis of the relative localization of the mitochondria and of the ER during store depletion failed to explain the differences in mitochondrial signaling. The kinetics of the cytosolic Ca<sup>2+</sup> increase induced by tunneling was however slower than ER release, presumably due to the slow pumping

in the ER by the SERCA as compared to Ca<sup>2+</sup> release through the IP<sub>3</sub>Rs. Conversely, when the target was a Ca<sup>2+</sup>-activated potassium channel, or the PM itself, tunneling was extremely effective in delivering Ca<sup>2+</sup> to these sites, whereas SOCE itself behaved as a poor activator. In contrast, on the opposite, when NFAT translocation was used as a reference for Ca<sup>2+</sup> signaling, SOCE was extremely potent while Ca<sup>2+</sup> release was ineffective and tunneling only induced a slight enhancement of the SOCE effect. We conclude that Ca<sup>2+</sup> entering through SOCE to be released by IP<sub>3</sub>Rs is preferentially released close to the source (*i.e* the PM) and generates a weaker rise deep in the cell unable to reach the required conditions to trigger Ca<sup>2+</sup> increase in the mitochondria. Recent findings suggest that active IP<sub>3</sub>Rs indeed located close to the PM surrounding and avoid the SOCE clusters(1). It is therefore clear the spatio-temporal properties of the Ca<sup>2+</sup> increase induced by tunneling contribute to activate selective intracellular Ca<sup>2+</sup> targets.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCA096

#### **Antioxidative Effects Of Chlorogenic Acid on Lung Injury Induced Renal Ischemia-Reperfusion**

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Renal ischemia/reperfusion (IR) injury resulting in acute renal failure is a major clinical problem due to the high mortality rate. Restoration of the blood flow along with the healing process causes paradoxical reperfusion damage while prolonged interruption of blood flow causes ischemic injury. Although the exact cause is unknown in the etiology of this damage, it is believed that oxidative stress and the formation of reactive oxygen play a key role. Renal IR causes local injury to the kidney, while increased pulmonary capillaries cause lung damage characterized by permeability, edema and neutrophil infiltration. Chlorogenic acid (5-O-caffeoylquinic acid, CGA) is a phenolic compound of the hydroxycinnamic acid family. This polyphenol has anti-oxidant, anti-inflammatory, antilipidemic, antidiabetic activities, among other biological properties. To evaluate the role of CGA in renal ischemia/reperfusion-induced acute lung injury, thirty-two Wistar Albino adult female rats were used. These rats were randomly divided into four equal groups (n=8). The groups were identified as: sham (S), IR, IR+5 mg/kg Chlorogenic Acid (CGA5), IR+10mg/kg Chlorogenic Acid (CGA10). Drug groups were subjected to surgery in the IR group. Rats in the Sham group underwent right dorsal side nephrectomy. Other groups were clamped in the left renal artery after right dorsal side nephrectomy. CGA was intraperitoneally applied in two doses 10 minutes before ischemia and reperfusion. After 1 hour of ischemia, 24 hours of reperfusion was applied to the left kidney. After the applications were completed, total antioxidant level (TAS), total oxidant level (TOS), superoxide dismutase (SOD), myeloperoxidase (MPO) and malondialdehyde (MDA) levels were measured spectrophotometrically. Oxidative stress index (OSI) was measured. In comparison to S, IR group's TAS and SOD values decreased, TOS, MDA and MPO values increased. An increase in TAS and

SOD levels, and a decrease in TOS, MDA and MPO values was observed in CGA groups. All of these data indicate that CGA has several roles in IR injury due to its antioxidative properties. In this study, effects of CGA on renal IR model were determined by measuring oxidative stress parameters SOD, TAS, TOS, OSI, MDA and MPO levels. This data demonstrates that CGA has protective effect against renal IR-induced oxidative injury and may be tried as a new therapeutic agent in the future.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA097

### Expression of aquaglyceroporins in the bovine gastrointestinal tract

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Urea nitrogen salvaging (UNS) is a crucial mechanism that ruminants evolved to conserve nitrogen. Facilitative UT-B urea transporters have been proven to be involved in urea transport across rumen epithelial layers and thus efficiently facilitate the UNS process. Recently, functional studies showed that apart from UT-B, aquaporins (AQPs) - probably aquaglyceroporins (AQGs), i.e. AQP3, AQP7, AQP9 and AQP10 - were also involved in urea transport across rumen. This current study aims to investigate how these AQGs are expressed in bovine gastrointestinal (GI) tract, especially in rumen, and hence provide evidence of for their physiological roles in UNS. Initial end-point RT-PCR analysis suggested that except for AQP10, all these AQGs genes were transcribed along ruminant GI tract but with varying abundance specific for genes and locations. In dorsal and ventral sacs of rumen, AQP3 was the most abundant transcript, followed by weak AQP7 expression and very weak AQP9 expression. In the lower GI tract, each gene, especially for AQP7 and AQP9, displayed a decreasing expression pattern downward the GI tract: abomasum > jejunum > colon. Using immunoblotting, AQP3 protein was detected in rumen, with a 45 kDa glycosylated form and a 25 kDa core unglycosylated protein. Further immunolocalization studies showed that like UT-B, AQP3 also mainly located at cell membranes of stratum basale layer but seemed to have a lower abundance. In conclusion, this study describes the transcripts expression patterns of AQP3, AQP7, AQP9 and AQP10 along ruminant GI tract, confirms that glycosylated AQP3 protein is present in rumen and characterizes, for the first time, the precise cellular location of AQP3 transporters in the bovine rumen epithelium.

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PCA098

### A proton antenna in carbonic anhydrase II supports lactate transport in cancer cells

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Tumor cells, especially those that reside in a hypoxic environment, produce vast amounts of lactate and acid, which have to be quickly removed from the cell to avoid intracellular lactacidosis and suffocation of metabolism. Lactate efflux from cancer cells is mediated by H<sup>+</sup>-coupled monocarboxylate transporters (MCTs). In the present study, we investigated whether lactate flux in MCF-7 breast cancer cells is facilitated by the intracellular carbonic anhydrase CAII. Knockdown of CAII, but not inhibition of CA catalytic activity with EZA, reduced H<sup>+</sup>-coupled lactate transport in normoxic and hypoxic MCF-7 cells, as determined by measuring changes in intracellular pH with the fluorescent dye SNARF-5F. Furthermore, knockdown of CAII resulted in a significant reduction in cell proliferation, as did inhibition of lactate transport with AR-C155858, while inhibition of CA catalytic activity with EZA did not influence proliferation. An *in situ* proximity ligation assay demonstrated that CAII is closely colocalized to MCT1, which suggests a direct interaction between the proteins. The mechanism behind this interaction was elucidated by coexpression of MCT1 and MCT4 with various CAII mutants in *Xenopus* oocytes. CAII facilitates MCT transport activity via CAII-Glu69 and CAII-Asp72, which have been suggested to function as surface H<sup>+</sup> antenna for the enzyme [1]. However, determination of CAII catalytic activity with gas-analysis mass spectrometry revealed that Glu69 and Asp72 are not involved in catalytic function. While CAII-Glu69 and CAII-Asp72 seem to mediate H<sup>+</sup> transfer between enzyme and transporter, CAII-His64, the central residue of the enzyme's intramolecular H<sup>+</sup> shuttle, is not involved in H<sup>+</sup> transfer between the two proteins, but mediates binding of CAII to the transporter's C-terminal tail, as shown by a pull-down assay with GST-fusion proteins. Taken together, the results suggest that CAII features a moiety that exclusively mediates H<sup>+</sup> exchange with the transporter, while the central residue of the intramolecular H<sup>+</sup> shuttle, His64, mediates binding to MCT. Thereby CAII drives the MCT-mediated efflux of H<sup>+</sup> and lactate from the cell to support cellular metabolism and proliferation (Fig. 1).



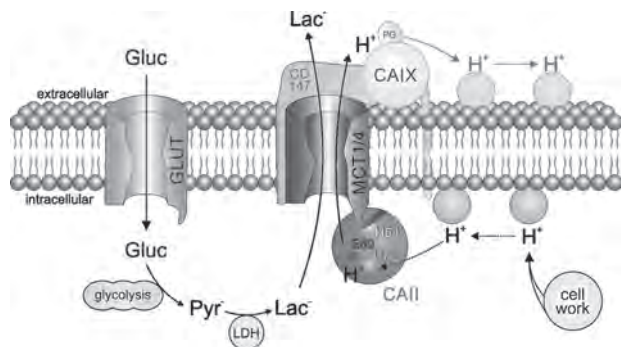


Figure 1: CAII functions as  $H^+$  antenna for MCTs. Intracellular CAII is anchored to MCT1/4 via CAII-His64. This binding brings CAII close enough to the MCT to shuttle  $H^+$  between transporter and surrounding protonatable residues.  $H^+$  transfer is mediated by CAII-Glu69 and CAII-Asp72. Under hypoxic conditions CAIX could bind to MCT1/4 via their chaperon CD147 to facilitate the exchange of  $H^+$  between transporter and extracellular protonatable residues in a similar fashion as CAII [2, 3]. By this non-catalytic mechanism intracellular and extracellular carbonic anhydrases could cooperate non-enzymatically to facilitate  $H^+$ -driven lactate flux across the cell membrane of cancer cells.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA099

#### Novel roles for the TRPM5 ion channel in regulating airway mucociliary clearance

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Mucociliary clearance (MC) is a major innate defense mechanism that removes pathogens from the airways. It is dependent on concerted ciliary beating and transepithelial ion transport. Cholinergic chemosensory brush cells are airway epithelial cells that express components of the canonical bitter taste signaling cascade such as bitter taste receptors, Phospholipase b2 (PLCb2) and the TRPM5 (transient receptor potential subfamily M channel 5) ion channel. We showed previously that these cells sense “bitter tasting” bacterial products and bitter substances, and evoke protective respiratory reflexes utilizing the canonical taste transduction cascade.

We here investigated the transcriptome of single tracheal ciliated and brush cells by single cell deep sequencing. The impact of various products from *Pseudomonas aeruginosa* (PA) on MC was estimated by the cilia-driven particle transport speed (PTS) on explanted mouse tracheas. PTS was visualized by tracking the transport of dynabeads on the surface of the mucosa before and after application of six PA products: PQS (3,4-dihydroxy-2-heptylquinoline, pseudomonas quinolone signal), 2-AA (2-aminoacetophenone), DHQ

(2,4-dihydroxyquinoline), HHQ (4-hydroxy-2-heptylquinoline), HQNO (2-n-heptyl-4-hydroxyquinoline N-oxide), and PyOC (pyocyanine). In addition, the influence of PQS and the bitter cascade agonist denatonium on transepithelial ion transport was examined with Ussing chamber experiments in murine isolated trachea.

The effect on MC differed between the investigated PA products. PQS, 2-AA and DHQ (all 100  $\mu$ M) significantly increased the PTS ( $p < 0.0001$ ,  $p = 0.0004$ ,  $p = 0.0217$ , respectively). The most prominent increase was observed with PQS. NGS of single tracheal epithelial cells revealed TRPM5 and PLC $\beta$ 2 as hallmark genes for brush cells. The PQS-induced increase was abolished with the TRPM5 antagonist TPPO (triphenylphosphine oxide, 100  $\mu$ M) and significantly reduced in TRPM5-deficient mice ( $p = 0.05$ ). HHQ and HQNO (both 100  $\mu$ M) decreased PTS ( $p = 0.0159$ ,  $p = 0.0005$ , respectively), while PyOC (100  $\mu$ M) had no effect. Furthermore, PQS (100  $\mu$ M, apical) transiently increased net transepithelial ion current ( $I_{sc}$ ) ( $p = 0.03$ ), whereas denatonium (1 mM) decreased  $I_{sc}$  ( $p < 0.001$ ). This effect was dose-dependent and repeatable without affecting the amplitude of the response. TPPO (basolateral, 100  $\mu$ M) significantly reduced the effect of denatonium (1 mM,  $p = 0.011$ ), indicating an activation of the bitter taste signaling cascade involving TRPM5 activation by denatonium. Here, we show that bitter “tasting” PA products alter both major components of MC, the transepithelial ion transport and the cilia-mediated PTS. Both are dependent on activation of the TRPM5 channel present only in chemosensory brush cells. Thus, the TRPM5 ion channel is a novel key player in regulation of MC and might play an important role in protection against bacterial colonization.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA100

#### Decorin modulates melanoma cell migration: Diverse effects caused by differential glycanation of the protein core

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The  $Na^+/H^+$  exchanger NHE1 controls cellular pH homeostasis and is required for directional cell migration and invasion of extracellular matrices. In the dermis, the predominant proteoglycan is decorin (DCN), and its N-terminal glycosaminoglycan (GAG) chain is either of a dermatan (DS) or a chondroitin sulfate (CS) type. DS-DCN and the GAG chondroitin 6-sulfate (CS6S) inhibit migration and invasion of murine melanoma cells (B16V) on 3D collagen-rich C57BL/6 fibroblast cultures. DS-DCN reversibly reduces NHE1 activity and acidifies the cytosol while CS6S does not affect NHE1 activity but leads to an irreversible cytosolic acidification (Stock et al., 2011). To discover whether the DCN protein core alone (core) or DCN with different GAG structures (DS-DCN and CS-DCN) would act differently on melanoma cells, we compared their effects on single cell migration, morphology, NHE1 expression, cell surface pH,  $\beta$ -integrin expression, metabolic activity and

apoptosis in B16V cells. Furthermore, we determined the DCN effect in 3D collagen-rich *Dcn*<sup>-/-</sup> fibroblast cultures supplemented with the different DCN preparations.

We discovered that both DS-DCN and CS-DCN decreased migration speed and total distance covered within 6 hours. In contrast, the core stimulated motility accompanied by a more dendritic or spindle-like morphology, which could be counteracted by the specific NHE1 inhibitor KR-33028. Interestingly, NHE1 expression was significantly reduced by DS-DCN, but significantly increased by the core. Cell surface pH, known to modulate cell adhesion and migration, was irreversibly acidified by all three DCN preparations. Remarkably, DS-DCN treatment caused a shift in integrin expression from  $\beta 3$  to  $\beta 1$  integrin, whereas CS-DCN had no effect, indicating a specific impact of the GAG chain. This is supported by the finding that the protein core induced a shift from  $\beta 1$  to  $\beta 3$  integrin. None of the three DCN preparations affected metabolic activity or induced caspase 3/7 activity. When incorporated in *Dcn*<sup>-/-</sup> matrices, none of the three DCN preparations had an effect on cell migration; however, CS-DCN and core reduced invasion significantly.

In summary, the core stimulated (i) NHE1 expression, (ii) acidification of the cell surface and (iii) cell motility in a 2D setting. However, both DS-DCN and CS-DCN, inhibited cell motility without affecting cell viability, and DS-DCN caused a significant decrease in NHE1 expression. In a more complex system where DCN was incorporated in a 3D matrix, differences in invasion depended on the GAG chain. This points to a possible therapeutic potential of DCN and GAGs and underscores the need to discover their (different) mechanism(s) of action. Also, the shifts in  $\beta$  integrin expression, induced by core and DS-DCN, deserve further study.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCA101

##### Modifying pentamidine blood-brain barrier transport through ion-pair formation

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Human African trypanosomiasis (HAT) is fatal unless treated. Pentamidine has been used to treat first-stage (hemolymphatic) HAT since the 1940s, but it is ineffective against second-stage (meningoencephalitic, or central nervous system) infection. Research has suggested that hOCT1 is involved in the transport pentamidine across the blood-brain barrier (BBB). The aim of this study was to investigate if through the formation of ion-pairs the hOCT1 mediated BBB transport of pentamidine could be improved. Structural properties (stoichiometry, binding sites and binding affinities) of ion-pairs formed between pentamidine and taurodeoxycholate, octanoate, xinafoate and quercetin (selected to generate an ion-pair with a 1 +ve charge) were examined by HPLC, NMR spectroscopy and fluorescence spectroscopy. The lipophilicity of pentamidine with excess of the counter-ions was measured

by octanol-water shake-flask method at pH 7.4. Pentamidine transport was studied using human brain endothelial cells (hCMEC/D3). Interactions with membrane transporter hOCT1 were studied using a *xenopus* oocytes system in which functional expression of hOCT1 was assessed by hOCT1 endogenous substrate acetylcholine (kinetic uptake, 1-120min). Data were analysed by two-way ANOVA and expressed as mean  $\pm$  S.E.M. The lipophilicity of pentamidine (log D7.4) in the presence of taurodeoxycholate, octanoate, xinafoate and quercetin increased by 1.15, 1.06, 0.82, 0.06 units compared to pentamidine respectively. NMR revealed that ion-pairing occurred through the pentamidine amine groups. The conditional binding affinity (pK<sub>ass</sub>) of pentamidine with xinafoate at pH 7.4 was 3.8 and only this ion-pair increase pentamidine accumulation in hCMEC/D3 cells compared to the drug alone (60min and 120min time points,  $p < 0.01$  and  $p < 0.001$  respectively,  $n = 3$ ). Uptake of acetylcholine confirmed the functional expression of hOCT1 in oocytes (hOCT1:  $2.23 \pm 0.73$  pmol/oocyte/h vs sham:  $1.19 \pm 0.28$  pmol/oocyte/h  $n = 28-32$ ). Pentamidine utilized hOCT1 to accumulate in the oocyte (hOCT1:  $86.35 \pm 18.99$  vs sham:  $40.00 \pm 12.06$  fmol/oocyte/h,  $n = 23-24$   $p < 0.5$ ) and its  $K_m$  was  $279 \mu M$  ( $n = 6-8$ ). The four counterions chosen in this study generated an ion-pair complex, which was more lipophilic than the parent drug, presumably due to the shielding of one of pentamidine's two +ve charges. This structural modification improved BB uptake. The hOCT1 expressed oocytes confirmed the role of hOCT1 pentamidine transport and future work will examine the ion-pair interaction with hOCT1 in the oocyte system.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCA102

##### Lubiprostone activates CFTR in human bronchial epithelial cells via an EPAC dependent process

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Cystic fibrosis transmembrane conductance regulator (CFTR) plays an important role in Cl<sup>-</sup> secretion across upper airway epithelia. When this secretion is disrupted, as seen in the inherited condition cystic fibrosis, there is defective mucociliary clearance and an increased risk of infection. This highlights the critical role CFTR plays in upper airway function. Previous work

in human bronchial epithelial cells [1] and ovine airway [2] has indicated that lubiprostone, the metabolite analogue of prostaglandin E1, activates CFTR via a prostaglandin receptor mediated process. In the current study the role that exchange protein directly activated by cAMP (EPAC) [3] plays in this activation mechanism was investigated.

(16HBE14o-) cells were grown on permeable supports until confluent and then mounted in an Ussing chamber, with standard Krebs on the basolateral side and low Cl<sup>-</sup> Krebs on the apical side (solutions bubbled with 5% CO<sub>2</sub>). The transepithelial potential (V<sub>te</sub>) was measured and the equivalent short circuit current (I<sub>SC</sub>) calculated by injection of 10 µA of current each minute. All measurements were blank insert corrected. Measurements over a 5-minute time period were taken as control, and then 1 µM lubiprostone was added to the apical side of the insert. Once steady-state was achieved 10 µM CFTR<sub>inh172</sub> was added to provide an indication of CFTR function. This was repeated in unpaired inserts in the presence of either DMSO vehicle or 25 µM ESI-09, an inhibitor of EPAC (both added 10 minutes before the start of the experiment). Statistical significance was tested using ANOVAs or Student's t test as appropriate and assumed at the 5% level.

With the vehicle controls, addition of lubiprostone shifted the V<sub>te</sub> from 7.00 ± 0.69 mV to 17.7 ± 1.86 mV (n=9), a mean increase of 10.7 ± 1.49 mV. CFTR<sub>inh172</sub> reversed this increase, with the V<sub>te</sub> falling by 10.3 ± 1.62 mV to 7.33 ± 0.84 mV. Pre-incubation with ESI-09 prior to the addition of lubiprostone prevented the shift in V<sub>te</sub>. V<sub>te</sub> was 5.30 ± 0.69 mV and 6.51 ± 1.40 mV (n=9), in the absence and presence of lubiprostone, respectively. There was no significant difference between these. Addition of the CFTR<sub>inh172</sub> was also without effect, V<sub>te</sub> was 3.09 ± 0.73 mV in the presence of CFTR<sub>inh172</sub>. These data support the idea that lubiprostone activates CFTR in human airway epithelial cells through a mechanism that requires EPAC. The specific action EPAC mediates in the activation process requires future work. However, given the previous literature one possibility is that EPAC acts to stabilise CFTR in the membrane [3].

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Both A. Hadjipanteli and S.I. Ming contributed equally to this work.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA103

### Screening colorectal cancer cell lines for connexin-dependent intercellular diffusive coupling

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**Background:** According to a widely held canon, most types of cancer have little or no cell-to-cell diffusive coupling involving gap junctions (GJs) (1). Moreover, connexin (Cx) proteins,

which assemble GJs, are suggested to be tumour suppressors, putatively because they restrict cell motility and limit the extent to which a cancer cell can regulate its cytoplasmic milieu independently of its neighbours (2). Recently, we have shown that some cancer cell lines, such as pancreatic Colo357, are diffusively coupled by Cx43 channels, which establishes a pathway for exchanging metabolites, including lactate and H<sup>+</sup> ions (3). Here, we screened a panel of colorectal cancer (CRC) cell lines for evidence of coupling.

**Methods:** A gene expression database from >100 CRC cell lines was searched for expression levels of connexin-coding genes (*GJA1* for Cx43, *GJB2* for Cx26, *GJB3* for Cx31). Measurements of cell-to-cell coupling were performed on a selection of CRC cell lines that included various combinations of Cx isoform expression, ranging from high to low/absent. The protein products were tested by western blot and immunofluorescence. Diffusive coupling was quantified in calcein-loaded monolayers by fluorescence recovery after photobleaching (FRAP), which involves bleaching one cell in a monolayer and measuring the time constant of fluorescence recovery, which is then converted into a permeability constant.

**Results:** The bioinformatics search revealed that the most substantial variation in expression was found for the gene coding for Cx43, a protein typically associated with neurons and cardiac muscle, and for Cx26, an isoform found in normal epithelia. CRC cells manifested different degrees of inter-cellular communication. Specifically, *GJB2*-expressing Cx26-positive CRC lines DLD1 and SNU1235 produced monolayers in which diffusive coupling was detected in the majority of the cells (permeability: 0.015±0.002, 0.012±0.002 µm/s, respectively). In contrast, *GJA1*-expressing Cx43-positive CRC lines CACO2, C10 and LOVO demonstrated heterogeneity, observed as distinct sub-populations with strong coupling or no coupling. The ratio of these sub-populations ranged from 1:1 in C10 and LOVO cells to 3:1 in CACO2 cells. Coupled cells demonstrated strong coupling, ranging from 0.010±0.001 µm/s in LOVO cells to 0.038±0.002 µm/s in CACO2 cells.

**Conclusions:** Our results show that cell-to-cell connectivity varies between CRC cells and even within subpopulations of a CRC line. The extent of diffusive coupling between connected cells is sufficient to permit a substantial flux of small molecules, including metabolites. This could be particularly important in solid tumours where large diffusion gradients of metabolites, formed along gradients of oxygen, could drive net fluxes through coupled cells and hence influence tissue physiology. Loewenstein WR, Kanno Y. *Nature* (1966) 209 (5029):1248-9.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.



## PCA104

**Enhanced  $\text{Ca}^{2+}$  signalling and over-expression of STIM1 are characteristic of human eccrine sweat gland secretory coil cells isolated from hyperhidrotic individuals**R.L. Evans<sup>1</sup>, S.R. Johnstone<sup>2</sup>, M. Elias<sup>2</sup>, J. Roberston<sup>2</sup>, D.L. Bovell<sup>3</sup> and P.E. Martin<sup>2</sup><sup>1</sup>Unilever Research & Development, Bebington, UK, <sup>2</sup>Life Sciences, Glasgow Caledonian University, Glasgow, UK and <sup>3</sup>Weill-Cornell Medicine-Qatar, Doha, Qatar

The condition of hyperhidrosis occurs when eccrine gland sweating exceeds that required for normal body temperature regulation. It can be primary (focal), where no underlying cause has been identified, or secondary (generalized), where an underlying cause is known. Primary hyperhidrosis typically occurs in the axillae, or on the forehead, palms and feet, and can be anxiety-inducing and disabling. The underlying cause of hyperhidrosis is largely unknown.

Eccrine gland sweat production is initiated by a biphasic increase in the level of intracellular  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ) consisting of an initial agonist-induced release of  $\text{Ca}^{2+}$  from internal stores, followed by an influx of extracellular  $\text{Ca}^{2+}$ , a process known as store-operated  $\text{Ca}^{2+}$  entry (SOCE)<sup>1</sup>. Recent studies of isolated human eccrine gland secretory coil cells have demonstrated that SOCE is regulated by the interaction of STIM and Orai proteins<sup>2</sup>.

The aim of this study was to investigate the role of SOCE during hyperhidrosis, using eccrine sweat gland secretory coil cells from individuals with and without hyperhidrosis. Sweat glands were isolated from skin samples donated with consent<sup>3</sup>. Intracellular  $\text{Ca}^{2+}$  responses were studied using  $\text{Ca}^{2+}$  imaging techniques, and the relative expression of various proteins associated with SOCE determined by mRNA expression, Western blot analysis and immunofluorescence. siRNA knockdown of STIM and Orai proteins, in conjunction with  $\text{Ca}^{2+}$  imaging of cells stimulated with the ER  $\text{Ca}^{2+}$ -ATPase inhibitor thapsigargin (Tg), was used to determine the relative contributions of various SOCE proteins to fluid secretion by hyperhidrotic cells.

Fluorescence imaging showed that the dose-response to stimulation with the muscarinic agonist carbachol was elevated at least 2-fold in hyperhidrotic eccrine gland secretory cells compared to those from non-hyperhidrotic cells. Expression of STIM1 mRNA was also significantly higher (~2-fold) in hyperhidrotic cells, whilst mRNA expression of the SOCE proteins Orai1, TRPC1, the muscarinic receptor m3,  $\text{IP}_3$  receptor isoforms 1,2 and 3, and the water channel AQP5, were not significantly elevated. Over-expression of STIM1 was also confirmed at protein level by Western blotting and immunohistochemistry. STIM2 was not over-expressed.  $\text{Ca}^{2+}$  imaging of hyperhidrotic eccrine glands showed that Tg-induced SOCE was reduced ~90% in a STIM1 knockdown, and by ~79% in an Orai1 knockdown.

This study suggests that the over-expression of STIM1 results in enhanced SOCE (and probably sweat secretion) in human hyperhidrotic eccrine gland secretory coil cells. Further mechanistic investigations will be required to fully determine the molecular physiology underlying this distressing human condition.

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2) Bovell et al., Proc Physiol Soc. 2016: 37: PCA137

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA105

**Metal oxide nanoparticles: size role in membrane interactions**D. Zanella<sup>3</sup>, E. Bossi<sup>3,1</sup>, R. Gornati<sup>3,1</sup>, N. Faria<sup>2</sup>, J. Powell<sup>2</sup> and G. Bernardini<sup>3,1</sup><sup>1</sup>The Protein Factory Research Center, Politecnico of Milano and University of Insubria, Milan, Italy, <sup>2</sup>Department of Veterinary Medicine, University of Cambridge, Cambridge, UK and <sup>3</sup>Department of Biotechnology and Life Sciences, University of Insubria, Varese, Italy

Nano scale modifies the physical-chemical characteristics of metal particles in comparison with the original bulk materials and alters the type and scale of the interactions with biological materials. The modified properties may alter interactions with cell membranes allowing non-endocytotic nanoparticles (NPs) entrance. This pathway has been recently documented for commercially available  $\text{Co}_3\text{O}_4$  and  $\text{Fe}_3\text{O}_4$  NPs<sup>1,2</sup>. Metal oxide NPs have therefore been analysed, to investigate this mechanism of permeation that can be important both under the toxicological and the translational point of view.

In the present work, cobalt and nickel-based NP interactions with *Xenopus laevis* oocyte membranes were characterized by Two-Electrode Voltage Clamp; resting potential, membrane capacitance, membrane resistance and current-voltage relationships were measured at different times from the beginning of the exposure to NPs, using the protocols described previously<sup>2</sup>.

Oocytes exposed to  $\text{Co}_3\text{O}_4$  NPs presented significant reductions of membrane resistance and resting potential within 5 min from the beginning of the treatment. These reductions disappeared in oocytes exposed for 20 min (Fig. 1A). NiO NPs failed to elicit any relevant change in membrane parameters. Oocytes were then exposed to  $\text{Co}_3\text{O}_4$  NPs in aggregated form (30 minutes after sonication NPs were aggregated), and no significant differences were measured in membrane resistance and resting potential, highlighting the role of particles characteristics in the first 5 min after sonication in determining the biological effects. The transmembrane currents at the voltage of -120 mV and +40 mV are reported in Fig. 1B-C.

Static Light Scattering (SLS) and Laser-Doppler Microelectrophoresis were used to determine the physical and chemical properties of the NPs in the experimental medium. Both NPs had neutral z-potentials ( $\text{Co}_3\text{O}_4 = 0.5 \pm 0.05$  mV and NiO  $= 0.045 \pm 0.018$  mV,  $n=3$ ,  $\text{mean} \pm \text{S.E.M.}$ ), thus aggregating in the medium. Hydrodynamic size distributions (Fig. 2) showed differences among the NPs:  $\text{Co}_3\text{O}_4$  NPs possessed a submicron population for at least 5 min and NiO NPs possessed a submicron population for 20 minutes. The submicron population of  $\text{Co}_3\text{O}_4$  NPs was smaller than 200 nm, while NiO NPs were always above 250 nm.

The main difference determined in physical and chemical properties between  $\text{Co}_3\text{O}_4$  and NiO NPs is colloidal size, which is most likely to play a significant role in passive permeation mechanisms.

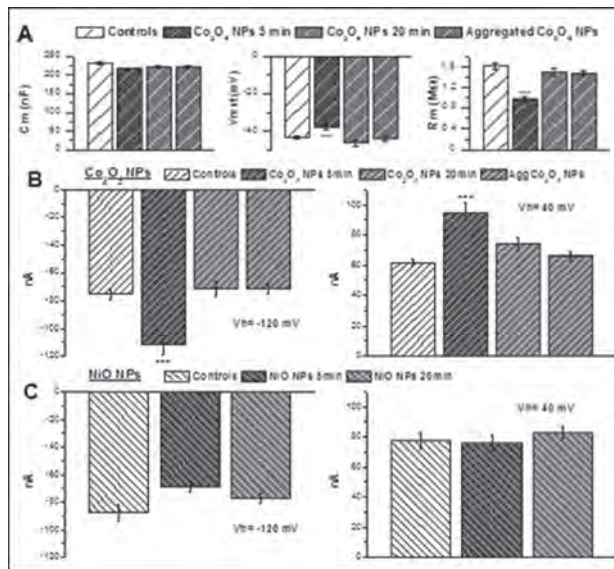


Fig. 1: A) Membrane Parameters. Co<sub>3</sub>O<sub>4</sub> NPs: Controls n=76, 5min n=25, 20 min n=30, Aggregated n=44, all from at least 2 batches. B) Co<sub>3</sub>O<sub>4</sub> NPs Transmembrane Current Values: same n values reported in A. C) NiO NPs Transmembrane Current Values: Controls n=53, 5min n=34, 20min n=47, from 2 batches. Statistical analysis: One-Way ANOVA/Bonferroni-Holm post-hoc test

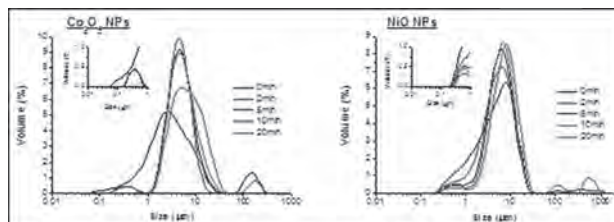


Fig. 2: Hydrodynamic size determined over time by SLS (representative experiments)

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA106

## The jejunum epithelium adapts quickly to hypoxic insults by modifying transepithelial glucose transport

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The gastrointestinal epithelium is known to tolerate huge variations in oxygen supply. Thus, it must command elaborate adaptation mechanisms to maintain its key functions like nutrient uptake. We hypothesized that the import of energy substrates into the intestinal epithelial cells must be tightly regulated under hypoxia and elucidating its adaptation might also shed light on the underlying mechanisms.

We incubated isolated lagomorph jejunum epithelia in Ussing chambers under short-circuit-conditions. After equilibration, we simulated hypoxia by gassing a group of epithelia with 1% O<sub>2</sub> + 99% N<sub>2</sub>. We assessed electrogenic transport of glucose and the dipeptide glycyl-glutamine, respectively by measuring the increase of short-circuit current (I<sub>sc</sub>) after mucosal

addition of each substrate. Transepithelial transport of glucose, orthomethyl-glucose (OMG) and mannitol was determined radioactively. Additionally, we incubated the epithelia with inhibitors for different glucose transport mechanisms and evaluated their effect on <sup>14</sup>C-glucose transport.

After only 45 minutes of hypoxia, a significant decrease in the glucose- and glycyl-glutamine-induced I<sub>sc</sub> was observed compared to the control group, i.e. a decreased activity of secondary active transport mechanisms. Despite the reduced electrogenic import of glucose, its transepithelial transport was not diminished by hypoxic conditions. Paracellular integrity was kept up as indicated by mannitol flux rates. Effects of (altered) intracellular metabolism on glucose permeation could be excluded by the use of OMG. Thus, the active import by SGLT 1 was presumably supplemented by other transport mechanisms. An inhibition of transepithelial glucose transport by a specific inhibitor of glucose transporter (GLUT) 1 detectable under hypoxic but not under control conditions revealed an involvement of GLUT 1. We assessed the influence of AMP-activated protein kinase (AMPK) by preincubating the epithelia with its antagonist compound C before submitting them to hypoxia. By doing so, we could abolish the decrease in SGLT 1 activity under hypoxia. Western blot studies revealed a phosphorylation, i.e. activation of AMPK under hypoxia that was also abolished by compound C.

Summing up, we could show that nutrient transport mechanisms in jejunum epithelium are modulated quickly under hypoxic conditions. A substitution of the energy-dependent import via SGLT 1 by GLUT 1 mediating facilitated diffusion might help to secure an energy-saving uptake of the cells' most important fuel and thus maintaining their function. This adaptation process seems to be mediated by AMPK.

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PCA107

## A new mode of binding between Calmodulin and the human Na<sup>+</sup>/H<sup>+</sup> exchanger SLC9A1 with implications for the SLC9A1 function

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The plasma membrane Na<sup>+</sup>/H<sup>+</sup> exchanger NHE1 is a ubiquitously expressed ion transporter with essential physiological roles. It functions as a dimeric hub protein with many binding partners, one of which is the calcium-binding protein calmodulin (CaM) (1). CaM is an important regulator of NHE1 activity (2) and is shown to bind two consecutive sites in the NHE1 C-terminal tail (1,3). The aim of the present work was to delineate the structural and functional roles of CaM for NHE1.

For *in vitro* analyses, we created two NHE1 peptides: CaM Site1 (residues 622-657) and Site1+2 (622-692). Using circular dichroism (CD) spectroscopy to monitor thermal unfolding, we showed that the N-lobe of Ca<sup>2+</sup>-saturated CaM was stabilized by both peptides (T<sub>m, CaM N-lobe alone</sub> = 44±2°C, T<sub>m, Site1</sub> = 77±1°C, T<sub>m, Site1+2</sub> = 82±2°C) (±SEM, n=1). Isothermal titration calorimetry (ITC) showed a CaM:peptide stoichiometry in solution of 1:2 (n=2) for the Site1 peptide. HSQC NMR spectra of <sup>15</sup>N-labeled CaM titrated with unlabeled Site1 peptide demonstrated that the peptide initially bound the CaM C-lobe

then the CaM N-lobe (n=1). Notably, addition of the Site1+2 peptide showed NMR peak saturation at a 1:2 stoichiometry of CaM:peptide similarly to Site1 spectra peaks. Importantly, this demonstrates that CaM can also bind two molecules of NHE1 *in vitro*. Two phosphorylation sites, S648 and T653, are located in the sequence covered by the Site1 peptide and have been assigned NHE1 regulatory roles. Exploiting the lack of endogenous NHE1 in the cell line PS120 (4), we generated six cell lines expressing WT NHE1 or mutant variants; 1K3R1D3E (K641D, R643E, R645E, R647E) and 1Q2R3E (Q640E, R636E, R632E) to disrupt CaM binding, and S648A, S648D and T653A to inhibit or mimic phosphorylation at these sites. BCECF-AM was used to measure intracellular pH in cells bathed in Ringer solution pH 7.4, followed by acidification using the NH<sub>4</sub>Cl prepulse technique. Untransfected cells were used as control. Preliminary results showed that WT NHE1 cells had the fastest rate of recovery ( $28.4 \pm 0.1 \times 10^{-3}$  pH/s) followed by the S648D ( $13.8 \pm 0.1 \times 10^{-3}$  pH/s), the S648A ( $12.3 \pm 0.2 \times 10^{-3}$  pH/s) and lastly the 1K3R1D3E ( $6.8 \pm 0.1 \times 10^{-3}$  pH/s) ( $\pm$ SEM, n=4). This suggested that CaM-binding is necessary for NHE1 activity and that substitution of S648 to Ala or Asp is important too, but less so. Ongoing work investigates the kinases involved. In conclusion, we characterize a new mode of binding between CaM and NHE1 with a stoichiometry of 1:2 and potential implications for Ca<sup>2+</sup> mediated regulation of NHE1 dimerization. Ongoing systematic work on the functional importance of CaM binding and phosphorylations around CaM binding site 1 in NHE1 will expand the current understanding of the physiological relevance of Ca<sup>2+</sup>/CaM signaling for NHE1.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA108

### Hypoxic inhibition of alveolar epithelial Na-transport is aggravated by hypocapnia

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Hypoxia stimulates ventilation resulting in exposure of alveolar epithelium to both hypoxia and hypocapnia. Hypoxia inhibits Na-reabsorption across the alveolar epithelium by decreasing the activity of epithelial Na channels (ENaC) and Na/K-ATPase, due to decreased mRNA and protein expression as well as their abundance in the plasma membrane. Effects of hypocapnia in combination with hypoxia on alveolar ion transport are not well understood. To test this, primary alveolar epithelial cells were isolated from male Sprague Dawley

rats (~150g), were cultured on transwell filters, and kept in normoxia and hypoxia (1.5% O<sub>2</sub>) in combination with normocapnia (5% CO<sub>2</sub>) and hypocapnia (3% CO<sub>2</sub>) for 24 hours in presence of various modulators (see below). Acute exposures were done by equilibrating cells cultured in normoxia with the respective gas mixture for 30 min in Ussing chambers prior to measuring transepithelial electrical currents (I<sub>eq</sub>).

Normocapnic hypoxia for 24h decreased ENaC (-80%) and Na/K-ATPase (-42%) activity measured as amiloride- and amphotericin B sensitive electrical currents, respectively. ENaC activity was even further decreased by hypocapnic hypoxia (-60%), whereas hypocapnia did not affect Na/K-ATPase. Effects were small after acute exposure but inhibition by hypocapnic hypoxia was already detectable. The decreased ENaC activity was not prevented by inhibition of carbonic anhydrase. In 24h experiments, addition of HEPES to bicarbonate-containing medium to keep extracellular pH independent of hypocapnia-induced changes in pH did not prevent aggravated inhibition of ENaC. However, culturing cells in bicarbonate-free, HEPES-buffered media for 24h decreased ENaC activity in normocapnic hypoxia but prevented the additional decrease in hypocapnic hypoxia. Culturing cells in bicarbonate-buffered media together with inhibitors of protein kinase C (chelerythrine and GÖ6976), which is involved in inhibition of alveolar epithelial Na-transport in hypercapnia (Vadasz & Snajder, Front Immunol; doi: 10.3389/fimmu.2017.00757), and of ERK-1/2 (U0126), did not prevent the inhibition of ENaC by normocapnic hypoxia nor its aggravation by hypocapnia. Hypoxia (24h) decreased  $\alpha$ -,  $\beta$ -, and  $\gamma$ - ENaC mRNA expression by ~70%, and decreased  $\alpha$ ENaC protein abundance in the apical membrane and in the intracellular compartment (measured after membrane biotinylation) by approximately 50%. However, hypocapnia did not affect mRNA and protein expression.

These results indicate that hypocapnia aggravates hypoxic inhibition of alveolar epithelial ENaC-mediated Na-reabsorption. Aggravated inhibition was independent of alkalosis, PKC, ERK, and of mRNA and protein expression of ENaC, but seems to be caused by increased intracellular chloride concentrations upon decreasing cellular bicarbonate in hypocapnia.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA109

### Localization of the Plasma Membrane Ca<sup>2+</sup> ATPase 1 (PMCA1) to Renal and Intestinal Epithelia

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Calcium (Ca<sup>2+</sup>) is essential for a wide range of physiological processes, such as neurological function, intracellular signaling and the stability of bone. As such, the systemic and intracellular Ca<sup>2+</sup> concentration must be tightly regulated within a narrow range for these physiological processes to occur. A stable Ca<sup>2+</sup> concentration in the blood is maintained by exchange from bone and by transcellular Ca<sup>2+</sup> transport in epithelia of intestine and kidney. The four Plasma membrane Ca<sup>2+</sup> ATPase (PMCA) genes are important for cellular Ca<sup>2+</sup>



transport. In previous studies, we have found high expression of the  $\text{Ca}^{2+}$  ATPase PMCA4 in the distal nephron of kidney, where high vectorial transport of  $\text{Ca}^{2+}$  occurs, while no PMCA4 expression was detected in intestinal epithelial cells. Using a pan-specific antibody against all PMCA forms, immunoreactivity in the intestinal epithelia was noticeable and we therefore hypothesized that PMCA1 could be the main basolateral transporter expressed in the intestine. To address this question, we raised monoclonal antibodies in mice directed specifically against PMCA1. In mice duodenum, PMCA1 antibodies stained lateral membranes of enterocytes in line with a role in intestinal  $\text{Ca}^{2+}$  transport. Unlike PMCA4, no major immunoreactivity was observed in basolateral membranes of tubular epithelial cells. Instead, we found discrete PMCA1 staining in sub-apical membrane domains of the proximal tubules, thick ascending limbs, distal convoluted tubules and collecting ducts. Notably, myogenic cells in peritubular capillaries and mesangial cells showed high intensity staining, and so did the smooth muscle layers in the renal and intestinal vessels. Similar strong immunoreactivity was found in the aorta. Our study finds that PMCA1 is most likely the main PMCA in the intestine, driving transcellular  $\text{Ca}^{2+}$  transport while maintaining a "housekeeping" function in renal epithelial cells. Abundant expression in smooth muscle cells points to a role in regulation of contractility, as supported by previous studies. Ongoing investigations aim to determine the cellular expression and regulation of PMCA1 in duodenal epithelial isolates, to provide novel insights into the role of this important calcium transporter.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCA110

### Role of neutral amino acid transporter LAT4 in mouse epithelia

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Amino acids (AAs) are not only building blocks of proteins, but also play key roles in energy metabolism and as (precursors of) neurotransmitters. To maintain amino acid homeostasis, small intestine (SI) and kidney play a major role by absorbing amino acids from food and preventing their loss in the urine, respectively. Given their zwitterionic nature, neutral AAs require transport proteins to cross cell membranes. LAT4 (Slc43a2), a facilitated diffusion uniporter specific for essential neutral amino acids (Leu, Ile, Val, Met and Phe) was first identified by Bodoy et al., 2005 and then shown by us to be highly expressed in the basolateral membranes of SI and kidney tubule epithelia. LAT4 global knockout caused a postnatal malnutrition-like condition leading to death within 10 days (Guetg et al. 2015). To test whether this phenotype was due to a defect in intestinal absorption, we generated an inducible intestine-specific LAT4 knockout mouse model using a Cre-LoxP system. After induction of recombination by I.P. injection of tamoxifen (3mg/ml per 25g body weight) for 5 days just after weaning or at adult stage, body weight and food intake were monitored for 10 days before mice were euthanized by cervical dislocation and intestines collected. Quantitative PCR, western blot and immunofluorescence data showed a nearly 99% efficient knockout of LAT4 throughout the SI (n=20). However, these mice displayed no visible phenotype. Amino acid transport along the SI was assessed

by gavaging an AA mix containing radioactively labelled LAT4 substrate  $^3\text{H}$ -Leu. Blood samples were collected from the tail vein 10' before and 3', 10' and 60' after gavage. Mice were then euthanized, SI divided into 4 equal parts and the intestinal tissue and its contents collected separately. Measuring the distribution of  $^3\text{H}$ -Leu using a  $\beta$ -counter revealed only a slightly impaired transport with accumulation of  $^3\text{H}$ -Leu in the initial segment of the SI of knockout mice (n=3, p<0.05 by ANOVA). Furthermore, expression of other AA transporters studied at the RNA level did not seem to be altered to compensate for the lack of intestinal LAT4 (n=10). Thus, we showed that LAT4 knockout in SI past the postnatal phase does not affect the general well-being of mice that displayed only a mild absorption defect. To test whether this defect could be more deleterious in earlier development, we are breeding constitutional intestine-specific LAT4 knockout mice. In parallel, we have generated a doxycycline-inducible kidney tubule-specific LAT4 knockout mouse model to assess the effects of LAT4 deletion in kidney, both in young and adult animals. Results obtained with these new models will allow us to determine whether the lethal phenotype of LAT4 global knockout mice was due to a defect in intestinal absorption and/or urinary loss of amino acids or whether it was caused by the defect of LAT4 in other tissues.

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PCA111

### Relative contribution of inhibition of sodium-hydrogen exchanger 3 (NHE3)-mediated fluid absorption and cystic fibrosis transmembrane conductance regulator (CFTR)-mediated fluid secretion to the increase in intraluminal alkalinity and fluidity induced by the heat-stable *Escherichia coli* enterotoxin (STa) analogue linaclotide

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**Background:** Constipation and intestinal obstructive episodes are major health problems in cystic fibrosis patients. The heat-stable *Escherichia coli* enterotoxin STa, the endogenous analogues guanylin and uroguanylin and the synthetic analogues linaclotide and plecatanide induce an increase in gut fluidity via binding to the guanylate cyclase C and eliciting cGMP and possibly cAMP production in the enterocyte. This results in stimulation of CFTR-mediated anion secretion and inhibition of NHE3-mediated fluid absorption. Theoretically, the effect of STa/guanylin/linacotide on NHE3 inhibition may be preserved in the CF gut, but this has not been studied. **Aim:** We investigated whether and how well the STa analogue linaclotide inhibits fluid absorption and increases alkaline secretion in the jejunum of CFTR null (CFTR KO), F508del mutant, and WT mice in vivo. **Methods and Results:**  $10^{-7}\text{M}$  of the STa analogue linaclotide was added to the jejunal perfusate during single pass perfusion of a jejunal segment in

isoflurane-anesthetized (5% for induction, 1,8-2,2% for maintaining), acid/base- and blood pressure controlled CFTR KO, F508del mutant, and WT mice. Net fluid balance before and after linaclotide application was assessed gravimetrically, and the net alkaline output into the perfusate was determined by pH-stat backtitration. Basal fluid absorptive rate was significantly higher in CFTR KO and F508del mutant than in WT mice ( $147 \pm 33$ ,  $148 \pm 34$ , and  $140 \pm 24$   $\mu\text{l}/\text{cm}/\text{h}$  at 10 min (t10), 20 (t20) and 30 (t30) min after stabilization in CFTR KO,  $130 \pm 26$ ,  $126 \pm 23$ , and  $130 \pm 19$   $\mu\text{l}/\text{cm}/\text{h}$  at t10, 20 and 30 in F508del, and  $113 \pm 20$ ,  $114 \pm 21$ ,  $114 \pm 21$   $\mu\text{l}/\text{cm}/\text{h}$  at t10, 20 and 30 in the WT jejunum. Basal  $\text{HCO}_3^-$  output was  $3,6 \pm 1,4$   $\mu\text{eq}/\text{cm}/\text{h}$  in WT,  $1,7 \pm 0,7$  in F508del, and  $1,28 \pm 0,4$  in CFTR KO jejunum.  $10^{-7}\text{M}$  linaclotide resulted in a change from jejunal fluid absorption to secretion by  $178 \pm 28$   $\mu\text{l}/\text{cm}/\text{h}$  in WT, by  $70 \pm 18$   $\mu\text{l}/\text{cm}/\text{h}$  in CFTR KO and by  $58 \pm 20$   $\mu\text{l}/\text{cm}/\text{h}$  in F508del jejunum, while it increased  $\text{HCO}_3^-$  output by  $5,6 \pm 1,3$   $\mu\text{eq}/\text{cm}/\text{h}$  in WT, by  $3,1 \pm 1,1$  in F508del, and by  $1,7 \pm 0,6$  in CFTR KO jejunum ( $n=5-7$ ). **Summary and conclusions:** The results demonstrate that activating the GCC receptor elicits a decrease in jejunal fluid absorption and an increase in luminal alkalinity in CFTR KO and F508del mutant mice. Targeting this pathway pharmacologically may be an effective method to improve gut fluidity and alkalinity in CF patients.

This project was funded by the Cystic Fibrosis Trust SRC-CFGI (speaker Prof. Soraya Shirazy-Beechey), the DFG SE460-19-1 and 21-1, and by the Volkswagen Foundation (VW-Vorab) to US.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCA112

##### **Genetic deletion of the Slc26a9 anion transporter results in reduced pancreatic fluid secretion and impaired glucose tolerance in young female mice**

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**Background:** Slc26a9 is a member of the Slc26 multifunctional anion transporter family. In expression systems, it interacts with CFTR and may enhance or inhibit CFTR function, depending on the expression system. Polymorphisms in Slc26a9 are associated with an increased incidence of diabetes in cystic fibrosis patients. **Aim:** We investigated the expression of Slc26a9 in the pancreas and elucidated its potential role in pancreatic ductal electrolyte and fluid secretion and in pancreatic endocrine function. **Methods and Results:** The mRNA expression of Slc26a9 was low in pancreatic parenchyma but 20-fold higher in microdissected pancreatic ducts. No Slc26a9 mRNA expression was detected in the liver, while bile ducts displayed low Slc26a9 expression. Pancreatic and biliary fluid and bicarbonate secretion were assessed in isoflurane (1.3-1.8%) anesthetized, artificially ventilated Slc29<sup>-/-</sup> and age- and sex-matched wildtype (WT) littermates. To test pancreatic endocrine function, blood glucose levels were measured after an i.p. glucose bolus over a period of 2 hours. Significantly reduced basal as well as secretin-stimulated pancreatic fluid

secretory rates were observed in young adult (6-8 weeks) female Slc26a9<sup>-/-</sup> mice, with no difference in the bicarbonate secretory rates. In young male mice, as well as male and female aged mice (>1 year), no significant difference in pancreatic ion secretion was observed. Blood glucose levels declined slightly but significantly slower after 2g/kg i.p. glucose bolus in Slc29a9<sup>-/-</sup> female mice. No difference in peripheral insulin resistance was observed in Slc26a9<sup>-/-</sup> compared to sex- and age-matched controls. Biliary fluid and bicarbonate secretion were not affected by loss of Slc26a9 expression. **Conclusions:** Deletion of Slc26a9 is associated with a reduction in pancreatic fluid secretion and impaired glucose tolerance in young female mice. The results underline the importance of Slc26a9 in pancreatic physiology particularly at young age.

This project was funded by a grant from the Deutsche Forschungsgemeinschaft DFG SE460/19-1

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCA113

##### **Salmo salar oligopeptide transporters PepT1a and PepT1b: a comparative electrophysiological characterization of partial and complete transport cycle**

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The H<sup>+</sup>-coupled peptide transporter 1 (PepT1) belongs to SoLute Carrier family 15 (SLC15A1) and is responsible for the absorption of di/tripeptides in enterocytes. Beside its nutritional role, it has been hypothesized that PepT1 functions as a transceptor, i.e. a peptide sensor/transporter involved in gut hormone release from entero-endocrine cell(s)<sup>1,2</sup>.

Studying the role of PepT1 in peptide absorption in the gut is relevant for the direct relation between dietary protein availability and fish growth<sup>3</sup>. In salmonids, PepT1 gene has been duplicated and two transporters, i.e. PepT1a and PepT1b, have been found in the intestine.

The partial and complete transport cycle of the two PepT1-type transporters of *Salmo salar*, ssPepT1a and ssPepT1b, heterologously expressed in *Xenopus laevis* oocytes, were studied using Two Electrode Voltage Clamp technique. The pre-steady state currents of ssPepT1b were like that of other fish orthologs<sup>4</sup> but differed from those of ssPepT1a. ssPepT1a showed a slower decaying currents, and the charge vs voltage (Q/V) and time constant vs voltage ( $\tau/V$ ) curves shifted to more positive potentials behaving as the mammalian transporter<sup>4</sup>. In both transporters, reducing external pH from 7.6 to 6.5 slowed the transients decay, shifting to more positive potential the Q/V and  $\tau/V$  curves (Fig. 1).

To evaluate the transport activity of ssPepT1a and ssPepT1b, the transport-associated currents were recorded in presence of 1mM of lysine(Lys)-containing peptides (KcPeps) as Lys is a limiting amino acid for animal growth<sup>3</sup>. KcPeps elicited

transport-associated currents of different amplitudes, for e.g. ssPepT1b generated large currents when exposed to peptides carrying Lys in the N-terminus (KG, KM), while ssPepT1a produced small currents independently of Lys position (Fig. 2). The current vs voltage ( $I/V$ ), in the presence of KcPeps, showed small and similar currents at two different pH (6.5, 7.6) in ssPepT1a. Conversely, in ssPepT1b the  $I/V$  curves differed at the most negative potentials with larger currents recorded at pH 7.6. KG dose-response experiments were also conducted, and while fitting with logistic equation allowed to obtain the kinetic parameters ( $K_{0.5}$  and  $I_{max}$ ) at each voltage for ssPepT1b, only an estimation was possible for ssPepT1a at -140 and -120mV.

The analysis on the transient and transport currents indicated important functional differences between ssPepT1a and ssPepT1b transporters. The dissimilar substrate specificity for KcPeps supports the idea of distinct roles in peptide recognition and transport for ssPepT1a and ssPepT1b.

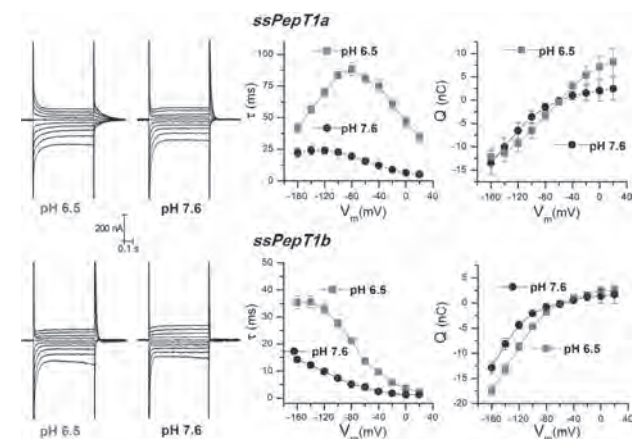


Fig. 1 Current traces and pre-steady state currents analysis for ssPepT1a and ssPepT1b.

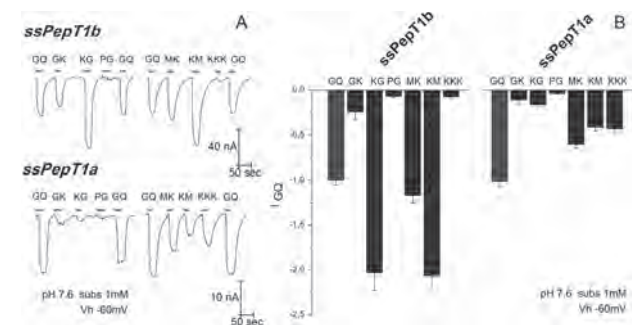


Fig. 2 Transport currents elicited by KcPeps. A: representative currents B: Means of transport-associated current normalized.

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PCA114

## Effects of knockout versus pharmacological inhibition of $\text{Na}^+/\text{H}^+$ exchanger NHE1 on growth and chemosensitivity of cancer cell spheroids

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Rapid proliferation of cancer cells is accompanied by an increased metabolic acid production, in turn increasing reliance on net-acid-extruding transporters. The  $\text{Na}^+/\text{H}^+$  exchanger NHE1 (SLC9A1) is upregulated in a variety of cancers including breast cancers, is a key player in intracellular pH homeostasis, and contributes to tumor growth. NHE1 inhibition has been found to synergize with cisplatin chemotherapy to induce cancer cell death, yet the importance of NHE1 for specific breast cancer subtypes is essentially unknown (1). The aim of this project is to investigate the dependence on NHE1 for cell growth and survival in Luminal-A and triple-negative (TNBC) breast cancer subtypes. MCF-7 (Luminal-A) and MDA-MB-231 (TNBC) human breast cancer cellswere grown as 3-dimensional spheroids. Under these conditions, NHE1 knockdown- or knockout (KO) delays spheroid growth (2). Spheroids were treated with NHE1 inhibitors EIPA or cariporide, alone or in combination with chemotherapy (Tamoxifen or a CAF regimen, respectively), every 2-3 days. Spheroid size was monitored by imaging and a final assessment of cell viability was performed on day 9. Cell viability was not affected by cariporide (10  $\mu\text{M}$ ) but was drastically reduced by EIPA (10  $\mu\text{M}$ ) in both cell lines. No synergy between NHE1 inhibitors and chemotherapy was observed. To investigate the dramatic effect of EIPA, an EIPA dose-response (0-10  $\mu\text{M}$ ) spheroid growth experiment was performed. EIPA potently reduced MCF-7 cell viability, with an EC50 of app. 3.2  $\mu\text{M}$  for WT, whereas MDA-MB-231 cell spheroids were only significantly affected at the highest dose (n= 3, One-way ANOVA,  $p>0.05$ ). To assess the NHE1-dependence of the response, this assay was next applied to two NHE1 CRISPR-KO cell lines for each cell type. Surprisingly, the response was identical between WT and NHE1 KO cell lines, consistent with a potent, NHE1-independent cytotoxic effect of EIPA. Propidium iodide staining of MDA-MB-231 and MCF-7 spheroids suggested that EIPA acted via a combination of antiproliferative effects and cell death in the spheroid core. This was confirmed by immunoblotting of MCF-7 spheroids lysed at day 2 of inhibitor treatment: EIPA (10  $\mu\text{M}$ ) essentially abolished pRb phosphorylation and increased cleavage of PARP and caspase-7 consistent with cell cycle arrest and apoptosis induction. Collectively, these results show that KO or cariporide-mediated inhibition of NHE1 in Luminal-A and TNBC breast cancer cells grown in spheroid culture has no effect on chemotherapy sensitivity. In contrast, the NHE1 inhibitor EIPA has potent growth-inhibitory, NHE1-independent effects involving increased apoptosis and decreased proliferation. This warrants reevaluation of previous work based on this compound. Future studies should assess whether EIPA may be of therapeutic interest despite its lack of specificity.



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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## PCA115

### Sodium-induced conformational changes in a glutamate transporter homologue

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Excitatory amino acid transporters (EAATs) ensure low synaptic glutamate concentration by secondary active transport of the released neurotransmitter back into neurons and glial cells. Glutamate uptake is coupled to the cotransport of three  $\text{Na}^+$  ions, one proton and the countertransport of one  $\text{K}^+$  ion; this coupling stoichiometry provides the driving force needed to maintain up to  $10^6$ -fold glutamate gradients across the membrane, essential to guarantee reliable signal transduction in excitatory synapses.

The transport cycle is characterized by two conformational rearrangements: closure of a gate shields the binding pocket from the bulk solution, while an elevator-like transmembrane translocation ensures alternating accessibility  $\leftarrow$  I tend to prefer this; or: ensures alternating access to the domain from either side of..  $\rightarrow$  of the substrate binding domain to either side of the membrane.

Additional regulation is provided by  $\text{Na}^+$  ion binding: two  $\text{Na}^+$  ions associate before the substrate and are correlated with the opening of the extracellular gate; binding of the substrate, followed by the third  $\text{Na}^+$ , induces gate closure, which in turn enables translocation across the membrane; the  $\text{K}^+$  ion then associates from the intracellular side to permit re-translation of the transport domain.

Here, we use all-atom molecular dynamics simulations and stopped-flow fluorescence spectroscopy experiments to characterize the coupling between  $\text{Na}^+$  binding and gate dynamics. We show that  $\text{Na}^+$  binding is preceded by two events: opening of the extracellular gate and reorganization of the binding pocket. Both are rare events in the *apo* state, but association of  $\text{Na}^+$  stabilizes the open, *bound-like* configuration primed for substrate binding; the coupling between  $\text{Na}^+$  binding and gate opening thus follows a conformational selection mechanism. In support of these findings, we experimentally demonstrate that two mutants, previously characterized as either affecting sodium coupling or substrate selectivity  $\leftarrow$  I feel the "respectively" would be grammatically incorrect here  $\rightarrow$  both exert their action by modifying the extracellular gate equilibrium between open and closed state.

Our results illustrate how an allosteric coupling mechanism between  $\text{Na}^+$  binding, gate dynamics and subsequent substrate binding enables glutamate transporters to fulfil their physiological task by simultaneously accomplishing high glutamate transport rates and high glutamate concentration gradients.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## PCA116

### The $\text{Na}^+/\text{HCO}_3^-$ cotransporter NBCn1 in cell cycle regulation – possible role for an unexpected localization?

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The net acid extruding  $\text{Na}^+/\text{HCO}_3^-$  cotransporter NBCn1 (Slc4a7) is widely expressed in the plasma membrane of mammalian tissues. Work from us and others has implicated NBCn1 in breast tumor development and NBCn1 knockdown or -knockout reduces tumor growth and proliferation *in vivo* (1;2). We recently demonstrated that stable knockdown of NBCn1 in MCF-7 breast cancer cells leads to slower cell cycle progression in synchronized cells, in particular in G2/M transition (3). Accordingly, doubling time was increased and Ki-67 staining decreased in asynchronous MCF-7 cells. Cell cycle regulatory proteins were affected in congruence with this pattern, but no mechanism could be identified (3). The aim of our ongoing work is to identify mechanisms involved in NBCn1-mediated cell cycle regulation. We performed a GST pulldown of the C-terminal of NBCn1 in MCF-7 cell lysates. Surprisingly, strong hits included End-binding protein (EB)1, associated with the mitotic spindle, and three centrosomal/ciliary base-located proteins, CEP135, CEP164, and CEP290. Co-immunoprecipitation of NBCn1 with CEP135 as well as the reverse pulldown confirmed this interaction. These data show that at least in cell lysates, NBCn1 interacts with centrosome- and spindle proteins. Fluorescence imaging using native and exogenously expressed NBCn1, and three different antibodies, as well as live imaging of GFP-tagged NBCn1, indicates localization to spindle, midbodies, and centrosome in a variety of cancer and non-cancer cells from several species (MCF-7, HeLa B, Panc-1, NIH-3T3, MDCK II, RPE-1). Ongoing work using a series of tagged and truncated NBCn1 variants is addressing the specificity of this pattern for NBCn1. If interaction can be validated in intact cells, we will test the hypothesis that NBCn1 may play a role in the regulation of pH in the spindle/centrosome region. This is in congruence with the known pH sensitivity of mitotic spindle formation, and with clear evidence from others that during mitosis a number of membrane proteins localize to the spindle and/or midbodies. Some membrane proteins appear to have secondary roles related to cytokinesis, but the role of most membrane proteins at the spindle proteome is unknown.

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PCA117

**Anti-apoptotic actions of lithocholic acid on colonic epithelial cells: implications for treatment of inflammatory bowel disease**

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**Introduction:** Although inflammatory bowel disease (IBD) represents a significant health and economic burden, current therapies are often ineffective. Dysregulated intestinal barrier function has been well-documented to contribute to IBD pathogenesis, with increased epithelial apoptosis being apparent. We have previously reported that ursodeoxycholic acid (UDCA) attenuates intestinal inflammation in the DSS model of colonic inflammation in mice, and that its efficacy is dependent on bacterial metabolism into lithocholic acid (LCA) (Ward et al, 2017). Here, we set out to examine the effects of LCA on apoptosis in colonic epithelial cells.

**Methods:** C57BL/6 mice were administered dextran sodium sulphate (DSS, 2.5%) in their drinking water for 5 days, with or without treatment with lithocholic acid (30 mg/kg) via IP injection each day. T<sub>84</sub> colonic epithelial cells were treated with LCA (30  $\mu$ M) or the farnesoid X receptor (FXR) agonist, GW4064. Apoptosis was induced by treatment of the cells with a combination TNF- $\alpha$  (20 ng/ml) and INF- $\gamma$  (40 ng/ml) for 24 hrs and was assessed by measuring levels of cleaved Poly (ADP-ribose) polymerase (PARP). miRNA profiling was performed on GW4064 (5  $\mu$ M; 6 hrs)-treated T<sub>84</sub> cells, with TargetScan being used to identify miRNA targets. Reverse transfection of T<sub>84</sub> cells was performed using lipofectamine RNAimax and protein expression was assessed by western blotting or qPCR.

**Results:** In the in vivo model of DSS-induced colonic inflammation, LCA treatment reduced levels of cleaved-PARP in the mucosa from  $19.9 \pm 10.9$  to  $3.9 \pm 1.1$  fold of that in non-DSS treated mice ( $n = 3-4$ ;  $p \leq 0.01$ ). Similarly, in cultured monolayers of T<sub>84</sub> cells, LCA prevented cytokine-induced apoptosis, an effect that was mimicked by the FXR agonist, GW4064. miRNA array profiling of GW4064-treated T<sub>84</sub> cells revealed increased levels of miR-29a-3p. miR-29a-3p is predicted to target PTEN, a pro-apoptotic protein, with a 92% context score as ascertained by TargetScan. Treatment of T<sub>84</sub> cells with GW4064 resulted in decreased PTEN mRNA ( $n = 8$ ;  $p \leq 0.05$ ) and PTEN protein levels ( $n = 3$ ;  $p \leq 0.05$ ). Similarly, transfection of T<sub>84</sub> cells with a miR-29a-3p mimic, but not a scrambled control, decreased PTEN levels ( $n=3$ ;  $p \leq 0.01$ ).

**Discussion / Conclusion:** Our data suggest that bacterial metabolism of UDCA to LCA in the colonic lumen may promote barrier function by prevention of cytokine-induced apoptosis. The mechanism by which LCA exerts anti-apoptotic actions may involve activation of FXR, induction of miR29a3p, and reduced expression of the pro-apoptotic protein, PTEN.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCA118

**ATP-induced constitutive mucus secretion requires TMEM16A for mucus secretion in large intestine**

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TMEM16A is a calcium activated chloride channel found in the intestine and has been associated with goblet cell metaplasia. Here we report for the first time that TMEM16A is essential for basal secretion of intestinal mucus. Intestinal epithelial knockout of TMEM16A leads to accumulation of mucus in intestinal goblet cells. Acute ATP-induced mucus secretion by colonic goblet cells is strongly compromised in the absence of TMEM16A and requires apical calcium. In contrast cholinergic mucus secretion due to compound exocytosis is independent of TMEM16A. The data demonstrate a previously unrecognized role of TMEM16A for membrane exocytosis in HEK293 cells overexpressing TMEM16A and describe a novel ATP-driven pathway for intestinal mucus secretion. We conclude that ATP-dependent constitutive mucus secretion in the intestine requires TMEM16A and apical calcium influx. The results provide the basis for a completely novel therapeutic approach for the treatment of intestinal mucus-dependent diseases.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCA119

**Inhibiting enteroendocrine T1R2/R3 glucose receptors reduces blood glucose and serum insulin responses**

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It is well established that T1R2 and T1R3 receptors, present in small intestine enteroendocrine cells, cause transient insertion of glucose transporter type 2 (GLUT2) into the apical membrane of enterocytes upon stimulation. This GLUT2 insertion facilitates glucose uptake and transport into the blood affecting blood glucose and insulin levels (1-3).

This study reports effects of glucose gavage in mice with (WT) and without (CALHM1 KO) functioning intestinal T1R2/R3 receptors. We report KO mice generally have lower blood glucose and serum insulin levels than WT mice, suggesting the absence of functional intestinal T1R2 or T1R3 receptors decreases glucose uptake. We also utilized *Gymnema sylvestre* (GA) extract containing the stable, inhibiting polypeptide, gurmardin, to further investigate T1R2/R3's enteroendocrine role in postprandial glucose and insulin responses. To this end, WT and KO mice received 10% glucose gavage with and without extract from GA leaves. Glucose gavage with GA

extract lowered blood glucose and serum insulin in WT mice but changed these parameters little in KO mice. Together, this data points to T1R2/R3's role in intestinal glucose absorption and implicates a possible target for lowering blood glucose and insulin with excessive carbohydrate consumption.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA120

### NoxO1 Controls Proliferation of Colon Epithelial Cells

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**Aim:** Reactive oxygen species (ROS) produced by enzymes of the NADPH oxidase family serve as second messengers for cellular signaling. Processes such as differentiation and proliferation are regulated by NADPH oxidases. In the intestine, due to the exceedingly fast and constant renewal of the epithelium both processes have to be highly controlled and balanced. Nox1 is the major NADPH oxidase expressed in the gut and its function is regulated by cytosolic subunits such as NoxO1. We hypothesize that the NoxO1-controlled activity of Nox1 contributes to a proper epithelial homeostasis and renewal in the gut.

**Results:** NoxO1 is highly expressed in the colon. Knockout of NoxO1 reduces the production of superoxide in colon crypts and is not subsidized by an elevated expression of its homologue p47phox. Knock out of NoxO1 increases the proliferative capacity and prevents apoptosis of colon epithelial cells. In mouse models of DSS-induced colitis and AOM/DSS induced colon cancer, NoxO1 has a protective role and may influence the population of natural killer cells.

**Conclusion:** NoxO1 affects colon epithelium homeostasis and prevents inflammation.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA121

### Importance of hypoxia-inducible factors (HIF-1 and HIF-2) for the pathophysiology of inflammatory bowel disease

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About 400,000 people in Germany suffer from inflammatory bowel diseases (IBD). The most common IBD types are Crohn's disease and ulcerative colitis. At present, these types

of disease are not curable and their causes are not completely known.

Inflammation is characterized by lack of oxygen (inflammatory hypoxia). The cellular adaptation to hypoxia is regulated by the transcription factors hypoxia-inducible factors (HIF-1 and HIF-2). In IBD the current state of knowledge is mainly limited to the function of HIF-1. Previous work of our group has shown that HIF-1 in myeloid cells plays a disease-promoting role in the animal model of dextran sodium sulfate (DSS)-induced colitis <sup>1</sup>.

Based on this work, first experiments were done with *HIF1<sup>+/f/+</sup> x HIF2<sup>+/f/+</sup>* mice (WT) and *Lyz2-Cre/HIF1<sup>+/f/+</sup> x HIF2<sup>+/f/+</sup>* mice (KO). For acute inflammation, mice received 2.5% (w/v) DSS (MP Biomedicals, MW 36–50 kDa) in drinking water for six days while control mice (C: n=8) received normal drinking water. The course of disease was recorded by body weight, stool consistency and occult blood in the feces being important for the Disease Activity Index (DAI) as clinical classification <sup>2</sup>. Afterwards, severity of disease was analyzed macroscopically (changes in weight and length of colon) and microscopically (antigen specific immunohistochemistry and histology score <sup>2</sup>. Also HIF-regulated gene expression in colon tissue was analyzed by quantitative PCR as well as by immuno blot.

The DAI during DSS-treatment increased on day 4 and indicated colitis while control mice remained unaffected. Also DSS-treated animals showed a 1.5 fold higher colon weight to length ratio and a 4-fold higher Histology Score for destroyed tissue than control mice, which are hallmarks for intestinal inflammation. Furthermore, treated animals showed a significantly lower mRNA expression of epithelial mucin *muc3* and a significant higher expression of cytokines like *tumor necrosis factor α (tnfα)* and *lipocalin-2 (lcn-2)* than control mice being an indication for inflammation. In KO mice, epithelial mucin *muc2* expression was significantly lower than in WT mice under treated and untreated conditions.

In further experiments, the influence of myeloid HIF-2 in the course of disease of DSS-induced colitis is to be studied *in vivo*. In addition, *in vitro* studies with murine myeloid cells carrying a *hif-2a* knockout or *hif-1a / hif-2a* double knockout should be performed. HIF-regulated gene expression will be studied in bone marrow-derived macrophages under normoxic (21% O<sub>2</sub>) and hypoxic (1% O<sub>2</sub>) conditions.

Our data suggest that additional knockout of HIF-2α might be able to reverse the disease ameliorating effect of myeloid *hif-1a* knockout in experimental colitis. HIF-2 could therefore play an antagonist role to HIF-1 but further experiments with animals carrying a *hif-2a* knockout in myeloid cells need to be done.

1. Bäcker, V., Cheung, F. Y., Siveke, J. T., Fandrey, J. & Winning, S. Knockdown of myeloid cell hypoxia-inducible factor-1α ameliorates the acute pathology in DSS-induced colitis. *PLoS One* 12, (2017).

2. Cooper, H. S., Murthy, S. N., Shah, R. S. & Sedergran, D. J. Clinico-pathologic study of dextran sulfate sodium experimental murine colitis. *Lab. Invest.* 69, 238–49 (1993).

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.



## PCA122

**The amino acid transporter Lat1 (SLC7A5) resides in intestinal crypts and affects villus development**

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Lat1 is a sodium-independent obligatory amino acid (AA) exchanger composed of two subunits: the catalytic permease SLC7A5 and its accessory glycoprotein SLC3A2. Lat1 transports large neutral AA including the essential AA leucine, which exerts a permissive effect on the mTORC1 pathway. By transporting leucine, Lat1 couples nutrient sensing and growth signals.

To study the impact of *Slc7a5* deletion in adult mice, we used a tamoxifen-inducible (*Rosa26-CreERT2*) mouse model. Tamoxifen (4mg/25g body weight) was administered orally for 3 consecutive days. This procedure produced >90% reduction in *Slc7a5* mRNA expression in peripheral tissues (spleen, lung, small intestine, skeletal muscle, pancreas, adipose tissue), and resulted in a progressive body weight loss over a 12-day period. Reduced skeletal muscle (~5%) and white adipose tissue mass (~50% epididymal fat pads) substantially contributed to this loss of weight. Consistently, the *Slc7a5* knockout mice exhibited a decreased respiratory exchange rate (RER) and higher remaining energy in faeces. These results prompted us to study intestinal absorption. An intestinal ring uptake approach showed reduction of leucine and glucose absorption in the ileum of *Slc7a5* knockout mice. This functional phenotype was associated with decreased mRNA levels for B<sup>0</sup>AT1 and SGLT1, respectively AA and glucose luminal transporters.

We used qPCR and *in situ* hybridization methods to show that *Slc7a5* mRNA was expressed within the crypts and co-localised with the stem cell markers LGR5 and Olfm4. Labelling ileum sections with Ki67 (cellular marker for proliferation) revealed an extended domain of transit/amplifying cells within the *Slc7a5* knockout crypts. Similarly, the BrdU incorporation method showed a higher proliferation rate in this cell population. To further dissect the molecular mechanisms underlying this phenotype, we established an *ex-vivo* culture model. However, organoids derived from *Slc7a5*<sup>fl/fl</sup> *Rosa26-CreERT2* intestines and treated transiently *in vitro* with 4-Hydroxy-tamoxifen (4-OHT, 1μM), failed to develop stable crypt-like structures and displayed a decreased 5-ethynyl-2'-deoxyuridine (EdU) incorporation despite an increase in Ki67 positive cells.

Taken together these data suggest that Lat1 (SLC7A5) expression within the intestinal crypts is required for adequate villus development. Further experiments including an RNAseq approach to compare wildtype and *Slc7a5* knockout crypts and villi will help clarify the role Lat1 plays for the control of the proliferation/differentiation balance in intestinal epithelium. *Work funded by the Swiss National Science Foundation, the University of Zurich and the Wellcome Trust.*

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## PCA123

**Tuft cell hyperplasia in diarrhoea-predominant Irritable Bowel Syndrome colonic biopsies.**

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Tuft cells are a rare subtype of gastrointestinal epithelial cell, which are thought to be activated by parasites and allergic reactions. Luminal stimulation causes release of pro-inflammatory cytokines, which can modulate gastrointestinal function. Irritable bowel syndrome (IBS), a heterogeneous functional bowel disorder, characterised by abdominal pain and altered bowel habit, exhibits altered circulating cytokine profiles. The aim of this study was to investigate if the number of colonic tuft cells was altered in human IBS or in a stress-sensitive animal model of IBS. The number of DCLK1 immuno-labelled tuft cells with a strong cytoskeleton labelled with Phalloidin-iFluor 488 was compared between mucosal biopsies from diarrhoea- and constipation-predominant IBS patients and healthy controls (n=6 per group). The number of tuft cells was also compared in colonic cross-sections from stress-sensitive Wister Kyoto rats and the control Sprague Dawley comparators (n=3 per group). As compared to healthy controls, colonic tuft cell hyperplasia was detected in diarrhoea-predominant IBS mucosal biopsies (p<0.05). Increased numbers of tuft cells were also detected in the colonic mucosa of Wister Kyoto rats (p<0.05) as compared to Sprague Dawley controls. These findings illustrate a previously unreported change in IBS colonic morphology. Gastroenteritis, which could stimulate tuft cell hyperplasia, is a strong predictor of developing IBS. However, none of this patient cohort was diagnosed with post-infectious IBS. Moreover, clean rodent housing indicates that a catalyst other than the known stimuli of protozoans and helminths caused the increase in tuft cells in Wister Kyoto rats. Activation of the stress response and its capacity to exacerbate and prolong symptom flares is an accepted contributing factor in human IBS pathophysiology, and as such, is worthy of further investigation as an underlying cause of colonic tuft cell hyperplasia in IBS.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## PCA124

**Melatonin in combination with antibiotherapy alleviates gastric oxidative injury in rats: role of microbiota**

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Gastric dysbiosis, by affecting the host inflammatory response, was suggested to have a critical role in gastric pathogenesis<sup>1</sup>. The pineal hormone melatonin is the modulator of circadian rhythm of both the host and gut bacteria<sup>2</sup>,

and exerts protective effects on acute gastric damage<sup>3</sup>. The present study was aimed to elucidate the contribution of gut dysbiosis and the effect of melatonin on the host's oxidative response to gastric ulcerogenesis. Following the ethical approval, Wistar albino rats of both sexes were randomized to control or ulcer groups. Under ketamine and chlorpromazine (100 and 10 mg/kg, intraperitoneally) anaesthesia, saline (control; n=6) or acetic acid (0.5 ml, 80 %) was applied on gastric serosa (ulcer; n=24). Before the induction of ulcer, rats were given either normal water, a cocktail of broad-spectrum antibiotics (neomycin+ampicillin+metronidazole; 1g/L each), melatonin (40 mg/L), or melatonin plus antibiotics combination in their drinking water for 12 days. All treatments were continued on the postsurgical 3 days until they were decapitated. In gastric tissue samples, myeloperoxidase (MPO) and caspase-3 activities, and the levels of glutathione, malondialdehyde, luminol- and lucigenin-enhanced chemiluminescence (CL), 8-hydroxy-2'-deoxyguanosine (8OHdG) were measured. Gastric tissues were stained with hematoxylin and eosin for microscopical analysis, and apoptosis was evaluated by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method. Statistical analysis was made using ANOVA test. Compared to control group, malondialdehyde, luminol- and lucigenin-CL, 8OHdG levels and MPO and caspase-3 activities were increased in the gastric tissues of non-treated ulcer group ( $p < 0.01-0.001$ ), while the antioxidant glutathione was depleted ( $p < 0.01$ ). The number of TUNEL-positive cells in the ulcer group was increased with respect to the control group ( $p < 0.05$ ). Pretreatments with antibiotics, melatonin or combination similarly reduced ulcer-induced elevations in malondialdehyde, CL, 8OHdG ( $p < 0.001$ ), while increased caspase-3 and MPO activities were depressed significantly only by the combination ( $p < 0.05$  and  $p < 0.001$ ). Ulcer-induced reduction in glutathione was replenished by both melatonin and combined therapies ( $p < 0.05-0.01$ ). Compared to the untreated ulcer group, less necrotic lesions and a relatively lower number of TUNEL-positive cells were observed in melatonin-, antibiotics- and melatonin+antibiotics-treated ulcer groups. The results show that antibiotics in combination with melatonin provide protection against oxidative gastric injury. Antibiotics and melatonin could be alleviating oxidative gastric injury by preventing bacterial colonization and supporting the gut microbiota in reconstructing its homeostatic state.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA125

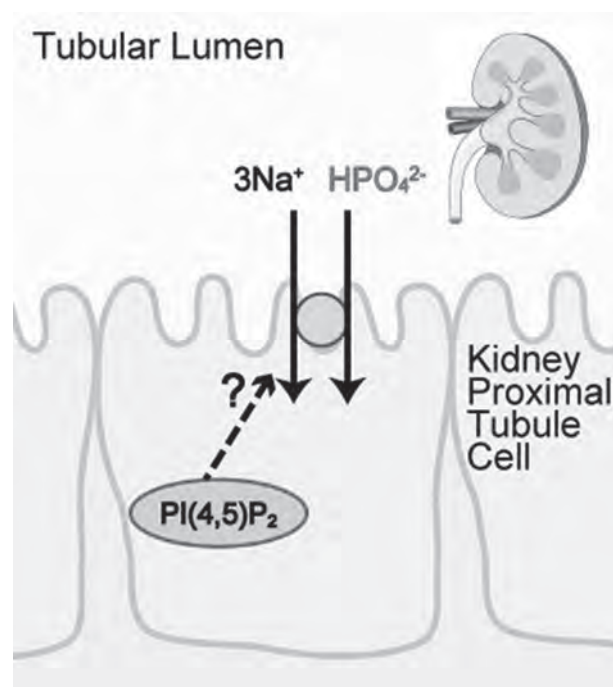
### The analysis of sensitivity of Na-Pi cotransporter activity to phosphoinositides

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Inorganic phosphate (Pi) is an essential constituent for maintenance of cell activity. Na-Pi cotransporter (SLC34) is the main molecule for Pi homeostasis in kidney. NaPi-IIa (SLC34A1) is localized at apical brush border membrane in the renal proximal tubule and reabsorbs Pi from glomerular filtrate (Forster et al, 2002). One of the regulation mechanisms of this transporter activity in the tubule is to reduce the molecule number by vesicular transport system through endocytosis.

However, other mechanisms, especially in situ modulation of the activity on plasma membrane, are not known. It is known that the activity of various ion channels and some transporters is regulated by phosphatidylinositol 4,5-bisphosphate (PI(4,5)P<sub>2</sub>) abundant in plasma membrane (Suh et al, 2008, Thornell et al, 2015). Because NaPi-IIa functions on plasma membrane, we hypothesized that the transporter activity may depend on PI(4,5)P<sub>2</sub>. To test this idea, we expressed mouse NaPi-IIa in *Xenopus* oocytes and evaluated its PI(4,5)P<sub>2</sub> dependence by using *Ciona intestinalis* voltage-sensing phosphatase (Ci-VSP) that has phosphatase activity for phosphoinositide depending on membrane potential. During NaPi-IIa current recording, depolarization pulse was applied to activate Ci-VSP to induce reduction of PI(4,5)P<sub>2</sub> on plasma membrane. PI(4,5)P<sub>2</sub> level was monitored by measuring rat KCNQ2/3 current coexpressed with mNaPi-IIa and Ci-VSP. Na-bicarbonate cotransporter (rb2NBC) was used as a positive control. We first measured PI(4,5)P<sub>2</sub> dependence of rb2NBC. After depolarization, rb2NBC current was significantly decreased (pre  $53.1 \pm 7.1$  nA, post  $40.5 \pm 6.6$  nA,  $n = 8$ , paired t-test;  $p < 0.0001$ , rate of change  $0.741 \pm 0.036$ ) where reduced PI(4,5)P<sub>2</sub> level was observed. However, NaPi-IIa current did not change before and after depolarization (pre  $105.5 \pm 21.2$  nA, post  $108.2 \pm 15.9$  nA,  $n = 4$ ,  $p = 0.733$ , rate of change  $1.064 \pm 0.077$ ). These results suggest that mouse NaPi-IIa has no or extremely low PI(4,5)P<sub>2</sub> dependence. Ci-VSP can dephosphorylate PI(4,5)P<sub>2</sub> upon membrane depolarization, but produces PI(4)P. However, the magnitude of NaPi-IIa current may not be affected by Ci-VSP if the activity of mNaPi-IIa both depend on PI(4)P and PI(4,5)P<sub>2</sub>. We are now examining PI(4)P dependence of mNaPi-IIa by using Pseudojanin, a chimera protein, which has both activities of 5-phosphatase and 4-phosphatase (Hammond et al, 2012).



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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA127

**The role of TMEM16A/ANO1  $\text{Ca}^{2+}$ -activated chloride channel in pancreatic cancer**Y. KIM<sup>1</sup> and H. Park<sup>2</sup><sup>1</sup>Pharmacology, Yonsei University College of Medicine, Seodaemun-gu, Korea (the Republic of) and <sup>2</sup>St. Vincent hospital, The catholic university of Korea, Suwon, Korea (the Republic of)

**Introduction:** Despite calcium-activated  $\text{Cl}^-$  channels (CaCCs) were first described over 3 decades ago, the molecular identity of CaCCs has recently been identified. These channels are widely expressed in various cell types and tissues which are expressed in various cell types including tracheal, intestinal, and glandular epithelia, smooth muscle cells, intestinal pacemaker cells, sensory neurons, and several tumors. Interestingly, over-expression of TMEM16A/ANO1 has been found in many tumor types including breast cancer, and head and neck cancer. However, the expression and function of TMEM16A in gastrointestinal cancer, such as pancreatic cancer, has not been revealed. Pancreatic cancer is the eighth leading cause of cancer death and is difficult to treat because clinical presentation is often late, and the disease is resistant to conventional chemotherapy. About 57% of pancreatic cancer patients are diagnosed at distant metastasis status and five-year survival rate is only 2%. More than 90% of pancreatic cancer has activating mutations in KRAS and recent exome sequencing studies have identified additional mutations in several kinds of genes such as AIRD1A, ARID1B, SMARCA1. However, most of mutational genes are tumor suppressor genes and these are no effective druggable target in pancreatic cancer. Hence, the study for new druggable target is unmet medical need.

**Method:** PANC-1, ASPC-1 and BXPC-3 cells were used as pancreatic tumor cell lines and hTERT-HPNE cell line was employed control pancreatic cell line from ATCC. The coding regions of human TMEM16A/ANO1 (ac isoform) sub-cloned into the pEGFP-N1 mammalian expressible plasmid and pLVX-Ac-GFP-N1 without a C-terminal GFP using BamH1 and Xho1 restriction enzymes. Intracellular chloride concentration was measured with a chloride sensing fluorescent, triple mutant YFP (H148Q/I152L/F46L) on PANC-1 cells.

**Results:** To explore the function and mechanism of TMEM16A/ANO1 in pancreatic cancer, we carried out MTT assay and immunoblotting at TMEM16A/ANO1 over-expressed pancreatic cells. Pancreatic tumor cells death and proliferation by TMEM16A/ANO1 was regulated by extracellular chloride concentration. In addition, the molecular mechanism of these effects was proved by immunoblotting in pancreatic cancer cell lines.

**Conclusion:** In this study, we show that TMEM16A/ANO1 channel promotes cell proliferation in pancreatic cancer cell lines and intracellular chloride through TMEM16A/ANO1 is important mechanism for cell proliferation. These results indicate that TMEM16A/ANO1 can be possible druggable targets using ANO1/TMEM16A inhibitor. Further, TMEM16A/ANO1 might play a role as prognostic factors for survival outcomes in pancreatic cancer.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA128

**Mechano-signaling in tumor-associated pancreatic stellate cells**

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Pancreatic ductal adenocarcinoma (PDAC) is the fourth most common leading cause of cancer-related deaths worldwide and is characterized by the formation of a dense fibrotic stroma. The excessive amount of extracellular matrix (ECM) is primarily formed by pancreatic stellate cells (PSCs). ECM contributes to a high pancreatic tissue pressure which itself also stimulates PSCs. The effect of increasing tissue pressure and altered biomechanics in the pathogenesis of PDAC, however, is poorly understood. Mechano-sensing and -transduction have been linked to opening of mechanosensitive ion channels such as PIEZO1, TRPV4 or TRPC1. We hypothesize that these channels whose expression we showed in PSCs, play crucial roles in sensing/transducing mechanical cues that are typical for the PDAC microenvironment.

We have shown that TRPC1 channels participate in the activation of mechanically stressed PSCs. TRPC1 knock-out is sufficient to attenuate the pressure-induced activation (Fels *et al.* 2016 *Eur Biophys*). We performed live-cell imaging migration experiments and monitored  $\text{Ca}^{2+}$  influx into PSCs by means of the  $\text{Mn}^{2+}$  quench technique in order to assess the function of PIEZO1 channels in PSCs. Stimulation of PIEZO1 channels with its activator Yoda1 leads to a doubling of the translocation of migrating PSCs ( $41.2 \pm 4.3 \mu\text{m}$  vs.  $20.4 \pm 2.2 \mu\text{m}$ ) and enhanced directionality by  $67 \pm 10 \%$ . Activation of PIEZO1 leads to a fourfold increase of calcium influx into the PSCs. We then focused on the PIEZO1-mediated effect on the 3D invasion of PSCs. Cell spheroids were embedded in a 3D desmoplastic matrix and invasion was analyzed for a time period of 48h with or without PIEZO1 stimulation. After 24h, invasion area of Yoda1-stimulated PSCs is 10% lower than that of control cells. However, PIEZO1 activation causes the number of detached cells from the spheroid core to increase by 68%. Additionally, upon PIEZO1 activation PSCs transmit more force to the surrounding ECM, as revealed by measuring the dislocation of microbeads embedded in the surrounding matrix (control:  $9.0 \pm 0.4 \mu\text{m/h}$ ; Yoda1:  $11.3 \pm 0.4 \mu\text{m/h}$ ).

In summary, stimulating PIEZO1 activates PSCs by inducing calcium influx. This results in increased motility within the ECM. Activated PSCs thereby transfer more force to the matrix, possibly align ECM fibers and subsequently improve their invasion capacity and detach from the primary spheroid. We hypothesize a strong interdependence between the mechanical output of PSCs and stromal mechanics, which promotes early local invasion of PDAC cells.

Fels, B.; Nielsen, N.; Schwab, A. Role of TRPC1 channels in pressure-mediated activation of murine pancreatic stellate cells. *Eur. Biophys. J.* 2016, 1–14, doi:10.1007/s00249-016-1176-4.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.



PCA129

### Gold nanoparticle-mediated laser perforation (GNOME LP)/dye transfer: a convenient method to analyse connexin channels in tissue-engineered cell culture systems

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Connexins form gap junction channels and hemichannels that participate in the formation of functional physiological units from individual cells and therefore are essential for many tissue functions. Tissue-engineered models can be used to analyse the integration of connexin channels in general signalling mechanisms in a tissue and thereby to understand the role of connexin channels in the physiology of a tissue and their involvement in pathological conditions. However, analysing connexin channels in tissue-like 3D cell culture is challenging. Simple and convenient methods to analyse gap junction coupling usually rely on dye transfer, e. g. after micro-injection or scrape loading. These techniques are invasive and almost impossible to apply to complex cell culture systems. We used the GNOME LP technique (Heinemann *et al.* 2013) to establish an improved method for gap junction coupling analysis via dye transfer (Begandt *et al.* 2015). The method is less invasive, allows a semi-automatic analysis of gap junction coupling and is applicable to complex cell culture models as shown for 3D cell culture of vascular endothelial cells in vessel-like structures (Begandt *et al.* 2015). Furthermore, a blood-brain barrier model with co-culture of cerebral microvascular endothelial cells, pericytes and astrocytes can be analysed. Adding a medium flow to mimic the circulating blood will allow to analyse the role of connexin channels in the blood-brain barrier function and their participation in pathological events such as ischemia or stroke. The connexin channels in each cell type involved in the formation of the blood-brain barrier can be analysed, while at the same time following intercellular cross-signalling based on other mediators such as purinergic signalling or inflammatory-related responses. The GNOME LP/dye transfer technique was also applied to lung epithelial cells cultured in transwell inserts, allowing a simultaneous analysis of connexin channels and transepithelial electrical resistance or permeability. Furthermore, implementation of air-liquid interface culture in this model allows to polarise the cells with separated apical and basolateral sides. Within this model, a crosstalk between connexin channels and signalling pathways activated from either side of the cells can be analysed. Connexin channels were shown to be involved in pathological events in tissue. It was proposed to target these channels for treatment of various pathologies such as inflammation or hypoxia-related impairments. Application of the GNOME LP/dye transfer technique to analyse connexin channels in tissue-engineered models will improve our understanding of the pivotal role of connexin channels in tissue physiology. It can further create a testing platform for pharmacological intervention of various pathologies.

Begandt *et al.* (2015), *J Bioenerg Biomembr* 47(5), 441–449.

Heinemann *et al.* (2013), *PLoS One* 8(3), e58604.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA130

### Delivery of epithelial sodium channel antisense oligonucleotides to primary human airway epithelial cells via chitosan as a non-toxic transfection reagent

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Cystic fibrosis (CF), the most common lethal genetic disorder in the Caucasian population, is caused by defects in the *cystic fibrosis transmembrane conductance regulator* (CFTR), a cAMP-dependent chloride channel expressed in the apical membrane of epithelial cells. The disease is associated with a Na<sup>+</sup> hyperabsorption mediated by the epithelial sodium channel (ENaC) causing an enhanced imbalance of ion and water transport. The dysregulation of these proteins leads to severe impairments in the metabolism making CF a multi-system disorder with airways and lungs being affected the most. Until now, therapy focuses on alleviating the symptoms of the disease as no treatment has been developed yet to cure the disease on a cellular level. Hence, we aim at designing a double-tracked strategy targeting both CFTR and ENaC to treat abnormal ion transports in airway epithelial cells. Recently, we established a non-viral delivery system for wtCFTR-mRNA based on chitosan (CS) to restore CFTR function<sup>1</sup>. In this work we are seeking to develop a similar system to deliver ENaC antisense oligonucleotides (ASO) to correct Na<sup>+</sup> hyperabsorption. CS-ASO complexes were harnessed at varying charge ratios (+/-) and characterized for their physicochemical properties (e.g. size, zeta potential, stability). Most promising complexes (+/- 90, 85 mM NaCl) had an average size of 150 nm, a zeta potential of approximately +30 mV, were relatively stable in transfection medium over 24 h and were therefore chosen for *in vitro* transfection experiments. Primary human nasal epithelial cells grown on Transwell® permeable filters were incubated with CS-ASO complexes for 24 h (0.45 µg ASO/cm<sup>2</sup>). Afterwards, functional Ussing chamber measurements were conducted. Measurements showed decreased Na<sup>+</sup> current mediated by ENaC (15,8%; n=6) confirming successful transfection with CS-ASO complexes. With the results presented herein we mark the start of the development of a double-tracked strategy to cure abnormal ion transports in CF.

Kolonko AK *et al.* (2016). *JSM Genet Genomics* 3(2), 1016

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA131

**A comparative study of the  $\text{NH}_4^+$  conductance of human and bovine TRPV3 channels as expressed in *Xenopus* Oocytes**

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**Introduction:** In Olmsted syndrome of humans, a gain of function mutation of TRPV3 (hTRPV3) leads to severe hyperkeratosis of the skin (1). Recently, it has been suggested that the bovine analogue (bTRPV3) is involved in the uptake of  $\text{NH}_4^+$  (2,3). Data for the human analogue are lacking. **Methods:** *Xenopus* oocytes were injected with a linearized strep-tagged cRNA of hTRPV3, bTRPV3, or with RNase-free water (control). The expression was verified using immunoblotting (n=8) and immunohistochemistry (n=3). Double-barreled pH-sensitive microelectrodes and the single channel patch clamp technique were used to investigate permeability to  $\text{NH}_4^+$ . Values are means  $\pm$  S.E.M. (ANOVA on Ranks).

**Results:** After switching from NMDGCl to NaCl solution, membrane potential depolarized in all groups ( $p \leq 0.005$ ). Relative to NMDG<sup>+</sup>, permeability of  $\text{Na}^+$  was similar ( $p > 0.4$ ) in hTRPV3 ( $1.94 \pm 0.17$ , n=16) and bTRPV3 ( $2.19 \pm 0.18$ , n=18), both significantly ( $p < 0.01$ ) higher than in controls ( $1.39 \pm 0.12$ , n=17). Intracellular pH ( $\text{pH}_i$ ) remained the same ( $p > 0.5$ ; hTRPV3:  $7.57 \pm 0.03$ , bTRPV3:  $7.55 \pm 0.05$ , control:  $7.62 \pm 0.03$ ). A switch to  $\text{NH}_4\text{Cl}$  induced a significant depolarization ( $p \leq 0.001$ ) and acidification ( $p \leq 0.001$ ). The relative permeability of  $\text{NH}_4^+$  versus NMDG<sup>+</sup> was significantly higher ( $p = 0.03$ ) in bTRPV3 ( $3.81 \pm 0.23$ ) than in hTRPV3 ( $3.32 \pm 0.28$ ) or controls ( $3.09 \pm 0.24$ ).  $\text{pH}_i$  was lower ( $p \leq 0.01$ ) in hTRPV3 ( $6.00 \pm 0.11$ ) than in bTRPV3 ( $6.33 \pm 0.07$ ) or controls ( $6.36 \pm 0.09$ ).

In patch clamp experiments, large single channel conductances ( $>100$  pS) for  $\text{NH}_4^+$  were determined in over 50% of patches from both types of overexpressing oocytes (hTRPV3:  $178 \pm 8$  pS, n=15 of 25; bTRPV3:  $158 \pm 12$  pS, n=11 of 20;  $p = 0.13$ ), but not in any patches from controls. Smaller channels ( $<100$  pS) were observed in 62% of control ( $46 \pm 9$  pS), 52% of hTRPV3 ( $63 \pm 8$  pS), and 35% of bTRPV3 patches ( $49 \pm 11$  pS). Interestingly, traces of eight hTRPV3 membrane patches simultaneously showed both large and small channels, not observed in bTRPV3. In 38% of control, 16% of hTRPV3, and 10% of bTRPV3 patches, no channel activity was seen. In conjunction, overexpression of hTRPV3 or bTRPV3 significantly enhanced single channel conductance ( $p \leq 0.005$ ), while smaller endogenous conductances were suppressed. **Conclusion:** Both in the human and bovine species, an involvement of TRPV3 channels in the transport of  $\text{NH}_4^+$  as a major product of protein metabolism appears likely. Given that TRPV3 is highly expressed by keratinocytes (1,3), implications follow both for the pathophysiology of certain skin diseases in humans and for dietary uptake mechanisms across the ruminant forestomach. We further report that *Xenopus* oocytes express a high endogenous conductance for  $\text{NH}_4^+$ , requiring care when this expression system is chosen for studies of ammonia transport.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA132

**Temperature-dependent regulation of ANO6 chloride channel variants**

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Anoctamin-6 (ANO6) belongs to a family of calcium ( $\text{Ca}^{2+}$ )-activated chloride channels (CaCCs), with three types of splicing variants (V1, V2, and V5) showing plasma membrane expression<sup>1</sup>. Unlike other CaCCs, ANO6 requires a non-physiological intracellular free calcium concentration ( $[\text{Ca}^{2+}]_i > 1 \mu\text{M}$ ), and needs several minutes for full activation under a whole-cell patch clamp<sup>2</sup>. Therefore, the physiological role of ANO6 as an ion channel is in doubt; it is more commonly considered as a  $\text{Ca}^{2+}$ -dependent phospholipid scramblase<sup>3</sup>. Here, we demonstrated that physiological temperature ( $37^\circ\text{C}$ ) increased the  $\text{Ca}^{2+}$  sensitivity of ANO6 under a whole-cell patch clamp; V1 was activated by  $1 \mu\text{M}$   $[\text{Ca}^{2+}]_i$ , whereas V2 and V5 were activated by  $300 \text{ nM}$   $[\text{Ca}^{2+}]_i$ . Upon temperature increase to  $42^\circ\text{C}$ , all ANO6 variants can be activated by  $100 \text{ nM}$   $[\text{Ca}^{2+}]_i$ . Moreover,  $37^\circ\text{C}$  condition significantly accelerated the delayed time to full activation the three variants. Notably, the temperature-dependent  $\text{Ca}^{2+}$ -sensitisation of ANO6 became insignificant under inside-out path clamp, suggesting a critical role of unknown cytosolic factors. Unlike the channel activity, physiological temperature did not induce the scramblase function of ANO6 with submicromolar  $[\text{Ca}^{2+}]_i$  ( $300 \text{ nM}$ ), irrespective of variant type. Our results reveal the physiologically meaningful anion conducting property of ANO6, which might precede the scramblase activity.

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We would like to thank Hyun Jong Kim (Dongguk University College of Medicine) for his interest and ideas related to this study. This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Ministry of Science, ICT & Future Planning (MSIP) of the Korean government (No. NRF-2015R1C1A1A02037738).

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

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PCA134

***Antiapoptotic and Anti-Inflammatory Effects of Urapidil Against Ovarian Tissue Damage In Experimental Ischemia-Reperfusion Model In Rats***

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**Background and Objectives:** To investigate the effects of urapidil, as an antioxidant and anti-inflammatory agent, on apoptosis and cell death on ischemia-reperfusion (IR) related ovarian injury.

**Materials and Methods:** Wistar Albino female rats (n=40) were divided into five groups: Sham operation (Group I), IR (Group II), IR plus %15 dimethyl sulfoxide (Group III), IR plus urapidil 0.5 mg/kg (Group IV) and IR plus urapidil 5 mg/kg (Group V). Bilateral adnexal 3-h period of ischemia was performed, followed by 3-h of reperfusion in study groups. Ovarian tissue TNF- $\alpha$ , IL-1 $\beta$ , NF- $\kappa$ B and Caspase-3 were measured using immunohistopathological methods.

**Results:** Histopathological examination showed that the ovarian tissues has normal histologic in Group I. In other treatment groups, interstitial tissue and corpus luteum were found in haemorrhages. In Group II and Group III haemorrhage were observed to be intense and decreased in treatment groups. The mildest haemorrhage was seen in group V.

Immunohistochemical staining of TNF $\alpha$ , IL-1 $\beta$ , NF $\kappa$ B, caspase-3 and LC3B immunopositivities were observed in study groups. The most intense immunopositivity for IL-1 $\beta$  and NF $\kappa$ B were found in Group III. The most intense TNF $\alpha$  immunopositivity was found in Group II. IL-1 $\beta$ , NF- $\kappa$ B, and TNF $\alpha$  immunopositivity decreased in the group IV and group V.

In the immunohistochemical staining for apoptotic cell death, in Group I, caspase 3 immunopositivity was not observed. The most intense caspase 3 immunopositivity was observed in Group III. It is observed that caspase 3 immunopositivity decreased in Group IV and Group V.

In the Group I, immunopositivity wasn't observed in immunohistochemical staining for autophagic cell death of LC3B. It is observed that immunopositivity was intense in Group II and Group III, but LC3B immunopositivity decreased in Group IV and Group V.

**Conclusions:** In conclusion, urapidil reduces I/R induced inflammation, apoptosis and autophagic cell death in a



dose-dependent manner. Our results are promising in the prevention of IR ovarian tissue damage.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA135

### Cation selectivity of the thick ascending limb depends on voltage, $\text{Ca}^{2+}$ and ionic strength

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The thick ascending limb of the loop of Henle (TAL) is one of the main reabsorption sites for divalent cations in the mammalian kidney. Transcellular salt transport generates a lumen positive transepithelial voltage ( $V_{te}$ ) which serves as a driving force for paracellular transport of the cations  $\text{Na}^+$ ,  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ . In addition the luminal fluid becomes more dilute towards the cortical part of the segment because of the water tightness of the TAL. As the paracellular pathway is highly cation selective a lumen positive diffusion potential (DP) can be generated by the  $\text{Na}^+$  concentration gradient towards the luminal side. This in turn provides a driving force for further reabsorption of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  in the cortical parts of the TAL where transcellular transport is already slowed down or even ceased because of reduced luminal  $\text{Cl}^-$  concentrations. The current study was designed to investigate the influence of increased luminal or basolateral  $\text{Ca}^{2+}$  concentrations on the paracellular cation selectivity of TAL and its dependence on voltage and ionic strength.

Microdissected TAL from the cortex (cTAL) or the inner stripe of outer medulla (ISOM-TAL) of kidneys of 6-12 week old C57/BL6/J mice were investigated in a double-barreled perfusion system using a standard solution containing 145 mM NaCl and 1 mM  $\text{Ca}^{2+}$ . Active transcellular transport was inhibited by application of 50  $\mu\text{M}$  furosemide. DPs were generated by either replacing the luminal solution or the basolateral solution by an isosmotic 30 mM NaCl solution to generate lumen positive or lumen negative potentials, respectively, as a measure of paracellular cation selectivity.  $\text{Ca}^{2+}$  was increased to 5 mM at one or on both sides of the epithelium. This maneuver was also repeated in the presence of symmetrical 145 mM or 30 mM NaCl solutions.

Under lumen negative conditions DPs were higher compared to the lumen positive situation. Basolateral increase of  $\text{Ca}^{2+}$  directly and reversibly attenuated the DP if lumen negative, but had less pronounced effects if DP was lumen positive. Luminal increase of  $\text{Ca}^{2+}$  directly and reversibly attenuated the lumen positive DP. These effects were not reversed by increasing  $\text{Ca}^{2+}$  symmetrically which indicates that the effect is not simply an additional  $\text{Ca}^{2+}$  diffusion potential. Generally the effects in the cTAL were much higher than in ISOM-TAL. These findings show voltage dependence of TAL cation selectivity. In addition  $\text{Ca}^{2+}$  attenuates cation selectivity from both luminal and basolateral side. The effect of  $\text{Ca}^{2+}$  again was voltage dependent and highest at low ionic strength at its site of action.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA136

### Intact and Furin-cleaved Epithelial Sodium Channel (ENaC) $\gamma$ - and $\alpha$ -subunit is detected in Human Kidney and Urinary Exosomes

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The amiloride-sensitive epithelial sodium channel ENaC, located at the apical membrane of the aldosterone-sensitive distal nephron segments and the collecting ducts establishes the "fine-tuning" of sodium reabsorption and is essential for sodium homeostasis and blood pressure regulation. The channel consists of three homologue subunits:  $\alpha$ ,  $\beta$  and  $\gamma$ (1). ENaC activity is regulated by proteolytic activation during biosynthesis by cleavage of the  $\alpha$  and  $\gamma$ -subunits by furin(2). Full activation is believed to occur extracellularly at the luminal membrane by a second cleavage of the  $\gamma$ -subunit by the GPI-anchored serine protease prostasin(3-5). The relevance of these proteolytic activation events *in vitro* is not fully elucidated in human. It was hypothesized that intracellular furin cleavage of the  $\alpha$  and  $\gamma$  subunit of ENaC can be detected in human kidney; that apical plasmin/prostasin-cleavage is associated with proteinuria and that predominantly cleaved products are detectable in human urinary exosomes.

Three sets of monoclonal antibodies were generated by immunizing balb/c mice with the relevant peptide synthesized sequence; end-specific against the  $\gamma$ -ENaC furin cleavage-site (mAb-furin, AA 138-147/EAESWNSVSC), overlying the non-cleaved  $\gamma$ -ENaC prostasin cleavage site (mAb-intact-RKRK, AA 173-190/CDFFTGRKRKVGGSIIHKA) and against the  $\alpha$ -ENaC-subunit (mAb-alpha, AA436-445/PRPQNVEYC) and tested on human kidney cortex tissue pool from nephrectomized patients and on urinary exosomes from healthy persons (all patients gave informed written consent and the use of the tissue was approved by the Institutional Review Board Ethics Committee).

A furin cleavage site-specific  $\gamma$ ENaC antibody revealed a protein at  $\approx 70$  kDa compatible with furin-cleavage in a human kidney cortex pool (n=4). An antibody overlapping and therefore requiring intact prostasin/plasmin cleavage site showed bands compatible with full-length ( $\approx 90$  kDa) and furin-cleaved ( $\approx 70$  kDa)  $\gamma$ ENaC. Deglycosylation resulted in a shift in these bands to 75 and 50 kDa respectively. The furin-cleaved protein was enriched within membrane fractions. The  $\alpha$ -ENaC antibody showed a dominant  $\approx 50$ kDa product compatible with predominance of furin-cleaved  $\alpha$ -ENaC in tissue. In urinary exosomes furin-cleaved, but not intact  $\gamma$ ENaC were detected.  $\alpha$ ENaC was detected in both its intact ( $\approx 80$ kDa) and furin-cleaved form in exosomes. No difference was detected with predicted low aldosterone (patients receiving ACE-inhibitor, n=4), predicted high aldosterone (patients receiving diuretics, n=3) or in patients with proteinuria (n=5) compared to controls (n=4).

The results indicate that proteolytic processing by furin of both alpha and gamma ENaC occurs in human kidney. The presence of prostasin-cleavage was not clearly present. Cleavage products of both  $\gamma$ ENaC and  $\alpha$ ENaC and intact  $\alpha$ ENaC were detectable in urinary exosomes.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

### PCA137

#### Endocytosis of NKCC2 is impaired in renal tubule in moesin knockout mice

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Tubular reabsorption of electrolytes in the kidney is an essential function in regulating fluid balance in the body. In the thick ascending limb of Henle (TAL), 20-40% Na<sup>+</sup> filtered by the glomeruli are reabsorbed by Na<sup>+</sup>-K<sup>+</sup>-2Cl<sup>-</sup> cotransporter type 2 (NKCC2). Mutations in the human gene coding for NKCC2 were identified in patients of Bartter syndrome type I, which is characterized by severe salt losing tubulopathy. Despite of the physiological importance of NKCC2 in NaCl homeostasis, the molecular mechanisms for its membrane trafficking have not been fully elucidated. An actin-binding protein, moesin was reported to play an important role in the apical recycling of NKCC2 by *in vitro* experiments (Carmosino *et al.* 2012). However, the physiological roles of moesin in the kidney remain unclear. Here, we examined the physiological roles of moesin in the regulation of renal function *in vivo* by using male moesin-null (*Msn<sup>-/-</sup>*) mice. Mice were anesthetized by isoflurane inhalation. Statistical analysis was performed by student T test or one way ANOVA.

To evaluate the cell surface NKCC2 expressions and endocytosis of NKCC2 in mouse TAL, TAL suspensions were prepared by collagenase treatment and filtering with nylon mesh. We measured surface expression of NKCC2 using protein biotinylation and found that apical surface expression of NKCC2 was significantly increased in *Msn<sup>-/-</sup>* TAL (n = 11, respectively. P < 0.05 vs. WT). We performed endocytosis assay with TAL suspensions. The endocytosis of NKCC2 was observed in both WT and *Msn<sup>-/-</sup>* tubules within 60 min incubation at 37 °C. However, internalized NKCC2 was significantly reduced in the *Msn<sup>-/-</sup>* TAL (n = 3, respectively. WT; 25.2±3.0 % internalized, *Msn<sup>-/-</sup>*; 10.5±3.8 %, values are means ± S.E.M. P < 0.05). We further investigated the distribution of NKCC2 in lipid raft fractions by the 10-40 % Optiprep-based density gradient centrifugation using renal medullary lysate, since lipid raft distribution of NKCC2 is important for the endocytosis

(Ares *et al.* 2010). In this result, lipid raft expression was significantly decreased in *Msn<sup>-/-</sup>* mice (n = 3, respectively. P < 0.05 vs. WT). To examine the renal physiological roles related to the NKCC2 in *Msn<sup>-/-</sup>* mice, we performed biochemical analysis of plasma and urine (WT: n = 12; *Msn<sup>-/-</sup>*: n = 13). Significant increase of plasma Cl<sup>-</sup> concentration (P<sub>Cl</sub>) concomitant with slight increase of plasma Na<sup>+</sup> concentration (P<sub>Na</sub>) was observed in *Msn<sup>-/-</sup>* mice (P<sub>Cl</sub>: 112.4±0.7 vs. 115.5±1.0 mM, P < 0.05; P<sub>Na</sub>: 150.6±0.7 vs. 151.9±0.5 mM). Urinary absolute excretions of Na<sup>+</sup> and Cl<sup>-</sup> (E<sub>Na</sub> and E<sub>Cl</sub>) in *Msn<sup>-/-</sup>* mice were lower than those of WT mice (E<sub>Na</sub>: 716.1±163.8 vs. 652.7±97.5 μmol/100 g BW/day; E<sub>Cl</sub>: 505.9±91.1 vs. 452.1±58.4 μmol/100 g BW/day). These results suggest that moesin regulates the apical surface expression level of NKCC2 by targeting NKCC2 to lipid raft and plays important roles in the renal electrolyte handling.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

### PCA138

#### TMEM16A activation by lipid peroxidation drives renal cyst growth

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In polycystic kidney disease (PKD) multiple bilateral renal cysts gradually enlarge causing a decline in renal function. Transepithelial Cl<sup>-</sup> secretion through cystic fibrosis transmembrane conductance regulator (CFTR) and TMEM16A (anoctamin 1) is known to drive cyst enlargement. Recently we found that Cl<sup>-</sup> currents by TMEM16A are not only controlled by intracellular Ca<sup>2+</sup> levels, but also by plasma membrane phospholipids. Modifications occurring in plasma membrane phospholipids by phospholipase A2 or ROS-induced lipid peroxidation could activate TMEM16A. We therefore examined whether membrane phospholipids are oxidized in polycystic kidneys and whether this activates TMEM16A. We show that plasma membrane phospholipids in human and mouse polycystic kidneys are strongly peroxidized, probably because of regional hypoxia. MDCK cysts *in vitro* also demonstrated enhanced levels of reactive oxygen species (ROS), which greatly correlated with cyst volume. ROS and lipid peroxidation strongly activated TMEM16A, leading to Ca<sup>2+</sup> store depletion. Store operated Ca<sup>2+</sup> influx augmented activation of CFTR, the essential secretory Cl<sup>-</sup> channel known to drive cyst growth. ROS scavengers such as glutathione or the analogue of coenzyme Q10, idebenone, as well as inhibition of lipid peroxidation by ferrostatin-1 largely attenuated activation of TMEM16A and thus reduced proliferation and fluid secretion *in vitro*. We propose inhibition of TMEM16A by antagonizing lipid peroxidation as a novel powerful therapeutic tool to delay cyst development in PKD.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA139

**Effects of tyrosine kinase inhibitor treatment on the renal transcriptome**J. Witte<sup>1</sup>, J. Golchert<sup>2</sup>, M. Mühlbauer<sup>1</sup>, R. Rettig<sup>1</sup> and O. Grisk<sup>1</sup><sup>1</sup>Physiology, University of Greifswald, Karlsburg, Germany and<sup>2</sup>Functional Genomics, University of Greifswald, Greifswald, Germany

Antiangiogenic tyrosine kinase inhibitors are used in tumor treatment and cause severe arterial hypertension. We have shown that rats treated with the angiogenesis inhibitor sunitinib develop arterial hypertension within four days which is associated with reduced renal fractional sodium excretion. Extensive hypothesis-driven studies excluded activation of distal convoluted tubule and collecting duct sodium reabsorption mechanisms as well as renal endothelial dysfunction as contributing factors. The present study was performed to identify further renal candidate mechanisms involved in sunitinib-induced hypertension.

Ten-week-old rats were instrumented with radiotelemetric devices for arterial pressure recordings under ketamine/xylazine anesthesia (100/10 mg\*kg<sup>-1</sup>, i.p.). After a seven-day recovery period, animals were treated with sunitinib [15 mg/(kg\*d)] or vehicle. After four days of treatment, animals were anesthetized (pentobarbital, 60 mg/kg, i.p.). Kidneys were removed, dissected into cortex and medulla and mRNA expression profiles were obtained using Affymetrix microarray technology.

Within four days, arterial pressure increased by 20 mmHg in sunitinib-treated rats. Using a  $p < 0.05$  and a ratio of expression levels  $> 1.5$  for analyses of the microarray data, we found that sunitinib treatment induced seven genes in the renal cortex and three genes in the renal medulla. Applying these cut-offs, the number of repressed genes was 24 in the cortex and 88 in the medulla. Pathway analyses revealed sunitinib-induced inductions of prostaglandin synthesis and B lymphocyte signaling pathways as well as repression of VEGF receptor signaling pathways. Within these pathways we identified and confirmed (qPCR) increased cortical cyclooxygenase 2 (COX2) and decreased cortical soluble guanylate cyclase (sGC) mRNA abundances. Renin mRNA abundance was suppressed in the cortex and medulla.

The number of differentially expressed renal genes is relatively small in early sunitinib-induced hypertension. Two enzymes, namely COX2 and sGC, known to be important regulators of renal sodium disposition were identified as candidates that may contribute to increased renal sodium reabsorption during the development of angiogenesis inhibitor-induced hypertension.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCA140

**Acute renal tubular lesions compromise the function of the glomerular filtration barrier: a multiphoton microscopy study**

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Albuminuria is a hallmark of kidney disease of various etiologies. Acute lesions of the renal tubular system (acute tubular necrosis) are frequently accompanied by albuminuria, which is considered to be the consequence of a compromised tubular reabsorptive function. Net urinary albumin excretion, however, is also determined by glomerular albumin filtration. Therefore, we hypothesized that in situations of insults to the tubular system, a tubular-glomerular crosstalk may lead to an increased filtration of albumin and consequently may contribute to the development of albuminuria.

To test this hypothesis, we used intravital multiphoton microscopy in Munich Wistar Froemter rats to determine the glomerular sieving coefficient (GSC) of albumin after ablation of single cells of the proximal tubule in an otherwise healthy kidney. For intravital imaging, rats were anesthetized with 100 mg/kg Ketamin, 3.45 mg/kg Xylazin and 2 mg/kg Midazolam *i.m.*. Single tubular cells were ablated by focused laser exposure. The GSC of albumin was determined after *i.v.* infusion of Alexa-594-labeled albumin as the ratio of the fluorescence intensities in Bowman's space/the glomerular capillary lumen, corrected by the background intensities. The density of the glomerular endothelial glycocalyx was determined after *i.v.* application of wheat germ agglutinin.

The GSC of albumin in the healthy rat kidney was low, averaging  $0.0008 \pm 0.0002$ . Within 20 min after laser ablation of a few proximal tubular cells, the albumin GSC of the affected nephron increased rapidly to  $908 \pm 287\%$  of baseline ( $p < 0.01$  vs. baseline;  $n=25$ ), whereas the albumin GSC of neighboring nephrons remained unchanged ( $p=ns$ ). In parallel with the albumin leakiness of the glomeruli of the affected nephrons, the density of the glomerular endothelial glycocalyx declined, averaging 18.3 rU in controls and 14.2 rU after tubular lesions ( $p < 0.0001$ ;  $n=215$  regions of interest). Furthermore, we occasionally observed changes in the shape of the podocyte cell bodies.

In summary, using intravital multiphoton microscopy, we found a nephron-specific marked increase in the GSC of albumin in response to acute insults to the proximal tubule. Our data further suggest a crucial involvement of the endothelial glycocalyx in the function of the glomerular filtration barrier and provide first evidence for a functional modulation of the integrity of the glomerular filtration barrier by the tubular system.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*



## PCA141

**Altered expression of the renal prostaglandin E<sub>2</sub> signaling system in kidney tissue from patients suffering from hydronephrosis and renal fibrosis**S.S. Tofteng<sup>1</sup>, A.K. Mogensen<sup>1</sup>, A. Toft<sup>3</sup>, B.L. Jensen<sup>1</sup> and K. Madsen<sup>1,2</sup><sup>1</sup>Cardiovascular and Renal Research, University of Southern Denmark, Odense, Denmark, <sup>2</sup>Department of Pathology, Odense University Hospital, Odense, Denmark and <sup>3</sup>Department of Urology, Odense University Hospital, Odense, Denmark

Renal fibrosis is the final common pathway in chronic kidney diseases (CKD). The mechanisms leading to renal fibrosis are only partly understood. Increased prostaglandin E<sub>2</sub>-EP<sub>4</sub> receptor signaling through increased EP<sub>4</sub> receptor expression has been suggested to attenuate renal fibrogenesis in mice using the unilateral ureteral obstruction (UUO) model of CKD. It is currently unknown if patients suffering from hydronephrosis and renal fibrosis show similar regulation of the renal prostaglandin E<sub>2</sub> signaling system. We tested the hypothesis that hydronephrosis and renal fibrosis is associated with increased renal COX-2 and EP<sub>4</sub> receptor expression in humans. Kidney tissue was collected from patients diagnosed with hydronephrosis undergoing unilateral nephrectomy (n=12) at the Dept. of Urology, Odense University Hospital. Normal appearing kidney tissue from age- and gender matched subjects undergoing nephrectomy due to renal cell carcinoma (n=12) was used as controls. Kidney tissue was collected within 60 minutes of removal from the patient and divided into cortex, outer and inner medulla. Tissue sections were Periodic Acid Schiff (PAS) stained and the percentage of the cortical region affected by interstitial fibrosis was scored according to Sethi *et al.* (Kidney Int 91:787-797,2017). Expression of the renal prostaglandin E<sub>2</sub> signaling system was determined by quantitative PCR. The study was approved by the Regional Committees on Health Research Ethics. Mean age of the patients was 54 years [range 26-78 years] in the hydronephrosis group and 55 years [range 28-78 years] in the control group. Male:female ratio was 1:1. In the control group, all patients showed minimal or no signs of renal fibrosis (less than 10%). In the hydronephrosis group, 4 patients showed mild (10-25%), 3 patients moderate (26-50%) and 2 patients a severe degree of fibrosis (>50%), whereas 3 patients showed minimal or no signs of renal fibrosis. No significant changes in COX-2, COX-1 or prostaglandin E<sub>2</sub> synthase mRNA abundance was detected in kidney regions. In kidney cortex, a significant upregulation of EP<sub>2</sub> receptor mRNA abundance was detected in the hydronephrosis group compared to controls and a concomitant downregulation of the EP<sub>3</sub> receptor mRNA level. No significant changes in EP<sub>1</sub> or EP<sub>4</sub> receptor mRNAs were seen. In outer medulla, a significantly reduced expression of the EP<sub>3</sub> receptor was seen in the hydronephrosis group. No changes in EP receptor expression were seen in the inner medulla. In conclusion, hydronephrosis and renal fibrosis in humans is associated with altered renal EP<sub>2</sub> and EP<sub>3</sub> receptor expression. No changes were detected in COX-2 and EP<sub>4</sub> receptor expression as has been described in mice. Results suggest the EP<sub>2</sub> and not the EP<sub>4</sub> receptor to be dominant in PGE<sub>2</sub> receptor signaling during renal fibrogenesis in humans. Sethi *et al.* (Kidney Int 91:787-797,2017)

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA142

**The pressure natriuresis response is not affected by SGLT2 inhibition in rats with Type 1 diabetes mellitus**

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Increasing blood pressure (BP) induces natriuresis due to downregulation of sodium transport in the proximal tubule. This pressure natriuresis (PN) response regulates BP<sup>1</sup>. Type-1 diabetic (T1DM) patients show enhanced sodium reabsorption and high BP. In T1DM rats, PN is impaired due to uncoupling of tubular sodium reabsorption from BP<sup>2</sup>. The molecular mechanism is unknown but the sodium-glucose cotransporter SGLT2 is a plausible candidate and inhibitors induce diuresis in T1DM rats<sup>3</sup>. This study tested the hypothesis that SGLT2 inhibition with dapagliflozin would improve the acute PN response in T1DM rats.

T1DM was induced in male Sprague-Dawley rats by streptozotocin (30-45mg/kg IP; plasma glucose 13.3->35mmol). After 3 weeks rats were anaesthetised with Inactin (120mg/kg, IP). The jugular vein was cannulated and isotonic saline (containing FITC-inulin and p-amino hippurate for measurement of glomerular filtration rate (GFR) and renal plasma flow (RPF)) was infused. Rats were randomly allocated to receive either dapagliflozin (50mg/kg; n=7) or saline vehicle (n=7) and after an equilibration period and baseline urine collection, BP was increased by sequential arterial ligation of first the coeliac and mesenteric arteries and then the distal aorta and the natriuretic response was measured. Experiments were performed under a single blind and rats were euthanised by an overdose of anaesthetic.

Data are mean +SD and comparisons were made by 2-way ANOVA. GFR and RPF were not different between treatment groups and were unaffected by the experimentally-induced rise in BP, consistent with autoregulation. In vehicle-treated rats, incremental rises in BP increased urine flow rate from a baseline of 6.2±3.0 to a peak of 51.4±27.7 µl/min/gkw (P<0.01), sodium excretion rate from 1.7±0.5 to 16.7±1.4 µmol/min/gkw (P<0.01) and fractional sodium excretion from 0.2±7.6 to 5.4±4.5% (P<0.01). The dapagliflozin rats responded to increasing pressure with a diuresis and natriuresis but the peak absolute (3.8±4.1 µmol/min/gkw; P=0.010) and fractional (15.2±8.0%; P=0.018) sodium excretion were significantly lower than in the vehicle-group. Dapagliflozin increased urinary glucose excretion from 0.1±0.2 to 0.4±0.2 µmol/min/gkw. However, although mean BP increased by ~30mmHg in both cohorts, it was lower in dapagliflozin-treated rats by ~14mmHg (P<0.01) and non-linear regression did not separate pressure diuresis (P=0.22) and natriuresis (P=0.53) curves in the two treatment groups.

SGLT2 inhibitors induce an increase in urine flow rate and glucose excretion when BP remains at steady state levels, but they do not enhance the acute pressure natriuresis response in anaesthetised T1DM rats. We conclude that impaired pressure natriuresis in T1DM does not reflect increased SGLT2 activity.

Ivy JR and Bailey MA. (2014). *J Physiol* 592, 3955-3967Culshaw GJ *et al.* (2014). *Int Soc Nephrol Intrinsic Renal Regulation*Thomson SC *et al.* (2012). *Am J Physiol Regul Integr Comp Physiol* 302, R75-R83

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## PCA143

### Na<sup>+</sup> and volume balance is maintained in AE4 (Slc4a9) knockout mice during Na<sup>+</sup> depletion

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The renal Na<sup>+</sup>/HCO<sub>3</sub><sup>-</sup> cotransporter AE4 (Slc4a9) is localized to the basolateral membrane of β-intercalated cells (β-ICs) in the renal collecting duct. It has been shown that β-ICs contribute not only substantially to acid-base homeostasis, but also to renal Na<sup>+</sup> reabsorption during Na<sup>+</sup> depletion. On the luminal side Na<sup>+</sup> uptake is mediated by the parallel action of the Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger pendrin (Slc26a4) and the Na<sup>+</sup>-driven Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger NDCBE (Slc4a8). The Na<sup>+</sup>/HCO<sub>3</sub><sup>-</sup> cotransporter AE4 participates in the basolateral Na<sup>+</sup> extrusion in the β-ICs, but it remained unclear whether AE4 is essential for the maintenance of Na<sup>+</sup> and intravascular volume balance during Na<sup>+</sup> depletion *in vivo*.

We therefore analyzed urinary Na<sup>+</sup> excretion, plasma volume, and activation of the renin-angiotensin-aldosterone system (RAAS) in AE4 wild-type (AE4<sup>+/+</sup>) and knockout mice (AE4<sup>-/-</sup>) during dietary Na<sup>+</sup> and Cl<sup>-</sup> depletion for 10 days. As the basolateral AE4 cotransporter mediates Na<sup>+</sup> and HCO<sub>3</sub><sup>-</sup> reabsorption, we also analyzed Na<sup>+</sup> and volume homeostasis after Na<sup>+</sup> and HCO<sub>3</sub><sup>-</sup> depleted diet.

Both genotypes exhibited an equally and strongly reduced urinary Na<sup>+</sup> excretion after changing from normal to Na<sup>+</sup> and Cl<sup>-</sup> depleted diet. In addition, plasma volume and hematocrit of AE4<sup>-/-</sup> mice did not differ from those of AE4<sup>+/+</sup> mice, and the RAAS was similarly activated in both genotypes after 10 days of Na<sup>+</sup> and Cl<sup>-</sup> depletion. As we found no difference with regard to renal Na<sup>+</sup> balance and plasma volume we examined, whether other renal Na<sup>+</sup> transporters of the ASDN were upregulated during NaCl depletion to compensate for the AE4 deficiency. Therefore, the abundance and phosphorylation of the Na<sup>+</sup>/H<sup>+</sup>-exchanger NHE3 and the thiazide-sensitive NaCl cotransporter NCC were analyzed by western blotting. Also the protein abundance of the α-, β-, and γ-subunit of the epithelial Na<sup>+</sup> channel ENaC was determined. No compensatory activation of these renal Na<sup>+</sup> reabsorption pathway was found in AE4<sup>-/-</sup> mice. Analysis of the natriuretic response to the diuretics hydrochlorothiazide and amiloride revealed an equal activity of the NCC and even a lower activity of the epithelial Na<sup>+</sup> channel ENaC in AE4<sup>-/-</sup> mice compared to AE4<sup>+/+</sup> mice. Also after Na<sup>+</sup> and HCO<sub>3</sub><sup>-</sup> depletion no evidence for disturbance of Na<sup>+</sup> and volume balance was found, as plasma Na<sup>+</sup> concentration and hematocrit of AE4<sup>-/-</sup> mice did not differ from those of AE4<sup>+/+</sup> mice.

These findings show that the Na<sup>+</sup>/HCO<sub>3</sub><sup>-</sup> cotransporter AE4 is not essential for maintaining Na<sup>+</sup> and intravascular volume balance during Na<sup>+</sup> depletion, independent of the accompanying ion. Our data suggest that AE4 deficiency can be compensated by another yet unidentified basolateral Na<sup>+</sup> efflux pathway in β-ICs, maintaining Na<sup>+</sup> balance during Na<sup>+</sup> and HCO<sub>3</sub><sup>-</sup> reabsorption by the apical transporter NDCBE.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA144

### Impairment of pressure natriuresis in a mouse model of ACTH-dependent Cushing Syndrome

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Abnormal activation of the hypothalamic-pituitary-adrenal axis increases circulating glucocorticoids (cortisol in humans, corticosterone in rodents) to cause metabolic and electrolyte abnormalities. Hypertension is a common feature of ACTH excess. We have previously used a mouse model of ACTH-dependent Cushing Syndrome to show that increased blood pressure (BP) is associated with enhanced ENaC-mediated sodium reabsorption in the distal nephron (1). In the present study, we used this model to determine whether ACTH-excess altered the acute pressure diuresis and natriuresis response, i.e. the positive relationship between arterial pressure and renal sodium and water excretion.

Osmotic minipumps were implanted s.c. into adult male C57BL/6J mice under isoflurane anaesthesia, delivering ACTH (2.5µg/d; n=6) or saline (n=6) for 10-12 days. Mice were then anaesthetised with Inactin (120mg/kg IP) and isotonic saline containing FITC-inulin (0.25%w/v) infused (0.3ml/h/10g) through the jugular vein. BP was measured via the carotid artery. A laparotomy was performed and ligatures loosely tied around the isolated mesenteric and coeliac arteries and the distal aorta. Urine was collected from the bladder. After a 30 minute baseline period, the mesenteric and coeliac arteries were ligated to induce a rise in BP. After 30 minutes of urine collection the aorta was ligated to further increase BP and urine again collected. Data are mean±SEM. BP was higher in ACTH-treated mice (98±3mmHg) than in controls (81±3mmHg; p=0.001, Student's t test, n=6 per group). The sequential ligation of arteries significantly increased BP in both groups (p<0.0001, 2way ANOVA). The first ligation increased BP by 37mmHg in ACTH and by 44mmHg in vehicle; aortic ligation further increased BP by 38mmHg (ACTH) and 33mmHg (vehicle). The applied increased BP induced a significant diuresis in both groups of mice, but this was less extensive in the ACTH group, particularly in the final period when urine flow rate was ~50% that of control mice (ACTH=10.6±1.6µl/min/gkw vs vehicle=21.1±3.5µl/min/gkw. p=0.0005, 2way ANOVA with Holm-Sidak's *post hoc* test, n=5/6 per group). Similarly, the sequential rise in pressure caused a significant increase in sodium excretion but the natriuresis was suppressed in the ACTH group (ACTH=2.9±0.6µmol/min vs vehicle= 7.8±2.3µmol/min. p=0.01, 2way ANOVA with Holm-Sidak's *post hoc* test, n=5/6 per group). Glomerular filtration rate did not change significantly with BP in either group, consistent with intact renal autoregulation. These data show that chronic glucocorticoid excess impairs the acute natriuretic and diuretic response to increased BP. The underlying mechanisms have not been investigated but sustained tubular sodium reabsorption is indicated.

1. Bailey MA *et al.* (2009). *Hypertension*. 54,890-6.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA145

**A single gastric K<sup>+</sup> load induces acute diuresis in mice**

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**Introduction:**

K<sup>+</sup> balance relies on regulated renal K<sup>+</sup> excretion to match variable dietary K<sup>+</sup> intake. Upon a K<sup>+</sup> rich meal, rapid and effective urinary K<sup>+</sup> excretion is obligatory. The renal adaptation to an acute K<sup>+</sup> load involves an increase of the driving force for K<sup>+</sup> secretion by shifting the distal tubular Na<sup>+</sup> reabsorption from being electroneutral (NCC) to electrogenic (ENaC). In addition, K<sup>+</sup> secretion in the CD is stimulated by increased luminal flow. Here we asked, if a high K<sup>+</sup> intake acutely increases urinary flow.

**Methods:**

Mice were K<sup>+</sup> challenged through gavage, diets or a combination of these. After K<sup>+</sup> loading urinary volume, [K<sup>+</sup>]<sub>u</sub> and [Na<sup>+</sup>]<sub>u</sub>, plasma osmolality, [K<sup>+</sup>]<sub>p</sub> and [Na<sup>+</sup>]<sub>p</sub> were measured.

**Results:**

1) Mice switched from a 1% to a 2% K<sup>+</sup> diet increased their diuresis markedly within 12h. 2) Mice switched from a 1% to a 0.01% K<sup>+</sup> diet had a biphasic diuretic response. During the first 12h their diuresis decreased, whereas it increased from 12 to 36h. 3) A single K<sup>+</sup> load, by gastric gavage, corresponding to 25-50% of daily K<sup>+</sup> intake induced diuresis within 30 min. This occurred despite augmented plasma osmolality. [K<sup>+</sup>]<sub>u</sub> remained unchanged and therefore the increased urinary K<sup>+</sup> excretion depended on the volume effect 4) K<sup>+</sup> gavage did not change urinary creatinine excretion suggesting a constant GFR. 5) Subsequently, a possible direct and acute effect of a plasma [K<sup>+</sup>] ([K<sup>+</sup>]<sub>pl</sub>) increase was tested in isolated perfused mTALs and CDs. An acute [K<sup>+</sup>]<sub>pl</sub> increase (from 3.6 to 6.5 mM) did not affect TAL NaCl absorption (measured 5 and 25 minutes post K<sup>+</sup> jump). In contrast, the same manoeuvre reduced the CDs sensitivity to stimulated AVP-mediated water absorption (measured 10 minutes post K<sup>+</sup> jump).

**Conclusion:**

Dietary K<sup>+</sup> load induces a rapidly on setting diuresis. This increase in urinary volume appears crucial for a powerful K<sup>+</sup> elimination since it appears prior to alteration in [K<sup>+</sup>]<sub>u</sub>. Based on preliminary data we suggest that the physiological mechanism of K<sup>+</sup>-induced diuresis involves AVP desensitization of the CD.

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## PCA146

**Mitochondrial aggregates of mutated glycine amidinotransferase (GATM) cause renal Fanconi syndrome and kidney failure.**

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Chronic kidney disease (CKD) affects 8-16% of the adult population. A histological hallmark of CKD is renal fibrosis, a process correlating with the progressive decline of kidney function. The pathophysiology of renal fibrosis is complex and often involves the release of profibrotic and inflammatory signals from damaged tubular cells. Here, we investigated the patho-mechanism of a novel form of hereditary renal Fanconi syndrome with kidney failure.

Members of five families with autosomal dominant renal Fanconi syndrome and kidney failure were clinically and genetically characterized. We performed genome-wide linkage analysis and DNA sequencing as well as immuno-localization studies, evaluation of mitochondrial morphology and cell biology assays. Structural studies examined the effects of recognized mutations.

In all our patients, novel mutations in the "glycine amidinotransferase" (GATM) gene, a renal proximal tubular enzyme in the creatine biosynthetic pathway, were identified. Mutant GATM proteins form fibrillary aggregates in mitochondria triggering an inflammasome response and profibrotic signals. All heterozygous missense mutations were located within the non-catalytic domain of GATM. Gatm knockout mice showed no renal abnormalities, rendering haploinsufficiency unlikely. Unexpectedly, proximal tubular cells expressing mutated GATM and patients' kidney biopsies showed massively enlarged mitochondria with fibrillary aggregates containing GATM. In fact, structure analysis predicted an additional interaction interface for mutated GATM enabling linear aggregation of GATM dimers. Once aggregated, GATM fibrils escaped degradation and resulted in aging of mitochondria. Aggregates-containing mitochondria were associated with increased ROS production, activation of the NLRP3 inflammasome, enhanced expression of the profibrotic cytokine IL-18 and increased cell death.

These data establish a link between pathological intramitochondrial GATM protein deposits, inflammation and kidney fibrosis.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*



PCA147

**Calcium-dependent contraction of renal tubules**S.L. Svendsen<sup>1</sup>, A. Hofherr<sup>2</sup>, M. Köttgen<sup>2</sup> and J. Leipziger<sup>1</sup><sup>1</sup>Biomedicine, Aarhus University, Aarhus, Denmark and <sup>2</sup>ZKF, University Hospital Freiburg, Freiburg, Germany

Over several years, we have observed, that perfused renal tubules show “odd movements” when exposed to extracellular ATP. Whether this observation was merely an artefact or a relevant biological observation remained elusive. It is well known that renal tubules express a contractile machinery localized closely to the apical membrane. The functionality of this structure is enigmatic. Plate grown kidney cells show a pattern on their apical membrane consistent with active contraction of the luminal membrane domain.

Here we test if isolated perfused renal tubules can contract. We measured the effect on tubular diameter in isolated murine thick ascending limbs (TAL), when inducing a cytosolic  $[Ca^{2+}]_i$  increase with uncaging ATP/stimulation of the apical P2Y2 receptors.

Uncaging luminal ATP elicited a marked increase of  $[Ca^{2+}]_i$ , which could be fully blocked by intracellular  $Ca^{2+}$  chelation following BAPTA-AM loading. Remarkably, within 40 seconds following apical P2Y2 receptor stimulation, both the inner and outer diameter of the perfused TAL were significantly reduced. The inner and outer diameter decreased by  $0.236\mu m$  (SEM  $\pm 0.041$ ,  $n=5$ ) and  $0.170\mu m$  (SEM  $\pm 0.022$ ,  $n=5$ ), respectively. This reduction could be largely prevented either blocking the  $[Ca^{2+}]_i$  increase with BAPTA or by hindering actin-myosin interaction with highly specific antagonists.

We report the remarkable observation that renal tubules in a  $[Ca^{2+}]_i$ -dependent manner can contract. This suggests that the diameter of renal tubules is a subject to active and acute regulation.

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whether this differed from that of lifelong (29 [28-40] years of training) endurance-trained runners ( $n = 13$ : age, 57 [54-59] years). Indices of spontaneous sympathetic baroreflex function were determined from beat-by-beat changes in diastolic blood pressure (DBP, photoplethysmography) and the corresponding MSNA (microneurography) during six minutes of supine rest. Aortic (Carotid-Femoral Pulse Wave Velocity via applanation tonometry) and carotid ( $\beta$  Stiffness Index via B-mode ultrasound) stiffness were also measured. Data presented are means [95% confidence intervals] and were compared using independent t-tests. Resting heart rate (electrocardiogram) was lower in runners (43 [38-47] vs 56 [49-62] bpm,  $P < 0.01$ ) but mean brachial artery pressure was not different (93 [90-96] vs 95 [89-101] mmHg,  $P = 0.54$ ). Aortic ( $6.8 [6.2-7.3]$  vs  $7.5 [6.9-8.1]$   $m/s^{-1}$ ,  $P = 0.02$ ) and carotid artery stiffness ( $4.06 [3.25-4.88]$  vs  $5.06 [3.94-6.19]$  AU,  $P = 0.04$ ) were also lower in runners. Spontaneous sympathetic baroreflex operating DBP was similar (77 [74-81] vs 79 [73-85] mmHg,  $P = 0.57$ ), as was MSNA burst frequency (31 [27-34] vs 28 [19-38] bursts/min<sup>-1</sup>,  $P = 0.55$ ) and sympathetic baroreflex gain (i.e. responsiveness) ( $-6.1 [-8.0 - -4.1]$  vs  $-6.9 [-9.8 - -4.0]$  %/mmHg,  $P = 0.55$ ). However, the corresponding MSNA burst incidence (i.e. 'set point') was higher in runners (72 [63-81] vs 50 [33-66] bursts/100hb<sup>-1</sup>,  $P < 0.01$ ). In conclusion, lifelong endurance runners had lower stiffness in barosensitive arteries, and an upward setting of the sympathetic baroreflex; meaning there was a higher likelihood of a burst of MSNA for a similar operating DBP. This was evident despite no difference in the responsiveness of MSNA to spontaneous fluctuations in blood pressure. We suggest that the greater MSNA burst incidence in lifelong runners is necessary to maintain resting blood pressure in the context of the training-induced bradycardia, skeletal muscle angiogenesis and possible reductions in  $\alpha$ -adrenergic sensitivity. Investigation is required to explore the mechanisms that underpin modified sympathetic baroreflex function following lifelong endurance training.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA148

**Lifelong endurance training modifies sympathetic baroreflex control of neural vasoconstrictor tone at rest**D.J. Wakeham<sup>1,2</sup>, R.N. Lord<sup>1</sup>, J.S. Tablot<sup>1</sup>, B. Curry<sup>1</sup>, T. Dawkins<sup>1</sup>, L.L. Simpson<sup>3,4</sup>, R. Shave<sup>5</sup>, J. Moore<sup>3</sup> and C.J. Pugh<sup>1</sup>

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Changes in blood pressure during middle age are associated with elevated muscle sympathetic nerve activity (MSNA), alterations in the sympathetic baroreflex, and stiffening of the barosensitive aortic and carotid arteries. It is well known that lifelong endurance-training attenuates the age-related increase in arterial stiffness; however, the impact on sympathetic baroreflex function (i.e. operating pressure, set point and gain) is less clear. We examined sympathetic baroreflex control of MSNA in age-matched recreationally active middle-aged men ( $n = 10$ : age, 53 [52-55] years), and determined

PCA149

**Acute effect of isometric handgrip exercise on arterial stiffness in older adults**S.-. NAKMAREONG<sup>1</sup>, M. Thasomboon<sup>1</sup>, B. Sangjan<sup>1</sup>, C. Prarat<sup>1</sup>, K. Apaijit<sup>2</sup> and U. Kukongviriyapan<sup>3</sup>

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Arterial stiffness is a major contributor to the development of atherosclerosis and consequently cardiovascular disease. Studies have shown a positive correlation between advancing age and arterial stiffness. Isometric handgrip exercise is a simple form of exercise, which recognized as antihypertensive effect. However, the effect of a single bout of isometric handgrip exercise on arterial stiffness remains unknown. The purpose of this study was to investigate the changes in acute arterial stiffness and post-exercise hypotension following a single bout of isometric handgrip exercise in older adults. Thirty healthy elderly (5 men, 25 women; age 60-80 years) were randomly allocated to either isometric exercise group (age  $69.4 \pm 7.4$  years, body mass index  $22.0 \pm 1.9$  kg/m<sup>2</sup>,

n=15) or control group (age  $69.7 \pm 5.2$  years, body mass index  $22.5 \pm 1.5$  kg/m<sup>2</sup>, n= 15). Participants in isometric exercise group performed sustained isometric handgrip exercise at intensity of 30% of maximal contraction (Handgrip dynamometer; T.K.K.5001 grip A, Japan) by holding 2 minutes with 1 minute rest for 4 times (Millar et al., 2014), whereas subjects in the control group were asked to hold handgrip without exercise. Eligible participants were familiarized to all testing protocols and equipments. Brachial-ankle pulse wave velocity (baPWV) was measured at baseline and after the exercise using Arterial Compliance Monitor (Barts and The London's School of Medicine and Dentistry, UK) (Phababpha el al., 2013). Resting blood pressure was measured using a calibrated Blood Pressure Bedside Monitor. This study protocol was approved by the Human Research Ethics Committee, Khon Kaen University (No. HE602295). Values are reported as means  $\pm$  S.D., compared by paired t-test and t-test. All baseline characteristics were no significant differences ( $P > 0.05$ ). Following acute isometric handgrip exercise, showed a significant reduction of  $16.5 \pm 8.6\%$  in mean baPWV when compared with baseline value. In addition, single bout of isometric handgrip exercise significantly decreased baPWV compared with control group ( $1040.5 \pm 96.3$  cm/sec vs.  $1263.1 \pm 115.4$  cm/sec,  $P < 0.05$ ). Concomitant significant alleviated of systolic blood pressure in exercise group (from  $119.4 \pm 10.3$  mmHg to  $108.6 \pm 6.8$  mmHg,  $P < 0.05$ ). However, diastolic blood pressure remained unchanged in either exercise (from  $68.3 \pm 5.3$  mmHg to  $68.5 \pm 4.4$  mmHg) or control group (from  $68.8 \pm 6.1$  mmHg to  $67.2 \pm 6.6$  mmHg). In conclusion, the acute effect of isometric handgrip exercise alleviates arterial stiffness, resulting in reduced resting blood pressure. Therefore, the finding of this study might be used as a preliminary data for setting the exercise protocol among older adults.

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This work was supported by the Faculty of Associated Medical Sciences.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCA150

##### High-intensity resistance exercise with low repetitions maintains endothelial function

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Abnormal endothelial function marked by reduced dilation of blood vessels as blood flow increases (endothelium-dependent flow-mediated dilation; FMD) is an early hallmark of cardiovascular disease. According to the line, resistance exercise impairs endothelial function, and this impairment is thought to be mediated by sustained elevation in blood pressure. Herein, we tested the hypothesis that resistance exercise-induced endothelial dysfunction would be prevented by high-intensity resistance exercise with low repetitions. This type of resistance exercise is known to

induce temporal elevation in blood pressure due to low repetitions and long resting period between sets. Thirteen young healthy subjects completed three randomized experimental trials: (1) moderate-intensity with moderate repetitions trial (moderate-moderate trial); (2) low-intensity with high repetitions trial (low-high trial); (3) high-intensity with low repetitions trial (high-low trial). Following baseline brachial artery FMD and blood pressure measurements, subjects performed resistance exercise according to the different types of trial. The intensity of moderate-moderate, low-high and high-low trials comprised of 10 repetitions for five sets at 70 % of 1RM, 40 repetitions for five sets at 30 % of 1RM, and 3 repetitions for five sets at 85 % of 1RM, respectively. Resting periods among all sets were 60 s, 60 s and 180 s, respectively. Thereafter, brachial artery FMD and blood pressure measurements were repeated 10, 30, and 60 min after the exercise. All experimental procedures and measurements were confirmed to Declaration of Helsinki and were approved by the Ethics Committee for Human Experiments at Sports Research Center, Hosei University, Japan [ID: 2017-003]. A two-way (time x trial) repeated measures analysis of variance (ANOVA) with Tukey posthoc testing was performed on all dependent variables between moderate-moderate trial and high-low trial or low-high trial and high-low trial. Significance was accepted at  $P \leq 0.05$ . Data are expressed as means  $\pm$  SE. Although systolic blood pressures were significantly elevated after exercise in all trials ( $P < 0.05$ ), the magnitudes of rise in blood pressure increase were significantly lower in the high-low trial than in the moderate-moderate and low-high trials ( $P < 0.05$ ). Importantly, moderate-moderate and high-low trials caused a significant impairment in brachial artery FMD (Baseline vs. 10 min after exercise:  $7.9 \pm 1.0\%$  vs.  $3.8\% \pm 1.1$  in the moderate-moderate trial,  $8.3 \pm 1.0\%$  vs.  $3.4 \pm 1.2\%$  in the low-high trial,  $P < 0.05$ ). However, high-low trial did not alter brachial artery FMD (Baseline vs. 10 min after exercise:  $7.5 \pm 0.7\%$  vs.  $8.2 \pm 1.4\%$ ). In conclusion, endothelial function was maintained by conducting high-intensity resistance exercise with low repetitions.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCA151

##### Novel redox-targets of NADPH oxidase 4 identified by the BIAM switch assay

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Nox-derived ROS have been implicated in redox-signaling as they can elicit cysteine oxidation in target proteins. Only a limited number of Nox-differentially oxidized proteins have been identified so far and particularly little is known concerning redox-targets of the constitutively active NADPH oxidase Nox4. We set out to identify redox-targets of Nox4 by redox-proteomics, therefore podocytes of WT and Nox4<sup>-/-</sup> were used. Redox-modified proteins were identified by the BIAM switch assay in combination with mass spectrometry. Increased Nox4 expression in response to TGF $\beta$ -1 was detected in podocytes of WT mice. In response to TGF $\beta$ -1, oxidation of 138 proteins increased in podocytes of wildtype but not in podocytes of Nox4<sup>-/-</sup> mice. Identified proteins clustered in

different groups of cellular processes like “cellular oxidant detoxification” and “activation of protein kinase activity”. The difference in oxidation of identified proteins could be validated for selected proteins by the BIAM-switch assay and western blot analysis and show up some interesting novel redox-targets of Nox4. The BIAM-Switch assay coupled to Mass spec is a powerful and versatile tool to identify differentially oxidized proteins. Nox4 is a source of ROS which changes the redox-state of numerous proteins.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA152

### Sphingolipid Signals Regulate Hypoxia-Inducible Factors in Macrophages and Fibroblasts

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Tumor hypoxia impairs efficacy of radiotherapy. Within hypoxic areas gene expression is controlled and mediated by the transcription factor complex hypoxia-inducible factor 1 (HIF-1). Additionally, the sphingolipid metabolism becomes increasingly recognized as target for cancer treatment. Preliminary data indicate interplay between sphingolipid signaling and HIF-1 activation. Acid sphingomyelinase (Asm), which hydrolyses sphingomyelin to ceramide, has recently been recognized to significantly impact tumor sensitivity to radiation therapy (1). In this context, Asm activation is required to induce endothelial damage after high single dose radiation therapy (SDRT).

Here, we investigated the molecular mechanisms that control HIF-1 $\alpha$  subunit expression by sphingolipids in different cell types with special regard to the Asm/ceramide system. Both, fibroblasts and macrophages are highly represented cell types in the tumor microenvironment. Induction of ceramide caused by treatment with compound C, an inducer of ceramide synthase, increased the expression of HIF-1 $\alpha$  in monocytic PLB and THP-1 cells under normoxia and hypoxia. Asm-deficient mouse embryonic fibroblasts (MEFs) treated with hypoxia and toll-like receptor 4 ligands show elevated HIF-1 $\alpha$  accumulation compared with wild type cells. An increase of HIF-1 $\alpha$  protein and mRNA expression was detected in MEFs coincident with increased levels of sphingosine-1-phosphate.

Our data thus far suggest interplay between lipid signaling and HIF-1 activation, and provide a basis for further *in vivo* studies on the function and regulation of the hypoxic response after SDRT.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA153

### Reactive oxygen species mediate endoplasmic reticulum stress-induced mitochondrial dysfunction in human skeletal muscle cells

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Myositis is an acquired autoimmune disease that causes profound skeletal muscle weakness and dysfunction (1). Historically thought to be predominantly immune-mediated, significant evidence now suggests that muscle weakness correlates poorly with the degree of inflammation. Moreover, weakness persists and in cases precedes inflammatory cell invasion into muscle. Chronic activation of the endoplasmic reticulum (ER) stress pathway is a characteristic hallmark of myositis and reported non-immune mediated mechanism involved in disease pathogenesis (2). ER stress is associated with modified reactive oxygen species (ROS) generation, altered mitochondrial bioenergetics, and oxidative damage (3); which is postulated as a potential mechanism involved in the underlying muscle weakness experienced by patients with myositis. In this study, we examine the impact of a superoxide dismutase/catalase mimetic (EUK-134) in an *in vitro* model of ER stress in skeletal muscle (4). Human skeletal muscle cells cultured *in vitro* were exposed to the ER stress inducer Tunicamycin (0.1mg/ml) with or without EUK-134 (2 $\mu$ M) for 24 hours. Quantitative PCR examined ER stress pathway activation and mitochondrial dynamics. Mitochondrial function was assessed using Seahorse extracellular flux analysis and mitochondrial membrane potential interrogated using the JC-1 fluorophore. ROS generation was measured using MitoSOX red (superoxide) alongside quantification of sulphhydryl and oxidised glutathione (GSSG) content. Data were analysed for statistical significance by ANOVA and post hoc Tukey test. Tunicamycin induced upregulation in gene expression of ER stress markers Grp78, CHOP, XBP-1, and ERDJ4, which was significantly reduced in the presence of EUK-134 ( $p \leq 0.05$ ). Data showed increased gene expression of mitochondrial-related markers, citrate synthase, FIS1, UCP-3, TFAM, DRP1, HSPA9, HSP60, SOD1, and MFN1, in response to Tunicamycin treatment ( $p \leq 0.05$ ). A significant reduction was seen in gene expression of UCP-3, HSPA9, citrate synthase, SOD1, FIS1, and TFAM in the presence of EUK-134 ( $p \leq 0.05$ ). Tunicamycin induced declines in basal, maximal, and ATP-linked mitochondrial respiration, alongside increased proton leak and reduced mitochondrial membrane potential; effects mitigated by EUK-134 ( $p \leq 0.05$ ). Tunicamycin increased mitochondrial superoxide and GSSG levels, and decreased overall sulphhydryl content, an effect ameliorated in the presence of EUK-134. These findings suggest that the quenching of ROS generation using the superoxide dismutase/catalase mimetic EUK-134 can ameliorate aspects of ER stress-induced mitochondrial dysfunction. Overall, this reinforces a role for ROS in mediating ER stress effects, and that in instances of chronic ER stress, such as in myositis, targeting ROS generation may be an avenue for beneficial impact.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA154

### **<sup>13</sup>C methacetin breath test for assessment of liver function: validation of a shortened collection time in healthy adults**

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<sup>13</sup>C breath tests for clinical assessment of physiological function have received much recent attention due to their non-invasive nature and their relatively low cost. The <sup>13</sup>C methacetin test assesses cytochrome P450 dependent liver function [1] and has been shown to accurately predict the degree of liver cirrhosis in chronic liver disease patients [2]. The traditional version of this test involves 120 minutes of breath sample collection, which may not always be practical in a clinical setting. However, Schneider et al. [3] have previously demonstrated that a shortened test with a single sample collection 15 minutes after ingestion may be sufficient for accurate assessment of liver function by <sup>13</sup>C breath excretion. There is still, nevertheless, confusion over what cut-off values should be used to assess liver function at this time point. Consequently, the purpose of the current study was to assess liver function in healthy individuals and determine normal values at the 15-minute sample collection using the <sup>13</sup>C methacetin test. Twenty six male and female adults completed this test. All were generally healthy, with no history of liver disease, as determined by a pre-participation medical screening questionnaire. Participants completed one experimental trial which involved arriving at the laboratory in a fasted state before drinking 100 mL of water with the addition of 75 mg of <sup>13</sup>C methacetin. End expiratory breath samples were collected immediately prior to ingestion and 10, 15, 20, 30, 40, 50, 60, 80, 100 and 120 minutes after ingestion. Samples were analysed for <sup>13</sup>CO<sub>2</sub>:<sup>12</sup>CO<sub>2</sub> ratio using infrared spectroscopy. The difference between values from baseline is expressed as delta over baseline (DOB). The median cumulative dose excretion after 120 minutes was 26.0 ‰<sup>13</sup>C with an upper and lower 95% confidence interval of 27.0 and 23.2 ‰<sup>13</sup>C respectively. DOB data (Figure 1) demonstrated that peak values for 54% of participants occurred 15 minutes after ingestion and peak values for 88% of participants occurring within 20 minutes of ingestion. Median DOB at 15 minutes was 20.2 with upper and lower confidence intervals of 16.3 and 21.0 respectively. The results of this study demonstrate that, in this small cohort of healthy participants, obtaining a breath sample 15 minutes after ingestion of <sup>13</sup>C methacetin is sufficient to provide an accurate assessment of liver function and that normal DOB values for this time point are likely to be between 16.3 and

21.0. This has potential clinical significance in reducing time and cost commitment for patients and healthcare providers, and merits further work to establish cut-off points for determination of liver function in clinical populations.

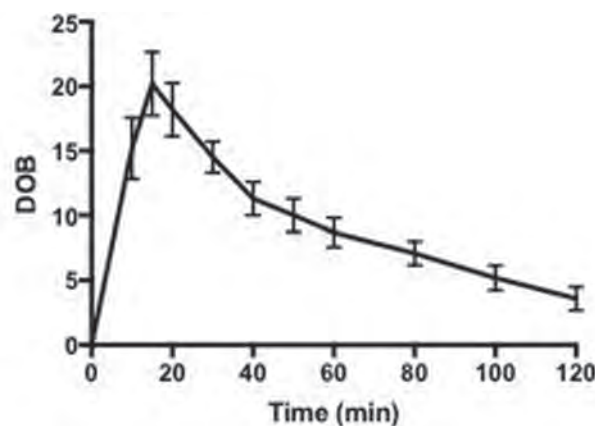


Figure 1: DOB values (median ± 95% CI) after ingestion of 75 mg <sup>13</sup>C methacetin

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA155

### **Low amplitude pressures in high resolution manometry**

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**Aim:** to investigate low amplitude pressures defined by distal contractile integral (DCI) <450mmHg/s/cm on high-resolution manometry (HRM)) in ≥50% of test swallows. **Materials and methods:** We searched retrospectively the database between January 2017 and May 2018 patients having in HRM a DCI<450mmHg/s/cm in ≥50% of test swallows. HRM results were interpreted using the Chicago Classification v3.0.

**Results:** A total of 92 patients (67.4% female) with a mean age of 49 ± 16 years were included. The main presenting symptom at time of manometry study was dysphagia in 50%. The mean of ICD in 10 deglutitions was 176.5mmHg/s/cm. The absence of contractility defined by the DCI < 100 mmHg/s/cm in all swallows was showed in 67% of cases with achalasia (n=40), scleroderma (n= 13), dysphagia (n= 8) and gastroesophageal reflux disease (GERD) (n=1). The ineffective esophageal motility defined by DCI<450mmHg/s/cm in ≥50% of test swallows was observed in 28 patients (30.4%) with 16 patients having absent contraction in more of 50% of deglutitions (7 scleroderma, 7 dysphagia and 2 GERD), 8 patients having weak contractions in > 50% of deglutitions (3 dysphagia, 2 GERD, 1 chest pain, 2 scleroderma) and 1 patient with 100%

of deglutitions were weak (GERD). Ineffective esophageal motility with mean of DCI > 450 mmHg/s/cm was observed in 10 patients (5 dysphagia, 3 GERD and 2 scleroderma).

Conclusion: Major disorders of peristalsis represented by absent contractility were observed in achalasia and scleroderma and minor disorders of peristalsis represented by ineffective esophageal motility in dysphagia and GERD.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

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#### PCA156

##### **Sphincters resting pressures in high resolution manometry**

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Aim: to evaluate the most appropriate time to measure the resting pressure of the upper esophageal sphincter (UES) and lower esophageal sphincter (LES) during high resolution manometry (HRM).

Methods: 70 patients who had HRM between December 2016 and March 2018 are recruited. The solid manometry catheter with 36 circumferential sensors was used. The measurement of resting pressure was done at the beginning and the end of procedures.

Results: The mean resting pressure of the UES decreased during HRM ( $p=0.013$ ). Decreasing in resting pressure of UES was observed in 62.9% of patients and increasing in 35.7% ( $p<10^{-4}$ ). The difference between time of recording is statistically significant ( $p<10^{-4}$ ). The correlation between LES pressure and the conclusion of exam was more significant when the pressure is measured in the end of the procedure ( $p=0.001$  vs  $p<10^{-4}$ ). Hypotonic and hypertonic LES was diagnosis at the beginning of test in 10% and 8.6% respectively and at the end in 13% and 11.4% ( $p<10^{-4}$ ). No correlation was observed between abnormal resting pressure of UES and LES in the both time of recording.

Conclusion: Measuring the basal pressures at the end of the test seems to be the best practice and better reflect the physiological sphincters function with a lesser effect of the vagus nerve in stress situation.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

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#### PCA157

##### **Esophageal abnormalities in patients with gastro-esophageal reflux disease on high resolution manometry as defined by Chicago classification**

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Aim: The aim of this study is to characterize esophageal dysmotility in patients with gastro-esophageal reflux disease (GERD) and compare demographic features, clinical symptoms and endoscopic findings across patients with different dysmotility patterns.

Methods: Patients with GERD who underwent high resolution manometry at our institution were identified. Data regarding demographic features, clinical symptoms and endoscopic findings were abstracted from medical records. High resolution manometry results were interpreted using the Chicago Classification, v3.0 criteria.

Results:

A total of 31 patients were colliged. There were 14 males and 17 females with mean age of  $56 \pm 15.2$  years. High-resolution esophageal manometry was normal in 22, showed ineffective esophageal motility in 6, absent contractility in 1, fragmented peristalsis in 1 and one case of type I achalasia. Esophagogastric junction (EGJ) resting pressure was within normal range (10 – 45 mmHg) in 23, low ( $< 10$  mmHg) in 6 patients and high ( $> 45$  mmHg) in 1 patient.

Dysphagia was significantly more frequently reported in patients with ineffective motility than patients with other dysmotility patterns (respectively 66.7% vs 16%;  $p=0.026$ ). Patients with hiatal hernia were older than patients who had not (respectively  $63.5 \pm 6$  vs  $50.1 \pm 15$  years;  $p=0.05$ ). Upper esophageal sphincter pressure was negatively correlated with age ( $r=0.3$ ;  $p=0.05$ ). There was no association between endoscopic findings and patterns of motility disorders.

Conclusion: The HRM has represented a major advance in defining and characterizing esophageal motility abnormalities in GERD patients. Ineffective esophageal motility is the most common and the most dysphagia-associated motility disorder in patients with gastro-esophageal reflux disease.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

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#### PCA158

##### **Rice bran protein hydrolysates exhibit antioxidant and modulatory effects on lymphocyte activation and proliferation**

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Maintenance of immune cell homeostasis is essential in protection of various diseases. Rice bran, the by-product from rice milling process contains a number of biologically active compounds. Protein is an important component of rice bran possessing nutritional and non-nutritional constituents. Protein from non-glutinous rice is suggested to be hypoallergenic and confers insulin sensitizing effect, vascular protection and antihypertension. The study was conducted to investigate whether protein from rice bran affect immunological functions of lymphocytes. Protein was extracted from a cold-pressed defatted Thai Sung-Yod rice bran. Rice bran was subjected to alkaline solubilization and proteolysis by protease enzyme. The rice bran protein hydrolysates (RBH) were freeze-dried for long-term storage. RBH showed the radical scavenging activity with EC<sub>50</sub> of  $0.35 \pm 0.09$  mg/mL, compared with ascorbate of  $5.0 \pm 2$  µg/mL by diphenylpicrylhydrazyl assay and possessed a reducing power as ascorbate equivalent to  $15 \pm 0.3$  mg/g RBH by ferric reducing antioxidant

power assay. RBH suppressed nitric oxide formation in murine RAW 264.7 cells induced by lipopolysaccharide and inhibited human lymphocyte proliferation induced by phytohemagglutinin (PHA) or combination of anti-CD3/anti-CD28 antibodies. Moreover, PHA remarkably induced increased expression of IL-2, IFN- $\gamma$  and IL-4 in lymphocytes by  $36.1 \pm 5.1$ ,  $64.0 \pm 24.2$  and  $12.7 \pm 8.4$  fold from controls at 6 h of incubation, where RBH significantly suppressed the induction of cytokines by  $42.1 \pm 2.5$ , and  $49.9 \pm 4.0$  and  $46.2 \pm 10.3$  % of the induced state, respectively. RBH from Sung-Yod rice bran have a potential effect in modulating the dysregulated immune cell function.

This work was supported by the Agricultural Research Development Agency, Thailand.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

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#### PCA159

### Four-weeks equipment-free high-intensity interval training does not improve the cardiorespiratory fitness of young healthy adults, but does elicit gains in muscle mass

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#### Introduction:

Low cardiorespiratory fitness (CRF) and skeletal muscle mass (MM) are both associated with increased morbidity and mortality (1). Despite exercise training being a proven method to improve both CRF and MM, a strikingly low proportion (2) of UK adults meet the current government guidelines for exercise (3), with "lack of time" one of the most-commonly cited barriers (4). One exercise training mode that has been proven to improve CRF with a reduced time-commitment is high-intensity interval training (HIIT) (5). However, the majority of HIIT research has involved training on a specialised cycle ergometer with intensity determined by a pre-training cardiopulmonary exercise test (CPET). In order to assess the efficacy of HIIT with a further barrier to exercise (equipment) removed, we investigated the efficacy of 4-weeks equipment-free HIIT for improving CRF and MM in young adults.

#### Methods:

Eight healthy volunteers aged 25 (18-35) y, were randomised to HIIT or a no-intervention control group. For the HIIT group, exercise sessions comprised a brief warm-up, five 1-minute bouts of high-intensity, equipment-free efforts (star-jumps, squats and static sprints) interspersed with 90-seconds recovery and a final recovery period. Each session lasted 15-minutes, with all volunteers completing 12 sessions over 4-weeks. CRF was assessed before and after the intervention by CPET, with muscle (*vastus lateralis* (VL)) thickness assessed by ultrasound.

#### Results:

HIIT did not elicit significant increases in either of our measures of CRF (AT: anaerobic threshold ( $13.7 \pm 2.0$  vs.  $15.7 \pm 2.7$  ml/kg/min,  $P=0.09$ ); or  $\text{VO}_2$  max ( $32.6 \pm 7.6$  vs.  $33.4 \pm 7.6$  ml/kg/min,  $P=0.6113$ )), with no change in either of these parameters in the control group (AT:  $19.6 \pm 7.9$  vs.  $19.9 \pm 9.2$  ml/kg/min,  $P=0.9$ ;  $\text{VO}_2$  max:  $40.7 \pm 12.2$  vs.  $40.6 \pm 13.7$  ml/kg/min,  $P=0.9$ ). Despite these findings, 75% of volunteers in the HIIT group did achieve an increase in AT above the threshold previously stated as clinically meaningful ( $>1.5$  ml/kg/min). HIIT did

elicit significant increases in VL muscle thickness ( $218.8 \pm 4.5$  vs.  $226.5 \pm 4.5$  mm,  $P=0.04$ ), with no change observed in the control group ( $246.3 \pm 6.0$  vs.  $247.3 \pm 7.4$  mm,  $P=0.9$ ).

#### Discussion:

Despite our time-efficient, equipment-free HIIT programme not being able to elicit significant improvements in CRF in this study, the trend towards an increase in AT and the significant increase in muscle thickness, suggest that this format of HIIT can be beneficial in improving physiological parameters associated with fitness and well-being. As the majority of individuals in the HIIT group did make meaningful gains in AT, further research in a larger cohort is required to assess the true utility of this intervention for improving CRF, and to determine the heterogeneity of adaptation to this mode of exercise.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

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#### PCA160

### Believe It or Not: Yoga and Yoga-Based Interventions

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#### Background:

Yoga has multiple facets which may include physiology, philosophy, psychology and spirituality. In today's world, there are lots of scientific literature available which show the widely acceptable role of yoga-based interventions in health and different diseases. Even though, many of us don't believe in yoga.

#### Objective:

To study the relationship between patient's belief in Yoga, and their satisfaction level with the yoga-based intervention.

#### Methods:

This study is a part of a larger study where yoga-based intervention was given to patients with glaucoma at a tertiary care hospital in Delhi. In this study, 41 glaucoma patients (16 females) of age 18-70 years were included and was conducted at Integral Health Clinic (IHC), All India Institute of Medical Sciences, New Delhi. Here, we used a self-designed feedback form, a part of ethically approved research project, which was filled by the participants at the end of 4 weeks of yoga-based intervention. The feedback included objective questions about their belief in Yoga, satisfaction level with the yoga-intervention, overall experience at IHC and willingness to revisit the IHC. Subjects rated their response on a score of 1 to 5 (Likert scale), where 1 being worst and 5 being best.

#### Results:

Thirty-seven subjects had significantly higher 'believe in Yoga score'. Subjective satisfaction score was significantly



high in 30 subjects. The 'overall experience score' was significantly higher in 38 subjects. There was no association of the frequency of Yoga practice in past with that of their satisfaction or overall experience at our yoga-facility. Belief of all 37 subjects had no relationship with overall experience. There was positive correlation of satisfaction score with overall experience score (except among males) and likely-to-revisit score. Conclusions:

Subjects with higher belief in yoga were more satisfied with the intervention. But, irrespective of their belief in yoga, the overall experience score was good and majority of them (40 subjects) were willing to revisit for yoga-intervention.

Correlation Among the Studied Scores.

| Variables                | Believe in Yoga score (Female/Male) | Satisfaction Score (Female/Male) | Overall Experience Score (Female/Male) | Likely-to-Revisit Score (Female/Male) |
|--------------------------|-------------------------------------|----------------------------------|----------------------------------------|---------------------------------------|
| Believe in Yoga Score    | 1.000<br>(1.000/1.000)              | 0.549**<br>(0.312/.825**)        | 0.247<br>(0.090/0.393)                 | 0.426**<br>(0.294/0.616**)            |
| Satisfaction Score       |                                     | 1.000<br>(1.000/1.000)           | 0.484**<br>(0.582*/0.386)              | 0.619**<br>(0.543*/0.663**)           |
| Overall Experience Score |                                     |                                  | 1.000<br>(1.000/1.000)                 | 0.632**<br>(0.645*/0.634**)           |
| Likely-to-Revisit        |                                     |                                  |                                        | 1.000<br>(1.000/1.000)                |

\*p-value≤0.05: Significant; \*\*p-value≤0.01: Highly significant. Spearman's rank correlation coefficient given.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA161

### Time course adaptations in cardiorespiratory fitness and fractional oxygen extraction with low-volume high-intensity interval training and moderate-intensity continuous training in people with type 2 diabetes

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Maximal oxygen uptake ( $VO_{2max}$ ) in people with type 2 diabetes (T2D) is ~20% lower than in healthy individuals and they display an over reliance in fractional oxygen extraction, reflected by an increased rate of muscle deoxygenation (i.e., deoxygenated haemoglobin and myoglobin, [HHb+Mb]) during ramp incremental exercise (RI) (Gildea *et al.* 2016). Moderate-intensity continuous training (MICT) and low-volume high-intensity interval training (LVHIIT) improve  $VO_{2max}$  in those with T2D (MacAnaney *et al.* 2011; Francois *et al.* 2017); however, the time course of changes in  $VO_{2max}$  and [HHb+Mb] following MICT or LVHIIT are not known. The aim of this study was to compare  $VO_{2max}$  and [HHb+Mb] responses pretraining and at 3, 6, 9 and 12 weeks of either MICT or LVHIIT in middle-aged individuals with T2D.

Thirty-one participants with T2D (18 men, mean ± SD: age 53 ± 9 yr, body mass index 30.1 ± 4.5 kg.m<sup>-2</sup>) were randomly assigned to MICT (n=13, 50 min of moderate-intensity cycling) or LVHIIT (n=9, 10 x 1 min at ~90% maximal heart rate interspersed by 1 min of 'unloaded' cycling) or to a non-exercising control group (n=9). Exercising groups trained 3 times/week and every 3 weeks exercise intensity was adjusted. Muscle

deoxygenation was measured using near-infrared spectroscopy (NIRS) at the *vastus lateralis* muscle. Normalised [HHb+Mb] profiles ([HHb+Mb]%) were plotted as a function of relative work rate (% $PO_{peak}$ ) using a bilinear model as proposed by Spencer *et al.* (2012). Breath-by-breath  $VO_2$  data were recorded using an online metabolic unit. Time point analyses of  $VO_{2max}$  and [HHb+Mb] response were performed using a 2-way repeated-measures ANOVA. Values are mean ± SD.  $VO_{2max}$  significantly increased after 3 weeks of MICT (+17%;  $P<0.05$ ) and LVHIIT (+8%;  $P<0.05$ ) with no further significant changes thereafter (total increases in  $VO_{2max}$  after 12 weeks of MICT and LVHIIT were 24% and 13% respectively). After 3 weeks there was a significant decrease in the slope of the first linear component of the delta[HHb+Mb]% vs delta $PO_{peak}$  profile ( $S_1$ ) in both the LVHIIT and MICT groups with no further changes observed thereafter. There were no changes in  $S_1$  or  $VO_{2max}$  in the control group at any time point. LVHIIT and MICT induced similar magnitude of improvements in  $VO_{2max}$  following a similar time course in participants with T2D. These improvements were, at least in part, due to a reduction in the over reliance on  $O_2$  extraction and improved microvascular  $O_2$  delivery.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA162

### The effect of high intensity interval training (HIIT) without dietary intervention on glucose regulation in sedentary aging men and masters athletes.

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The aim of the present investigation was to establish whether masters athletes (LEX) exhibited improved glucose regulation (fasting glucose, fasting insulin, and the homeostatic model assessment of insulin resistance and insulin sensitivity [HOMA-IR and HOMA-IS respectively]) compared to sedentary older males (SED). A secondary aim was to investigate whether 6 weeks' high intensity interval training (HIIT; 6 x 30 s sprints at 40% peak power, once every five days) preceded by 6 weeks' aerobic preconditioning would affect glucose regulation. Twenty-two males (62 ± 2 years) comprised the SED group and 17 males (60 ± 5 years) were enrolled as LEX. Insulin,

HOMA-IR, and HOMA-IS were lower in LEX than SED ( $P < 0.05$ ). HIIT did not alter glucose, insulin, HOMA-IR, or HOMA-IS in LEX ( $P > 0.05$ ). HIIT reduced SED glucose compared to baseline ( $P = 0.013$ , Cohen's  $d = 0.36$ ) and compared to post-preconditioning ( $P = 0.009$ , Cohen's  $d = 0.35$ ). HIIT reduced HOMA-IR in SED compared to baseline ( $P = 0.027$ , Cohen's  $d = 0.25$ ), and compared to after preconditioning ( $P = 0.069$ , Cohen's  $d = 0.36$ ). Results of this study indicate that HIIT preceded by preconditioning without dietary intervention improve glucose regulation in SED, as evidenced by reduced fasting glucose and HOMA-IR.

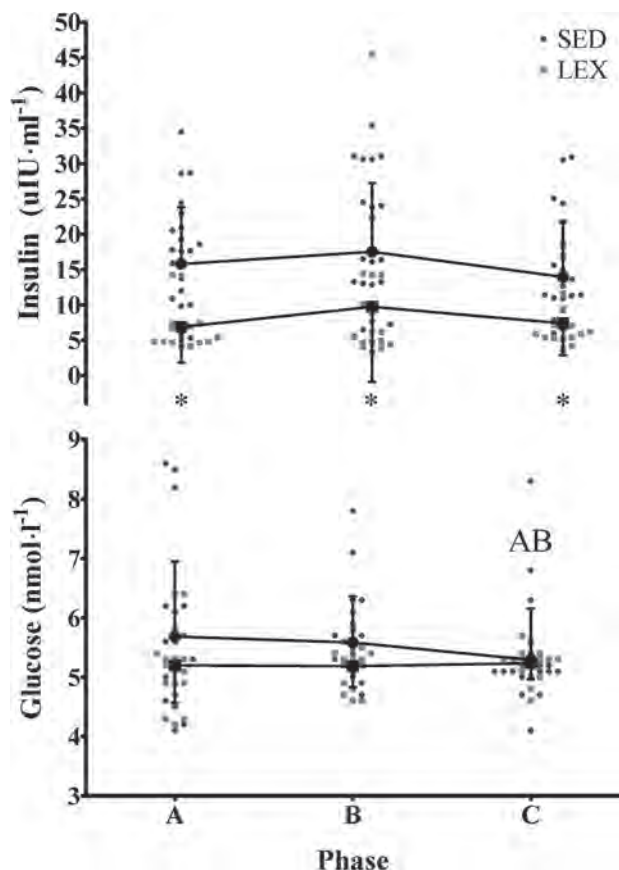


Figure 1: (upper panel) Insulin, and (lower panel) glucose, in a group of sedentary (SED) and lifelong exercising (LEX) older males. Data are presented as mean  $\pm$  SD, plus individual data points. \*Denotes significant differences between groups at this experimental phase ( $P < 0.05$ ). A = significantly different from phase A in SED ( $P < 0.05$ ). B = significantly different from phase B in SED ( $P < 0.05$ ).

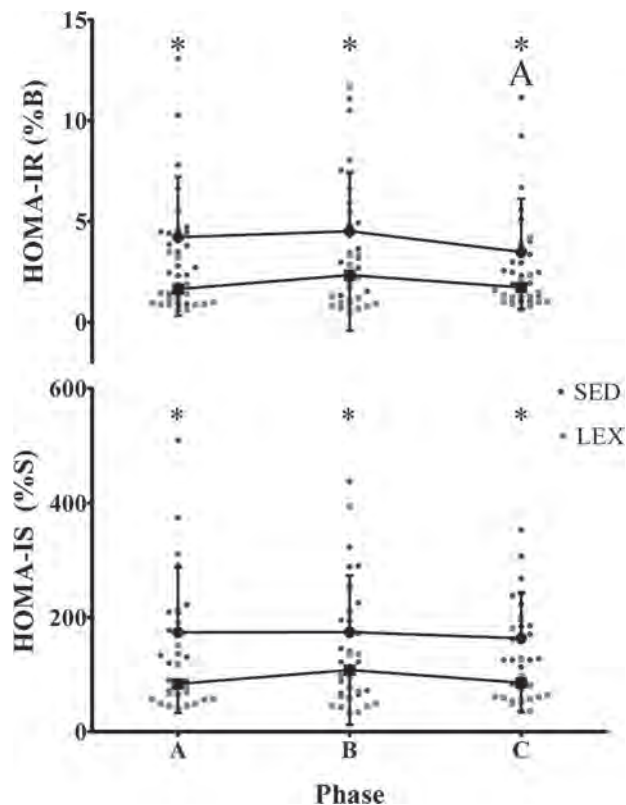


Figure 2: (upper panel) HOMA-IR, and (lower panel) HOMA-IS, in a group of sedentary (SED) and lifelong exercising (LEX) older males. Data are presented as mean  $\pm$  SD, plus individual data points. \*Denotes significant differences between groups at this experimental phase ( $P < 0.05$ ). A = significantly different from phase A in SED ( $P < 0.05$ ).

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA163

#### Cardiovascular effects of capsaicin application on human skin at rest in temperate and warm conditions

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The heat-sensitive TRP vanilloid 1 (TRPV1) channels are stimulated at the heat-pain temperature of 43°C (1) or by capsaicin (2). It has been shown that capsaicin reduces the synthesis of prostaglandins, which stimulate the release of calcitonin gene-related peptide, a potent endogenous vasodilator (3), which, in turn, enhances Na<sup>+</sup> excretion and nitric oxide release in endothelial cells (4). Thus, TRPV1 activation through capsaicin may have important potential for promoting cardiovascular health (5). To date, no study has elucidated whether TRPV1 stimulation has any effect on humans' blood pressure regulation. Therefore, we explored cardiovascular responses at rest in temperate (20°C) and warm (30°C) environment without and with application of capsaicin on the skin of human subjects. Ten healthy males were exposed, while seated, for 30 min to 20°C or 30°C on two occasions: without (NCA) and with (CA) skin application of capsaicin patches (12 x 18 cm), each one impregnated with of 4.0g capsaicin. In CA, one patch was applied on right

pectoralis major muscle, another one on right trapezius and two more patches on the two vastus lateralis muscles. Mean arterial (MAP), systolic (SYS) and diastolic pressure (DIA), cardiac output (CO) and total peripheral resistance (TPR) were continuously measured via a photoplethysmometer (Finometer 2003, FMS, The Netherlands). Data were analyzed by 2-way ANOVA and the level of significance was set at  $p < 0.05$ . Values are presented as mean  $\pm$  SD. MAP was lower ( $p \leq 0.01$ ) when subjects were exposed at  $30^\circ\text{C}$  ( $98.6 \pm 7.7$  mmHg) compared to  $20^\circ\text{C}$  ( $103.1 \pm 5.7$  mmHg). Moreover, it was lower ( $p < 0.01$ ) in CA ( $98.7 \pm 7.9$  mmHg) than in NCA condition ( $103.0 \pm 5.4$  mmHg). Both SYS and DIA were lower ( $p \leq 0.01$ ) in  $30^\circ\text{C}$  ( $131.0 \pm 9.8$  and  $79.0 \pm 7.7$  mmHg, respectively) than in  $20^\circ\text{C}$  ( $140.3 \pm 9.2$  and  $81.5 \pm 4.6$  mmHg, respectively) and in CA ( $133.3 \pm 12.4$  and  $78.1 \pm 6.8$  mmHg, respectively) than in NCA condition ( $138.0 \pm 7.8$  and  $82.4 \pm 5.3$  mmHg, respectively). No differences were detected in SYS and DIA among  $20^\circ\text{C}$  NCA,  $20^\circ\text{C}$  CA and  $30^\circ\text{C}$  NCA. Nonetheless, the mean values of SYS and DIA were lower ( $p < 0.05$ ) on average by 13.9 and 9.2 mmHg, respectively, in  $30^\circ\text{C}$  CA compared with the other three conditions. Regardless of capsaicin application, TPR was similar between  $20^\circ\text{C}$  and  $30^\circ\text{C}$ ; it was lower ( $p = 0.02$ ), however, in CA than in NCA condition, with significant differences being evident only in  $30^\circ\text{C}$ . In addition, no differences were observed in CO among the experimental trials. At  $30^\circ\text{C}$  capsaicin induced arterial hypotension associated with vasodilation as indicated by the lower TPR. The absence of differences in blood pressure in CA at  $20^\circ\text{C}$  indicates a possible role of other heat-sensitive receptors on vascular relaxation. We conclude that the hypotensive effect of capsaicin is temperature-dependent.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## Hypoxia compounds exercise-induced free radical formation in humans; partitioning contributions from the cerebral and femoral circulation

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Historically considered as toxic, mutagenic “accidents” of in-vivo chemistry constrained to cellular oxidative damage and pathophysiology, it has become increasingly clear that at physiological, albeit undefined concentrations, free radicals and associated reactive oxygen species formation during hypoxia and exercise can equally serve as important signal transductants that collectively serve to defend cellular oxygen ( $\text{O}_2$ ) homeostasis (Bailey *et al*, 2017). We designed the first human study to simultaneously measure free radical exchange across the cerebral and femoral circulation to evaluate the dynamic interplay taking place as a function of altered  $\text{O}_2$  demand at rest and during exercise-induced responses to hypoxia. This was considered an ideal model system characterized by physiological extremes of  $\text{O}_2$  flux (low in brain, high in muscle) facilitating experimental manipulation of local  $\text{O}_2$  tension ( $\text{PO}_2$ ) independently of flux ( $\text{O}_2$ ). Healthy participants (5♂, 5♀) were randomly assigned single-blinded to normoxic (21%  $\text{O}_2$ ) and hypoxic (10%  $\text{O}_2$ ) trials with measurements taken at rest and 30 min after cycling at 70% of maximal power output in hypoxia and equivalent relative and absolute intensities in normoxia. Blood was sampled from the brachial artery (a), internal jugular and femoral veins (v) for non-enzymatic antioxidants (HPLC), ascorbate radical ( $\text{A}^\bullet$ , electron paramagnetic resonance spectroscopy), lipid hydroperoxides (LOOH) and low density lipoprotein (LDL) oxidation (spectrophotometry). Cerebral and femoral venous blood flow was evaluated by transcranial Doppler ultrasound (CBF) and constant infusion thermodilution (FBF). With 3 participants lost to follow up (final  $n = 4♂, 3♀$ ), hypoxia increased CBF and FBF ( $P = 0.041$  vs. normoxia) with further elevations in FBF during exercise ( $P = 0.002$  vs. rest). Cerebral and femoral ascorbate and  $\alpha$ -tocopherol consumption ( $v < a$ ) was accompanied by  $\text{A}^\bullet$ /LOOH formation ( $v > a$ ) and increased LDL oxidation during hypoxia ( $P < 0.043$  to  $0.049$  vs. normoxia) implying free radical-mediated lipid peroxidation subsequent to inadequate antioxidant defense. This was pronounced during exercise across the femoral circulation in proportion to the increase in local  $\text{O}_2$  uptake ( $r = -0.397$  to  $-0.459$ ,  $P = 0.037$  to  $0.045$ ) but unrelated to any reduction in  $\text{PO}_2$ . These findings highlight considerable regional heterogeneity in the oxidative stress response to hypoxia that may be more attributable to local differences in  $\text{O}_2$  flux than to  $\text{O}_2$  tension. Since arterial hypoxaemia is a hallmark feature of circulatory disease, mapping the dynamic transvascular interplay of free radicals as a function of changing  $\text{O}_2$  demand may help define sensitive biomarkers of vascular health and monitor the success of interventions designed to optimise tissue oxygenation in the critically ill.

Bailey, DM *et al*. (2017). *Circulation* 135:166-176



Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA165

### Hyperoxia increases critical power but does not speed pulmonary oxygen uptake kinetics during upright cycling

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We have previously demonstrated that critical power (CP) is mechanistically related to the phase II time constant ( $\tau_{VO_2}$ ) of oxygen uptake ( $VO_2$ ) kinetics (1). CP is enhanced in hyperoxia compared to normoxia (2), however during upright cycle exercise, pulmonary  $VO_2$  kinetics are not normally speeded by hyperoxia (3), which suggests that the improved  $O_2$  delivery afforded by hyperoxia should increase CP independently of any changes in  $\tau_{VO_2}$ . Hence, whether the enhanced CP in hyperoxia is related to faster  $VO_2$  kinetics or improved microvascular  $O_2$  delivery *per se* is presently unclear. We therefore examined the effect of hyperoxia on CP and  $\tau_{VO_2}$  during upright cycle exercise in healthy, recreationally active males ( $n = 9$ ). CP was determined in normoxia (N) and hyperoxia (H; fraction of inspired  $O_2 = 0.5$ ) via 4 severe-intensity constant load exercise tests to exhaustion on a cycle ergometer, repeated once in each condition. Additionally, 6-minute bouts of exercise were repeated 4 times in each condition at 70% of the gas exchange threshold, in order to characterise  $\tau_{VO_2}$  and the time constant of muscle deoxyhaemoglobin kinetics ( $\tau_{[HHb]}$ ), alongside absolute concentrations of muscle oxy- and total-haemoglobin ( $[HbO_2]$ ,  $[THb]$ ) in each condition, the latter measurements via near infrared spectroscopy. Values are means  $\pm$  SD, compared by paired samples *t*-test (CP,  $\tau_{VO_2}$ ) and repeated measures ANOVA ( $\tau_{[HHb]}$ ,  $[HbO_2]$ ,  $[THb]$ ). CP was greater in H compared to N (H:  $216 \pm 30$  vs. N:  $197 \pm 29$  W;  $P < 0.001$ ), however  $\tau_{VO_2}$  was unchanged between conditions (H:  $25 \pm 6$  s vs. N:  $24 \pm 9$  s;  $P = 0.49$ ).  $\tau_{[HHb]}$  (H:  $11 \pm 6$  s vs. N:  $8 \pm 6$  s;  $P = 0.93$ ) and  $[THb]$  (H:  $99 \pm 18$  vs. N:  $99 \pm 21$   $\mu$ M;  $P = 0.98$ ) were unchanged, whereas  $[HbO_2]$  during exercise was greater in H versus N (H:  $74 \pm 21$  vs. N:  $68 \pm 18$   $\mu$ M;  $P < 0.001$ ). In conclusion, this study provides novel insights into the physiological determinants of critical power and by extension, exercise tolerance. Microvascular oxygenation (assessed via NIRS) and CP were improved during exercise in hyperoxia compared with normoxia. Importantly, the improved microvascular oxygenation afforded by hyperoxia did not alter  $\tau_{VO_2}$ , suggesting that microvascular  $O_2$  delivery is an independent determinant of the upper limit for steady-state exercise, i.e. critical power.

Goulding RP, Roche DM, Marwood S. Prior exercise speeds pulmonary oxygen uptake kinetics and increases critical power during supine but not upright cycling. *Exp Physiol*. 2017 Sep 1;102(9):1158–76.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA166

### Urinary orosomucoid (alpha-1 acid glycoprotein) latex immunoassay is effective for the detection of increased proteinuria associated with altitude and sea-level exercise.

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**Background:** Proteinuria is an established physiologic consequence occurring with exposure to altitude and with exercise. We have recently demonstrated that urinary alpha-1 acid glycoprotein ( $\alpha$ 1-AGP) correlates better with ascent to altitude compared with albuminuria; however, little research exists regarding the pharmacologic treatment of this pathophysiology at altitude. **Objective:** To examine the effects of losartan (angiotensin II receptor blocker) on  $\alpha$ 1-AGP excretion at altitude and with altitude exercise. **Methods:** Twenty participants took part in this study and were pair-matched for sex, age, and ACE genotype with losartan (100 mg/day) randomly assigned to one participant in each pair. Incremental exercise tests to exhaustion were conducted on a supine cycle ergometer at sea-level (SL) and at altitude (5035 m) in all participants. Urine samples were collected pre-exercise, post-60 min, post-120 min, and post-180 min. Urine samples were analyzed for  $\alpha$ 1-AGP using a novel latex immunoassay with a range of 0.077–4.936 mg/L on an Optilite nephelometer. Detectable  $\alpha$ 1-AGP concentrations were converted to micrograms/minute ( $\mu$ g/min) to account for sample volumes and time elapsed. Independent *t*-tests and paired *t*-tests were used to determine differences between groups and within groups, respectively. **Results:** The immunoassay detected  $\alpha$ 1-AGP in 97% of the neat samples with only 10% of samples requiring dilution.  $\alpha$ 1-AGP increased with exercise at SL in both groups (from  $3.5 \pm 3.9$  to  $12.3 \pm 9.1$   $\mu$ g/min in placebo group,  $p=0.04$ ; from  $1.6 \pm 1.6$  to  $17.9 \pm 14.2$   $\mu$ g/min in losartan group,  $p=0.01$ ), with no significant difference between groups. Pre-exercise  $\alpha$ 1-AGP increased at altitude in both groups (placebo,  $\Delta 1.7 \pm 4.0$   $\mu$ g/min; losartan,  $\Delta 0.5 \pm 1.0$   $\mu$ g/min), but was not significantly different between groups. Peak exercise power was reduced by  $\sim 40\%$  at altitude in both groups ( $p=0.89$ ).  $\alpha$ 1-AGP excretion increased with altitude exercise in both groups (placebo,  $\Delta 9.33 \pm 9.67$   $\mu$ g/min,  $p=0.01$ ; losartan,  $\Delta 6.19 \pm 6.04$   $\mu$ g/min,  $p=0.01$ ). Exercise associated increases in  $\alpha$ 1-AGP at altitude trended towards being lower ( $p=0.098$ ) than SL exercise in the losartan group only (58% mean reduction). Odds ratio for exhibiting a reduction in  $\alpha$ 1-AGP with altitude exercise (compared to SL exercise) was 2.25 greater with losartan. **Discussion:** The latex immunoassay is an effective method for detecting changes in  $\alpha$ 1-AGP associated with exercise both at SL and at altitude. Losartan (non-significantly) reduces exercise associated increases in  $\alpha$ 1-AGP at altitude, possibly due to blocking the AT1 receptor and creating the potential for reductions in heparin sulphate proteoglycans by podocytes, which results in retention of negative charges at the glomerular basement membrane.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA167

**Exercise proteinuria at altitude is reduced by acetazolamide.**

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**Background:** Proteinuria is exhibited with both sea-level exercise and ascent to altitude. Acetazolamide (Az) is a carbonic anhydrase inhibitor that is typically prescribed prior to altitude exposure and has been shown to reduce albuminuria upon ascent. The effects of Az on proteinuria when administered at altitude are unknown. Recently, urinary  $\alpha$ 1-acid glycoprotein ( $\alpha$ 1-AGP) has proven to correlate better with ascent than albumin. The combination of exercise and altitude should be a potent stimulus to proteinuria that might be better assessed with urinary  $\alpha$ 1-AGP concentrations. **Objective:** To examine the effects of Az on exercise induced  $\alpha$ 1-AGP excretion at altitude. **Methods:** Ten subjects participated in this study. An incremental exercise test to exhaustion was conducted at sea-level (SL) and at two separate time points at altitude (both at 5035 m, pre-Az and Az) on a supine cycle ergometer. Az, 125 mg twice daily, was administered immediately after the 1<sup>st</sup> altitude exercise test for four doses before the 2<sup>nd</sup> altitude exercise test. Urine samples were collected pre-exercise, post-60 min, post-120 min, and post-180 min. Urine samples were analysed for  $\alpha$ 1-AGP using a novel latex immunoassay with a sensitivity range of 0.077 – 4.936 mg/L on an Optilite nephelometer and albumin using radial immunodiffusion. Rates of excretion are reported in order to account for urine sample volumes and time elapsed. Paired samples t-tests were used to evaluate differences in  $\alpha$ 1-AGP between altitude exercise tests (with a significance threshold of  $p \leq 0.05$ ). **Results:** The immunoassay successfully detected  $\alpha$ 1-AGP in 99% of neat samples with only 16% requiring dilution. Mean  $\alpha$ 1-AGP at post-60 min was significantly different between pre-Az and Az altitude exercise tests ( $14.8 \pm 10.2$  vs.  $10.7 \pm 8.6$   $\mu$ g/min;  $p=0.05$ ). A significant difference ( $p=0.029$ ) was also observed between the exercise associated  $\alpha$ 1-AGP increase (pre-ex to post-60 min) between pre-Az ( $9.3 \pm 9.7$   $\mu$ g/min) and Az ( $3.6 \pm 6.0$   $\mu$ g/min) exercise tests (3-fold reduction), with no difference in peak power between tests. Mean albuminuria at post-60 min was reduced with Az (by 15 %), albeit not a significant difference from pre-Az ( $p>0.05$ ). Peripheral oxygen saturation at rest ( $75.1 \pm 5.8\%$  vs.  $84.0 \pm 4.4\%$ ;  $p<0.001$ ) and at exhaustion ( $69.1 \pm 4.5\%$  vs.  $75.8 \pm 3.8\%$ ;  $p=0.001$ ) were elevated with Az. **Discussion:** The latex immunoassay is an effective method for detecting changes in urinary  $\alpha$ 1-AGP, and is a more sensitive marker than albumin for the detection of changes in proteinuria during altitude exercise. Az significantly reduces  $\alpha$ 1-AGP with altitude exercise; however, further research is warranted to elucidate the mechanisms that are responsible. **Conclusion:** Increases in  $\alpha$ 1-AGP associated with altitude exercise are reduced by Az and can be successfully detected using a novel latex immunoassay.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA168

**Effect of period during sinusoidal leg cycling exercise on the blood flow dynamics of the brachial artery in humans**

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**BACKGROUND and PURPOSE:** Recent human studies have indicated the increased blood flow (BF) to inactive muscles during exercise (e.g., Padilla et al., 2011). This seems to be the key factor for endothelial adaptation of the inactive limb (the upper limb) beyond the active muscles (the lower exercising limb) (e.g., Green et al., 2017). However, the dynamic property of BF to the inactive arm (through the brachial artery; BA-BF) with respect to the non-steady state change in the work rate (WR) during leg exercise remains unclear. We first quantified the dynamics of BA-BF during leg cycling exercise using a sinusoidal WR forcing with a 4-min period and found that the sinusoidally changing BA-BF response showed an anti-phasic delay ( $\sim 180^\circ$ ), unlike other cardiorespiratory variables (Fukuba et al., 2017). However, since this anti-phasic response was observed in the BA-BF in the 4-min period, it should be clarified as to whether sinusoidal WR forcing with other periods would induce the similar phasic response. We, therefore, measured the dynamics of BA-BF in response to sinusoidal WR leg cycling exercises of 6-, 4-, and 2-min periods. **METHODS:** Seven healthy, young, male subjects (19–22 years) performed upright leg ergometer exercise with a constant WR (mean of the sinusoidal WR) for 30 min followed by three different periods of sinusoidal WR exercises (number of the repetitions); 6-min (3), 4-min (4) and 2-min (7), where the WR fluctuated in the range of 20 W to peak WR, corresponding to the 60% of  $\dot{V}O_{2\max}$ . During protocols, we continuously measured the pulmonary gas exchange, heart rate (HR), mean arterial blood pressure, stroke volume, blood velocity and cross sectional area of the BA, and forearm skin BF (SBF) and sweating rate (SR). The measured and calculated variables:  $y(t)$  were fitted as  $y(t) = M + A \cdot \sin((2\pi/T) \cdot t - \theta)$ , where  $t$ : time,  $M$ : mean level,  $A/M$ : relative amplitude,  $T$ : period (of sinusoidal WR),  $\theta$ : phase. **RESULTS:** All variables traced as the sinusoid form adequately. The phases and  $A/M$ s of the variables for gas exchange and central circulation, such as  $\dot{V}O_2$  and HR, to WR forcing were similar (e.g., HR, phase [ $^\circ$ ]: 6-min,  $37 \pm 8$ , 4-min,  $45 \pm 10$ , 2-min,  $60 \pm 7$ ;  $A/M$  (%): 6-min,  $12.3 \pm 2.8$ ; 4-min,  $10.3 \pm 2.4$ ; 2-min,  $8.0 \pm 1.1$ , mean  $\pm$  SD). Similar to previous studies with sinusoidal WR forcing (e.g., Casaburi et al., 1977), a longer period showed a shorter phase and larger amplitude of responses. In contrast, the response of BA-BF showed an approximately anti-phase ( $165$ – $195^\circ$ ) and a relatively constant  $A/M$  ( $\sim 28\%$ ) regardless of the period, while the forearm SBF and SR were similar to those with the gas exchange and central circulation. **CONCLUSION:** The BF in the non-active limb is out of phase relative to the active muscle, reflecting a redistribution of BF and oxygen transport.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA169

### Effects of long-term dark chocolate intake on cognitive function in healthy young subjects

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In our prior research in mice, orally-administered theobromine for about 1-month enhanced motor learning that was assessed using the three-lever task, suggesting improvement of cognitive function. However, it is not known how orally-ingested theobromine affects cerebral function in humans. There are various types of theobromine-enriched foods and beverages. Among them, chocolate is a remarkable commercially-available food. This study therefore investigated whether long-term intake of dark chocolate improves cognitive function in humans. Eighteen healthy young men (n=13) and women (n=5) (22 ± 3 (SD) years-old) were randomly divided into two groups; dark chocolate intake group (DC; n=10) and theobromine-free white chocolate intake group (WC; n=8, control). Subjects in each group took in dark or white chocolate every day for 30 ± 4 (SD) days. These chocolates were kindly provided by Dr. H. Inagaki, Morinaga & Co., Ltd. Before and after chocolate intake, cognitive function and prefrontal cortex function of all subjects were assessed using a modified Stroop color word test (modified SCWT) and Digit cancellation test (D-CAT). Blood samples were also taken for routine biochemical analysis. For modified SCWT, the subject's task is to look at a paper with color words written in a variety of random colors. The subject must read words (Words test) or name the ink colors (Colors test) as quickly and accurately as possible for 1 minute. The number of correct answers and errors were counted in both Words and Colors tests. The D-CAT was carried out with a standard method after finishing the modified SCWT. In addition, we continuously measured prefrontal cerebral blood flow (FCBF) with Near infrared spectroscopy (NIRS) and middle cerebral artery blood flow (MCABF) with an ultrasonic Doppler probe before and during the tests. In both groups, the same tests were repeated 3 weeks after the end of chocolate intake (Post). Body weight and body fat did not differ between the DC and WC groups

through the intervention period (all, P>0.32). In the DC group, the number of correct answers in the Words test was significantly increased by chocolate intake (P=0.017). Furthermore, the high performance appeared to be persistent in the Post. Similar results were shown in the Colors test. White chocolate intake had no significant effects on both the Words tests and Colors tests (all, P>0.09). The FCBF and MCACBF during modified SCWT did not differ between the DC and WC groups (all, P>0.50). In D-CAT, similar improvement of performance was observed only in the DC group. There were no adverse changes in liver function and electrolyte values in both DC and WC groups. These results suggest that long-term dark chocolate intake may improve cognitive function in young subjects. Interestingly, this effect seemed to last for at least 3 weeks after the end chocolate intake. (COI: No)

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PCA170

### Cytoplasmic diffusivities of CO<sub>2</sub> gas and H<sup>+</sup> ions are reduced in diseases of human red cells that raise haemoglobin concentration

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**Background:** Physiologically-adapted red blood cells (RBCs) must have adequate capacity to carry gases around the body and facilitate rapid gas exchange during their brief capillary transit time. A higher concentration of haemoglobin (Hb) will increase RBC gas carriage, but also produces a more tortuous environment for smaller molecules to diffuse in, which could restrict gas exchange. Our recent findings on wild-type RBCs from various animal species have demonstrated that higher Hb concentrations reduce the diffusivity of CO<sub>2</sub> gas and H<sup>+</sup> ions (1). The extent of this diffusive restriction can explain why RBCs with a higher mean corpuscular Hb concentration (MCHC) must also be thinner in order for gases to exchange rapidly and for cytoplasm to respond promptly to ambient changes in plasma pH (e.g. to execute the Bohr effect). Diseases of human RBCs are often linked to changes in cell shape and MCHC, which could have a hitherto uncharted effect on gas and H<sup>+</sup> ion diffusion.

**Methods:** Blood samples were obtained from hereditary spherocytosis (HS; n=3) and pyruvate kinase deficiency (PKD; n=1) patients (ethical approval 13-WA-0371), manifesting a raised (360, 358, 343 g/L) and reduced (299 g/L) MCHC, respectively, relative to control subjects (n=2; MCHC 330 g/L). RBCs were washed in EDTA buffer and settled in a superfusion chamber mounted on a confocal microscope. Diffusive properties in cytoplasm were interrogated by measuring the diffusive spread of H<sup>+</sup> ions (reported with the fluorescent dye cSNARF1) in response to photolytic H<sup>+</sup> uncaging (from 6-nitrobenzaldehyde; 1mM) applied to one end of the cell. The apparent H<sup>+</sup> diffusion coefficient (D<sub>H</sub><sup>app</sup>) was measured in Hepes-buffered (CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>-free) superfusates to probe Hb-facilitated H<sup>+</sup> diffusion, and in 5% CO<sub>2</sub>/55 mM HCO<sub>3</sub><sup>-</sup>-buffered superfusates to estimate the additional facilitation by cytoplasmic CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>.

**Results:** Hb-facilitated D<sub>H</sub><sup>app</sup> was slower in HS patients (4.4±0.4; 5.3±0.7; 4.2±0.4 μm<sup>2</sup>/s) and faster in the PKD patient



( $9.6 \pm 0.8 \mu\text{m}^2/\text{s}$ ), compared to wild-type RBCs. The addition of  $\text{CO}_2/\text{HCO}_3^-$  buffer to cytoplasm produced a smaller acceleration of  $\text{D}_\text{H}^{\text{app}}$  in HS cells ( $5.3 \pm 0.6$ ;  $5.9 \pm 0.5$ ;  $6.7 \pm 0.8 \mu\text{m}^2/\text{s}$ ) compared to PKD cells ( $12.8 \pm 1.3 \mu\text{m}^2/\text{s}$ ). Thus, a higher MCHC restricts Hb-assisted  $\text{H}^+$  diffusivity and impairs the ability of cytoplasmic  $\text{CO}_2/\text{HCO}_3^-$  to shuttle  $\text{H}^+$  ions. The latter effect is attributable to reduced  $\text{CO}_2$  (and  $\text{HCO}_3^-$ ) diffusivity in cytoplasm owing to Hb-dependent tortuosity. In combination with the increased diffusion distance arising from spherical geometry, RBCs from HS patients will have substantially slowed gas exchange which could contribute to reduced exercise tolerance. Physiological fitness of RBCs is thus a compromise between high gas-carrying capacity (favoured by high MCHC) and rapid cytoplasmic diffusivity (favoured by low MCHC). Richardson & Swietach (2016) *Sci Rep* 6:36018

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA171

### Effects of temperature on repeated local skin heating induced desensitization in humans

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Thermoregulatory and cutaneous thermosensory reflexes are vital for survival (1). Rapid skin heating induces a biphasic microvascular hyperaemic response: an initial axon-mediated vasodilation, followed by a sustained increase and plateau of skin blood flow (2). Skin heating responses maybe subject to desensitization phenomenon, whereby a previously heated site has reduced microvascular function on subsequent thermal provocation (3), but findings are mixed (4). Desensitization has important implications for persons undergoing repeated thermal stress, such as firefighters or athletes in heat-based competitions, as well as for methodological considerations. A key element of desensitization maybe the magnitude of heating employed. The aim of this study was to investigate the effect of temperature on skin microvessel responses to repeated local heating.

12 participants (1 female) had forearm skin microvessel function assessed by laser Doppler flowmetry (LDF) responses to 3 site-specific levels of local heating: 36°C, 39°C, and 42°C (PRE). Participants were exposed to 2 more bouts of local heating 30 and 90 minutes later (POST30, POST90). Skin blood flux was indexed as cutaneous vascular conductance ( $\text{CVC} = \text{LDF flux} / \text{mean arterial pressure, PU.mmHg}^{-1}$ ), normalised to maximal vasodilation (44°C heating at end of each stage of testing, %CVCmax). Data were compared by ANOVA per timepoint (baseline/axon peak/axon nadir/plateau/maximum) with Bonferroni correction.

Local skin heating responses were graded according to target temperature at each stage of testing (Table 1). There was an increased axon-peak response POST30 at 42°C (vs PRE,  $P=0.005$ ), and a decreased axon-nadir at 42°C (vs PRE,  $P=0.005$ ). There were no changes to axon reflex responses at 36°C or 39°C POST30 ( $P>0.05$  both). There was a reduced plateau response POST30 at all 3 sites (vs PRE: 42°C,  $P<0.001$ ; 39°C,  $P=0.003$ ; 36°C,  $P<0.001$ ). POST90 plateau responses were also attenuated, but only significantly at 42°C (vs PRE,  $P=0.002$ ).

Repeated local skin heating causes an altered axon reflex response at temperatures  $>40^\circ\text{C}$ , and reduces endothelium-dependent 'plateau' vasodilation at temperatures as low as 36°C, indicating cutaneous heat dissipation maybe impaired during subsequent exposure to elevated skin temperature.

| SITE | TIMEPOINT OF HEATING PROTOCOL |         |          |           |          |         |            |          |         |         |          |          |
|------|-------------------------------|---------|----------|-----------|----------|---------|------------|----------|---------|---------|----------|----------|
|      | BASELINE                      |         |          | AXON PEAK |          |         | AXON NADIR |          |         | PLATEAU |          |          |
|      | PRE                           | POST30  | POST90   | PRE       | POST30   | POST90  | PRE        | POST30   | POST90  | PRE     | POST30   | POST90   |
| 42°C | 7 ± 2                         | 11 ± 4* | 97 ± 10  | 62 ± 15   | 72 ± 15* | 33 ± 13 | 50 ± 14    | 38 ± 12* | 78 ± 14 | 87 ± 9  | 63 ± 14* | 71 ± 11* |
| 39°C | 9 ± 5                         | 16 ± 5* | 102 ± 14 | 43 ± 13   | 45 ± 14  | 32 ± 16 | 22 ± 9     | 23 ± 10  | 56 ± 15 | 47 ± 17 | 28 ± 16* | 37 ± 14  |
| 36°C | 7 ± 3                         | 11 ± 7  | 109 ± 16 | 13 ± 4    | 15 ± 6   | 16 ± 24 | 11 ± 4     | 11 ± 4   | 33 ± 8  | 22 ± 7  | 13 ± 6*  | 16 ± 8   |

Table 1: Skin microvascular responses to repeated local heating, expressed as %CVCmax ( $\pm$ SD). #, POST90 baseline and axon reflex responses not included in statistical analyses due to spuriously elevated blood flow from POST30. \*,  $P<0.05$  vs PRE. Abbreviations: PRE, initial local heating protocol; POST30, first bout of repeated heating, 30 mins after PRE; POST90, second bout of repeated heating, 90 mins after PRE.

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## PCA172

### Mechanism of the Hypotensive Effect of Hibiscus sabdariffa Tea: A Double-Blind Study

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*Hibiscus sabdariffa* tea (HST) has been reported to lower blood pressure (BP) in pre-hypertensive and mildly hypertensive subjects<sup>1</sup> due to its anthocyanin, polyphenol and hibiscus acid contents.<sup>2</sup> Findings from an open study suggest that *Hibiscus sabdariffa* extract lowers BP by dampening the sympathetic nervous system (SNS).<sup>3</sup> Since open studies may be subjective, the present study was designed to examine this problem using a randomized double-blind placebo-controlled approach. Following ethical approval and informed consent, the cold pressor test (CPT) was performed in healthy human subjects before and after the oral administration of 200mg/kg HST ( $n=20$ ) or a food colourant (FC;  $n=20$ ) which served as placebo. The basal BP and pulse rate (PR) were determined, then each subject immersed one hand into iced water (4°C) just above the wrist for 1-2 minutes and the BP and PR responses were measured. Experiments were performed in accordance with the Principles of the Declaration of Helsinki. Mean arterial pressure (MAP) was taken as representative BP. Results are expressed as Mean $\pm$ SEM. Paired T test was used to analyze the data within the HST and FC groups while unpaired T test was

used to analyze the difference between the HST and FC groups. When three or more groups were compared, ANOVA with repeated measures was used.  $P < 0.05$  was considered significant. The peak MAP and PR obtained during the CPT in the absence of FC ( $94.2 \pm 1.7$  mmHg and  $77.6 \pm 1.2$  beats/min) were significantly higher than the basal values ( $83.3 \pm 1.8$  mmHg and  $69.2 \pm 1.4$  respectively;  $P < 0.05$ ). In the presence of FC these parameters rose significantly ( $100 \pm 1.8$  mmHg and  $82.9 \pm 1.2$  beats/min;  $P < 0.05$ ) compared to its absence. The peak MAP and PR obtained during the CPT without HST ( $105.1 \pm 2.8$  mmHg and  $82.3 \pm 2.0$  beats/min) were significantly higher than the corresponding basal values ( $90.8 \pm 3.0$  mmHg and  $72.8 \pm 2.0$  beats/min;  $P < 0.05$ ). These parameters fell significantly when the subjects received HST ( $97.3 \pm 2.6$  mmHg and  $77.1 \pm 2.0$  beats/min;  $P < 0.05$ ) compared to its absence. In the presence of HST, CPT-induced changes ( $\Delta\text{MAP} = 7.0 \pm 1.3$  mmHg;  $\Delta\text{PR} = 4.3 \pm 0.6$  beats/min) were significantly reduced compared to its absence ( $\Delta\text{MAP} = 14.4 \pm 1.6$  mmHg,  $\Delta\text{PR} = 9.5 \pm 1.1$  beats/min;  $P < 0.0001$  respectively). However, in the presence of FC, the changes ( $\Delta\text{MAP} = 16.9 \pm 1.7$  mmHg,  $\Delta\text{PR} = 14.0 \pm 1.4$  beats/min) were significantly higher than in its absence ( $\Delta\text{MAP} = 10.4 \pm 1.4$  mmHg,  $\Delta\text{PR} = 8.4 \pm 1.1$  beats/min;  $P < 0.0001$  respectively). These results suggest that CPT-induced activation of the SNS was attenuated by HST but aggravated by FC. It is concluded that the hypotensive effect of HST may occur through the reduction of SNS activation while FC may further activate it. Hence FC may neither be an inert placebo nor be innocuous.

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#### PCA173

### Mobile Physiologic Laboratory (mobPhysioLab™) Infield Monitoring of Humans Maintaining Employability, Human Performance, and Welfare

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Introduction: “Mobile health” or “mobile telemedicine” (eHealth) is not to be imagined as not existing in the today’s world anymore. In many respects, the most modern and latest information and communication systems are also used being applied in the area of the health system. In some areas, it does not go without telemedical transference at all, namely in Space. Here, the NASA has developed medical telemetry programs since the nineteen sixties and has brought them to an accordingly high level but must again leave in the absence of financial support. In medical centres, such systems are used for a long time to the monitoring by patients’, for example, on

intensive care units or by the handing over of patients from the surgery to the peripheral stations.

Methods: Within the German armed forces, the temporal and qualitative optimization of the rescue chain has been handed over by the introduction of the SAFES only recently. This computer and software supporting system allows the patients’ transport on-line to monitors and the best for the soldier of the place of the injury up to the final care place. With American soldiers so-called “soldier of the future” with the possibility of the on-line monitoring of his performance data is for a long time by the USARIEM during the application in the development. However, the aim should not be grasped it to make only the vital parameters and these for him useful, but to raise status in the other future cognitive, metabolic, and “combat ready” status.

Discussion: Remote Health monitoring in hazardous environments has the potential to improve operational safety. It can identify exposures that influence short and long-term health conditions and thus save lives of professionals such as, e.g., firefighters or military personnel. Until now it is up to impossible for medical advisors to analyze and identify the causes of health complaints from these individuals. The mobile physiologic laboratory (mobPhysioLab™) with its core technology the HealthLab system consists of a smart textile sensor garment that monitors the health status of professionals. In combination with the Human-Centric Cyber-Physical System (HCCPS), it provides user interfaces for commanders, medical advisors, and participants.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCA174

### Endothelial function in healthy postmenopausal women: effects of chronic and acute exercise

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Aerobic exercise training is associated with decreased cardiovascular disease risk, including attenuation of the age-associated decline in endothelial function. However, growing evidence suggests that this vascular benefit may be obviated in post-menopausal women (PMW). To examine this further, we compared endothelial function in habitually exercise trained and untrained PMW, both before and after an acute bout of exercise.

Two groups of healthy, age-matched ( $56.5 \pm 1$  years) PMW were studied: habitually exercise trained PMW ( $\geq 2$  hours moderate-to-vigorous intensity exercise/week for  $\geq 5$  years; TrPMW;  $n=6$ ) and untrained PMW (no regular exercise for  $\geq 5$  years; UntrPMW;  $n=7$ ). Resting and peak calf blood

flow (CBF), an index of endothelial function, were assessed using strain-gauge plethysmography. Vascular resistance (VR; mean arterial pressure/blood flow) was calculated. Serum measures of 17 beta-estradiol, sex-hormone binding globulin (SHBG) and testosterone were determined. Vascular measures were assessed before and 60 minutes after an acute bout of dynamic exercise (45 minutes walking at 60%  $\text{VO}_{2\text{peak}}$ ).

**Baseline:** Groups did not differ ( $p>0.05$ ; one-way ANOVA) in height ( $163.4\pm1.5$  cm; overall mean $\pm$ SEM), weight ( $61.7\pm2.3$  kg), heart rate ( $60\pm2$  beats/min), systolic blood pressure (BP;  $118.6\pm3.1$  mmHg), and serum measures of testosterone ( $0.9\pm0.1$  nmol/L), SHBG ( $55.5\pm5.8$  nmol/L), and 17 beta-estradiol ( $72.8\pm1.3$  pmol/L). Resting and peak CBF and VR were also similar ( $p>0.05$ ) between groups (Table 1). In contrast,  $\text{VO}_{2\text{peak}}$  was higher ( $36.8\pm3.4$  vs.  $28.3\pm1.4$  ml/kg/min;  $p<0.05$ ), and BMI ( $21.5\pm1.2$  vs.  $24.4\pm1.0$  kg/m<sup>2</sup>) and resting diastolic BP ( $73.6\pm1.0$  vs.  $78.8\pm1.8$  mmHg) lower ( $p<0.05$ ) in the TrPMW versus UntrPMW. **Post-exercise:** Using repeated measures ANOVA (group x condition), within-group measures of CBF (Table 1), heart rate, and systolic and diastolic BP were increased ( $p<0.05$ ) and VR decreased ( $p<0.05$ ) in both groups. In contrast, no between-group differences ( $p>0.05$ ) were detected.

Our preliminary findings demonstrate that resting and peak peripheral blood flow and VR do not differ between habitually trained and untrained PMW. A single bout of dynamic exercise similarly increased blood flow and decreased VR in both groups. These observations suggest that while a single bout of dynamic exercise acutely improves endothelial function, chronic aerobic exercise training does not attenuate the age-associated decline in endothelial function in healthy PMW.

Table 1. Resting and peak peripheral blood flow values pre- and post-exercise.

|                            | TrPMW (n=6)       |                   | UntrPMW (n=7)     |                   |
|----------------------------|-------------------|-------------------|-------------------|-------------------|
|                            | PreEx             | PostEx            | PreEx             | PostEx            |
| Resting CBF (mL/100mL/min) | 1.51 $\pm$ .26    | 2.26 $\pm$ .45*   | 1.57 $\pm$ .24    | 2.84 $\pm$ .42*   |
| Peak CBF (mL/100mL/min)    | 32.53 $\pm$ 2.93  | 35.84 $\pm$ 3.11* | 27.45 $\pm$ 2.67  | 31.46 $\pm$ 2.79* |
| Resting VR (U)             | 62.52 $\pm$ 10.98 | 43.52 $\pm$ 8.29* | 71.16 $\pm$ 10.17 | 37.73 $\pm$ 7.67* |
| Peak VR (U)                | 2.77 $\pm$ .29    | 2.50 $\pm$ .26*   | 3.57 $\pm$ .27    | 2.85 $\pm$ .24*   |

All values are mean $\pm$ SEM, compared by two-way repeated measures ANOVA (Group x Condition)

\* Within-groups, significant effect of exercise,  $p<0.05$

CBF, calf blood flow; VR, vascular resistance

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA175

## Modulation of Oxygen Sensing in Breast Cancer Cells by Myoglobin

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Myoglobin is well known as an oxygen-binding protein in cardiac and skeletal myocytes. It is believed to function as a transport and temporary storage protein for  $\text{O}_2$ . However, myoglobin has recently also been identified as a hallmark in luminal breast cancer and was shown to be robustly induced

under hypoxia. In general, cellular responses to hypoxia are regulated by the transcription factor hypoxia-inducible factor (HIF). Here, we hypothesize that myoglobin in breast cancer cells has a function under normoxic (21%  $\text{O}_2$ ) and hypoxic (1%  $\text{O}_2$ ) conditions with respect to HIF activation.

For exploring the function of myoglobin in breast cancer, we employed the human cell line MDA-MB-468, grown in a 3D multicellular spheroid model that mimics the in vivo avascular tumor architecture and physiology. Myoglobin expression was knocked down by siRNA (kindly provided by Dr. Thomas Gorr, University of Zurich) and the ability of adaptation to hypoxia was investigated. Myoglobin knockdown was successful both in monolayer cultures and in the tumor spheroids with efficiencies over 90%.

Due to their three dimensional architecture, spheroids have a heterogeneous cell population of proliferating cells in the rim, hypoxic cells in the middle region and non-cycling or necrotic cells in the core region. In the core region hypoxia to anoxia results in cell death, called central necrosis. This central necrosis was increased after myoglobin knockdown. Another potential function of myoglobin is scavenging of reactive oxygen species (ROS) that can initiate cell death. We found that myoglobin knockdown lead to higher ROS levels in the cells after treatment with  $\text{H}_2\text{O}_2$ . In addition, we showed that hypoxia inducible factors (HIFs) accumulated under conditions of hypoxia, indicating that MDA-MB-468 cells can adapt to hypoxic conditions. Moreover, myoglobin knockdown causes higher levels of HIF-1 $\alpha$  after treatment with NO.

Our data indicate that myoglobin plays an important role in HIF activation and in survival of breast cancer cells due to its ROS and NO scavenging properties.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA176

## The influence of matched increases in cerebral artery shear stress induced via hypercapnia and exercise on neurovascular coupling

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Increases in arterial carbon dioxide tension (i.e., hypercapnia) and exercise both lead to increases in cerebral perfusion, and consequently, increase shear rate on the wall of cerebral arteries (Smith et al. 2017). The nature of how these blood flow patterns may influence the matching of cerebral blood flow to cerebral metabolism (termed neurovascular coupling, NVC) has not been explored. Exercise leads to improved vascular health, likely due to these shear stress responses [reviewed in: (Green & Smith 2017)]; therefore, we hypothesized that matched increases in cerebral artery shear stress induced via hypercapnia and exercise would both improve NVC. Ten healthy male individuals (25 $\pm$ 6 yrs) participated in three 30-min experimental interventions, each separated by >48 hrs: 1) hypercapnia ( $\text{CO}_2$ ; 4.5%  $\text{CO}_2$ , 21%  $\text{O}_2$ , and balanced  $\text{N}_2$ ); 2) submaximal cycling (EX; 60% of heart-rate reserve); and 3) resting (CTR; time-matched control). Transcranial Doppler



ultrasound was used to assess mean posterior cerebral artery blood velocity (PCAv). Overall, the NVC response appeared to favour improved NVC following CO<sub>2</sub> but not EX or CTR (relative peak response of PCAv pre- vs. post-; CO<sub>2</sub>: 20.2±6.5% vs. 22.6±9.3%; EX: 21.6±10.4% vs. 18.7±12.6%; CTR: 19.1±9.4% vs. 17.6±7.8%; P=0.52); however, there were no significant differences between these NVC responses irrespective of matched increases in extracranial blood flow, vasodilation, and shear rate following CO<sub>2</sub> and EX conditions. The influence of exercise and hypercapnia-mediated changes in NVC will be explored in a larger sample size to further elucidate this relationship.

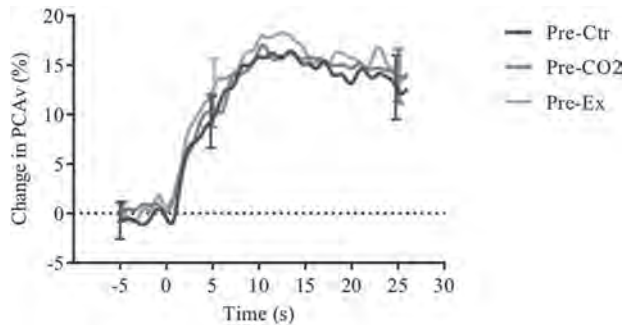


Fig. 1. Cerebrovascular response to neurovascular coupling pre-exposure to control (Ctr), hypercapnia (CO<sub>2</sub>), and exercise (Ex) conditions. Relative change in posterior cerebral artery velocity (PCAv); at time 0s there is cerebral activation during eyes-open following resting eyes-closed. There were no significant differences in NVC pre-intervention (P>0.05). Data are mean ± SEM for n=10.

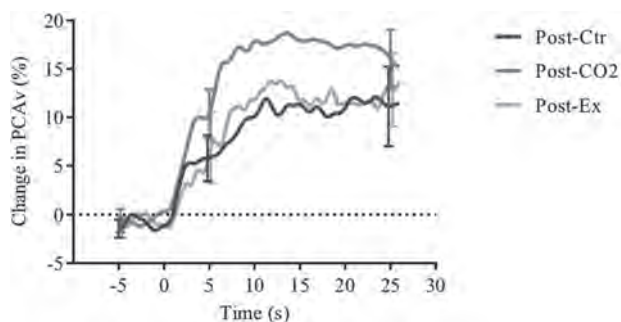


Fig. 2. Cerebrovascular response to neurovascular coupling post-exposure to control (Ctr), hypercapnia (CO<sub>2</sub>), and exercise (Ex) conditions. Relative change in posterior cerebral artery velocity (PCAv); at time 0s there is cerebral activation during eyes-open following resting eyes-closed. There were no significant differences in NVC post-intervention (P>0.05); however, there is an observable response favouring an increase in NVC following CO<sub>2</sub>. Data are mean ± SEM for n=10.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## Hypoxia related pathways in the pathomechanism of bladder cancer

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Bladder cancer (BC) ranges among the tenth most cancers worldwide. Two main forms of BC exist, superficial (sBC) and muscle invasive BC (miBC). Different therapies are in use but the treatment success is not always convincing. For different treatment opportunities every second patient with miBC died within the next five years after diagnosis and up to 40% of patients with sBC get relapses after standard therapy like Bacillus-Calmette-Guarin (BCG)-instillation. The decision for the right therapy is challenging because prediction of therapy success is difficult. (1)

This is why there is necessity of new prognostic markers for a successful treatment and better understanding of the pathomechanism of BC.

Hypoxia often occurs in big solid tumors, likewise in BC, and it seems to play a role in progression and metastatic spread of BC. Hypoxia is also one hallmark of inflammation.

Repeated inflammation is suspected to favor the development of BC. But the immune system seems to play a paradox role in the pathomechanism of BC. For example BCG-instillation induces a massive immune-response which prevents the recurrence of sBC. But repeated infections of the urinary tract seem to promote BC while the frequent use of NSAID (non-steroid anti-inflammatory drugs) seems to be protective. (1)

The aim of the study is to discover new coherences between hypoxia related pathways and growth behavior including metastatic potential of BC cells (BCCs) and interaction of BCCs with immune cells.

Therefore different human cell lines were used: superficial (RT4) and transitional (J82) BCCs, one immortalized normal urothelial cell line, and immune cells.

The present data characterize J82 and RT4 cells under normoxia (21% O<sub>2</sub>) and hypoxia (1% O<sub>2</sub>) for proliferation and migration, hypoxia inducible factors (HIFs)- and target gene-mRNA expression and HIF-protein stabilization. Hypoxia inducible factors are the main regulatory transcription factors for the adaptation of mammalian cells to hypoxia. HIFs and their target genes are used as parameters for cellular reaction to hypoxia.

All used cell lines react to hypoxia by stabilizing HIF-1α protein and increased expression of HIF-1α target genes. Interestingly proliferation and migration are not affected by hypoxia. Studies are underway with co-culture of BCCs (monolayer or spheroids) with different immune cell types under varying conditions (hypoxia and HIF-1α knock down or inhibition) to better understand the behavior of BCCs in interaction with immune cells with special focus on hypoxia related pathways.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA178

**Characterizations of human pancreatic tumor cell lines under normoxic and hypoxic conditions**

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With a median survival rate of about 5 %, over a period of 5 years after diagnosis, pancreatic cancer is one of the most abrasive cancer types (1). Until today curative therapy is still missing. To develop a successful treatment deeper understanding of the cellular processes involved in pancreatic tumor development and growth is indispensable. Since hypoxia has an important role in tumor development (2), we investigated hypoxia inducible factors (HIF) in human pancreatic cancer cell lines (hPCCs). Because even less is known about the interaction between pancreatic tumor cells and infiltrating immune cells under hypoxic conditions we started experiments under conditions of inflammatory hypoxia. i.e. a process of combined hypoxia and immune cell activation, and identified suitable pancreatic tumor cell lines for detailed investigation of HIF and the connected pathways.

Four different hPCCs (HPAC, PSN-1, KP-4, HuP-T4) were used. Expression of mRNA and protein levels as well as proliferation and migration under normoxic (20% O<sub>2</sub>) and hypoxic (1% O<sub>2</sub>) conditions were analyzed. Comparison of the different hPCCs under the applied conditions showed similar behavior with varying extent. The hypoxic status of the hPCCs was verified by qPCR analysis of HIF1 $\alpha$  mRNA expression and immunoblot of stabilized HIF1 $\alpha$  protein. Under hypoxia hPCCs revealed increasing HIF1 $\alpha$  mRNA levels and stabilized HIF1 $\alpha$  protein compared to control cells under normoxia. Furthermore, tumor cell proliferation was decreased compared to that under normoxic conditions. In almost the same manner hPCCs displayed a better migration under normoxia compared to hypoxia.

The present data demonstrate that the investigated hPCCs (HPAC, PSN-1, KP-4, HuP-T4) are influenced by hypoxia. In conclusion, these first results indicate that the characterized hPCCs are useful for further observations with regard to cellular processes which are associated to pancreatic tumor development and growth. Further experiments will investigate interaction of hPCCs with different immune cells under normoxic and hypoxic conditions, to get a deeper understanding of the cellular events involved in the pancreatic tumor development and growth.

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## PCA179

**Important role of the erythrocyte as a regulator of neurovascular coupling in humans**R.L. Hoiland<sup>1</sup>, D. Nowak-Flück<sup>1</sup>, C.K. Willie<sup>1</sup>, M. Tymko<sup>1</sup>, J.C. Tremblay<sup>2</sup>, C.A. Howe<sup>1</sup>, J. Donnelly<sup>3</sup>, M. Stembridge<sup>4</sup>, D.M. Bailey<sup>5</sup>, D. Macleod<sup>6</sup> and P. Ainslie<sup>1</sup>

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The erythrocyte has emerged as an integral oxygen sensor and response effector for the regulation of oxygen delivery throughout the vasculature. *In vivo* rodent models have demonstrated that erythrocyte signaling underscores the coupling of cerebral blood flow to neural activity [i.e. neurovascular coupling (NVC)]; however, this has yet to be examined in humans. In eight healthy males (27 $\pm$ 6 years, 23 $\pm$ 2 kg/m<sup>2</sup>) (mean  $\pm$  standard deviation), NVC was assessed in response to an eyes open visual stimulus using transcranial Doppler ultrasound prior to and following isovolumic haemodilution whereby 20% of blood volume was removed and replaced with an equal volume of 5% human serum albumin. This NVC test assesses the coupling of posterior cerebral neural activity, which is manipulated with the aforementioned visual stimulus, and the corresponding change in posterior cerebral blood flow. Paired t-tests were used to test for statistical significance ( $\alpha$ =0.05). Haemodilution resulted in a decreased haemoglobin concentration (14.0  $\pm$  0.9 vs 11.4  $\pm$  0.5 g/dL; P<0.01), hematocrit (42.8  $\pm$  2.5 vs. 35.0  $\pm$  1.5 %; P<0.01) and arterial oxygen content (CaO<sub>2</sub>; 19.1  $\pm$  1.6 vs. 15.6  $\pm$  0.7 mL/dL; P<0.01). Posterior cerebral artery blood velocity (PCAv) was correspondingly elevated (39  $\pm$  8 to 43  $\pm$  11 cm/s; P<0.01) in the face of unaltered mean intra-arterial pressure (99  $\pm$  9 vs. 97  $\pm$  4 mmHg; P = 0.67) and partial pressure of end-tidal CO<sub>2</sub> (43  $\pm$  2 vs. 42  $\pm$  2 mmHg; P = 0.20). Collectively, these changes in CaO<sub>2</sub> and PCAv led to a reduction in estimated cerebral oxygen delivery (PCAv  $\times$  CaO<sub>2</sub>, arbitrary units) at rest (751 $\pm$ 185 vs. 693 $\pm$ 178; P=0.03). The absolute peak change in PCAv (8.5 $\pm$ 4.7 vs. 8.5 $\pm$ 3.3 cm/s; P=0.29) and the absolute average change in PCAv (3.4 $\pm$ 2.4 vs. 2.9 $\pm$ 2.0 cm/s; P=0.20) across the eyes open period were unaltered; however, given the reduced CaO<sub>2</sub> following haemodilution, both the peak (177 $\pm$ 83 vs. 133 $\pm$ 63; P=0.01) and average (65 $\pm$ 45 vs. 45 $\pm$ 31; P=0.04) increases in estimated cerebral oxygen delivery were reduced during NVC. The attenuated oxygen delivery response is attributable to a reduced peak (24  $\pm$  9 vs. 19  $\pm$  9%; P = 0.02) and average (9  $\pm$  5 vs. 6  $\pm$  4%; P = 0.04) percent increases in PCAv as well as smaller peak (-0.48 $\pm$ 0.25 vs. -0.36 $\pm$ 0.17 mmHg/cm/s; P<0.01) and average (-0.20 $\pm$ 0.11 vs. -0.13 $\pm$ 0.09 mmHg/cm/s; P<0.01) reductions in cerebrovascular resistance. These data indicate that reductions in vasomotor tone during NVC in humans are dependent upon erythrocyte-mediated signaling, and that acute experimental reductions in haemoglobin concentration constrain increases in oxygen delivery during neural activation.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## PCA180

**Elevated systemic oxidative-nitrosative stress and cerebrovascular function in professional rugby union players: the link to impaired cognition**

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Sports-related concussion (SRC) represents a growing public health concern in rugby union, yet remains one of the least understood injuries facing the community today. There is concern that prior SRC may contribute to long-term neurologic sequelae in later-life(1). This may be due to an accelerated decline in cerebral perfusion, a major risk factor for neurocognitive decline (2), though the underlying mechanisms remain inconclusive. It was hypothesised that recurrent SRC in current professional rugby union players would result in elevated systemic oxidative-nitrosative stress(3), reflected by a free radical-mediated reduction in bioactive nitric oxide (NO) metabolite bioavailability and impaired cerebrovascular and cognitive function.

A longitudinal study design was adopted across the 2017-2018 rugby season. Ethical approval was obtained from the University of South Wales Ethics Committee. Data collection is ongoing and the current report documents result from the pre-season data collection. Participants were divided into two separate groups; 23 professional rugby union players (aged  $26 \pm 5$  years) and 22, age- and physical activity-matched non-concussed controls ( $27 \pm 8$  years). Pre-season measurements were performed for cerebrovascular function (Doppler ultrasound of middle cerebral artery velocity (MCAv) at rest and in response to hypocapnia/hypercapnia), venous concentrations of the ascorbate radical (electron paramagnetic resonance spectroscopy), and bioactive NO metabolites (nitrite and S-nitrosothiols, ozone-based chemiluminescence) including cognition (neuropsychometric tests).

The rugby players expressed greater oxidative-nitrosative stress confirmed by a systemic elevation in ascorbate radical ( $P < 0.05$  vs. control) and reduction in cumulative bioactive NO ( $P < 0.05$  vs. control). The players performed worse in the Rey Auditory Verbal Learning Test B (learning and memory) and the Grooved Pegboard test using both the dominant and non-dominant hands (visuomotor coordination,  $P < 0.05$  vs. control). No between-group differences in cerebral perfusion at rest (MCAv:  $54 \pm 13$  cm.s<sup>-1</sup> vs.  $59 \pm 12$  cm.s<sup>-1</sup>,  $P > 0.05$ ) or in response to the CO<sub>2</sub> challenges were observed (CVR<sub>CO<sub>2</sub>Hypo</sub>:  $2.58 \pm 1.01$  cm.s<sup>-1</sup> vs.  $2.58 \pm 0.75$  cm.s<sup>-1</sup>,  $P > 0.05$  and CVR<sub>CO<sub>2</sub>Hyper</sub>:  $2.69 \pm 1.07$  cm.s<sup>-1</sup> vs.  $3.35 \pm 1.28$  cm.s<sup>-1</sup>,  $P > 0.05$ ).

The present study identified that the rugby players are characterised by impaired cognitive function subsequent to elevated systemic-oxidative-nitrosative stress. This appears to be independent of any functional impairment in cerebrovascular function. Given the potential long term trajectory towards accelerated cognitive decline following SRC, prophylaxis to increase NO bioavailability warrants consideration (4).

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D.M. Bailey is a Royal Society Wolfson Research Fellow (#WM170007).

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA181

**Hemoconcentration reduces cerebral blood flow during acclimatization to high-altitude in humans**

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At high-altitude, increases in haematocrit (HCT) are achieved acutely through altitude-induced diuresis and chronically through erythropoiesis, both of which increase arterial oxygen content (CaO<sub>2</sub>). Given the impact of alterations in HCT on CaO<sub>2</sub>, haemoconcentration during acclimatization has been hypothesized to partly explain the reductions in cerebral blood flow (CBF) at high-altitude. However, the degree to which this response mitigates the decrease in CBF has not been experimentally explored. To test this hypothesis, healthy males (n=13) ascended to 5050 m over nine days without the aid of prophylactic acclimatization medications. Following one week of acclimatization at 5050 m, participants were haemodiluted by rapid saline infusion ( $2.10 \pm 0.28$  L) to return HCT towards pre-acclimatized levels. Arterial blood gases, HCT, global CBF (duplex ultrasound), and haemodynamic variables were measured following initial arrival to 5050 m and after one week of acclimatization at high-altitude, prior to and following the hypervolemic haemodilution protocol. Following one week at high-altitude, HCT increased from  $42.5 \pm 2.5$  to  $49.6 \pm 2.5$  % ( $P < 0.001$ ) and was subsequently reduced to  $45.6 \pm 2.3$  % ( $P < 0.001$ ) following haemodilution. Global CBF decreased from  $844 \pm 160$  to  $619 \pm 136$  mL/min ( $P = 0.033$ ) following one week of acclimatization and increased to  $714 \pm 204$  mL/min ( $P = 0.045$ ) following haemodilution. Despite the significant



changes in HCT, and thus  $\text{CaO}_2$ , cerebral oxygen delivery was unchanged at all time points. Further, these observations occurred in the absence of any changes in mean arterial pressure, cardiac output, pH or partial pressure of arterial oxygen pre and post haemodilution, highlighting the influence of HCT in the regulation of CBF. These findings are the first to experimentally demonstrate, via manipulation of HCT, that haemoconcentration contributes to the reduction in CBF during acclimatization to high-altitude.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

#### PCA182

### **The promotion of erythrocyte deformability after dark chocolate ingestion in healthy humans.**

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Erythrocyte deformability is important property of erythrocytes that considerably affects blood flow and hemodynamics in general. The high content of polyphenols present in dark chocolate has been reported to play a protective role in functionality of erythrocytes. We hypothesized that chocolate might influence erythrocytes not only after repeated chronic intake, but also immediately after its ingestion. Thus, we determined the acute effect of dark chocolate and milk (with lower content of biologically active substances) chocolate intake on erythrocyte deformability. We were also focused on selected factors that may affect erythrocyte deformability, specifically nitric oxide (NO) production in erythrocytes and total antioxidant capacity of plasma. We determined post-treatment changes in mentioned parameters 2 hours after consumption of chocolate compared with their levels before consumption of chocolate. Erythrocyte deformability was determined using filtration method and NO production in erythrocytes using fluorescent NO probe DAF-2 DA. The total antioxidant capacity of plasma was measured by determining the trolox equivalent antioxidant capacity. The study was approved by the Ethics Committee of Faculty of Medicine, Comenius University and University Hospital in Bratislava, Slovak Republic. Written informed consent was obtained from all the subjects. The study conformed to the code of ethics stated in the Declaration of Helsinki. In contrary to milk chocolate intake, the dark chocolate led to significantly higher increase in erythrocyte deformability ( $3.5 \pm 0.7\%$  dark vs.  $0.4 \pm 1.1\%$  milk,  $p=0.016$ ). NO production in erythrocytes was not changed after dark chocolate intake ( $6437 \pm 344$  before vs.  $6353 \pm 385$  after,  $p=0.77$ ), but significantly decreased after milk chocolate ( $6430 \pm 436$  after vs.  $7426 \pm 636$  before,  $p=0.003$ ). The plasma total antioxidant capacity remained unaffected after ingestion of both chocolates. We may conclude that our hypothesis was confirmed. Single ingestion of dark chocolate improved erythrocyte deformability despite unchanged NO production and antioxidant capacity of plasma. Increased deformability of erythrocytes may considerably improve rheological properties of

blood and thus hemodynamics in humans, resulting in better tissue oxygenation.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

#### PCA183

### **The effect of kinesiology tape on pain and muscle contraction in healthy participants.**

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It has been suggested that the elastic properties of kinesiology taping (KT) contributes to improvements in musculoskeletal pain and function. Delayed onset muscle soreness (DOMS) is an experimental model used to evaluate the effect of treatments on musculoskeletal pain in human participants. To date, studies using DOMS have found that KT reduced musculoskeletal pain and improved function. These studies have used isokinetic dynamometry which measures maximal voluntary contraction of muscle groups and relies on the participant's ability to exert maximal voluntary effort. Tensiomyography (TMG) has been used to assess the contractile properties of muscle in response to electrical stimuli, such as maximal muscle displacement (Dm) and contraction time ( $T_c$ ). An advantage of TMG is that the technique does not rely on volitional effort. There is a paucity of studies that have assessed the effect of KT on contractile properties of individual muscles in response to an involuntary contraction. The aim of this study was to compare the effects of KT, non-elastic surgical tape (NET) and no tape (nT) on musculoskeletal pain and muscle contractile properties of the biceps brachii, following the induction of experimental DOMS.

This was a randomised, repeated measures, parallel group study. Participants received 1 of 3 possible interventions (KT,  $n=10$ ; NET,  $n=10$ ; nT,  $n=10$ ). Day 1 consisted of baseline (pre-DOMS) measurements, induction of DOMS (eccentric muscle exercises), application of intervention (10mins) and immediate post-intervention measurements. Tape was then re-applied for participants in the tape groups and remained in-situ until the next study visits on Day 3 and Day 5. At these visits, measurements were taken 20mins after tape had been removed. Primary measurements at each study visit were: pain intensity during full flexion and extension (mPI, numerical pain rating scale, 0=no pain, 10=worst pain imaginable), and Dm by incrementally increasing the amplitude of the electrical stimulus until Dm reached a plateau. Primary outcomes were differences in mPI and Dm at Day 3 relative to baseline (Day 3 - baseline) and analysed using a one-way ANOVA.

Thirty participants completed the study (mean  $\pm$  SD age:  $25.97 \pm 4.98$  years; height:  $171.58 \pm 8.96$  cm; weight:  $76.97 \pm 15.47$  kg; 16 females). Paired t-tests revealed that mPI increased ( $P=0.00$ ) and Dm decreased ( $P=0.00$ ) at Day 3 relative to baseline. There were no differences in the magnitude of this change between groups for mPI (mean  $\pm$  SD KT =  $5.00 \pm 2.05$ , NET =  $5.40 \pm 2.50$ , nT =  $5.20 \pm 2.15$ ,  $P=0.92$ ) or Dm (KT =  $-2.33 \pm 4.68$ , NET =  $-2.97 \pm 4.51$ , nT =  $-3.23 \pm 5.39$ ,  $P=0.91$ ).

In conclusion, KT and NET did not alter the increase in mPI and decrease in Dm associated with DOMS.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

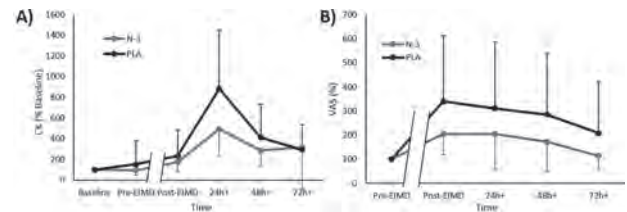
PCA184

### The effect of omega-3 supplementation on exercise-induced muscle damage

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Exercise-induced muscle damage (EIMD) results in transient muscle inflammation, strength loss, muscle soreness (Damas et al., 2016) and can result in subsequent exercise avoidance. Omega-3 (n-3) supplementation has been proposed to minimise EIMD via its anti-inflammatory properties (Jakeman et al., 2017), however its action remains unclear. We aimed to examine the effects of n-3 supplementation on exercise-induced inflammatory response following muscle-damaging exercise. Physically active, healthy Caucasian males ( $n = 14$ ,  $25.07 \pm 4.05$  years) provided written informed consent, then were single blind randomised to either receive 3 g/day n-3 supplementation (N-3,  $n = 7$ ) or placebo (PLA,  $n = 7$ ). Following 4 weeks n-3 supplementation, a downhill running protocol (60 minutes at 65%  $\text{VO}_2\text{max}$ , -10% gradient) was performed. Before supplementation (baseline), prior to EIMD, immediately after EIMD, and at 24, 48, and 72 hours post-EIMD, venous plasma was collected for creatine kinase (CK), interleukin (IL)-6 and tumour necrosis factor (TNF)- $\alpha$ , and maximal voluntary isometric contraction (MVIC), anaerobic power and perceived muscle soreness were also quantified. Results are presented here as 'mean  $\pm$  SD'. No significant differences in CK activity were found between N-3 and PLA group ( $p = 0.363$ ), however, PLA showed a larger increase in CK levels (baseline- vs 24h post-EIMD) compared to N-3 ( $882.8\% \pm 564.8$  vs  $496.1\% \pm 275.4$ , respectively). PLA group showed a larger increase in IL-6 compared to N-3 immediately post-EIMD ( $162.3\% \pm 68.6$  vs  $123.3\% \pm 15.1$ , respectively), however, there was no significant difference between the two groups ( $p = 0.422$ ). TNF- $\alpha$  showed a smaller increase for the N-3 group compared to the PLA, again, there were no significant differences between the two groups ( $p = 0.320$ ). No significant difference in MVIC between N-3 and PLA group ( $p = 0.883$ ) was found, however, there was a significant main effect for time ( $p = 0.008$ ) with both groups showing a reduction in muscle strength immediately post-EIMD. No differences in peak power were found between N-3 and PLA group across time ( $p = 0.798$ ). No significant difference in muscle soreness was found between N-3 and PLA ( $p = 0.3$ ), however, the latter showed a larger increase in muscle soreness (pre- vs post-EIMD) compared to the N-3 group ( $>165.1\%$  vs N-3). In this study, even though we recorded some reduction in the plasma markers for the N-3 group, there was no statistically significant decrease to allow us to draw any definitive conclusions about the n-3 supplementation. Additionally, there was no impact on muscle function nor power output. Future studies might compare the dosage and duration of n-3 supplementation on muscle function or examine the effect of n-3 supplementation on EIMD during ageing-associated muscle function loss, where increased basal inflammation is seen.



A) Creatine kinase (CK) and B) Visual analogue scale (VAS) between omega-3 (N-3) and placebo (PLA) group across timepoints following EIMD. Baseline collected 1 month prior to exercise-induced muscle damage (EIMD). Error bars indicate standard deviation. Note B) (VAS) does not include baseline.

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Jakeman, J. R., Lambrick, D. M., Wooley, B., Babraj, J. A. and Faulkner, J. A. (2017). Effect of an acute dose of omega-3 fish oil following exercise-induced muscle damage. *European Journal of Applied Physiology*, 117 (03), pp.575-582

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA185

### Effect of Post-Prandial Hyperlipidaemia on Cerebrovascular Function: Gender Difference?

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**Background:** The consumption of a high-fat meal is characterised by a state of post-prandial hyperlipidaemia (PPH) with an exaggerated increase in triglycerides (Tg) that peaks at 4 hours<sup>1</sup>. We recently demonstrated that PPH was associated with impaired cerebrovascular reactivity in aged, but not young males<sup>2</sup>. However, to what extent PPH impacts the cerebral circulation in females, who are more prone to cognitive decline and dementia in later life<sup>3</sup>, remains to be established. **Methods:** Eighteen males (age:  $24 \pm 6$  years; body mass index (BMI):  $23.2 \pm 3.6$  kg.m<sup>2</sup>) and 8 females (age:  $21 \pm 2$  years; BMI:  $23.7 \pm 3.1$  kg.m<sup>2</sup>) participated in the study. Cerebrovascular function and Tg were assessed prior to, and 4 hours after the consumption of a standardised high-fat meal<sup>1</sup>. Middle cerebral artery velocity (MCAv; transcranial Doppler ultrasound), mean arterial pressure (MAP; photoplethysmography) and end-tidal CO<sub>2</sub> (capnography) were continuously recorded throughout each testing session. Serum Tg were determined via established methods from venous samples obtained from an indwelling cannula. MCAv and MAP were assessed following 5 minutes of seated rest. Cerebrovascular reactivity to carbon dioxide was assessed in response to 3 minutes of breathing 5% CO<sub>2</sub> (balanced air;  $\text{CVR}_{\text{CO}_2\text{HYPER}}$ ) and following 3 minutes of controlled hyperventilation (15 breaths per minute;  $\text{CVR}_{\text{CO}_2\text{HYPO}}$ ). Cerebrovascular range ( $\text{CVR}_{\text{CO}_2\text{RANGE}}$ ) was calculated as  $\text{CVR}_{\text{CO}_2\text{HYPER}} + \text{CVR}_{\text{CO}_2\text{HYPO}}$ . Data were analysed using a 2-way repeated measures ANOVA and Bonferroni corrected paired sample t-tests and independent sample t-tests. Significance was established at  $P < 0.05$  and data are expressed as mean  $\pm$  SD.

**Results:** At baseline, females were characterised by elevated MCAv, CVR<sub>CO2HYPER</sub>, CVR<sub>CO2HYPO</sub> and CVR<sub>CO2RANGE</sub> compared to the males (Table; all  $P < 0.05$ ). During PPH, Tg increased relative to baseline in both groups and was associated with impaired CVR<sub>CO2HYPER</sub> and CVR<sub>CO2RANGE</sub> (Table;  $P < 0.05$ ). Though this was independent of gender (Table;  $P > 0.05$ ). Furthermore, PPH did not influence changes in resting MCAv or MAP (Table;  $P > 0.05$ ).

**Conclusion:** Contrary to our previous findings<sup>2</sup>, PPH has the capacity to impair cerebrovascular function in young adults. Though, it appears to be independent of gender. These observations are important given that a reduction in CVR<sub>CO2</sub> may enhance the risk of stroke and neurodegenerative disease<sup>4</sup>.

**Table.** Changes in metabolic and cerebrovascular function following a high-fat meal.

| Gender                                          | Males (n = 18) |              | Females (n = 8) |              | P Value     |             |             |
|-------------------------------------------------|----------------|--------------|-----------------|--------------|-------------|-------------|-------------|
|                                                 | Pre-meal       | Post-meal    | Pre-meal        | Post-meal    | Gender      | Meal        | Interaction |
| Triglycerides (mmol/L)                          | 0.88 ± 0.50    | 2.32 ± 1.49* | 0.97 ± 0.43     | 1.48 ± 0.34* | 0.30        | <b>0.00</b> | <b>0.03</b> |
| MCAv (cm.s <sup>-1</sup> )                      | 62 ± 13        | 61 ± 10      | 73 ± 11         | 71 ± 10      | <b>0.04</b> | 0.45        | 0.71        |
| MAP (mmHg)                                      | 88 ± 9         | 86 ± 7       | 80 ± 11         | 81 ± 11      | 0.09        | 0.73        | 0.39        |
| CVR <sub>CO2HYPER</sub> (%.mmHg <sup>-1</sup> ) | 2.77 ± 0.77    | 2.40 ± 0.85  | 3.80 ± 0.96     | 3.19 ± 0.66  | <b>0.01</b> | <b>0.01</b> | 0.52        |
| CVR <sub>CO2HYPO</sub> (%.mmHg <sup>-1</sup> )  | 2.47 ± 0.46    | 2.05 ± 0.50  | 3.45 ± 0.94     | 3.39 ± 0.76  | <b>0.00</b> | 0.24        | 0.36        |
| CVR <sub>CO2RANGE</sub> (%.mmHg <sup>-1</sup> ) | 5.24 ± 0.87    | 4.45 ± 1.13  | 7.24 ± 1.77     | 6.59 ± 0.98  | <b>0.00</b> | <b>0.01</b> | 0.81        |

Values are mean ± SD; \* =  $P < 0.05$  vs. pre-meal.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA186

### Heat shock response and apoptosis signaling following a single bout of high intensity intermittent exercise in physically active and inactive men

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The heat shock protein families (HSPs) are known regulatory protein, which were related with protein folding, transport and aggregation in the cells after heat, mechanical or metabolic stress and exercise. HSP responses to exercise in men who have different levels of physical activity are not clearly defined. The aim of this study was to compare blood HSPs mRNA levels following one bout of high intensity interval training (HIIT) in physically active (PA) and inactive (I) men. Healthy young men (aged between 22 and 38 years) who defined the exercise duration more than 4 hours per week at least one year was accepted as PA group (n=9) and lower than 1.5 hours per week was accepted as I group (n=9). Peak oxygen consumption (VO<sub>2</sub> peak) was measured at the first day, HIIT comprising 4 repeats of a Wingate test (0.050 kg load/kg) was performed at the second day by a cycle ergometer. mRNA levels of HSP27, HSP60, HSP70, HSP90, TNFα, IL6, P53, BCL2, BAX, Caspase 3, Caspase 8 were determined by quantitative real-time PCR analysis, and lactate levels were measured by enzymatic method in blood samples taken before HIIT, 5th min and 24 h after the HIIT. Values are means ± SD. The relative fold changes compared by using one-way ANOVA, Duncan

test, differences with in groups were analysed independent samples t test and analysis of covariance. VO<sub>2</sub> peak was higher in PA group than I group (37.0±6.0 ml/min/kg vs. 25.5±4.1 ml/min/kg;  $p=0.023$ , respectively). Lactate levels at 5 min in both groups were significantly higher than baseline and 24 h lactate levels ( $p<0.001$  for all comparisons). Relative fold changes of HSP27 were elevated in PA and I groups at 5<sup>th</sup> min and 24 h of post-exercise ( $P_{PA}=0.020$ ,  $P_I=0.001$ ). HSP60 values were higher than baseline at 5<sup>th</sup> min and 24 h after the HIIT in I group ( $P_I=0.0001$ ). HSP70 was higher at 5<sup>th</sup> min in comparison to the baseline in PA group ( $P_{PA}=0.024$ ). When compare two groups, relative fold change of HSP70 was higher in PA group than I group ( $P=0.026$ ) and HSP60 was higher in I group than PA group ( $P=0.017$ ) at 5<sup>th</sup> min after the HIIT. HSP90 were similar to baseline after post-exercise in both groups. At 5<sup>th</sup> min, elevated P53, BCL2, BAX, Caspase 3, Caspase 8 have been shown both in PA and I groups and elevated TNFα and IL6 were found only in the I group ( $p<0.05$  for all comparisons). The findings of this study indicate that physical activity status may alter HSP response in men. HSP60 that is known as mitochondrial HSP, may be more effective on apoptotic and inflammatory changes following HIIT in inactive men but HSP70 may be effective on apoptotic function in physically active men.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA187

### Roles of C-terminal amino acids in the PI(4,5)P<sub>2</sub>-dependent regulation of TWIK-related two-pore domain K<sup>+</sup> channel

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TWIK-related two-pore domain K<sup>+</sup> channels (TREKs) contribute to the negative resting membrane potential as a partial 'leak' K<sup>+</sup> conductance. TREKs are activated by various physicochemical conditions including membrane stretch, unsaturated fatty acid, temperature and acidic intracellular pH. For the proper activation of TREKs, tonic inhibited state is necessary. However, the determining factors and related molecular structure are not clearly understood yet. Especially, there has been controversy over the direction of regulation by PI(4,5)P<sub>2</sub> that are dynamically generated by phosphatidylinositol (PI) kinases using ATP.

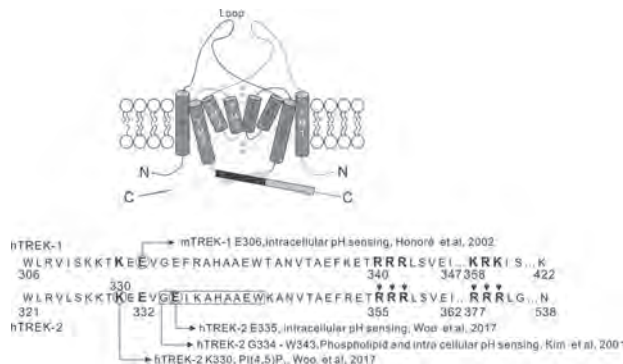
TREK-1 and TREK-2 currents are commonly increased spontaneously by dialysis with ATP-free pipette solution for whole-cell recording ( $I_{TREK,w-c}$ ) or by membrane excision for inside-out patch clamp recording. Consistently, both  $I_{TREK-1,i-o}$  and  $I_{TREK-2,i-o}$  were inhibited by ATP to the cytoplasmic side. The ATP-dependent inhibition was prevented by PI kinase inhibitor. Also, direct application of PI(4,5)P<sub>2</sub> strongly inhibited  $I_{TREK,i-o}$ . The putative PI(4,5)P<sub>2</sub>-mediated inhibition of TREK-2 by intracellular ATP was also observed in the intrinsically expressed cells such as astrocytes and B cells. Treatment with polycationic poly-L-lysine (PLL) totally abolished  $I_{TREK-2,i-o}$ , then the application of PI(4,5)P<sub>2</sub> showed dose-dependent dual effects; activation and inhibition by relatively low and high ranges, respectively. When co-expressed with voltage-sensitive PI(4,5)P<sub>2</sub> phosphatase (Dr-VSP),  $I_{TREK-2,w-c}$  was initially activated and then inhibited by sustained activation of Dr-VSP, further indicating the dual effects of PI(4,5)P<sub>2</sub> on TREK-2.

To identify the residue(s) of pCt for the ATP-dependent TREK-2 inhibition, site-directed substitution of caionic residues with

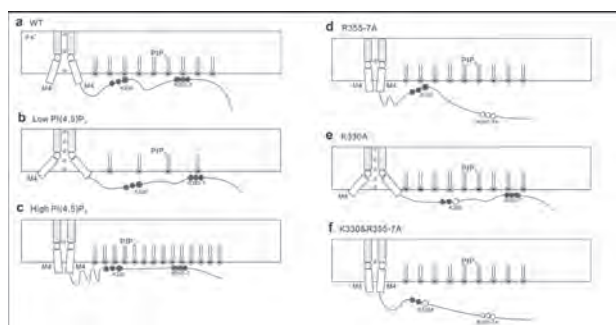


Ala was introduced to human TREK-2. Neutralization of Lys330 (K330A) showed higher basal activity and was not inhibited by ATP. In whole-cell recordings, K330A also showed higher basal activity and the further activation by 2APB, arachidonic acid or acidic pH<sub>i</sub> (6.9) was less significant. On the contrary, neutralization of the three consecutive Arg distal to Lys330 (R355-7A) showed very low basal activity even without ATP. Finally, I investigate the acidic pHi-sensing residues. Consistent with the previous study of TREK-1, neutralization of a Glu in the pCt of hTREK-2 (E332A) showed higher basal activity and insensitivity to acidic pHi. However, different from hTREK-1, an additional Glu335 of hTREK-2 was contributing to the acidic pHi-sensitivity; E335A showed partial attenuation of the pHi effect.

Taken together, I suggest that the ATP-dependent intrinsic PI(4,5)P<sub>2</sub> induces tonic inhibition of TREKs, which might be beneficial for the wide changes of ITREK modulation for physiological responses.



Sequence alignment of proximal C-terminal (pCt) domains in hTREK-1 and hTREK-2



Cartoon summary of TREK-2 gating regulated by interaction between pCt Lys 330, Arg 355-7 and PI(4,5)P<sub>2</sub>

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Woo JH et al. (2018). *Pflugers Arch.* 470(2):327-337

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PCA188

## Role of Xin (Xirp1) in microdamage and repair after moderate eccentric exercise in human m. vastus lateralis

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Exercise involving repeated high strain contractions results in muscle micro-damage. Adaptations to eccentric exercise reduce susceptibility to micro-damage even after a single bout

(Paulsen et al., 2009), presumably due to repair and improvement of susceptible structures.

Xin (Xirp1) is an adapter protein involved in the growth of actin filaments. A constituent of adult muscle fibres, it is found at high levels in nascent and regenerating fibres. Focal spots with high signals for Xin have been linked to constitutive local repair in rat muscle fibres (Eulitz et al., 2013). However, no studies have examined Xin during repair of exercise-induced micro-damage in human muscle.

We monitored structure bound Xin in a series of vastus lateralis biopsies from six male subjects (age 25.0 ± 10.7) after 3 sets of 25 repetitions (5 min rest in between) of single-leg maximal eccentric contractions. Biopsies were obtained before, immediately, 3h and 48 h after exercise. With indirect immunohistochemistry, binding of Xin and other marker proteins to insoluble structures (myofibrils, cytoskeleton) was monitored in each biopsy, in ~100 muscle fibres that were followed through 3 consecutive sections stained with different combinations of antibodies, to αB crystallin, Hsp27 (small chaperones that bind to unfolded proteins), p70S6K phosphorylated at Thr-421/Ser-424 (linked to increasing protein synthesis), the myofibrillar proteins type II myosin, actinin and desmin and to Xin. The sections were scanned on an Axioscan Z1 (Zeiss) at 20 x and selected fibres photographed at 63x.

Median bound Xin, αB crystallin and Hsp27 and were significantly increased (p<0.05, Wilcoxon) immediately after exercise and decreased again by 48h (Figure 1).

There was considerable inter-individual variability, as expected in an eccentric exercise study. No consistent differences were found between type I and II fibres.

In all immediate and 3h post exercise biopsies, significant correlations were found between Xin, αB crystallin and p70S6K-p421/424 bound per fibre (p<0.001, Spearman); αB crystallin and Hsp27 correlated significantly in 11 out of the 12 biopsies.

A minority of fibres in the immediate and 3h post exercise biopsies had small areas with very strong staining which we interpret as damage/repair “hot spots”. Pixel by pixel analysis of αB crystallin-hot spots in 45 fibres showed that they all had significantly enhanced signals for the other 3 markers (p<0.001, t-test). In longitudinally cut fibres, analysis of larger magnification (63x) images indicated disturbed myofibrillar structures at hot spots.

Our results indicate for the first time in humans that Xin is involved in the microdamage/repair cycle following a bout of eccentric exercise. Damage is largely in the sub-microscopic range and repaired locally within a few hours.

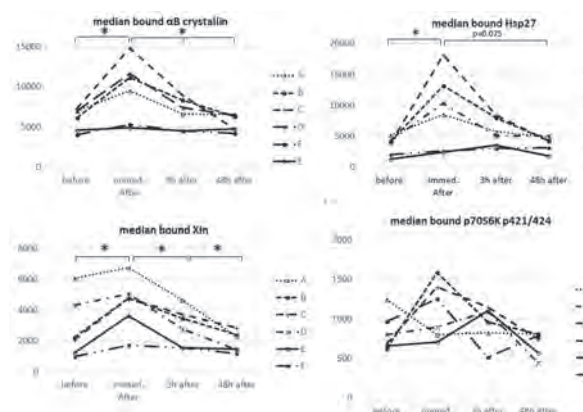


Figure 1: Median values for bound αB crystallin, Hsp27, Xin and p70S6K p421/424 in ~100 fibres per biopsy before and after eccentric exercise. The lines connect biopsies from individual subjects (A-F). \* indicates significant differences (p<0.05, Wilcoxon).

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA189

### Systemic molecular adaptations to intermittent hypoxia: impact on microvascular oxygenation and hypoxic exercise performance.

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Intermittent hypoxia (IH) presents a less arduous training tool than chronic exposure for athletes seeking to improve cardio-respiratory fitness. While the sea-level (normoxic) performance benefits of IH remain equivocal (1, 2), less is known regarding its impact on hypoxic performance. Therefore, we examined if IH had the capacity to improve cardiorespiratory fitness in hypoxia and if so, identify the haemodynamic and molecular responses driving this adaptation. Eighteen healthy human male participants ( $22 \pm 4$  years) were randomly assigned single-blind to an intermittent normoxia (IN; 21% O<sub>2</sub>, n = 9) or IH (10% O<sub>2</sub>, n = 9) group. They completed ten sessions in a normobaric environmental chamber, each of which incorporated nine exposure periods (in normoxia or hypoxia depending on group). Each period was 5-minutes in length and separated by 5-minutes of exposure to normal ambient air (21% O<sub>2</sub>). Peak oxygen uptake (VO<sub>2peak</sub>) was assessed in hypoxia (10% O<sub>2</sub>) via online respiratory gas analysis during an incremental cycling test to exhaustion prior to and following the IH/IN programme. Continuous wave near-infrared spectroscopy was employed before and during exercise to monitor concentration changes in cerebral and muscular (vastus lateralis) oxy- and deoxyhaemoglobin (O<sub>2</sub>Hb and HHb), which also served as an index of regional changes in blood volume (O<sub>2</sub>Hb + HHb). Antecubital blood samples obtained at rest and VO<sub>2peak</sub> were analysed to assess oxidative (ascorbate radical (A<sup>•</sup>)) by electron paramagnetic resonance spectroscopy, nitrosative (nitric oxide metabolites by ozone-based chemiluminescence) and inflammatory (sVCAM-1 and sICAM-1 by enzyme-linked immunosorbent assay) stress. Data were analysed using a three-way repeated measures ANOVA. VO<sub>2peak</sub> increased by 2.3% following IH, whereas no changes were observed following IN. Furthermore, submaximal VO<sub>2</sub> at a workload of 60 watts decreased by 4.5% following IH, compared to a decrease of 1.6% following IN. Notably, improvements in exercise performance subsequent to IH were accompanied by significant increases in nitric oxide at rest and VO<sub>2peak</sub> ( $P < 0.05$ ), and augmented levels of sVCAM-1 and sICAM-1 at VO<sub>2peak</sub> ( $P < 0.05$ ). Moreover, A<sup>•</sup> accumulation was attenuated at VO<sub>2peak</sub> succeeding IH, however this difference was non-significant ( $P = 0.10$ ). Additionally, significant increases in cerebral oxygenation at rest and VO<sub>2peak</sub> ( $P < 0.05$ ) and elevated blood flow at the muscle site during exercise ( $P < 0.05$ ) were observed following IH. This

is paramount given that hypoxic exercise performance is limited by cerebral and muscular deoxygenation (3). Collectively, these findings indicate that enhanced hypoxic exercise performance subsequent to IH may be due to attenuated oxidative-nitrosative-inflammatory stress and consequential improvements in microvascular oxygenation.

Tadibi V, Dehnert C, Menold E, Bartsch P. Unchanged anaerobic and aerobic performance after short-term intermittent hypoxia. *Medicine and science in sports and exercise*. 2007;39(5):858-64.

Hamlin MJ, Hellemans J. Effect of intermittent normobaric hypoxic exposure at rest on haematological, physiological, and performance parameters in multi-sport athletes. *Journal of sports sciences*. 2007;25(4):431-41.

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D.M Bailey is a Royal Society Wolfson Research Fellow.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA190

### Mechanosensitive mechanisms responsible for the exercise-induced increased autoregulatory range of coronary arterioles.

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Exercise elicits early adaptations of coronary vessels enabling the coronary circulation to provide greater blood flow during higher demands. Systolic blood pressure and diastolic blood flow greatly increase during exercise. Thus we hypothesized that short-term daily exercise induces biomechanical and functional remodeling of the coronary resistance arteries related to mechanosensitivity: pressure and flow/shear stress.

Male rats were subjected to progressively increasing four-week treadmill exercise program (over 60min/day, one mph in the final step EX) whereas the other group remained sedentary (SED). Coronary arterioles (~120 µm in diameter at 50 mmHg) were isolated and their diameters were measured by television-microscopy. The biomechanical characteristics, the magnitude of myogenic and flow/shear stress responses and the contribution of endogenous nitric oxide and prostanoid production to diameter were assessed, by specific inhibitors. Arterioles isolated from exercised animals - compared to their sedentary mates - had thicker walls (~10 µm), increased distensibility (0.5 vs. 0.8 kPa<sup>-1</sup>), decreased elastic modulus (in low-pressure range) as a result of reduced wall stress (by ~15%). Arterioles of exercised rats exhibited a significantly greater myogenic constriction (SED: 102.1 vs. EX 80.1 µm) and flow/shear stress-induced dilation (diameter increase: 52.4±7.8 vs. 30.3±3.9 µm). Inhibition of nitric oxide synthase and cyclooxygenases reduced the diameter at the lower pressure range and eliminated flow/shear stress-induced dilation.

Indomethacin increased the diameter of arterioles at higher pressure range.

In conclusion, short-term daily exercise induces morphological and functional remodeling of rat intramural coronary arterioles involving smooth muscle and endothelial mechanosensitive mechanisms, resulting in a greater constrictor and dilator reserves extending the range of coronary autoregulation contributing thereby to the optimization of coronary blood flow during exercise.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCA191

### Low temperature affects the morphology and impairs glutamine-induced hypertrophic response in human primary myotubes

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Cold water immersion and other strategies are extensively used by athletes to reduce muscle temperature (TEMP) and are believed to limit muscle soreness and improve recovery after strenuous exercise. However, it remains unclear whether low TEMP affects skeletal muscle hypertrophy and protein synthesis in response to anabolic stimuli. In this study, we used an *in vitro* model to investigate whether a reduced TEMP (32°C, TEMP in skeletal muscle during cold water immersion vs. 37°C, core TEMP) affects the growth and impairs glutamine (GLUT)-induced hypertrophic response in human myotubes. Myoblasts from human muscle biopsies (n=8) were first differentiated into myotubes during 48h at 37°C. Then, myotubes were treated with GLUT to induce hypertrophy and were incubated at either 37 or 32°C during 1h (T1), 8h (T8) or 48h (T48). Myotube area (a marker of myotube size) was assessed at T48 by using immunocytology. Protein synthesis (puromycin incorporation) and phosphorylation levels of two components of the mechanistic target of rapamycin (mTOR) pathway [P70 S6 kinase (P70S6K) and eukaryotic translation initiation factor 4E (4E-BP1)] were assessed at T1 and T8 from western blot analysis. Ribosome biogenesis was evaluated at T8 by determining total RNA concentration, and by assessing the levels of 18S ribosomal RNA (18S rRNA), precursor 45S rRNA (pre-45S rRNA) and MYC mRNA from quantitative polymerase chain reaction. Data were analysed by a two-way ANOVA for repeated measures. A first major observation was that low TEMP induced morphological alterations (short myotube segments and larger myotube area). Importantly, GLUT-induced myotube hypertrophy was only observed at 37°C (+40%,  $P < 0.01$  at 37°C; +4%, NS at 32°C). Similarly, GLUT stimulated protein synthesis at T1 (main effect,  $P < 0.01$ ), with a 53% increase at 37°C ( $P < 0.05$ ) and a 23% increase at 32°C (NS). Protein synthesis was reduced at 32°C at T8 (main effect,  $P < 0.01$ ), while GLUT increased protein synthesis at 37°C (+17%,  $P < 0.01$ ) but not at 32°C (-7%, NS). GLUT-induced 4E-BP1 phosphorylation at T1 was found at 37°C (+16%,  $P < 0.01$ ) but not at 32°C (-1%, NS). Moreover, GLUT increased the phosphorylation of P70S6K at T8 by 50% at 37°C ( $P < 0.01$ ) and by 26% at 32°C ( $P < 0.05$ ). Overall, total RNA concentration, levels of MYC mRNA, 18S rRNA and pre-45S rRNA were reduced at 32°C. In conclusion, this study indicates that low TEMP affects

myotube morphology. In addition, we demonstrate that low TEMP impairs GLUT-induced myotube hypertrophy and protein synthesis, a result accompanied by a minor activation of the mTOR pathway. The impaired ribosome biogenesis observed at 32°C at T8 may also contribute to the reduced protein synthesis at this time point. These findings suggest that reducing muscle TEMP after resistance exercise may be detrimental for muscle hypertrophy and training adaptations.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCA192

### Preferential skeletal muscle myosin loss is prevented by neuromuscular electrical stimulation in steroid-denervation rats

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Steroid has been shown to induce preferential depletion of myosin and severe muscle weakness in denervated skeletal muscle (Rouleau et al, 1987). We here investigated whether neuromuscular electrical stimulation (ES) treatment could prevent these deleterious changes in steroid-denervation (S-D) rat. All experimental procedures were approved by the Committee on Animal Experiments of Sapporo Medical University (No. 16-076). Wistar rats (9 week old, n=24) were assigned into control (CNT) (n=12) and S-D (n=12) group. Under 2% isoflurane anesthesia, S-D was induced by cutting the sciatic nerve and subsequent daily injection of dexamethasone (5 mg/kg) for 7 days. Throughout the ES treatment, rats were anesthetized by isoflurane inhalation and their left foot was secured in a foot plate connected to a torque sensor (S-14154, Takei Scientific Instruments). Plantarflexor muscles were electrically stimulated supramaximally (45 V) using a pair of surface electrodes to produce four sets of five isometric contractions each day. After the experimental periods, rats were killed by cervical dislocation under isoflurane anesthesia and gastrocnemius (GAS) muscle weight, skinned muscle fiber force, and protein and mRNA expression were measured. Data are presented as mean  $\pm$  SEM. A two-way ANOVA followed by Tukey-Kramer post hoc test was used to examine the statistical significance. ES treatment partly prevented the S-D-induced decreases in plantarflexor *in situ* maximum isometric torque (CNT:  $125 \pm 2$ , CNT+ES:  $116 \pm 5$ , S-D:  $15 \pm 5$ , S-D+ES:  $43 \pm 3$  mNm [n=5-6];  $P < 0.05$ : S-D vs S-D+ES) and GAS muscle weight (CNT:  $1461 \pm 43$ , CNT+ES:  $1447 \pm 43$ , S-D:  $769 \pm 21$ , S-D+ES:  $938 \pm 23$  mg [n=5-6];  $P < 0.05$ : S-D vs S-D+ES). ES treatment fully prevented S-D-induced decreases in maximum force in skinned fiber (CNT:  $264 \pm 16$ , CNT+ES:  $275 \pm 16$ , S-D:  $134 \pm 16$ , S-D+ES:  $258 \pm 19$  kN/m<sup>2</sup> [n=14-18];  $P < 0.05$ : S-D vs S-D+ES) and myosin heavy chain (MyHC) expression ([n=5-6]; S-D vs S-D+ES,  $P < 0.05$ ), as well as increases in the reactive oxygen/nitrogen species-generating enzymes NADPH oxidase 2 and 4, autolyzed active calpain-1 and mRNA expression of the muscle-specific ubiquitin ligases muscle ring finger-1 (MuRF-1) and atrogin-1 ([n=5-6]; S-D vs S-D+ES,  $P < 0.05$ ). In conclusion, ES treatment fully prevented the decrease in myofibrillar force production due to decreased MyHC expression and partly



prevented muscle atrophy in S-D muscle. The S-D-induced protein degradation involves reactive oxygen species-dependent activation of calpain-1 and the muscle-specific ubiquitin ligases MuRF-1 and atrogin-1.

Rouleau, G., Karpati, G., Carpenter, S., Soza, M., Prescott, S., and Holland, P. (1987). Glucocorticoid excess induces preferential depletion of myosin in denervated skeletal muscle fibers. *Muscle Nerve* 10, 428-438.

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## PCA193

### Current insights in the use of platelets as biomaterials in regenerative medicine of skeletal muscle: assimilating the molecular physiology

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Platelet-based applications such as platelet-rich plasma and platelet releasate have recently gained unprecedented attention as autologous biomaterials in regenerative medicine across a variety of tissues (1). The rationale behind utilising platelet-based applications originates in the delivery of numerous cytokines and growth factors from  $\alpha$ -granules to the targeted area, which in turn act as cell cycle regulators and promote the healing process across a variety of tissues (2). Due to increasing understanding in cell signalling and growth factor biology, research and clinical attention has been drawn to the use of autologous applications as a novel means of delivering growth factors to injured tissue such as liver, bone and skeletal muscle. However, at present, there is inevitably a large discrepancy in the effectiveness of platelet-based applications in regenerative medicine, especially between human and experimental animal studies (3). Therefore, we aim here to critically discuss and assimilate current experimental evidence on the role of platelets as biomaterials in tissue regeneration, particularly in skeletal muscle, by integrating findings from human, animal and cell studies.

This presentation is composed of three parts: firstly, we review key aspects of platelet biology that precede the preparation and use of platelet-related applications for tissue regeneration. Secondly, we critically discuss relevant evidence on platelet-mediated regeneration in skeletal muscle focusing on findings from a) clinical trials, b) experimental animal studies and c) cell culture studies; and thirdly, we discuss the application of platelets in the regeneration of several other tissues including tendon, bone, liver, vessels and nerve. Importantly, we identify key technical variations in platelet preparation that may account for the large discrepancy in outcomes from different studies and we make suggestions on how to standardise them. Finally, we aim to assimilate novel insights in the molecular mechanisms linking platelet biology to stem cell regulation and tissue regeneration and provide an up-to-date discussion for physiologists as well as biomedical and clinical scientists involved in platelet-mediated regenerative applications. The current consensus with the use of platelet-rich plasma and especially modified plasma (where individual factors are depleted) in skeletal muscle regeneration remains promising. Most importantly, the molecular mechanisms linking platelet

biology to skeletal muscle regeneration, have just begun to unravel and are expected to transform our understanding in using platelets as a biomaterial for tissue healing.

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(2) Qian et al. (2017) *Front Chem*, 5:89

(3) Scully et al. (2018) *Acta Physiol*, Apr 6:e13071

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA194

### L-arginine ingestion inhibits eccentric contraction-induced proteolysis and force deficit via S-nitrosylation of calpain

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[Introduction] It has been shown that eccentric contraction (ECC), in which skeletal muscles are stretched while contracting, results in long-lasting force depression, at least partly, through calpain-mediated proteolysis of  $\text{Ca}^{2+}$ -regulatory proteins (1) and that exogenously applied nitric oxide decreases the calpain-induced proteolysis (2). The aim of this study was to investigate whether ingestion of L-arginine (ARG), a nitric oxide precursor, inhibits ECC-induced proteolysis of  $\text{Ca}^{2+}$ -regulatory proteins and force deficit by decreasing the proteolytic function of calpain. [Methods] All experimental procedures were approved by the Animal Experimental Committee of Okayama Prefectural University and Hiroshima University. Male Wistar rats (8-9 weeks old) were assigned to a control (CON) or an ARG group ( $n=7$  for each group). The ARG rats were provided water containing 0.3% ARG ad libitum for 7 days starting from 3 days before the ECC protocol (average ingestion 611 mg/kg body wt/day). Under anesthesia by an intraperitoneal injection of a mixture of medetomidine (0.4 mg/kg body wt), midazolam (2.0 mg/kg body wt) and butorphanol (2.5 mg/kg body wt), tibialis anterior (TA) muscles underwent 200 repeated ECCs *in situ*. The rested muscles of the contralateral legs were used as controls. Three days after ECC, dorsiflexion torque was measured under anesthesia and subsequent biochemical analyses were performed in excised TA muscles. Data are presented as means  $\pm$  SEM. A two-way ANOVA followed by Holm-Sidak post hoc test was used to examine the statistical significance. [Results] ARG ingestion attenuated ECC-induced force deficit (torque at 20 Hz:  $57 \pm 3$  for CON-rest,  $34 \pm 3$  for CON-ECC,  $63 \pm 2$  for ARG-rest, and  $49 \pm 2$  mNm for ARG-ECC.  $P < 0.05$ , CON-ECC vs ARG-ECC) and autolysis of calpain-1 ( $12 \pm 2$  for CON-rest,  $20 \pm 3$  for CON-ECC,  $11 \pm 1$  for ARG-rest, and  $13 \pm 1$  % for ARG-ECC,  $P < 0.05$ , CON-ECC vs ARG-ECC), and increased the amounts of S-nitrosylated calpain-1 (CON-rest:  $100 \pm 11$ , CON-ECC:  $142 \pm 47$ , ARG-rest:  $245 \pm 31$ , ARG-ECC:  $247 \pm 34$  %;  $P < 0.05$ : CON-ECC vs ARG-ECC). ARG ingestion completely inhibited proteolysis of ryanodine receptor (CON-rest:  $100 \pm 7$ , CON-ECC:  $65 \pm 7$ , ARG-rest:  $107 \pm 5$ , ARG-ECC:  $93 \pm 10$  %;

$P < 0.05$ : CON-ECC vs ARG-ECC) and dihydropyridine receptor (CON-rest:  $100 \pm 9$ , CON-ECC:  $60 \pm 7$ , ARG-rest:  $97 \pm 5$ , ARG-ECC:  $87 \pm 7$  %;  $P < 0.05$ : CON-ECC vs ARG-ECC) and partially inhibited proteolysis of junctophilin-1 (CON-rest:  $100 \pm 8$ , CON-ECC:  $21 \pm 6$ , ARG-rest:  $107 \pm 5$ , ARG-ECC:  $51 \pm 14$  %;  $P < 0.05$ : CON-ECC vs ARG-ECC). [Conclusion] These results indicate that ARG ingestion can attenuate ECC-induced proteolysis of  $\text{Ca}^{2+}$  regulatory proteins and force deficit by decreasing calpain activation via S-nitrosylation.

Kanzaki K, Watanabe D, Kuratani M, Yamada T, Matsunaga S & Wada M. (2017). Role of calpain in eccentric contraction-induced proteolysis of  $\text{Ca}^{2+}$ -regulatory proteins and force depression in rat fast-twitch skeletal muscle. *J Appl Physiol* 122, 396-405.

Liu R, Li Y, Wang M, Zhou G & Zhang W. (2016). Effect of protein S-nitrosylation on autolysis and catalytic ability of mu-calpain. *Food Chem* 213, 470-477.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCA195

**The magnitude of compression-induced hyperaemia is affected by the driving pressure, more than by the muscle pump.**

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A short-lasting muscle compression (MC), as can be achieved by rapidly inflating and deflating a pneumatic cuff wrapped around a limb, is known to elicit a rapid and transient increase in blood flow to the affected tissues. The determinants of such compression-induced hyperaemia are still largely unknown. The observation that a larger response takes place if the limb is held below as compared to above heart level supports the involvement of the muscle pump (Credeur et al., 2015). On the other hand, other studies have argued against this hypothesis (Jasperse et al 2015). Aiming to identify the determinants of the compression-induced hyperaemia, this study investigates the effects of modulating the pressure levels that precede (pre-compression) and follow (post-compression) the MC stimulus, as a mean to affect vascular filling and perfusion pressure, respectively.

In order to deliver the intended pressure profiles, a new a pneumatic system was developed and tested

In 10 healthy subjects, haemodynamic changes were recorded in response to the MC stimulus (200 mmHg, 1-s duration) preceded or followed by pre- and post- compressions in the range 0-50 mmHg, and 50-s duration.

The subjects sat on a chair with the leg extended horizontally, laying on a pillow. The haemodynamic response to MC was assessed by recording both the arterial inflow and venous outflow, by Doppler echography at the level of the femoral artery and vein, respectively. The magnitude of the hyperaemic response was quantified as the excess blood volume supplied to the lower leg during the hyperaemia (time integral of blood flow increase over the duration of the hyperaemia), while the displaced blood volume was measured as the time integral of venous blood flow over the duration of the MC.

Increasing the pre-compression level from 10 to 50 mmHg progressively reduced the venous blood volume displaced by

the MC, (by  $70 \pm 16$  % at 50 mmHg,  $P < 0.05$ ), as compared to the MC without pre-compression. However, the magnitude of the hyperaemic response to MC (excess blood volume =  $48.8 \pm 14.1$  ml) was not affected by pre-compression at any of the tested pressure levels.

On the contrary, the hyperaemic response was progressively and strongly reduced by the application of post-compressions (by  $85 \pm 18$  % at 50 mmHg,  $P < 0.05$ ).

A new pneumatic prototype has been developed and validated allowing to deliver customizable compressive stimuli with pre-and post-compressions of adjustable pressure level. As expected, pre-compression reduced vascular filling, as indicated by the decrease in displaced blood volume, thus reducing the efficacy of the muscle pump. The independence of the hyperaemic response to MC on pre-compression level indicates a negligible role of the muscle pump. On the contrary, its strong dependence on the post-compression level suggests that changes in driving pressure are a major determinant of compression-induced hyperaemia.

Credeur DP, Holwerda SW, Restaino RM, King PM, Crutcher KL, Laughlin MH, Padilla J & Fadel PJ. (2015). Characterizing rapid-onset vasodilation to single muscle contractions in the human leg. *J Appl Physiol* (1985) 118:455-464.

Jasperse JL, Shoemaker JK, Gray EJ & Clifford PS. (2015). Positional differences in reactive hyperemia provide insight into initial phase of exercise hyperemia. *J Appl Physiol* (1985) 119:569-575.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCA196

**Effects of eccentric exercise on myofibrillar function and amount of  $\alpha$ B-crystallin binding to myofibrils in skeletal muscle from adjuvant-induced arthritis rats**

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Patients with rheumatoid arthritis (RA) suffer from muscle weakness. We have previously demonstrated that this abnormality is attributed to oxidative stress-induced myofibrillar dysfunction in adjuvant-induced arthritis (AIA) rats, a widely used animal model for RA (Yamada et al, 2015). In response to eccentric (ECC) contractions, but not concentric contractions, the small heat shock protein  $\alpha$ B-crystallin binds to myofibrils and protect them from degeneration (Frankenberg et al, 2014). The aim of this study was to determine whether ECC exercise prevents myofibrillar dysfunction in skeletal muscles from AIA rats. All experimental procedures were approved by the Committee on Animal Experiments of Sapporo Medical University (No. 15-083). Wistar rats (10 week old,  $n=18$ ) were assigned into control (CNT) ( $n=6$ ), AIA ( $n=6$ ), and AIA+ECC ( $n=6$ ) groups. Under 2% isoflurane anesthesia, AIA was induced in the knees of rats by an injection of complete Freund's adjuvant. Throughout the ECC exercise, rats were anesthetized by isoflurane inhalation. To induce ECC contractions, planter flexors were electrically stimulated via surface electrodes (45 V, 30 Hz, and 2 s / 4 s duty cycle) while the ankle was forcibly dorsiflexed by servomotor ( $20 \text{ deg s}^{-1}$ ). ECC exercise was applied every other day for 3 weeks and consisted of 4 sets of 5 contractions. After the intervention period, rats were killed by cervical dislocation under isoflurane

anesthesia and gastrocnemius muscles were excised for mechanical and biochemical analyses. Data are presented as mean  $\pm$  SEM. One-way ANOVA followed by Bonferroni post hoc test was used to examine the statistical significance. ECC exercise prevented the AIA-induced decreases in maximum  $\text{Ca}^{2+}$ -activated force in skinned fibers (CNT:  $362 \pm 8$ , AIA:  $239 \pm 11$ , AIA+ECC:  $344 \pm 11$  mN/mm<sup>2</sup> [ $n=40-51$ ];  $p < 0.05$ : AIA vs AIA+ECC) and myosin heavy chain (MyHC) and actin expression ([ $n=9$ ]; AIA vs AIA+ECC,  $p < 0.05$ ). Moreover, AIA muscles showed increased expression levels of malondialdehyde-protein adducts, NADPH oxidase, and neuronal nitric oxide synthase ([ $n=5-6$ ]; CNT vs AIA,  $p < 0.05$ ). ECC exercise markedly increased the steady-state levels of  $\alpha$ B-crystallin and its binding to the myofibrils in AIA muscles ([ $n=6-9$ ]; AIA vs AIA+ECC,  $p < 0.05$ ). In conclusion, ECC exercise preserves myofibrillar function by preventing the loss of MyHC and actin in AIA rats. These beneficial effects can be ascribed to the protective effect of  $\alpha$ B-crystallin on the myofibrils against oxidative stress-mediated protein degradation.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

#### PCA197

##### **The muscle relaxant methocarbamol blocks the muscular voltage-gated $\text{Na}^+$ channel $\text{Na}_v1.4$ and affects force characteristics of isolated mouse soleus muscles**

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The muscle relaxant methocarbamol is widely used to treat skeletal muscle spasms and pain syndromes. Besides its central actions, there are reports about peripheral effects of methocarbamol, e.g. a prolongation of the refractory period of muscles. The current study was designed to clarify peripheral effects of methocarbamol and to detect possible molecular targets in skeletal muscle. The refractory period is mainly determined by voltage-gated  $\text{Na}^+$  channels. Thus, we investigated effects of methocarbamol on  $\text{Na}_v1.4$  channels and studied its action on electrically stimulated mouse muscles. Voltage-gated  $\text{Na}^+$ -currents were recorded from HEK 293 cells stably expressing the human  $\text{Na}_v1.4$ . I/V curves, steady-state inactivation curves and recovery of the channels from inactivation were studied in the presence and absence of 2 mM methocarbamol. Muscle force and fatigue were studied on isolated soleus muscles from 100 d old mice. Muscles were stimulated to produce twitches and tetani. Then, in a second run twitches and tetani were recorded the presence and absence of methocarbamol. Subsequently, a protocol of sustained repetitive stimulation was applied.

Transient  $\text{Na}^+$  inward currents could be stably recorded from HEK 293 cells expressing the  $\text{Na}_v1.4$  (voltage pulses from -85 mV to -10 mV). On application of methocarbamol the  $\text{Na}^+$  current amplitudes were inhibited on average by  $37 \pm 8\%$  ( $n=16$ ). The effect occurred within 10 s and was reversible. While I/V curves were only marginally affected by methocarbamol, inactivation curves were shifted on average by -8.4 mV (from -71.6 mV to -80.0 mV,  $n=16$ ). Methocarbamol slowed recovery from inactivation of  $\text{Na}_v1.4$  channels. The mean recovery time constant was 3.2 ms in external solution. In the presence of methocarbamol a second slow recovery phase with a time constant of  $55 \pm 20$  ms ( $n=12$ ) appeared. Methocarbamol did not affect twitches, but tetanic force at 120 Hz. Normalized tetanic force was reduced to  $0.88 \pm 0.01$  by methocarbamol ( $p < 0.001$ ). In agreement with the mentioned findings, the tetanus/twitch ratio was lowered by methocarbamol by 15%. The fatigue process was accelerated. The time to reach 50% of initial force was reduced from  $180 \pm 29$  s (control) to  $149 \pm 37$  s (2 mM methocarbamol,  $p < 0.05$ ). The muscle relaxant methocarbamol has marked effects on force characteristics of isolated mouse muscles. It reduced tetanic force and accelerated muscle fatigue during long-term stimulation. These observations agree with the effect of methocarbamol on  $\text{Na}_v1.4$  channels, responsible for the refractory period. The findings are also in line with the observation that recovery from inactivation of  $\text{Na}_v1.4$  channels was slowed by methocarbamol. The study shows evidence that  $\text{Na}_v1.4$  channels are molecular targets of methocarbamol, explaining some of its peripheral effects.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

#### PCA198

##### **Standardized scores show similar gender differences in jumping ability and inspiratory strength in healthy elderly**

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Increasing importance of Rate-of-Force-Development (RFD) in the elderly (1) motivates the introduction of some new variables. As a consequence, functional training and testing may include the Maximum Inspiratory Pressure (MIP) and the performance in a Countermovement Jump (CMJ) when talking about respiratory or functional capacity respectively. Previous studies have already shown the influence of gender in these strength capacities, but normative data are still scarce in healthy and active elderly, mainly regarding the elastic power (height and relative power (2) in the CMJ). Moreover, to our knowledge, this is the first study to combine their testing, and to standardize the scores so as to look for gender similarities and differences on both neuromuscular capacities. 24 non-smokers healthy elderly (9 men:  $69.81 \pm 4.19$  years; 15 women;  $72.41 \pm 4.21$  years), undergoing a multicomponent exercise training program for at least one year, were assessed for body composition, blood pressure and MIP (3), and for CMJ (4) one week later. Student-T tests for independent samples were conducted after testing for normality, and once standardized, the Confidence Intervals (95%) were calculated and



graphically plot for gender and muscular capacity comparisons. As expected, despite the aging process, men displayed always significantly larger strength (MIP:  $84.78 \pm 16.33$  vs  $54.13 \pm 15.39$  cmH<sub>2</sub>O; Height:  $18.46 \pm 4.60$  vs  $11.15 \pm 3.00$  cm; and Relative Power:  $35.71 \pm 3.52$  vs  $25.47 \pm 3.88$  w\*kg<sup>-1</sup>;  $p=0.000$ ). Standardized data and the plot of CI (95%) showed how similar were the scores and the influence of gender despite assessing RFD through such different muscular capacities (figure 1). Moreover, relative power seems more appropriate to assess elastic power due to gender differences in body composition. Standardizing the scores allowed us to find out similarities in the muscle strength from different muscular systems, although MIP for men and women in our study was a bit lower considering the average population for their age and gender (normative ranges: 71-135 vs 39-91 cmH<sub>2</sub>O respectively) (5). New studies will display normative data for the jumping capacity in active elderly, helping researchers to understand this finding.

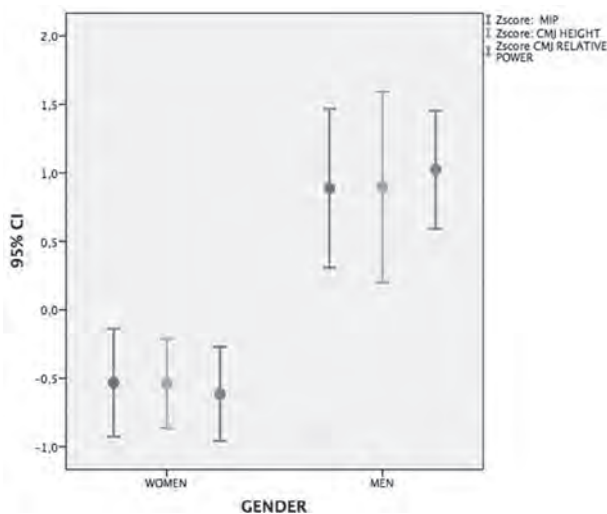


Figure 1. Confidence Interval (95%) plot for MIP, CMJ Height and relative power comparing men and women (standardized scores).

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## Evidence from a mouse model that high levels of circulating dihydrotestosterone (DHT) improves power and speed but reduces endurance in isolated fast- and slow-twitch muscles in males and females

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It is well known that in humans' men have more muscle mass and strength than women. There is strong, direct evidence that the effects of testosterone on muscle mass and strength are dose-dependent on circulating testosterone. However, these studies were largely performed in men and it is neither feasible nor ethical to subject women to high circulating testosterone levels for sufficiently long periods to test this dose-dependency in healthy women. In this study, we use a mouse model to investigate the effects of high circulating concentrations of the potent pure androgen DHT directly on muscle mass and function in both mature male and female wildtype mice. Each mouse was treated with DHT by subdermally implanted silastic tube containing ~10mg crystalline DHT which provide prolonged, steady-state DHT delivery for months if required. DHT was implanted into sexually mature females and orchidectomized males to equalize endogenous androgen exposure. All operative procedures were performed under anesthesia administered by intraperitoneal injection of a 4 mg/ml solution of ketamine and xylazine 100microliter/10 g body wt. Fast-twitch EDL muscle and slow-twitch soleus muscle were dissected from the hind limb and tied to a dual force transducer/linear tissue puller. Each muscle was then placed in a bath containing Krebs solution and bubbled continuously with carbogen. The muscle was stimulated by delivering a current between two parallel platinum electrodes. Isolated muscle contractile properties were analysed using the 615A Dynamic Muscle Control and Analysis software (Aurora Sci, Ins.). Muscles were trimmed and weighed at the end of the protocol. In DHT treated animals there was a significant increase in mass of EDL muscle from males and females. Male Blank  $9.7\text{mg} \pm 0.3$  n=8 Male DHT  $11.5\text{mg} \pm 0.4$  n=8 ( $P<0.001$ ); Female Blank  $8.7\text{mg} \pm 0.1$  n=10, Female DHT  $12.0\text{mg} \pm 0.2$  n=7 ( $P<0.0001$ ). In soleus muscles, only treated females showed a significant increase in mass (Male Blank  $10.0\text{mg} \pm 0.5$  n=10 Male DHT  $11\text{mg} \pm 0.3$  n=8 (ns); Female Blank  $9.0\text{mg} \pm 0.5$  n=8 and Female DHT  $11.5\text{mg} \pm 0.4$  n=8 ( $P<0.01$ ). There was also a very significant increase in maximum force output from DHT EDL muscles in both males and females (Male blank  $339\text{mN} \pm 6$  n=6 c.f. Male DHT  $386\text{mN} \pm 6$  n=8 ( $P<0.0001$ ); Female Blank  $292\text{mN} \pm 3$  n=10 and Female DHT  $377\text{mN} \pm 7$  n=7 ( $P<0.0001$ ); t-test/SEM/n=muscles. Interestingly, in DHT-treated female mice both soleus and EDL muscles showed a significant ( $P<0.0001$ )  $16\% \pm 5$  &  $18\% \pm 4$ , slowing of recovery from fatigue, which was not present in DHT-treated male mice. Increasing circulating androgen levels in female mice and orchidectomized male mice appears beneficial for power activities but reduces endurance performance in females.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA200

**MuRF1 and MuRF2 independently act to mediate muscle wasting in mice with cardiac cachexia**T. Bowen<sup>1</sup>, T. Nguyen<sup>2</sup>, S. Werner<sup>2</sup>, S. Labeit<sup>3</sup> and V. Adams<sup>4</sup><sup>1</sup>School of Biomedical Sciences, University of Leeds, Leeds, UK, <sup>2</sup>Leipzig Heart Center, Leipzig, Germany, <sup>3</sup>Universitätsklinikum Mannheim, Mannheim, Germany and <sup>4</sup>Dresden Heart Center, Dresden, Germany

Patients with cardiac cachexia are characterized by a severe loss of muscle mass, which reduces strength to limit mobility and quality of life. The mechanisms mediating muscle loss in cardiac cachexia however remain poorly established. Evidence indicates a key role for the muscle-specific ubiquitin E3 ligase muscle RING finger 1 (MuRF1), which is supported by this atrogen being consistently activated in acute wasting conditions. We have collected initial data to also show the second MuRF family member, MuRF2, is activated during cardiac cachexia. The current study, therefore, aimed to investigate whether both MuRF1 and MuRF2 can act independently to mediate muscle atrophy during cardiac cachexia. C57BL/6 wild-type (WT) mice (n=21) were compared to global MuRF1 (n=23) or MuRF2 (n=20) knockouts. Mice in each group were randomized to either weekly saline or monocrotaline (600 mg/kg) treatment (both i.p.). After 8 weeks, fibre cross-sectional area was measured using standard histology, while *in vitro* contractile function was assessed by direct stimulation. Protein expression (arbitrary units; a.u.) was determined by Western blot from muscle homogenates. Values are presented as mean±SEM and were assessed by unpaired t-test. Compared to saline-treated controls, mice treated with monocrotaline developed cardiac cachexia, with evidence of weight loss, pulmonary congestion, and right-ventricular hypertrophy (P<0.05). Compared to controls, the protein expression of both MuRF1 (0.12±0.01 vs. 0.21±0.02 a.u.) and MuRF2 (0.22±0.04 vs. 0.38±0.04 a.u.) was elevated (P<0.05) in muscle of WT mice with cardiac cachexia. Only WT mice with cardiac cachexia demonstrated muscle atrophy compared to saline-treated controls: muscle wet-weights of the soleus and tibialis anterior were reduced (P<0.05) by 11±3% and 13±3% respectively, while TA fibre cross-sectional area was reduced (P<0.05) by 32±6%. No differences (P>0.05) in muscle wet-weights or fibre cross-sectional area were observed in MuRF1/2 knockout mice with cardiac cachexia when compared to matched-controls. Maximal contractile force in the soleus of WT mice with cardiac cachexia was reduced (P<0.05) by 16±2% compared to controls, yet no impairments were developed in MuRF1/2 knockouts. Loss of muscle mass and function induced by cardiac cachexia was prevented in mice with a genetic deletion in MuRF1 or MuRF2. Overall, therefore, these data indicate that both MuRF family members, 1 and 2, can act independently to mediate muscle loss in cardiac cachexia.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## PCA201

**Myopenia in Obstructive Pulmonary Disease: an indicator of poor prognosis**

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**Background:** Muscle Mass (MM) wasting, frequently observed in advanced stages of COPD is currently considered as a major predictor of mortality.

**Aim:** The aim of this study was to investigate possible correlations between MM wasting, severity of airflow limitation and tolerance to sub-maximal physical exertion in a group of COPD patients.

**Methods:** This prospective study included 46 male COPD patients. Slow and forced spirometry, bronchial reversibility test with salbutamol, COPD Assessment Test (CAT), bioelectric impedance measurement and Six-Minute walk Test (6MWT) were performed for all patients.

**Results:** The average values of age and MM were respectively 60 ± 19 years and 35,5 ± 3,8%. Spirometric data revealed that 76% of patients were in stages 3 and 4 of disease (GOLD 2017). Significant correlations (p <0.05) were found between MM and these following parameters: Forced Expiratory Volume in one second (FEV1) post bronchodilator as indicator of severity of airway limitation, difference between Slow and Forced Vital Capacity (SVC - FVC) as indicator of trapping air volume and 6 MWT distance as indicator of global tolerance to physical exertion.

Our data revealed also a significant correlation between myopenia and multiparametric BODE index (Body Mass Index, Airflow Obstruction, Dyspnea, Exercise Capacity) as indicator of poor prognosis.

**Conclusion:** Regular assessment of body composition in particular MM should be systematically performed in all COPD patients to ensure early diagnosis of myopenia and to start as soon as possible nutritional management and respiratory rehabilitation.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## PCA202

**Carotid body chemosensitivity correlates with insulinemia and insulin resistance but not with blood pressure in metabolic syndrome patients**S.V. Conde<sup>1</sup>, J.P. Cunha-Guimaraes<sup>1</sup>, A.T. Timoteo<sup>2</sup>, I. Caires<sup>1</sup>, J.F. Sacramento<sup>1</sup>, M.J. Ribeiro<sup>1</sup>, M. Selas<sup>2</sup>, M.P. Guarino<sup>1,3</sup>, J. Santiago<sup>1</sup> and M. Mota-Carmo<sup>1,2</sup><sup>1</sup>CEDOC, NOVA Medical School, Faculdade Ciências Médicas, Lisboa, Portugal, <sup>2</sup>Serviço de Cardiologia, Hospital Santa Marta, Centro Hospital Lisboa Central, EPE, Lisboa, Portugal and <sup>3</sup>Escola Superior de Saúde de Leiria- Instituto Politécnico de Leiria, Leiria, Portugal

We previously showed that the carotid body (CB) is involved in the genesis of metabolic syndrome (MS) since abolishment of its activity prevents and reverses insulin resistance (IR) and hypertension (HT) in metabolic syndrome and type 2 diabetes rat models(1,2). Additionally, we described that CB activity is increased in rodent models of MS(1). Herein,

we have investigated if CB chemosensitivity is correlated with insulinemia, insulin resistance and enhanced blood pressure in MS and HT patients.

MS and HT patients were recruited at the Cardiology Service, Hospital Santa Marta, Centro Hospitalar Lisboa Central, EPE. Inclusion criteria for MS and HT were those defined by the American Heart Association. The study was approved the Ethics Committee and performed in accordance with the Helsinki Declaration. Written informed consent was obtained from all individuals. Peripheral chemosensitivity was assessed by the Dejours test, which measures the decrease in respiratory frequency (Rf) caused by two breaths of 100%O<sub>2</sub>. Intima-media thickness, blood pressure and glucose tolerance were evaluated as well as plasma insulin, glucose, HbA1c and lipid profile. Correlations between the variables were obtained by Spearman correlations, stepwise multiple linear regression analysis by SPSS and 2-way ANOVA. Data are means±SD.

Thirty-two MS (Age=60.5±11.8 years) and 17 HT patients (Age=67.7±10.4 years) were included. Systolic blood pressure was 150.6±20.8mmHg and 142.5±14.7mmHg in MS and HT patients. Abdominal perimeter was 100.3±12.9cm and 87.8±13.4cm in MS and HT patients (p<0.01). Fasting and 2-hours glucose following OGTT were, respectively, 98.6±41.8mg/dl and 180.2±65.8 mg/dl in MS (p<0.05) and 76.9±21.7 mg/dl and 131.6±22.9 mg/dl in HT. Fasting insulin and HOMA-IR were, respectively, 10.6±7.7 µU/ml and 2.7±2.7 (p<0.05) in MS and 7.0±4.1 µU/ml and 1.3±1.1 in HT patients. No significant differences were observed in the lipid profile between MS and HT patients except for HDL-c (p<0.01). Additionally, a non-statistical increase in CB chemosensitivity was found in MS patients, since the % of decrease in Rf produced by 100%O<sub>2</sub> is higher than in HT patients (MS= -5.5±12.6 vs HT= 0.4±21.4). Significant correlations were found between CB chemosensitivity and abdominal perimeter (r= -0.47, p<0.05), insulin levels (r=-0.72, p< 0.001) and HOMA-IR (r =-0.54, p< 0.01), in MS patients, but not with the others variables tested. No correlations were found between CB chemosensitivity and all the variables tested in HT patients. This work confirms our preclinical data showing that MS patients exhibit an increased CB chemosensitivity and that insulin levels and sensitivity are inversely correlated with CB activity. Additionally, our data suggest that CB chemosensitivity is not altered in HT.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA203

### Upregulation of the anti-apoptotic protein, humanin (HN), is present in multiple human cancer cell lines and tumor tissues.

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Despite rapid progress in the understanding and development of novel therapies for human cancers, there remains significant need for additional therapeutic opportunities, as all forms of cancer are responsible for millions of deaths worldwide and tens of millions additionally sickened.<sup>1</sup> As such the identification of new targets of therapy and modifiers of disease will continue to provide additional avenues for improvement for targeted therapies and outcomes. One such novel target for therapy that has been revealed is humanin (HN), a 24-amino acid peptide encoded in mammalian mitochondrial genomes with known anti-apoptotic activity.<sup>2</sup> The anti-apoptotic activity of HN implies potential pro-oncogenic phenotypes if overexpressed or otherwise inappropriately upregulated in cells. In order to ascertain the potential of HN as an oncogene, we first examined cell lysates of cultured non-malignant human ovarian surface epithelial (HOSE) cells and malignant SKOV3 and OVCAR-3 ovarian carcinoma cell lines *in vitro*. We found significantly (p≤ 0.05) increased HN protein expression in both malignant cell lines compared to non-malignant cells. We then expanded our immunoblot study to include multiple paired tissue lysates from biopsied human tissues-both normal and malignant. Of the 17 different tissue types we examined, six tissues showed a significantly higher (p ≤ 0.05 vs. non-tumor samples) expression of HN protein in tumorous tissue. This included adrenal, bladder, kidney, liver, lung, and thyroid. Other tissues, including ovarian, prostate, stomach and uterine, trended to higher levels, but did not reach statistical significance in this experiment. To corroborate these findings *in vivo*, we utilized arrays of tumor and normal tissue biopsies to statistically quantify HN protein expression using double-blind methodology. Our results indicate that HN is frequently upregulated in human cervical and thyroid cancers versus normal tissue biopsies, with additional studies are underway. We thus conclude that HN functionally contributes to the ability of cells to resist signals that would induce death in normal cells and tissues. Therefore, we conclude that HN represents a new target for anti-cancer therapeutic investigation.

Cancer Research UK (2018) <http://www.cancerresearchuk.org/health-professional/cancer-statistics/worldwide-cancer>

Yen K, et al. (2013) *Mol Endocrinol*. 11

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.



PCA204

### Effect of bitter melon extract, kuguacin-J and cisplatin on MCF-10A, MCF-7 and MDAMB-231 breast cancer cell lines *in vitro*

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*Momordica charantia* possess anti-carcinogenic properties and it can modulate its effect via calcium overloading-induced oxidative stress resulting in cancer cell death. This study investigated the potential chemotherapeutic effect of an ethanol soluble extract of *M. charantia*, kuguacin-J (K-J), an isolated compound from *M charantia* and cisplatin, a commercial anticancer drug on MCF-10A (normal human mammary primary epithelial healthy cells), MCF-7 (human mammary primary epithelial cancer cells) and MDAMB-231 (human mammary primary epithelial –triple negative (TNBC) cancer cells) breast cancer cell lines. The different cells were treated with varying concentrations (8 µg - 800 µg/ml) of either the extract, K-J or cisplatin (all dissolved in DMSO and diluted in the incubating medium) for 48 hours. Furthermore, cisplatin was combined with either the ethanol extract or K-J to investigate any combined effects on cell death. Cell viability and caspase activity were measured using established methods. The results show that either the extract of *M. charantia* or K-J had no effect on MCF-10A normal cell line compared to cisplatin which killed the healthy cells, especially at high concentrations. MCF-10 cell viability was 100 %, 100 % and 3.2 % for 80 µg/ml of the extract, K-J and cisplatin, respectively, compared to untreated cells (100%). With MCF-7, either the ethanol extract or K-J killed the cells only at high concentrations (80 µg - 800 µg/ml) compared to cisplatin which killed the cells using 8 µg - 800 µg/ml either alone or when combined with the extract or K-J. Typically, the cell viability for MCF-7 was 18.5 %, 4.6 % and 5.1 % for 800 µg/ml of the extract, K-J and cisplatin, respectively compared to untreated cells (100%). In contrast, all concentrations (8 µg - 800 µg/ml) of either the extract, K-J or cisplatin either alone or when combined killed MDAMB-231 cell line almost by 100%. MDAMB-231 cell viability was 1.58 %, 1.81% and 0.74 % for 8 µg/ml of the extract, K-J and cisplatin, respectively when compared to non-treated cells (100%). Furthermore, either the extract of *M. charantia*, K-J or cisplatin (800 µg/ml) elicited significant (Student's t-test;  $p < 0.05$ ) increases in caspase - 3 activity from MCF-7 and MDAMB-231 breast cancer cells compared to untreated cells. The results demonstrated that either the ethanol soluble extract of *M charantia* or K-J can be used effectively to treat breast cancer, especially MDAMB-231 breast cancer cells.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA205

### Stimulus induced elevations in lactate in mouse optic nerve are fuelled by glucose, not glycogen

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Increased neural activity in the mammalian nervous system is accompanied by transient elevations in interstitial  $[K^+]$  and lactate. In the mouse optic nerve (MON) high intensity stimulus produced frequency dependent transient elevations in  $[lac^-]_o$ , whose amplitude was logarithmically related to the accompanying increases in interstitial  $[K^+]$ . We demonstrate that the source of the lactate is glucose and not glycogen, despite MON astrocytes containing glycogen reserves.

All procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986 under appropriate authority of project and personal licenses. Adult male CD-1 mice were killed by cervical dislocation and decapitated. Optic nerves were dissected, placed in a perfusion chamber, superfused with aerated aCSF containing 10 mM glucose. The stimulus evoked compound action potential was evoked with supra-maximal stimuli. Lactate biosensors (sarissa.co.uk) recorded  $[lac^-]_o$ .

Lactate is continuously released from the mouse optic nerve with  $[lac^-]_o$  of  $72.8 \pm 11.4$  microM ( $n = 27$ ) recorded at the periphery of the MON bathed in 10 mM glucose aCSF. Decreasing the aCSF [glucose] to below 5 mM resulted in step wise decreases in  $[lac^-]_o$ , but increasing [glucose] above 5 mM had no effect on  $[lac^-]_o$ . The consonant relationship between [glucose] and  $[lac^-]_o$  implies glucose is the primary source of lactate. To test this we withdrew glucose from aCSF bathing the MON and found a rapid decrease in  $[lac^-]_o$ , followed shortly thereafter by the CAP fall. We next tested the source of  $[lac^-]_o$ . MONs were stimulated at 50 Hz for 2 minutes, while changing aCSF [glucose]. There was no difference in the stimulus induced  $[lac^-]_o$  peak when perfused in either 10 mM or 30 mM glucose, but with 5 mM glucose there was a significant drop in the lactate spike amplitude, that recovered upon switching to 30 mM glucose. Changing the [glucose] from 10 mM to 2 mM caused a decrease in basal  $[lac^-]_o$  as well as severely attenuating the stimulus evoked lactate transient. Qualitatively consistent results were obtained when the [glucose] was switched to 3 mM or 4 mM. In MONs perfused with 10 mM glucose aCSF, repeated stimuli evoked reproducible transient  $[lac^-]_o$  increases. However when [glucose] was changed from 10 mM to 2 mM, the stimulus evoked  $[lac^-]_o$  transients disappeared rapidly, as the baseline  $[lac^-]_o$  began to fall.

The lactate appears to come from glucose and not glycogen since decreasing glucose availability abolished lactate release and DAB had no effect on stimulus-evoked  $[lac^-]_o$  increases. The profile of the stimulus evoked lactate release suggested that the onset of stimulus promoted axonal uptake of lactate, followed by a substantial lactate release. On cessation of the stimulus the lactate continued to increase, consistent with instantaneous decrease in axonal lactate requirements but continued production.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA206

### Differential regulation of mRNA expression by the transcription factors WT1 and GATA4 during murine gonad development

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Gonad morphogenesis relies on the correct spatiotemporal expression of a number of genes that together fulfill the differentiation of the bipotential gonad into either testes or ovaries. Mutations in the human genes encoding the transcription factors GATA4 and WT1 cause malformations of the genitourinary system. Furthermore, mice with disrupted *Gata4* or *Wt1* genes are embryonic lethal and fail to develop gonads. WT1 and GATA4 are both expressed in the bipotential embryonic gonad and in the adult ovaries and testes. To compare the influence of WT1 and GATA4 on gene expression in male and female gonads during embryonic development, we combined a morpholino-induced gene knockdown technique with ex vivo embryonic organ cultures. For this purpose, WT1 and GATA4 protein translation were inhibited in cultured murine embryonic gonads by transfection with specific antisense vivo-morpholinos. Samples were analyzed by next generation mRNA sequencing (RNASeq). Our data analysis revealed striking differences between WT1 and GATA4 in the regulation of mRNA expression in male and female gonads. WT1 knockdown decreased testis promoting mRNAs and increased ovarian promoting transcripts in developing XY-gonads. Furthermore, antisense inhibition of WT1 in embryonic XX-gonads reduced mRNAs favoring ovary formation. This sex-specific mRNA expression pattern was not observed upon knockdown of GATA4, which regulates the same set of genes in male and female gonads.

These data demonstrate that WT1 and GATA4 play different roles in the embryonic gonads. While WT1 accounts for a sex-specific gene expression pattern, GATA4 may be required to adjust the mRNA levels that are necessary for normal gonad differentiation in both sexes.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA207

### Role of JmJc Histone Demethylase in the Development of Non-Alcoholic Fatty Liver Disease

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Non-alcoholic fatty liver disease (NAFLD) is caused by excessive fat accumulation in the hepatocytes. Mild steatosis may develop into aggressive forms of NAFLD including hepatic fibrosis, cirrhosis and carcinoma. Sterol Regulatory element-binding transcription factor 1c (SREBP1c) is master transcription factor of the lipogenesis and the major regulators of NAFLD. JHDM, a JmJc histone demethylase, is known as the demethylase of the Histone H3K9 while it binds to H3K4me3 with the Plant-Homeodomain (PHD). In the current study,

JHDM can bind and directly demethylate target protein. JHDM is known to have associated with metabolism-related transcription factors. In this study, we investigated the role of JHDM in the progression of NAFLD. For in vivo study, WT and JHDM overexpressed TG mice were fed normal diet or high fat diet for 8 weeks. And then, liver steatosis and blood chemistry were analyzed. Interestingly body weight and fat content was significantly decreased in TG mice and hepatic steatosis was improved. In HepG2 cells, overexpression of JHDM decreases SREBP1c protein and mRNA level and target gene mRNA level of SREBP1c. H3K9 demethylation is regarded as transcription-active, we considered another effects of JHDM. Using immunoprecipitation, we tested protein interaction between JHDM and SREBP1c. JHDM directly interacts with SREBP1c at PHD domain of JHDM and Helix-loop-Helix domain of SREBP1c. In addition, we found that overexpression of JHDM attenuates lipid accumulation through oil red o staining. These results suggest that JHDM plays a role as a repressor in the progression of NAFLD through the association with SREBP1c.

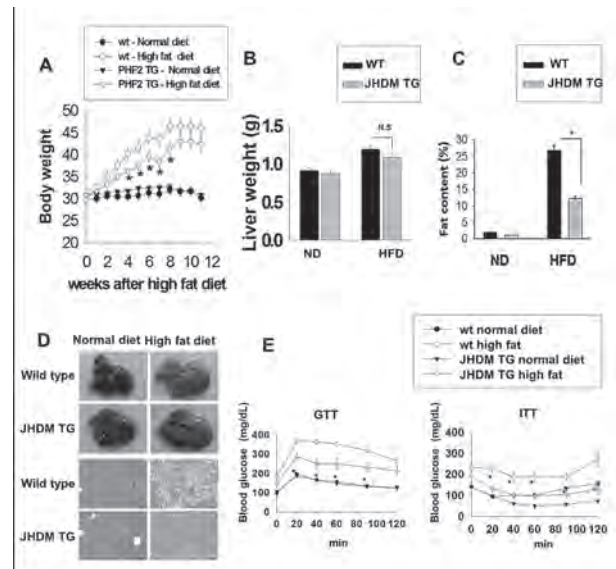


Figure 1. JHDM TG mice fed with HFD gain less weight and insulin Resistance.

(A) Body weight of each group was measured for 8 weeks. (B) Liver weight of WT and TG mice fed HFD were measured after 8 weeks. Data shown represent the mean  $\pm$  SEM and \* denotes  $P < 0.05$ . (C) Total fat contents were analyzed in liver tissues. (D) Represent pictures of WT and TG liver fed HFD for 8 weeks and H&E staining of Liver section. (E) GTT was performed after 12 h fasting and ITT was 6 h fasting. Compared to WT mice, TG mice displayed less glucose intolerance and insulin resistance. Data shown represent the mean  $\pm$  SEM ( $n=8$  mice/group) and \* denotes  $P < 0.05$

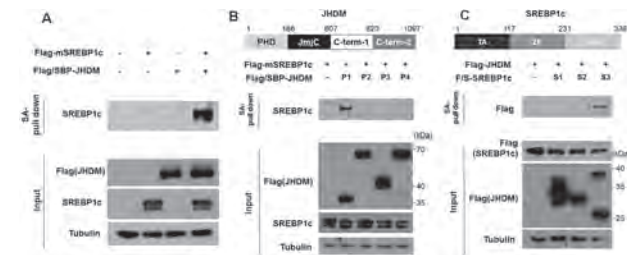


Figure 2. JHDM directly interacts with SREBP1c.

(A) HEK293T cells were co-transfected with Flag-mSREBP1c, Flag/SBP-JHDM. Cell lysates were pulled down using streptavidin beads and the precipitated proteins were immunoblotted with SREBP1c antibody. (B) The top panel shows four domain of JHDM. HEK293T cells were co-transfected with Flag-mSREBP1c, Flag/SBP-JHDM domain fragments. Cell lysates were pulled down using streptavidin beads and the precipitated proteins were immunoblotted with SREBP1c antibody. P1, Plant homeodomain; P2, Jumonji-C domain; P3, P4, C-terminal domain. (C) The top panel shows three domain of SREBP1c. HEK293T cells were co-transfected with Flag-JHDM, Flag/SBP-SREBP1c domain fragments. Cell lysates were pulled down using streptavidin beads and the precipitated proteins were immunoblotted with Flag antibody. S1, Transactivation domain; S2, Zinc-Finger domain; S3, helix-loop-helix leucine zipper domain.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA208

### Development of a human fungiform taste bud cell line: characterization and cell physiological studies

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Recently, there has been an upsurge of publications in the field of taste physiology, in order to screen sweeteners or other taste enhancers. There are five basic taste modalities sweet, umami, bitter, salt and sour. There is increasing evidence that there might be a new sixth taste modality, involved in oro-sensory perception of dietary fat. The taste receptors (TR) are localized on the apical region of lingual taste bud cells which are the units of taste papillae. There is approximately 5,000 taste buds in human. Each taste bud contains 50–100 taste cells, which are renewed after every 10 days. Taste bud cells contain 4 cell types with distinct functions: Type I (glial-like) cells, Types II (taste receptor) cells, Type III (neuronal-like) cells and Type IV (progenitor) cells.

Taste receptors for sweet, umami, fat, and bitter are mainly expressed by type II cells. T2R is involved in the perception of the bitter whereas the heterodimers, T1R2/T1R3 and T1R2/T1R3, detect umami and sweet tastes, respectively. Recently it has been reported that Fat taste is detected by CD36, and GPR120 and GPR40. The T1R2 is coupled to G-protein whereas

T1R3 is associated with PLCβ. It is very difficult to obtain human taste bud cells as this implies ethical approval, though the interest is very large. There is an indispensable requirement to immortalize human taste bud cells. We isolated human papillae from the donors that were admitted to the Stomatology department of the CHU. Human fungiform papillae were picked off by using a scissors under local anaesthesia and subjected to enzymatic digestion that contained elastase and dispase mixture. The isolated cells were cultured for 24 h in a mixture of Iscove's Modified Dulbecco's medium and MCDB 153, supplemented with 10% fetal bovine serum and antibiotics. Furthermore, the cells were transfected for 8 hours with h-tert lentivirus and after 15 days of culture, some clones were picked off and expanded until their characterization.

By employing western blot and RT-qPCR techniques, we observed that some clones expressed the receptors of all the 6 taste modalities, i.e., T1R1/T1R2/T1R3, T2R16/T2R38 and CD36 and GPR120. In addition, these cells expressed both alpha gustducin and PLC involved in downstream signal transduction. By employing Fura-2/AM, we noted that the selected positive clones also responded to all the taste modalities by inducing an increase in free intracellular Ca<sup>2+</sup> concentrations. Finally, we report that our immortalized human taste bud cells resemble to endogenous gustatory taste cells phenotypically and physiologically, and can be used for in vitro investigations. All the experimental procedures were performed as per Helsinki declarations and the protocol was approved by the Regional Human Ethical Committee

We greatly acknowledge the financial assistance sanctioned by the SATT-Grand Est (France).

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA209

### Immortalization of mouse fungiform and circumvallate taste bud cell lines: physiological characterization

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Taste buds, localized in the lingual papillae, are responsible for the gustatory perception of several taste modalities such as bitter, sweet, sour, salt and umami. The taste buds are principally localized in three lingual papillae, i.e., fungiform, foliate and circumvallate. The taste buds are composed of 4 taste bud cells types (TBCs) with distinct functions: Type I (glial-like) cells, Types II (taste receptor) cells, Type III (neuronal-like) cell and Type IV (progenitor) cells. The taste receptors for sweet, umami, fat, and bitter are mainly expressed by type II cells. The type 2 taste receptor (T2R) is involved in the perception of bitter taste whereas the heterodimers like T1R2/T1R3 and T1R2/T1R3 detect umami and sweet tastes, respectively. Fat taste, which represents the new sixth taste modality, is detected by CD36, GPR120 and GPR40. T1R and T2R are coupled to a G-protein, called gustducin. Generally, in order to study the physiological aspects of taste signaling, the TBCs are used as a primary cell culture. However, these cells degenerate and lose their properties as a function of time in the



cultured conditions. To the best of our knowledge, there is no mouse taste bud cell line available for conducting in vitro experiments. Therefore, there is a dire need to establish an immortalized mouse taste bud cell line that must express all the physiological characteristics.

In the present piece of investigation, we isolated the TBCs from circumvallate and fungiform papillae from 2-month old male mice of C57BL6 strain. The freshly isolated TBCs were treated by elastase and dispase mixtures, then cultured for 24 h in a medium that contained Iscove's DMEM and MCDB-153 media with 10% fetal bovine serum and a cocktail of antibiotics. The cells were transfected with m-tert retrovirus. After 15 day of culture, several clones were picked up and expanded until their characterization. We employed western blot and RT-qPCR techniques for the analysis of taste bud cell markers. We observed that some clones expressed sweet and umami (T1R1/T1R2/T1R3), bitter (T2R16/T2R38) and lipid receptors (CD36 and GPR120). In addition, these cells expressed both gustducin protein, responsible for signal transduction. Furthermore, we conducted calcium signaling studies in Fura-2/AM loaded cells and observed that the immortalized mouse taste bud cells responded to the agonists of all the taste modalities. In conclusion, the phenotype of the immortalized mouse taste bud cells closely resembles to taste-responsive type II TBCs. As the mouse model is largely used for laboratory experiments, the immortalized mouse TBCs, developed and characterized by us, for the first time, can be used for in vitro investigations to study the mechanism of action of tastants.

We greatly acknowledge the financial assistance sanctioned by the SATT-Grand Est (France).

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## PCA210

### Purinergic Signaling in Testicular Peritubular Cells

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Generation of spermatozoa is the most fundamental process for male fertility. However, the underlying physiological processes are still largely unknown. Seminiferous tubules represent the functional units of the testis. Their wall is composed of extracellular matrix proteins and flat smooth-muscle-like cells, the testicular peritubular cells. These cells are ideally positioned to serve multiple paracrine functions and thus contribute to spermatogenesis. We recently identified ATP-sensitive P2 receptors in peritubular cells (1), adding them to the complex testicular purinergic signaling network (2, 3). Here, we investigate ATP-dependent signaling mechanisms in both human and mouse testicular peritubular cells (HTPCs and MTPCs, respectively) utilizing electrophysiological and live cell imaging techniques in both primary cultures and acute *in situ* preparations.

Electrophysiological investigation of HTPCs reveal ATP-induced currents that show strong rectification and moderate desensitization. Together with ivermectin-mediated current potentiation, our data suggest expression of P2X4 receptors in HTPCs. In MTPCs (C57BL/6J), electrophysiological recordings reveal similar ATP-induced currents with moderate desensitization. On-going experiments aim to identify the underlying P2X receptor isoforms. Moreover,  $\text{Ca}^{2+}$  imaging

in acute seminiferous tubule slices reveals ATP-induced cytosolic  $\text{Ca}^{2+}$  transients in MTPCs. Signals are dose-dependent with an activation threshold of 1  $\mu\text{M}$ , supporting functional expression of P2X receptors. When examining the source of ATP-induced  $\text{Ca}^{2+}$  signals by either buffering extracellular  $\text{Ca}^{2+}$  or by depletion of internal  $\text{Ca}^{2+}$  stores,  $\text{Ca}^{2+}$  signals are significantly reduced under buffering condition and abolished by store depletion.

Together, this research provides insight into physiological signaling mechanisms in both HTPCs and MTPCs. In the long-term, our data will help to elucidate mechanisms that regulate germ cell development and might identify reasons for male infertility.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA211

### Characterization of the membrane progesterone receptor beta (mPR $\beta$ ) using the *Xenopus laevis* oocyte model

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Membrane progesterone receptors (mPRs) are 7-transmembranes integral membrane proteins that belong to the progestin and AdipoQ receptor (PAQR) family. Upon progesterone (P4) binding, mPRs rapidly alter cell signaling via non-genomic modulation of intracellular signaling cascades, including cAMP, MAPK, and  $\text{Ca}^{2+}$  signals. Emerging physiological roles for mPRs have been documented in different tissues, including oocytes, testis, uterus, placenta, breast, brain and immune cells. This raises the profile of mPRs as potential therapeutic targets for different diseases; however, the immediate mechanisms of action downstream of mPRs remain in question. One of the best-studied models of P4-mPR non-genomic action is the activation of oocyte maturation in the *Xenopus laevis* specie. In this study, we used mPR $\beta$  overexpression in Chinese Hamster Ovary (CHO) cells and P4-dependent release of meiotic arrest in *Xenopus* oocytes to characterize mPR $\beta$  (PAQR8) structure and function. We show that mPR $\beta$  has an extracellular C-terminus and a cytosolic N-terminus, a topology that is opposite to the typical G-protein-coupled receptors (GPCRs). We further show through deletions of the N- or C-terminus domain that the N-terminal domain is essential for mPR $\beta$  signaling, whereas the C-terminal domain is crucial for mPR $\beta$  trafficking and plasma membrane residence. Since mPR $\beta$  shares sequence similarity with alkaline ceramidase, we initiated studies to test whether mPR $\beta$  in the oocytes has ceramidase activity. We used pharmacological and metabolomics approaches and showed that the ceramidase inhibitor, ceranib1, negatively regulates P4-induced oocyte maturation, whereas preliminary metabolomics data revealed changes in free fatty acids in response to P4 treatment. Taking together, these results supports the hypothesis that XmPR might possess an intrinsic ceramidase activity. Collectively, our data provide new insights into the structure-function of mPR $\beta$ .

non-genomic P4-dependent signaling, that will pave the way to a more in depth studies to uncover fully its mechanisms of action.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA212

### Opioid-mediated Sertoli cells apoptosis is involved in testicular homeostasis and/or reproductive dysfunction

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The opioid system may exert positive direct and/or indirect effects on spermatogenesis at multiple levels including levels of the central nervous system and at the testes/sperm levels. However, long term opioid use could be associated with several reproductive complications that place the users at risk of hypogonadism and even infertility. The mechanisms underlying the contribution of opioids in testicular homeostasis and/or reproductive dysfunction are still unknown. There is also little available information regarding their apoptotic effects on testis and testicular cells including Sertoli cells. In the present study, the effects of different concentrations of mu, delta and kappa opioid receptors' selective agonists on Sertoli cell viability and apoptosis were investigated. Cultured Sertoli cells (TM4) were exposed to different concentrations of each agonist (0.1-100  $\mu$ M) for duration of 24 or 48 hours and their effects on TM4 cell viability and apoptosis were investigated by MTT and TUNEL assays. Cell viability decreased and apoptosis increased in cells exposed to DAMGO (MOR selective agonist) in a concentration-dependent manner in both 24 and 48 h treated cells. Accordingly, apoptotic index significantly increased from  $13.86 \pm 1.38$  % in control group to  $28.92 \pm 3.3$  % and  $38.78 \pm 2.22$  %, in cells exposed to 1 and 100  $\mu$ M DAMGO, respectively ( $P < 0.001$ ), in 24 h exposure period. Apoptotic index also significantly increased from  $22.06 \pm 3.15$  % in control group to  $32.08 \pm 3.1$  % in cells exposed to 1  $\mu$ M DAMGO, and  $35.25 \pm 1.52$  % in cells exposed to 100  $\mu$ M DAMGO ( $P < 0.01$ ), in 48 h exposure period. However, in cells exposed to DPDPE (DOR selective agonist) no significant changes in cell viability and apoptosis observed. In cells exposed to DYN 1-9 (KOR selective agonist), the viability did not significantly changed, however apoptosis increased significantly, following exposure to high concentration of DYN 1-9. These data suggest that MOR and KOR but not DOR mediated apoptosis in Sertoli cells may be involved, at least in part, in testicular homeostasis and/or Sertoli cell-based reproductive dysfunction.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA213

### Acidic extracellular pH increases cholesterol ester, sphingomyelin and hexosylceramide levels in metastatic breast cancer cells

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Cancer cells in solid tumors exhibit profound changes in lipid metabolism and dysregulation of pH homeostasis, including a very acidic extracellular pH ( $pH_e$ ) (1, 2). Both changes play a causal role in cancer development, however, the possible role of the altered  $pH_e$  in controlling lipid metabolic pathways is poorly understood. The aim of this study was to determine whether changes in  $pH_e$  impact overall lipid metabolism, and to investigate how this in turn may impact the phenotype of metastatic breast cancer cells. Metastatic breast cancer cells (triple negative, MDA-MB-231) were cultured under acidic ( $pH=6.5$ ) and alkaline ( $pH=7.6$ )  $pH_e$  for 24 or 72 h. Medium pH was adjusted by changing  $HCO_3^-$  at 5%  $CO_2$ . Under these conditions, intracellular pH ( $pH_i$ ) was  $6.6 \pm 0.1$  and  $6.7 \pm 0.1$  ( $pH_e$  6.5) compared to  $7.2 \pm 0.1$  and  $7.3 \pm 0.1$  ( $pH_e$  7.6) after 24 and 72 h, respectively, measured by live imaging of BCECF fluorescence ( $n=3$ ). Quantitative shotgun lipidomics were employed to analyze the cellular lipid profile. Notably, the cholesterol ester content was  $140 \pm 11$  % higher at  $pH$  6.5 than at 7.6 after 24 h, while the total amount of cholesterol did not change ( $n=3$ ). The content of sphingomyelins and hexosylceramides was also elevated at acid pH, whereas the total content of ceramides did not change. Changes were qualitatively similar after 72 h. To determine whether lipid location was affected under acidic  $pH_e$ , lipid droplets, cholesterol, and sphingomyelin, using Oil Red O (lipid droplets), fluorescent reporters of outer and inner leaflet cholesterol, and lysenin were used to assess the specific location of these lipid classes. To determine the possible impact of lipidomic changes, cellular cholesterol, cholesterol ester, and sphingomyelin content were manipulated and cell proliferation, migration and invasion quantified. Total cholesterol was decreased  $35 \pm 15$  % and increased  $235 \pm 50$  % after 16 h incubation with methyl  $\beta$  cyclodextrin (M $\beta$ CD) or M $\beta$ CD saturated with cholesterol, respectively ( $n=3$ ). This was associated with corresponding changes in lipid droplet content and plasma membrane cholesterol level. Proliferation (BrdU assay) was reduced by  $62 \pm 24$  % under cholesterol depletion, and recovered upon cholesterol enrichment (24 h,  $n=4$ ). Invasion (Boyden chamber assay) was decreased by  $40 \pm 6$  % under cholesterol depletion and increased to  $127 \pm 25$  % after cholesterol enrichment (24 h,  $n=4$ ). Depletion of sphingomyelin (myriocin or neutral sphingomyelinase) had similar effect showing a decrease of  $57 \pm 8$  %. Ongoing studies address the exact metabolic pathways affected. We conclude that the acidic tumor microenvironment may contribute to the metastatic breast cancer phenotype by impacting lipid metabolism.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA214

**Gold and silver nanoparticles affect endocrine regulation of reproductive system in male rats**

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Metallic nanoparticles are recognized as valuable agents used in different practical applications. However, their toxic properties are not fully understood (Lu et al., 2013). In vivo investigations of reproductive toxicity of nanometals often lead to controversial result (Ema et al., 2017; Jia et al., 2017). Thus, we estimated influence of gold and silver nanoparticles (nano-Au and nano-Ag, respectively) on the regulatory processes in the reproductive system of male rats.

The experiment was conducted on the male albino rats *Rattus norvegicus* (two age groups; aged 1 and 6 month; n= 55 each). Experimental procedures lasted for 10 days. Nano-Au and nano-Ag (spherical, 8-12 nm average diameter) were injected intraperitoneally (i.p.) in the dose of 1 mg/kg. Stimulation and inhibition of reproductive system were achieved through 3µg/kg intracerebroventricular (i.c.v.) injections of kisspeptin-10 (Kp-10) and peptide-234 (P-234) respectively (Qureshi & Abbas, 2013). Intraperitoneal injections were made on days 1-10, i.c.v. – days 8-10. To perform i.c.v. injections, animals were anesthetized with a ketamine-xylazine solution (80mg/kg + 10 mg/kg i.m.). On the day 10, 1 hour post-injection, animals were sacrificed, their testicles and pituitaries taken and histologically processed. We measured cross-sectional area of Leydig cell's nuclei, as well as cross-sectional area of nuclei and cytoplasm of pituitary gonadotrophs. Also, we estimated testosterone (T) concentration in plasma. Means were compared using analysis of variance and Student's t-test. We found no significant changes in T concentration in younger, while in 6-month-old animals nano-Au and nano-Ag caused a 60% drop, comparing to controls. Kisspeptin-mediated stimulated stimulation was verified in both age groups. Kp-10 retained its stimulatory potential in combination with nanoparticles and T levels increased significantly: by 502% - 60,35±1,27 nmol/L vs 10,02±1,55 nmol/L (p<0.05, 6 month, nano-Au+Kp-10 vs nano-Au), 462% - 57,23±1,73 nmol/L vs 10,17±1,14nmol/L (p<0.05, 6 month, nano-Ag+Kp-10 vs nano-Ag), 586% - 33,97±20,80 nmol/L vs 4,95±0,44 nmol/L (p<0.05, 1 month, nano-Au+Kp-10 vs nano-Au), and 398% - 21,82±3,24 nmol/L vs 4,38±0,43 nmol/L (p<0.05, 1 month, nano-Ag+Kp-10 vs nano-Ag). However, no supporting increase in Leydig cell's nuclei cross-sectional area was found. Injections of P-234 (combined and solely) caused significant drop of T levels in 6-month-old animals only. Nanoparticle-induced decrease in testicular activity was not supported by findings in pituitary. In contrary to expected increase in pituitary activity due to the realization of negative feedback, we found downregulation of gonadotrophs by both types of nanometals. Therefore, nanoparticles disrupt endocrine regulatory networks in reproductive system of rats, although exact mechanisms are still to be estimated.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA215

**The effects of polyphenols supplementation in feed on redox balance parameters in testes of male rats exposed to diesel exhaust**

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Exposure to diesel exhaust emission (DEE) is an important source of ambient air pollution with negative health outcomes in humans. Studies demonstrate that exposure to emission from diesel engines increases oxidative stress in the reproductive system (RS). Little is known, whether feed supplementation with polyphenols could reverse the negative impact of DEE on antioxidative defense and oxidative stress parameters in RS. The adult male Fisher 344 rats (308±14g; n=28) were exposed to diesel fuel exhaust (diluted to 2% in air) in the whole body inhalation chambers (exposure: 6h/day, 5 days/week, 4 weeks). The rats were divided into 2 main groups depending on the presence (F+, n=14) or absence (F-, n=14) diesel particle filter (DPF) application. Next, the rats from F+ and F- groups were divided into 2 dietary subgroups and were fed AIN-93 diet supplemented (F+/b, F-/b, n=7) or not (F+/0, F-/0, n=7) with lyophilized black currant marc (80 mg of total polyphenols/100 g feed). After 28 days the rats were anesthetized (by 1-minute inhalation of Isoflurane). The experimental procedures were approved by the 3<sup>rd</sup> Local Animal Care and Use Committee in Warsaw, Poland. Antioxidant defense indices (total antioxidant status, TAS; superoxide dismutase, SOD; glutathione reductase GR; peroxidase, GPx; and catalase, CAT activities; 2 forms of glutathione, GSH, GSSG), and the levels of oxidative stress markers (lipid hydroperoxides, LPO; 25-hydroxycholesterol, 25-HC, and 7-ketocholesterol, 7-KC) were assessed in testes homogenates. All the values are presented as means ± S.E.M., compared by post hoc Fisher LSD test (ANOVA). The results showed that rats from F-/b group had higher TAS concentration, SOD and GPx activities, and lower GR activity and LPO concentration in testis compared with F-/0 group (TAS: 1.352±0.032 vs. 1.170±0.044 µmol g<sup>-1</sup>, p<0.001; SOD: 0.301±0.014 U g<sup>-1</sup>



vs.  $0.271 \pm 0.009 \text{ U g}^{-1}$ ;  $p < 0.05$ ; GPx:  $0.171 \pm 0.034 \text{ U g}^{-1}$  vs.  $0.118 \pm 0.005$ ;  $p < 0.05$ ; GR:  $0.350 \pm 0.028 \text{ U g}^{-1}$  vs.  $0.551 \pm 0.019 \text{ U g}^{-1}$ ;  $p < 0.01$ ; LPO:  $231.5 \pm 7.5$  vs.  $282.9 \pm 6.2$ ,  $p < 0.001$ ). Higher SOD and lower GR, LPO, 7-KC and GSSG values were observed in F+/b versus F+/0 group (SOD:  $0.348 \pm 0.056 \text{ U g}^{-1}$  vs.  $0.224 \pm 0.006$ ;  $p < 0.01$ ; GR:  $0.558 \pm 0.030$  vs.  $0.637 \pm 0.007$ ,  $p < 0.05$ ; LPO:  $244.3 \pm 4.1$  vs.  $273.3 \pm 5.3$ ,  $p < 0.01$ ; 7-KC:  $13.0 \pm 0.6$  vs.  $15.8 \pm 0.3$ ,  $p < 0.001$ ; GSSG:  $2.33 \pm 0.07$  vs.  $2.98 \pm 0.25$ ,  $p < 0.05$ ). Catalase (CAT) activity, GSH, and 25-HC concentrations in testis did not significantly differ between groups. The results suggest that exposure to DEE regardless of DPF application disturbs the antioxidant defense balance in rats testes. Intake of black currant cause protecting effects on testes by increasing the anti-oxidative defense and attenuate oxidative stress.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA216

### Chronic Mild Cold Exposure Promotes Weight Gain and Deposition of Subcutaneous Adipose Tissue in Animals Raised at Thermoneutrality

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Background: The therapeutic activation of thermogenic brown adipose tissue (BAT) is a current 'hot' topic in the field of obesity and metabolic disease. It is now recognised that studies examining BAT physiology are carried out at sub-thermoneutral temperatures (e.g.  $\sim 20^\circ\text{C}$ ). As such, these studies are not translationally relevant to humans as BAT is 'hyperactive'. Therefore, the aim of this study was to determine the effect of common regulators of BAT metabolism when animals were raised at thermoneutrality (Tn,  $28^\circ\text{C}$ ).

Methods: Eighteen weanling Sprague-Dawley rats were housed at Tn ( $28^\circ\text{C}$ ) and fed a high-fat diet (HFD,  $n=18$ ) from 3-weeks of age. At 12 weeks, subgroups of HFD were randomised to either mild-cold exposure ( $20^\circ\text{C}$ , Cold,  $n=6$ ) or Mirabegron, a selective  $\beta_3$ -agonist ( $0.75\text{mg/kg/day}$ ,  $\beta_3$ ,  $n=6$ ). Metabolic assessment was undertaken in CLAMS during the last 48h and the acute response to administration of Mirabegron ( $0.75\text{mg/kg}$ ) was determined. Key thermogenic and metabolic genes were analysed by qPCR and targeted Insulin Resistance PCR Arrays (86 key genes,  $n=3$ ) in addition to quantitative proteomics.

Results: None of the interventions reduced body weight or total/visceral adiposity. Intriguingly however, mild-cold exposure significantly increased weight-gain during the 4-week intervention period ( $78.6$  vs.  $119.8$ ) which was accompanied by a significant increase in inguinal AT ( $7.14$  vs  $16.14\text{g}$ ,  $p=0.02$ ). This occurred despite no difference in energy expenditure, intake of physical activity between groups. First we analysed BAT mass which was unaffected with no induction of key thermogenic genes (e.g. UCP1 and PGC1 $\alpha$ ) with similar results in perivascular BAT (PVAT). Targeted PCR arrays demonstrated a divergent effect on these thermogenic tissues. ACACA and ACACB, PGC1 $\alpha$ , IRS1, PDK2 and RBP4 were all upregulated in

PVAT but not BAT suggesting an increased metabolic activity in PVAT. We then sought to determine whether there was evidence of 'browning' in IWAT and found no increase in thermogenic markers. There was also no upregulation of common adipogenic genes (e.g. Pparg) however there was increased expression of Perilipin which may, in part explain the increase in IWAT mass. We are now performing quantitative proteomics on interscapular BAT and IWAT and investigating central regulators of energy homeostasis to a) determine which metabolic pathways are affected in this scenario and b) to understand potential mechanisms contributing to weight gain and the deposition of fat mass in response to the cold. Conclusion: We show there is no consistent upregulation of key thermogenic genes in response to common stimuli when animals are raised at Tn. We propose that the effects of interventions carried out at sub-Tn are artificial and a consequence of a permanent mild-cold stress. Further, we hypothesise that deposition of subcutaneous fat is a centrally regulated insulative mechanism driven by insufficient thermogenic capacity of BAT in animals raised at Tn.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA217

### The selective mineralcorticoid receptor (MR) antagonist eplerenone prevents decompensation of liver cirrhosis.

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Background & aims: The MR contributes to fibrosis in various tissues and MR antagonists, like eplerenone, are used to prevent fibrosis. The role of MR antagonists for hepatic fibrosis and cirrhosis is unknown. Here, we investigated the role of MR and eplerenone in cirrhosis development.

Methods: Liver fibrosis (5 weeks), cirrhosis without (8w) and with ascites (12w) were induced by CCl<sub>4</sub> in rats and comprehensively analyzed. In addition the effect of eplerenone (MR antagonist) on the development of cirrhosis with ascites was assessed. With respect to MR expression, cellular and subcellular distribution as well as impact of hypoxia were investigated in vivo and ex vivo. Primary rat hepatocytes and cell lines were used to investigate MR trafficking and transcriptional activity mechanistically.

Results: In the liver the main celltype expressing the MR are hepatocytes. Blood plasma aldosterone levels increased significantly already during the first 5w and remained stable thereafter for 8w and 12w of CCl<sub>4</sub> treatment. In cirrhosis with ascites MR-mRNA and -protein expression were reduced in hepatocytes of hypoxic areas. While in normoxic areas MR was mainly cytosolic, the remaining MR in hypoxic areas was mainly localized in the nuclei, indicating activation followed by translocation and degradation. We also analyzed the expression of the glucocorticoid receptor, the nearest relative to the MR. GR revealed no change in expression during cirrhosis with ascites. The decrease of MR was detectable only in liver tissue. We did not observe any differences in MR mRNA or protein in renal or heart tissue from cirrhotic compared to control animals. Exposing hepatocytes ex vivo to hypoxia also induced nuclear MR translocation and enhanced transcriptional MR-activity at response elements of the NF $\kappa$ B pathway.

Visual evaluation of the liver surface showed significant less nodules, less shunts and less ascites in the eplerenone treated group compared to cirrhotic animals without eplerenone. Quantification of fibrosis using Sirius red staining revealed significantly less fibrosis in animals treated with eplerenone. Furthermore, in treated animals the liver weight was significantly higher, the spleen weight and the heart weight significantly lower compared to non-treated group. Accordingly, eplerenone treatment prevented nuclear MR translocation and worsening of cirrhosis.

Conclusion: We show for the first time that hypoxia leads to a pathogenetic relevant ligand independent activation of hepatic MR during cirrhosis with subsequent nuclear translocation and transcriptional activation of the NFkB pathway. Treatment with the MR antagonist eplerenone prevented worsening of cirrhosis by blocking the ligand independent activation of the MR.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCA218

### Intestinal modulate of glucose homeostasis in mongrel dogs

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Previous studies (Alada and Oyebola, 1996; Grayson and Oyebola, 1983) have shown that the intestine increased its glucose uptake during hyperglycemia and released glucose into circulation during insulin-induced hypoglycemia. This study was designed to investigate the metabolic fate of the increased glucose taken up by the intestine in response to hyperglycemia and the source of glucose released into circulation during hypoglycemia. Experiments were carried out on fasted, male, adult mongrel dogs anaesthetized with intravenous injection of sodium pentobarbitone (30.0mg/kg) and divided into three groups with 5 dogs per group. Groups I-III received i.v infusion of normal saline (0.1 ml/kg/min), glucose (20mg/kg/min) and insulin (8 iu/kg/min) respectively. Through a midline laparotomy, the upper jejunum was secured and cannulated for blood flow measurement. Blood samples were obtained for measurement of glucose and lactate from the upper jejunal segment. The plasma glucose and lactate were enzymatically determined. Intestinal glucose/lactate uptake was calculated as the product of jejunal blood flow and arterio-venous glucose /lactate difference. Values are means  $\pm$  SEM, compared by ANOVA and Student t-test. Jejunal tissue glycogen was determined by Anthrone method while activities of glycogen synthase, phosphorylase and glucose-6-phosphatase were measured spectrophotometrically. Glucose increased the intestinal glucose uptake from  $14.5 \pm 6.4$  mg/min to  $142.2 \pm 17.9$  mg/min while insulin caused a negative uptake of  $71.8 \pm 10.6$  mg/min. Intestinal lactate uptake increased from  $36.4 \pm 5.8$  mg/min to  $77.8 \pm 12.3$  mg/min and  $60.8 \pm 9.2$  for glucose and insulin respectively. Jejunal glycogen content increased from  $138.7 \pm 4.6$  mg/100g to  $165.5 \pm 10.6$  mg/100g for glucose and reduced to  $97.4 \pm 4.3$  mg/100g for insulin. Intestinal phosphorylase activity increased in response to insulin and decreased significantly in response to glucose. While glucose had no effect on intestinal glycogen synthase activity, insulin significantly reduced it. Also, insulin increased glucose-6-phosphatase activity while glucose reduced it. The results suggest that the small intestine modulates blood

glucose levels through oxidation, glycogen formation and conversion of lactate to glucose.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCA219

### Factor-inhibiting HIF (FIH) knockout enables an anti-inflammatory effect of HIFs in a mouse model of colitis-induced colon cancer

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Colorectal cancer is the fourth most common cause of cancer mortality worldwide (1) and can be promoted by inflammatory bowel diseases as chronic colitis. Inflammation as well as tumors induce hypoxia and enforce a gene expression response controlled by hypoxia-inducible factors (HIFs). HIF is regulated by prolyl hydroxylases and the asparaginyl hydroxylase FIH. HIF stabilization leads to a less severe inflammation in an acute colitis mouse model (2), but the role of HIF in colon cancer development and the importance of its regulator FIH are unknown.

In our study we used a mouse model for chronic intestinal inflammation and colon cancer development. Mice are treated twice with the oncogene azoxymethane (AOM, 10 mg/kg body weight, i.p.) and with two 5-day/one 4-day phase of 1.5% dextran sodium sulfate (DSS) in the drinking water, which induces intestinal inflammation by destruction of the epithelial barrier. In a continuative experiment, the hydroxylase inhibitor dimethylxaloylglycine (DMOG) is administered every second day of DSS-treatment (320 mg/kg body weight, i.p.) to study the effect of hydroxylase inhibition, which is protective in acute inflammation (2), in a chronic colorectal cancer model. We recorded the disease activity index (DAI) comprising weight loss, stool consistency, and fecal (pellet) bleeding (3) each day of inflammation and took samples for RNA and immunohistochemical analysis after 10 weeks. FIH<sup>fl/fl</sup> x VillinCre<sup>-/-</sup> (WT) and FIH<sup>fl/fl</sup> x VillinCre<sup>+/+</sup> (KO) mice were used to study the importance of FIH in epithelial cells. Values are means  $\pm$  SEM, compared by unpaired t-test. We observed a strong increase in tumor development in mice treated with DSS compared to control mice that only received AOM administration (tumor number:  $21.1 \pm 3.2$  n=6 vs.  $6.2 \pm 1.3$  n=14, p<0.01), but no differences between WT and KO mice. However, the analysis of the DAI in the third DSS phase indicates a diverse DMOG-dependent disease progress: The total DAI of WT mice increased due to the DMOG-treatment ( $23.5 \pm 3.5$  n=14 vs.  $34.2 \pm 1.8$  n=5, p<0.1), whereas the disease state of the KO mice was unaffected. On RNA level, WT mice treated with DSS and DMOG showed a more than 2-fold increase in *Il-10* expression compared to control mice while there was no difference in KO mice. Additionally, DSS/DMOG-treated WT mice showed a higher infiltration of macrophages immunohistochemically detected by *F4/80* expression than KO mice of the same experimental group.

FIH knockout in colon epithelial cells might influence the inflammation status in chronic colitis under chemical pan-hydroxylase inhibition during acute inflammation phases. With these data we can conclude that stabilized HIF has an

anti-inflammatory effect not only in acute colitis (2) but also in chronic colitis and colorectal cancer development as simulated in our AOM/DSS mouse model.

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## PCA220

### Matrix effects on the intestinal concentration of maslinic acid and its effects on colonic preneoplastic lesions induced by 1,2-dimethylhydrazine in rats

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Maslinic acid (MA) is a pentacyclic triterpene recognized as a bioactive compound that elicits different health-protecting properties without harmful effects, standing out among others, antitumoral activity. The low oral bioavailability together with a poor intestinal absorption leads to a high concentration in the large intestine, thus favouring its potential chemopreventive activity against colon cancer. Bearing in mind the high content of MA in table olives, we aimed at studying their effect on preneoplastic lesions in rats. Therefore, male Sprague-Dawley rats (7-week-old) were randomly divided into three groups according to the treatment orally administered by gavage: a MA group that received pure MA at 10 mg/kg (n=6), an olive group that was given Arbequina table olives at 3.8 g/kg which contained MA at 8.6 mg/kg (n=6) and a control group provided only with the solvent (n=6). At one week of treatment, preneoplastic lesions were induced in the colon by three weekly injection of 1,2-dimethylhydrazine (20 mg/kg; intraperitoneally). After 49 days, overnight fasted rats were anesthetized intraperitoneally with ketamine and xylazine (90 and 10 mg/kg, respectively) and blood was withdrawn by cardiac puncture and placed in EDTA-K2 tubes. Then, the colon was excised, its content collected and the tissue fixed in formalin. MA in plasma was extracted twice with ethyl acetate and vortex-mixing, whereas the determination in colon content included Polytron grinding with methanol 80% prior to HPLC-APCI-MS analysis. Aberrant crypt foci (ACF) were counted at the light microscope at 10x magnification after staining with methylene blue. MA was not found neither in animal feed nor in plasma and colon content of the control group. In contrast, this pentacyclic triterpene was detected in plasma of the MA group at  $23.4 \pm 7.0$  nM and olive group at  $10.1 \pm 2.7$  nM, being measured 20 h after the last oral administration. MA when administered as pure compound reached the colon at  $90.7 \pm 36.9$  nmol/g, whereas in the olive group was  $307.3 \pm 80.2$  nmol/g. Rats in the control group developed ACF that were reduced in a 17.8% in the MA group and a 54.1% in the olive group. In view of the results, it can be concluded that despite similar MA doses, the antitumoral activity is higher in the rats fed with table olives than in the animals receiving the pure compound. Noteworthy, the intestinal content of MA from the group administered with olives was 3.4-times higher than in the group receiving the pure compound. The lesser bioavailability of MA from table olives favours to attain higher concentrations in the colon which elicits a higher chemopreventive activity.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA221

### Is progesterone antagonism responsible for the abortifacient effect of Alligator pepper (*Zingiberaceae Aframomum Melegueta*) in pregnant Sprague Dawley rats?

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Alligator pepper (AP), a dietary spice used as food flavor enhancer in Nigeria and some other parts of the world, is also ingested by pregnant women in foods. Seeds of AP have capacity to terminate first trimester pregnancy in Sprague Dawley rats, 1 in a manner similar to the drug, mifepristone. 2 This study was to determine if AP effect on pregnancy is related to progesterone antagonism. 45 female Sprague Dawley rats were acclimatized for 2 weeks and then allowed to mate with male rats for 3 days. Thereafter, the pregnant rats were randomly separated into 3 groups (A - C) of 15 rats each. Grp A had intra-peritoneal injection of 2ml of normal saline, while grps B and C had 6.7mg/kg body weight and 13.4mg/kg body weight of saline extract of AP respectively on day 4 of pregnancy. Each group was randomly divided into 3 subgroups ( $A_1, A_2, A_3$ ;  $B_1, B_2, B_3$  and  $C_1, C_2, C_3$ ) of 5 rats each. Blood was collected from  $A_1, B_1, C_1$  on day 7;  $A_2, B_2, C_2$  on day 14 and  $A_3, B_3, C_3$  on day 21 of pregnancy by intra-ventricular aspiration and assayed for progesterone by ELISA technique. The study was done in line with guidelines, 3 and results expressed as mean $\pm$ SD, and P – values at 5% level of significance was calculated using Daniel Soper's statistical calculator online. 4 Serum progesterone levels were higher in the normal saline treated rats than in grps B and C. These were significant for the grps  $B_1, B_3$  and  $C_1$ . Progesterone level in grp  $C_1$  was significantly lower ( $P = 0.002$ ) than grp  $B_1$  rats. Deficiency in progesterone is probably the basis of the abortifacient effect of AP on pregnancy, and beta-caryophyllene is the most probable active ingredient of AP involved. 5 The mechanism of action is most probably the inhibition of progesterone synthesis by beta-caryophyllene component of the sesquiterpene constituent of AP.

Effect of different doses of Alligator pepper on Blood Progesterone Levels in Sprague Dawley Rats on days 7, 14 and 21 of gestation.

| Group          | No. of Rats | Pregnancy Status | Dose of Alligator Pepper in kg/body wt | Blood Progesterone Level $\pm$ SD ng/ml |                               |                             |
|----------------|-------------|------------------|----------------------------------------|-----------------------------------------|-------------------------------|-----------------------------|
|                |             |                  |                                        | Day 7 First trimester                   | Day 14 Second trimester       | Day 21 Third trimester      |
| A Control      | 15          | Pregnant         | Nil                                    | 35.08 $\pm$ 14.37 Subgroup A1           | 34.95 $\pm$ 22.03 Subgroup A2 | 45.6 $\pm$ 3.58 Subgroup A3 |
| B Experimental | 15          | Pregnant         | 6.7mg                                  | 10.55 $\pm$ 2.94 Subgroup B1            | 22.8 $\pm$ 12.47 Subgroup B2  | 39.3 $\pm$ 1.04 Subgroup B3 |
|                |             |                  | P value (A and B)                      | 0.006**                                 | 0.314                         | 0.005**                     |
| C Experimental | 15          | Pregnant         | 13.3mg                                 | 3.45 $\pm$ 2.02 Subgroup C1             | 13.55 $\pm$ 6.87 Subgroup C2  | 44.3 $\pm$ 7.04 Subgroup C3 |
|                |             |                  | P value (A and C)                      |                                         | 0.072                         | 0.722                       |
|                |             |                  | P value (B and C)                      | 0.002**                                 | 0.184                         | 0.155                       |

\*\* Significant decrease in progesterone

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA222

### Gum Arabic supplementation decreased inflammatory markers levels and Disease Activity Score among Rheumatoid arthritis patients

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**Background:** Rheumatoid Arthritis (RA) is autoimmune inflammatory disease attacks the synovium of the joints. Both TNF $\alpha$  and interleukin-1 play crucial roles in the pathogenesis of RA. Gum Arabic (GA) is gummy exudates from Acacia Senegal tree. Gum Arabic as a soluble fibre its fermentation by colonic bacteria increases serum butyrate concentrations, so it is considered as prebiotic agent. Gum Arabic (GA) has anti-inflammatory activity through its derivative butyrate.

**Patients and methods:** This is clinical trial phase II in which 40 patients were enrolled aged 18 to 70 years. Patients received 30g/day GA for 12 weeks. TNF  $\alpha$ , ESR, and complete blood count were measured and DAS-28 was calculated before and after regular GA consumption. Study approved by the Ethical committee of National Medicines and Poisons Board. Clinical this clinical trial was registered in Trials.gov Identifier: NCT02804581 Registered 19th June 2016. Prospective registration.

**Results:** This study showed significant decrease in level of serum TNF  $\alpha$  (p value 0.05) [95% CI, 0.65 -16.5], ESR (p value 0.011) [95% CI, 2.6 -18.89], and number of swollen and tender joints in RA patients after 12 weeks of GA intake which reflected as significant decrease in disease severity score DAS 28 P.V:0.00 [95% CI, 1.25 -1.99]. On the other hand, GA had trivial change in blood indices.

**Conclusion:** Gum Arabic has favorable immune modulator effect on Rheumatoid Arthritis. It can be utilized in clinical practice as adjuvant therapy.

**Trial Registration:** ClinicalTrials.gov Identifier: NCT02804581 Registered 19th June 2016. Prospective registration

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA223

### Neonatal administration of curcumin protects against high fructose diet-induced non-alcoholic steatohepatitis in male adolescent Sprague Dawley rats

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The increased prevalence of non-alcoholic fatty liver disease (NAFLD) which comprises non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH), is driven by an increased consumption of fructose. The suckling period in altricial species is characterised by rapid growth and developmental plasticity. Interventions with polyphenols such as S-allyl-l-cysteine in this period have proven benefits in the protection of the animals against adverse consequences of a high fructose diet. Curcumin is a dietary polyphenol with antisteatotic and antifibrotic effects in the liver. We aimed to determine whether neonatal administration of curcumin would protect growing rats against the development of NASH in adolescence when they are fed a high fructose diet.

A total of 65, 6-day old male suckling Sprague Dawley pups were used in the study which was conducted in two phases. In the first phase which extended from postnatal day (PN) 6 to PN 21, the pups were allocated to four groups and received treatments as follows: 10ml.kg<sup>-1</sup> of a 0.5% dimethyl sulfoxide solution (n=16), curcumin 500mg.kg<sup>-1</sup> (n=17), fructose (20%, w/v) (n=16) and a combination of curcumin and fructose (n=16). The treatments were administered daily via oral gavage. At weaning, which marked the beginning of the second study phase, the pups in each group were further subdivided into two; one group had normal rat chow and plain tap water while the other received normal rat chow and fructose (20%, w/v) as their drinking fluid. These treatments continued *ad libitum* for six weeks until PN 63 when the rats were terminated using sodium pentobarbital (150mg.kg<sup>-1</sup>, i.p). The liver was excised, and a section stored in 10% buffered formalin for histomorphometry whilst the rest was frozen for later lipid extraction. Hepatic lipid content was determined using the solvent (chloroform and methanol) extraction method as described by Bligh and Dyer (1959). The formalin preserved liver samples were processed for histology, stained with haematoxylin and eosin (HE) to assess hepatocellular changes and Masson's trichrome (MT) to assess fibrosis according to standard protocols as described by Bancroft and Gamble (2008).

The hepatic lipid content was similar across the treatment groups in the rats (2.1-3.3%, p>0.05, ANOVA). The livers of the rats that had fructose either as neonates and/or in the growing period had marked inflammation (3/3 vs 0/3 NAS score, p= 0.0015, Kruskal Wallis) and significant fibrosis (7.5 vs 1.0%, p<0.0001, ANOVA) with sinusoidal dilatation which were attenuated by curcumin.

Curcumin administration to pups attenuated the fructose-induced hepatic inflammation and fibrosis in livers of adolescent male rats and thus is a potential for prevention of non-alcoholic steatohepatitis.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA224

### Variations in plasma electrolytes and thirst perception during the menstrual cycle

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**Introduction:** Electrolyte-dependent water retention associated with hormonal changes during the menstrual cycle may alter the normal reference intervals of plasma electrolytes<sup>1</sup> with important effects on central nervous system, cardiovascular and renal functions.<sup>2,4</sup> Hence, we sought to determine and compare the concentrations of plasma electrolytes (sodium, potassium, bicarbonate, chloride and urea) and thirst perception during different phases of the menstrual cycle.

**Methods:** Blood samples obtained from the anti-cubital veins of 40 healthy females (18 – 25 years, 27 - 33 days menstrual cycle) on the 2nd (early follicular/menstrual, n = 10), 8th (late follicular, n = 10), 14th (ovulatory, n = 10) and 22nd (luteal, n = 10) day of the menstrual cycle were analysed for plasma electrolytes concentrations by photoelectric flame photometry. Thirst perception (TP) ratings were also obtained at the time of sample collection using the Visual Analogue Scale.

**Results:** Plasma [Na<sup>+</sup>] ( $P = 0.003$ ) and [Cl<sup>-</sup>] ( $P = 0.02$ ) were highest during ovulation while the late follicular and luteal phases had the least [Na<sup>+</sup>] and [Cl<sup>-</sup>] respectively. Plasma [K<sup>+</sup>], [HCO<sub>3</sub><sup>-</sup>], and [urea] did not differ significantly in relation to the phase of menstrual cycle. In addition, TP increased in a phase-dependent non-significant manner until after ovulation (Table 1).

**Conclusion:** Plasma concentrations of sodium and chloride are increased during ovulation. This may be attributed to estrogen-related salt retention, whereas the lower sodium and chloride concentrations observed in the luteal phase (compared to ovulation especially) could be due to the natriuretic action of progesterone.

Table 1. Plasma electrolytes concentration and thirst perception in different phases of menstrual cycle

|                                  | Early follicular | Late Follicular | Ovulation      | Luteal       |
|----------------------------------|------------------|-----------------|----------------|--------------|
| Plasma electrolyte concentration |                  |                 |                |              |
| Sodium, mmol/l                   | 138.2 ± 2.13     | 121.6 ± 7.42    | 143.8 ± 2.81** | 139.4 ± 1.30 |
| Chloride, mmol/l                 | 106.0 ± 1.35     | 109.3 ± 0.85    | 110.7 ± 1.85*  | 105.8 ± 0.63 |
| Potassium, mmol/l                | 4.0 ± 0.12       | 4.0 ± 0.07      | 4.12 ± 0.19    | 4.1 ± 0.07   |
| Bicarbonate, mmol/l              | 20.8 ± 0.51      | 19.7 ± 0.45     | 20.5 ± 0.40    | 20.4 ± 0.22  |
| Urea, mg/dl                      | 17.3 ± 0.97      | 19.0 ± 0.99     | 26.00 ± 4.26   | 21.1 ± 1.56  |
| Thirst Perception, cm            |                  |                 |                |              |
|                                  | 3.5 ± 0.58       | 4.4 ± 0.72      | 5.1 ± 1.22     | 3.6 ± 0.60   |

Data presented as mean ± standard error, n = 10 in each group. \*\*  $P < 0.01$ , \*  $P < 0.05$  (One-way ANOVA).

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## PCA225

### Protective Effects of Calcitriol against Fructose-induced Hyperglycaemia and Dyslipidaemia in male Albino Wistar rats

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Hyperglycaemia and lipidaemia produced adverse metabolic alterations in glucose and lipid metabolism. The study investigated the effects of calcitriol administration on glucose and lipid profiles in fructose-drinking male Albino Wistar rats. Twenty five (25) male Albino rats were used in this research work. The rats were divided into 5 groups of 5 animals each. *Group I:* Control; received normal rat feed + distilled water, *Group II:* Received normal rat feed + 125 µg/kg of Calcitriol, *Group III:* Received normal rat feed + 10% fructose solution, *Group IV:* Received normal rat feed + 10% fructose solution + 125 µg/kg of Calcitriol, *Group V:* Received normal rat feed + 10% fructose solution + 0.3 mg/kg of glibenclamide. Data were expressed as mean ± SEM, were analysed using one way ANOVA followed by Turkey-Kramer multiple comparison post-hoc tests to show multiple comparisons versus control. Fructose-drinking rats (Group III) exhibited significant increase in total cholesterol ( $4.90 \pm 0.23$  vs  $3.88 \pm 0.19$  mmol/L), triglycerides ( $1.90 \pm 0.27$  vs  $1.20 \pm 0.16$  mmol/L), LDL-cholesterol ( $2.80 \pm 0.26$  vs  $2.16 \pm 0.17$  mmol/L) compared to control. However, HDL in fructose-drinking rats was similar to that of the control ( $1.22 \pm 0.07$  vs  $1.32 \pm 0.22$  mmol/L) compared to control. Normal rats treated with calcitriol exhibited no significant changes in total cholesterol, triglycerides, LDL and HDL. In conclusion, the result showed that cholesterol supplementation in fructose-drinking rats protects against hyperglycaemia and dyslipidaemia.

**Keywords:** Calcitriol, fructose, cholesterol, hyperglycaemia, dyslipidaemia

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PCA226

### Impact of Green Tea and Exercise on Hyperthyroidism-Induced Haemostatic Alterations in Rats

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**Aim:** The present work aimed to clarify the potential beneficial effects of green tea and exercise training in alleviating hemostatic derangements in experimental hyperthyroidism highlighting the possible underlying mechanism(s). **Methods:** 46 adult male Wistar rats were divided into four groups: control, hyperthyroid, green tea supplemented hyperthyroid and exercise trained hyperthyroid. Rats (260-330g) were anaesthetized with thiopental sodium (40mg kg<sup>-1</sup>) and were subjected to determination of body weight (BW), body mass index (BMI), serum levels of free triiodothyronine (FT<sub>3</sub>), free thyroxine (FT<sub>4</sub>) and thyroid stimulating hormone, complete blood picture, prothrombin time (PT), activated partial thromboplastin time (APTT), plasma levels of fibrin degradation products (FDPs), malondialdehyde (MDA) and catalase activity and C-reactive protein (CRP). **Results:** Hyperthyroid group showed significantly decreased BW, BMI and their percentage change, prolonged PT and APTT, elevated plasma FDPs, reduced platelet count and plateletcrit, increased white blood cell (WBC) count and plasma MDA level and decreased catalase activity. Green tea supplementation significantly decreased serum FT<sub>3</sub>, increased BW and BMI and their percentage changes, shortened PT and APTT, decreased WBC count and CRP and increased catalase activity as compared to hyperthyroid group. Exercise-training significantly reduced serum FT<sub>3</sub> and FT<sub>4</sub>, shortened PT and decreased plasma MDA, WBCs count and serum CRP as compared to the hyperthyroid group. Platelet count and plateletcrit were increased in both green tea and exercise groups and reached control values. **Conclusion:** Both green tea and exercise training attenuated the hemostatic abnormalities mediated by hyperthyroidism, an effect probably owed to its antithyroid, antioxidant and anti-inflammatory activities.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA227

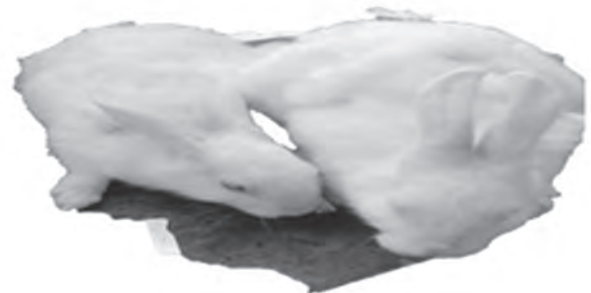
### Adipokines and Resveratrol Interactions in Cholesterol Diet Fed Rabbits

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Serum adiponectin concentration is inversely associated with leptin and insulin concentration. There is little paucity of information in the literature on the adipokine levels and regulation by

resveratrol in cholesterol diet-fed animals. Thirty rabbits were randomly divided into six groups, each of five animals: Group 1 = control (C), Group 2 = cholesterol diet (CD) only, Group 3 = resveratrol 200 mg/kg (R200), Group 4 = resveratrol 400 mg/kg (R400), Group 5 = CD + R200 and group 6 = CD + R400. The preparations were administered for 8 weeks of experimental protocol. Weight and blood glucose level were measured on week zero and the 8<sup>th</sup> week of the treatment. At the end of the study period, the rabbits were placed under light anaesthesia (ketamine) to remain unconscious. Blood sample of about 3ml was drawn via cardiac puncture for serum extraction which were evaluated for serum concentration of metabolites assays (adiponectin, leptin and insulin). Blood glucose level and body weight were significantly higher in CD group compared to groups that received resveratrol supplement. Serum adiponectin significantly ( $P < 0.05$ ) increased in resveratrol + CD groups, compared to CD group only. Leptin and insulin significantly ( $P < 0.05$ ) decreased in CD groups co-administered with resveratrol, compared with CD group only. In conclusion, the increase in adiponectin level and decrease in leptin and insulin level following resveratrol administration elucidated the therapeutic potential of resveratrol, despite cholesterol diet consumptions.



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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.



PCA228

**Increased water intake reduces copeptin and fasting plasma glucose in high copeptin healthy adults – the hydration to optimize metabolism (H2O Metabolism) pilot study**

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**Background:** Elevated concentrations of plasma copeptin, a marker for vasopressin and a known risk factor for diabetes development, is observed in individuals with low water intake. In this study, we hypothesized that water supplementation (1.5 L day<sup>-1</sup>) over a period of six weeks could lower fasting plasma copeptin (P<sub>Cop</sub>) and possibly impact glucose (P<sub>Gluc</sub>) concentrations in habitual low-drinkers.

**Design and method:** Stored plasma samples collected between 2013 and 2015 in the ongoing population study Malmö Offspring Study (MOS) were screened for high copeptin concentrations (P<sub>Cop</sub> ≥ 10.7 pmol L<sup>-1</sup> in men and ≥ 6.1 pmol L<sup>-1</sup> in women). Twenty-four percent of the MOS population presented high concentrations of copeptin; among these, thirty-one healthy adults with low fluid intake habits at the baseline of the current study (24h urine volume ≤ 1.5L and 24h urine osmolality ≥ 600 mOsm kg<sup>-1</sup>) were enrolled 2.8 ± 0.8 years after MOS screening. At baseline and at the end of the six-week intervention, fasting plasma samples, food and fluid diaries, and 24h urine samples were collected. Pre- vs post-intervention differences were assessed with paired t-test or Wilcoxon's paired rank test where appropriate. Unless stated otherwise, data are presented as median [25<sup>th</sup>; 75<sup>th</sup> percentiles].

**Results:** The water intervention succeeded in increasing mean (95% confidence interval) plain water intake from 0.43 (0.27; 0.58) to 1.35 (1.00; 1.71) L day<sup>-1</sup> (P < 0.001), on top of habitual fluid intake, with no other changes in diet. Urine osmolality, as a marker of urine concentration, was reduced from 879 [705; 996] to 384 [319; 502] mOsm kg<sup>-1</sup> (P < 0.001), and urine volume increased from 1.06 [0.90; 1.20] to 2.27 [1.52; 2.67] L d<sup>-1</sup>; P < 0.001.

At baseline, P<sub>Cop</sub> was 12.9 [7.4; 21.9] pmol L<sup>-1</sup> and did not differ from the values obtained during the population screening of MOS three years earlier. At the end of intervention, P<sub>Cop</sub> was significantly reduced to 7.8 [4.6; 11.3] pmol L<sup>-1</sup>; P < 0.001. The reduction of P<sub>Cop</sub> was most pronounced in subjects belonging to the top tertile of baseline P<sub>Cop</sub> (ΔP<sub>Cop</sub> 28.2 (8.1 - 58.6)), as compared to subjects in P<sub>Cop</sub> tertiles 1+2 (ΔP<sub>Cop</sub> 1.6 (-0.3 - 4.5)), P < 0.001. Finally, water supplementation significantly reduced mean (SD) plasma glucose (P<sub>Gluc</sub>) from 5.94 (0.44) to 5.74 (0.51) mmol L<sup>-1</sup>; P < 0.05. The reduction of P<sub>Gluc</sub> was most pronounced in subjects belonging to the top tertile of baseline P<sub>Cop</sub> (Figure 1).

**Conclusion:** Water supplementation in habitual low-drinkers with high copeptin appears as an effective and safe way to lower copeptin concentration. Given the suggested role of vasopressin in diabetes development, increasing water may be a promising intervention with potential of lowering fasting plasma glucose. Further investigations are warranted to support the present findings.

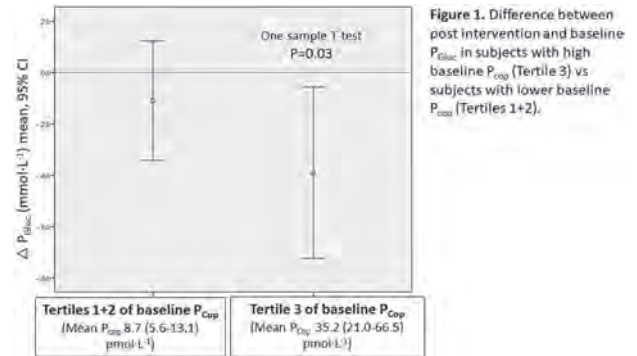


**Figure 1.** Difference between post intervention and baseline P<sub>Gluc</sub> in subjects with high baseline P<sub>Cop</sub> (Tertile 3) vs subjects with lower baseline P<sub>Cop</sub> (Tertiles 1+2).

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PCA229

**Modulatory Role Of Clove And Fermented Ginger Supplements On Oxidative Stress And Lipid Peroxidation Biomarkers In High Fat Diet Induced Type 2 Diabetes In Rabbits**

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Type 2 diabetes mellitus (T2DM) has become a global health challenge (Baynest, 2015). The search for it remedy across different available agents with medicinal value may elucidate the health burden imposed by the disease. Studies on clove and ginger revealed their medicinal potency in ameliorating metabolic disorders. However the effects of these supplements on oxidative stress biomarkers in type 2 diabetes still suffers' elusive scientific explanations (Tapsell *et al.*, 2006). Oxidative stress plays a pivotal role in progress and complications of the disease. This study aimed to evaluate the effects of clove and fermented ginger supplements on oxidative stress and lipid peroxidation biomarkers in high fat diet (HFD) induced type 2 diabetic rabbits.

**Method**

Rabbits were handled in accordance with the principles guiding the use and handling of experimental animals, ABU, Zaria. Ethical approval was obtained, with Approval No: ABUCAUC/2017/048. HFD was fed to rabbits (New Zealand breed) for eleven weeks to ascertain diabetic animal model (DAM) (Jimoh *et al.*, 2015). Thereafter, DAM were treated with supplements for six weeks. Thirty male rabbits (8-10 weeks of age) divided into six groups (n=5) were used for the study. Group I (Normal control) fed on standard animal feed (SAF) only, Group II-VI were (DAM groups): [Group II, fed SAF only (diabetic control), Group III = SAF + cholestran (0.26 g/kg), Group IV = SAF + clove buds (12.5%) supplements, Group V = SAF + fermented ginger (12.5%) supplements, and Group VI = SAF + clove buds (12.5%) + fermented ginger (12.5%)]. At the end, animals were humanely sacrifice and serum was used for assessments of superoxide dismutase (SOD), catalase

(CAT) and malondialdehyde (MDA) concentrations using standard laboratory procedures (Schmidt and Schmidt, 1976). One way ANOVA, with Tukey's Post-Hoc Test was used to analysed the data generated, the results were presented as mean  $\pm$  SEM.

The results shows a significant increase ( $P < 0.05$ ) in CAT concentration in group V compared to diabetic control group and also indicate a significant ( $P < 0.05$ ) increased SOD and CAT concentrations, and a decrease in MDA level in group VI compared to diabetic control group.

In conclusion, the observed increased oxidative stress enzymes and decreased lipid peroxidation marker in the treatment groups indicates ameliorative activity of the individual supplements on oxidative stress and importantly a synergistic effect was observed when they are combined, this may be attributed to presence of flavonoids, phenolic and other active constituents (Park *et al.*, 2011; Rahmani *et al.*, 2014; Hsu *et al.*, 2015) in the supplements. Hence, the combination of these supplements should be further studied for their consideration among diets content to ameliorate diabetes pathologies.

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PCA230

# **GABA differentially modulates cytokine secretion and proliferation of peripheral blood mononuclear cells and CD4<sup>+</sup> T cells from nondiabetic individuals and type 1 diabetic patients**

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The  $\gamma$ -aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the brain but also acts as an

immunomodulatory molecule in the blood (1). GABA is synthesized from glutamate via the enzyme glutamic acid decarboxylase (GAD, two isoforms 65 and 67). GAD65 is the major autoantigen in type 1 diabetes (T1D), however, it is still not known how the level of GABA changes in the blood and if it affects the cytokine release and proliferation of immune cells in T1D.

Nondiabetic (ND) individuals (n=30) and T1D patients (n=64) were included in the study. Plasma, peripheral blood mononuclear cells (PBMCs) and CD3<sup>+</sup> T cells were isolated from fresh blood samples and CD4<sup>+</sup> T cells from buffy coats. The GABA concentration and 92 cytokines were measured in plasma from the subjects by GABA ELISA and multiplex Proximity Extension Assay (PEA) assays, respectively. Twenty-six cytokines were significantly increased in plasma from T1D patients as compared to ND individuals. The plasma GABA concentration was significantly higher in T1D patients (mean  $\pm$  SEM, ND: 501 $\pm$ 32 nM; T1D: 649 $\pm$ 42 nM, two-tailed Student's t-test,  $p < 0.05$ ) and correlated with levels of 10 cytokines in plasma. The transcriptome analysis of isolated CD3<sup>+</sup> T cells using RNA-seq revealed that the expression of 6 genes involved in the cholesterol biosynthesis pathway was significantly reduced in T1D samples. The expression levels of 2 out of these 6 genes (*MSMO1* and *CYP51A1*) were negatively correlated with plasma GABA concentration (Spearman rank correlation,  $p < 0.05$ ). Isolated PBMCs and CD4<sup>+</sup> T cells were stimulated with anti-CD3 antibody to examine the effect of GABA on cell proliferation and cytokine secretion in the culture media. GABA (100 nM) significantly inhibited proliferation of PBMCs from T1D patients but not ND individuals. In addition, GABA (100 nM) decreased the release of 47 and 16 cytokines in the medium from stimulated PBMCs of T1D patients and ND individuals, respectively. Interestingly, stimulated CD4<sup>+</sup> T cells from ND individuals can be grouped into GABA responder and non-responder T cells based on the response to GABA (100 and 500 nM) in the proliferation assay. In the non-responder T cells, GABA only regulated the release of 8 cytokines. In contrast, in the responder T cells, GABA decreased cell proliferation and release of 37 cytokines.

These results suggest the immunomodulatory function of GABA is concentration-dependent, varies among immune cell subpopulations and is altered in T1D (2).

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA231

**Functional characterisation of GABA<sub>A</sub> receptors in human pancreatic  $\beta$  cells from nondiabetic and type 2 diabetic donors**

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Human pancreatic islets release many signal molecules including neurotransmitters like  $\gamma$ -aminobutyric acid (GABA) (1, 2). In the present study, the native GABA<sub>A</sub> receptors (GABA<sub>A</sub>Rs) in intact human pancreatic islets from nondiabetic (ND) and type 2 diabetic (T2D) donors were studied and used as biological sensors for GABA. We examined the biophysical properties of the GABA<sub>A</sub>Rs in the  $\beta$  cells and then, the functional implications of their activation.

By using the patch-clamp technique in the whole-cell configuration, we identified distinct patterns of GABA<sub>A</sub>R-mediated currents in human islet  $\alpha$ ,  $\beta$  and  $\delta$  cells. Based on single-channel current distributions registered in  $\beta$  cells, GABA<sub>A</sub>Rs can be divided into two groups that were termed islet-GABA<sub>A</sub> receptor I and II (iGABA<sub>A</sub>RI and iGABA<sub>A</sub>RII) with the single-channel conductance (as a mean  $\pm$  S.E.M.) of  $30 \pm 3$  pS ( $n = 11$ ) and  $72 \pm 2$  pS ( $n = 9$ ), respectively. Current-voltage relationships, open probability ( $P_o$ ) and mean current ( $I_{mean}$ ) as function of the membrane potential were analysed for the iGABA<sub>A</sub>Rs. We also compared the  $P_o$  and  $I_{mean}$  for the iGABA<sub>A</sub>Rs in  $\beta$  cells as a function of GABA concentrations from ND and T2D donors. The results showed that the frequency of iGABA<sub>A</sub>R single-channel openings was highest in 100 to 1000 nM GABA.

The kinetics of iGABA<sub>A</sub>Rs were temperature sensitive for both ND and T2D. Modelling the data revealed significantly lower GABA apparent affinity ( $K_a$  values) for  $\beta$  cell iGABA<sub>A</sub>Rs from T2D donors as compared to ND donors at room temperature as well as 34°C. The iGABA<sub>A</sub>Rs in T2D  $\beta$  cells are thus super-sensitive to GABA. At the level of insulin-containing single vesicles, 100 nM GABA decreased the rate of exocytosis in  $\beta$  cells. On the other hand, the effect of GABA on glucose-stimulated insulin secretion from groups of 10–15 islets was more variable.

We examined the effects of several commonly used medicines. The results demonstrate that the benzodiazepine diazepam and the anaesthetics propofol and pentobarbital but, interestingly, not the hypnotic zolpidem, significantly increased  $I_{mean}$  and the frequency of iGABA<sub>A</sub>R single-channel openings. Further, the incretin glucagon-like peptide-1 (GLP-1) significantly enhanced the frequency of channel openings by a factor of  $3.3 \pm 1.1$  (Mann-Whitney rank sum test,  $P < 0.05$ ,  $n = 7$ ). However, although the frequency decreased after GLP-1 washout, it remained significantly higher than before the GLP-1 application.

In summary, in human islets GABA activates  $\beta$  cell-specific GABA<sub>A</sub>Rs that become highly sensitive to GABA in T2D. GABA modulates rate of insulin granule exocytosis and glucose-stimulated insulin secretion. iGABA<sub>A</sub>R activity in  $\beta$  cells is enhanced by diazepam, anaesthetics, GLP-1 but not zolpidem (3).

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA232

**TRPC3 channels are indispensable for oro-sensory perception of dietary lipids, and involved in obesity in mice and human**

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Transient receptor potential (TRP) canonical (TRPC) channels play a critical role in sensory physiology. We assessed the implication of diacylglycerol (DAG)-responsive TRPC channels like TRPC3, TRPC6 and TRPC7, in oro-sensory perception of dietary lipids. We employed different biochemical techniques including, RT-qPCR, western blotting, and calcium imaging studies. All procedures on mice or humans were performed according to standard guidelines and were approved by the institutional ethical committees. We observed that mouse lingual taste bud cells (mTBC) expressed only TRPC3, but not TRPC6 or TRPC7, channels. Linoleic acid (LA), a long-chain fatty acid, triggered  $\text{Ca}^{2+}$  influx via the opening of TRPC3 channels in mTBC. Mass spectroscopy revealed that 1-stearoyl-2-linoleoyl-*sn*-glycerol (SLG), a DAG species, was produced by LA stimulation of mTBC. Consequently, an increase in  $[\text{Ca}^{2+}]_i$ , triggered by LA, SLG and 1,2-dioctanoyl-*sn*-glycerol (*DiC8*), an exogenous DAG analogue, was abolished in mTBC, treated with pyrazole-3 (Pyr-3), a TRPC3 channel blocker. Similarly, DAGs-induced increases in  $[\text{Ca}^{2+}]_i$  were curtailed in mTBC from *TRPC3*<sup>-/-</sup> mice, and also in mTBC in which TRPC3 expression was decreased by siRNA. Behavioral studies showed that spontaneous preference for a fatty acid was abolished in *TRPC3*<sup>-/-</sup> mice. Furthermore, while maintained on a high-fat diet or normal diet, the *TRPC3*<sup>-/-</sup> mice developed more severe obesity than wild-type animals, and TRPC3-signaling in mTBC was altered in obese mice. To study the implications of these findings in humans, we employed the “dominant model” to for genotyping analysis of rs11732666 TRPC3 single nucleotide polymorphism (SNP) in Algerian obese ( $n = 57$ ) and lean ( $n = 59$ ) children ( $n = 116$ ). It was revealed that Algerian obese children with heterozygous or mutated AA/AG genotype ( $n = 24$ ) exhibited significantly higher ( $p < 0.05$ ) fatty acid detection thresholds than homozygous GG genotype ( $n = 33$ ). Our study shows, for the first time, that lingual TRPC3 channels are implicated in lipid taste perception and, consequently, in obesity in mice and humans.

Authors are thankful to technical assistance of Miss Alexia Mathou (INRA, Dijon) and Miss Bettina Braun (LMU Munich). Thanks are also due to Higher Education Committee (HEC) Pakistan that sanctioned the PhD scholarship to one of the authors (BM). This work was also supported in part by the Intramural Research Program of the NIH (Project ES-Z01-101684 to LB).



Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

# PCA233

## Does glucose-dependent cross-linking influence *in vivo* toxicity and compatibility of albumin-derived perfluorocarbon-based artificial oxygen carriers?

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Within the last years several studies with albumin-derived perfluorocarbon-based artificial oxygen carriers (A-AOCs) with different shell materials were carried out successfully (1-3). In order to further prolong the circulation half-time of the capsules a new approach, the synthesis of glucose-cross-linked albumin-derived artificial oxygen carriers (gcA-AOCs), was tested for its *in vivo* compatibility.

To demonstrate safety of our artificial oxygen carriers in a top load model (TL), we applied such solutions (+ 1/6 of blood volume) in the presence or in the absence of gcA-AOCs (control), respectively. 16 healthy male Wistar Rats were anesthetized with isoflurane (2.0 % in 100 % medical O<sub>2</sub> at 4.0 l/min for induction, 1.5–2.0 % isoflurane in 100 % medical O<sub>2</sub> at 1.0 l/min throughout the experiment) through face masks connected to a vaporizer. For analgesia, they received ketamine (50 mg/kg body weight) and lidocaine (5 mg/kg body weight) subcutaneously into the right chest wall. Portex catheters were placed within the right femoral artery (biomonitoring) and the right femoral vein (infusion of gcA-AOCs or 0.9 % normal saline (NS), 10 ml/kg body weight). After the infusion period (30 min) rats were further observed up to 180 min. During TL systemic parameters, plasma enzyme activities and acid base status were continuously monitored. To determine the therapeutically relevant oxygen capacities, oxygen content donated by gcA-AOCs was discovered using a respirometer. Oxygen resolved in buffer was removed by adding yeast that was subsequently inactivated by adding potassium cyanide to prevent aerobic metabolism of the yeast in the processing experiment. After oxygen loading, gcA-AOCs or 0.9 % NS were applied to the oxygen-free measuring chambers of the respirometer under exclusion of air.

Results of 8 vol. % gcA-AOCs were compared to 8 vol. % A-AOCs in 0.9 % NS/ 5 % human serum albumin (5 % HSA)(4). During infusion systemic parameters (e.g. blood pressure, pH, pO<sub>2</sub> and pCO<sub>2</sub>) were unaffected in all groups (gcA-AOCs, A-AOCs and controls). Compared to all other groups, gcA-AOCs displayed increased plasma enzyme activities. Likewise gcA-AOCs released considerably more oxygen compared to A-AOCs.

The present study demonstrates that toxicity of equal doses of A-AOCs depends on the structure of the shell material. Using the same molecule (albumin) to construct the shell does not guarantee to sustain safety profiles and oxygen release as proven by the glucose-dependent cross-linking of A-AOCs. Because of the high oxygen release we are currently looking for more detailed information on the physico-chemical properties of gcA-AOCs.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

# PCA234

## “Alloxan”, a drug of technique.

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The greatest increase in the prevalence of diabetes mellitus (DM) is expected to occur in Africa and Asia by the year 2030 (Wild *et al.*, 2000). Animal models of DM represent an important tool in DM investigation and chemical methods of alloxan and streptozotocin-induced DM represent the most important and highly preferable experimental models for this pathological condition (Radenković *et al.*, 2016). There has been a controversy concerning the techniques of induction with alloxan and the duration of the induced hyperglycemia. Sprague Dawley rats were used and divided into two separate experiments. Experiment 1: to determine the most appropriate technique for induction of DM with alloxan; and Experiment 2 served as confirmation as well as a means to determine the duration of the induced hyperglycemia. Following a 24 hour and a 48 hour pre-induction fast, freshly dissolved alloxan at concentrations of 20 mg/ml, 10 mg/ml, 5 mg/ml and 4 mg/ml was administered to rats in 4 experimental groups; each group was based on the dose of alloxan administered per kg of body weight of rat (Group 1- 100 mg/kg, Group 2- 120 mg/kg, Group 3- 150 mg/kg and Group 4- 170 mg/kg). 72 hours post induction results showed that a pre-induction fast of 48 hours and administration of freshly prepared alloxan at 150 mg/kg of body weight, at a concentration of 20 mg/ml, would achieve the highest induction rate (70%), with the least mortality (20%). The duration of hyperglycemia lasted for 8 weeks. This study provides a simple model for alloxan-induced DM rats and implies that the technique used in induction is the most important factor in alloxan-induced DM.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA235

**Protective Effects of Magnesium Chloride on Liver Enzymes and Oxidative Stress Biomarkers in Cholesterol diet fed Rats**

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The excessive consumption of high cholesterol diet has been associated with an increased incidence of obesity. This is because obesity induced pathologies with high mortality, such as complications of dyslipidaemia, diabetes mellitus, arthritis, hypertension, myocardial infarction, and hepatocellular carcinoma. Although the associated, disease are enhanced by formation of oxidative stress, lipid peroxidation and hypercholesterolaemia. Magnesium chloride is found to be beneficial in a wide range of diseases. Magnesium is one of the most neglected mineral in human body. It is crucial for a healthy and lasting life. Magnesium is responsible for the activation of more than 300 enzymes in the body. The present study intends to determine the protective effect of magnesium chloride on liver enzyme and oxidative stress biomarkers in cholesterol diet fed rats. Twenty (20) adult male Wistar rats weighing (180 – 200) grams randomly divided into three treatment and one control groups of five rats each (n = 5). Group I Normal control receive normal feed only for 6weeks, Group II received high fat diet only for 6weeks, Group III received high fat diet with 250 mg/kg for 6weeks of mgcl<sub>2</sub> and Group IV received 500 mg/kg for 6weeks of mgcl<sub>2</sub> respectively all treatments were administered via oral route, and at the end of the sixth week rats were euthanized and blood samples were drawn from the heart by cardiac puncture and used to estimate oxidative stress biomarkers (Superoxide dismutase, Catalase and Gluthation peroxidase) and lipid peroxidation biomarkers (Malondealdehyde), and liver enzymes. Analysis of variance (ANOVA) and Turkey's post hoc test were used to analyze the data obtained. For the oxidative stress biomarkers assessed, the results showed that there was significant decrease (P < 0.05) in the SOD, CAT and GP<sub>x</sub> level of the HFD groups co-administered with mgcl<sub>2</sub> when compared with the HFD fed group only. Also the lipid peroxidation shows significant (p<0.05) decrease in the value of MDA administered with mgcl<sub>2</sub> and HFD when compared with HFD group only. For the liver enzyme, The result showed that there was statistical significant (p<0.05) decrease in value of AST, ALT and ALP in the group co-administered with mgcl<sub>2</sub> and high fat diet for both 250 mg/kg and 500mg/kg mgcl<sub>2</sub>, when compared to the HFD group only. The result showed that high-fat diet induces ROS, dyslipidaemia and release of biological metabolite, as evidenced by the rise in oxidative stress and activities of liver enzymes. Mgcl<sub>2</sub> increase compensatory mechanisms by inhibiting the rise in biological metabolites in groups treated with it. Mgcl<sub>2</sub> administration also protected the body against rise in the metabolites despite consumption of high-fat diet by the Wister Rats.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA236

**Effects of opioid (tramadol) treatment on testicular functions in adult male rats: The role of nitric oxide and oxidative stress**

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Tramadol hydrochloride is frequently used as a pain reliever, and for the treatment of premature ejaculation. Decreased semen quality was noted in chronic tramadol users. The present study aimed to elucidate the effects of tramadol on the testicular functions of adult male rats.

2.Methods: A total of 40 albino adult male rats were divided into control and tramadol groups, with 20 rats for each group. Rats of the tramadol group were subcutaneously injected with 40 mg/kg three times per week for 8 weeks. The control group received normal saline 0.9%. Blood samples from each animal were obtained. Plasma levels of different biochemical substances were determined. Nitric oxide was measured in testicular tissue samples. Those samples together with epididymal tissue samples were processed for histopathological examination.

3.Results: Tramadol significantly reduced plasma levels of luteinizing hormone, follicle-stimulating hormone, testosterone and total cholesterol, but elevated prolactin and estradiol levels compared with the control group. In addition, tramadol increased the testicular levels of nitric oxide and lipid peroxidation, and decreased the anti-oxidant enzymes activities significantly compared with the control group. The tramadol group showed decreased sperm count and motility, and numbers of primary spermatocytes, rounded spermatid and Leydig cells. Immunohistochemical examinations showed that tramadol increased the expression of endothelial nitric oxide synthase in testicular tissues.

4.Conclusion: The present study showed that tramadol treatment affects the testicular function of adult male rats, and these effects might be through the overproduction of nitric oxide and oxidative stress induced by this drug.

Key words: nitric oxide synthase, oxidative stress, testicular function, tramadol.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA237

### Does Irisin Affect the Hypothalamic Regulation of Feeding Behavior in High-Fat-Diet-Induced Obese Rats

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The hypothalamus is a key brain region in the regulation of food intake where many factors such as leptin and ghrelin are involved in this control mechanism. Irisin, a novel myokine-derived hormone, has been suggested to play an important role in regulation of feeding behavior and development of obesity. This study aimed to determine the role of irisin on the central regulation of feeding behavior in high-fat-diet-induced obese rats.

Sixty-three adults male Wistar-albino rats were used in the study. The rats were randomly divided into 7 groups (n=9) and fed with high-fat-diet-chow (HFDC) for 12 weeks. After development of obesity was confirmed, 10 and 100 nM concentrations of irisin were infused to animals (except control and sham groups) as intracerebroventricularly<sup>[1]</sup> or intraperitoneally for 14 days. The mRNA gene expression/protein levels of AgRP and CART in the hypothalamus and serum leptin and ghrelin levels were determined.

Neither intracerebroventricularly nor intraperitoneally administration of the two different concentrations of irisin in animal fed with HFDC caused any change in body weights, serum leptin and ghrelin levels while irisin caused significant decreases in hypothalamic AgRP and CART gene expression/protein levels in all experiment groups (p<0.05). Our results suggested that irisin can exert a central modulatory role on regulation of feeding behavior and thereby can provide to remain constant of body weight.

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PCA238

### Carotid sinus nerve resection reduces weight gain and improves metabolic function in high-fat animals via an increase in brown adipose tissue function and decrease in its inflammation

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Background and aim: Obesity has reached epidemic proportions, being defined by WHO as the “21<sup>st</sup> Century World Epidemic”(1) and the therapeutic solutions to treat this pandemic clearly scarce. Recently, the carotid body (CB), a

chemoreceptor organ classically defined as an oxygen sensor, has been shown to be involved in metabolic deregulation. It was demonstrated that an overactivation of the CB is present in animal models of prediabetes and type 2 diabetes and that the abolishment of CB activity, through the resection of the carotid sinus nerve (CSN) prevents and reverses insulin resistance and glucose intolerance (2,3), an effect mainly due to the restore of glucose uptake by the adipose tissue (3). Also, we have shown that CSN resection decreased weight gain and total body fat mass and increased the amount of brown adipose tissue (BAT) in rats submitted to a high-fat diet (4). Taking this into account, the main goal of this work was to investigate if the effect of CSN on weight gain and metabolic function in obesity is due to an increase in BAT amount and function.

**Materials and Methods:** Experiments have been performed in male Wistar rats randomly divided into 2 groups and submitted to 10 weeks of hypercaloric (HF, 5.1Kcal/g) or control (CTL, 2.56Kcal/g) diet. After 10 weeks of diet, animals were submitted to either bilateral CSN ablation or to a sham surgery. After CSN surgery, animals were maintained on their respective diets for 9 weeks and at the end of which they were anesthetized with pentobarbital (60 mg/kg i.p.) and tissues were collected, weighed and stored for further analysis. Caloric intake and body weight were monitored throughout the experiments. The number of mitochondrias and their activity were investigated in BAT by the use of Mitotracker and by UCP1 expression through immunohistochemistry. The expression of proteins involved in insulin signalling pathways, tissue hypoxia factors and inflammation was evaluated by Western blot. In a parallel experiment using the same animal model of obesity, *in vivo* glucose uptake was evaluated by an intravenous glucose tolerance test (IVGTT).

**Results:** HF diet decreased insulin receptor (IR) expression by 68% and increase IL-1 and IL-6 expression by 30% and by 179% in BAT. Bilateral CSN resection increases IR expression in BAT by 68% and by 157% in control and HF animals, respectively and decreased significantly the inflammation markers, IL-1 and IL-6 by 57% and 47%, respectively. Moreover, CSN resection improved glucose uptake in BAT by 125% in control animal and by 67% in HF animals.

**Conclusion:** We can conclude that CSN denervation positively impacts weight gain and metabolic function in a hypercaloric obese animal model via an improvement of BAT function and glucose uptake and a reduction in BAT inflammation.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.



PCA239

**Beneficial effects of carotid sinus nerve resection on white adipose tissue metabolism**

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**Background and aims:** The therapeutics to treat obesity are scarce(1). We have proposed a new role for the carotid body (CB) in the pathogenesis of metabolic disturbances, demonstrating that the abolishment of its activity, through resection of carotid sinus nerve (CSN), its sensitive nerve, prevents and reverses insulin resistance and glucose intolerance in animals with metabolic diseases(2,3). Furthermore, CSN resection decreased weight gain and improved metabolic function in an obese animal model, with metabolic dysfunction(4). Herein, we investigated if the effects on weight gain and glucose metabolism are related to a reduction on white adipose tissue (WAT) amount and an improvement on its metabolism.

**Materials and Methods:** Experiments were performed in 9 weeks male *Wistar* rats. Animals were submitted to 10 weeks of high-fat diet (HF) (5.1Kcal/g) or to a standard diet (2.56Kcal/g). After 10 weeks of diet, rats were submitted to either bilateral CSN resection or to a sham procedure and kept under the respective diets for 9 weeks. Insulin sensitivity, glucose tolerance, caloric intake and body weight were monitored throughout the experimental protocol. At terminal experiment rats were anaesthetised with pentobarbital (60mg/kg i.p.) and WAT depots were collected, weighted and stored for analysis of proteins involved in WAT metabolism and quantification of UCP-1 protein, mitochondrial density and adipocytes perimeter. In a parallel study *in vivo* glucose uptake was evaluated by an intravenous glucose tolerance test. **Results:** HF diet increased total WAT amount by 120% as well as adipocyte perimeter in the perienteric depot by 151%. Also, HF diet induced whole-body insulin resistance ( $K_{ITT}$  HF before surgery =  $4.4 \pm 0.9$  %glucose/min;  $K_{ITT}$  HF after 10 weeks of diet =  $1.8 \pm 1.0$  %glucose/min), glucose intolerance (AUC controls =  $20016.8 \pm 450.4$  mg/dlxmin; AUC HF after 10 weeks of diet =  $25415.3 \pm 689.3$  mg/dlxmin) and decreased the uptake of glucose in perienteric WAT depot by 48%. CSN resection in HF animals decreased perinephric (18%), epididymal (22%), perienteric (38%) and subcutaneous (24%) as well as total WAT amount (24%). Also, CSN resection restored insulin sensitivity and glucose tolerance 1 week after surgery, an effect that was maintained until the last week of evaluation. Furthermore, it was observed a decrease in adipocyte perimeter by 56% and an increase in the uptake of glucose in the perinephric, epididymal and perienteric WAT depots by 51%, 77% and 97%, respectively. **Conclusion:** We conclude that the beneficial effects of CSN resection in weight gain involve a reduction in the total amount of WAT, contributing to a decrease in the size of the adipocytes and an improvement of glucose metabolism in this tissue.

Zafriir, B. Brown adipose tissue: Research milestones of a potential player in human energy balance and obesity. *Hormone and Metabolic Research*, (11), 774–785.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA240

**Acute vs chronic administration of leptin in obese rats: effects on blood pressure, sympathetic activity and glucose homeostasis**

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Leptin is an adipocyte hormone essential in energy homeostasis regulation acting centrally and peripherally. In addition to its generalized sympathoexcitatory activity that influence cardiovascular function leptin is involved in glucose homeostasis. This hormone is one of the key players involved in the development of obesity induced-hypertension via sympathetic activation; however there is some controversy regarding the acute vs chronic leptin effects on BP (1). Chronic intravenous and carotid infusion of leptin increases BP in conscious rats (2) an effect that could be mediated in part by the carotid body (CB). Recently, we have reported that leptin might be involved in the trigger of CB (3), a peripheral chemo and metabolic sensor, contributing to the genesis of insulin resistance and hypertension in metabolic syndrome animals via sympathetic nervous system (SNS) activation (4). Also, it is known that chronic leptin administration improves insulin sensitivity and normalizes plasma glucose in type 2 diabetes rats, independent of energy intake via peripheral and possibility centrally mediated actions (5). Therefore, we aimed to evaluate the effect of acute vs leptin administration on mean arterial pressure (MAP), SNS activity and glucose homeostasis in a metabolic syndrome animal model. Experiments were performed in male *Wistar* rats (8-9 weeks) fed during 3 weeks with a standard (CTR) or 60% lipid-rich (HF) diet. Acute leptin administration (90-360 ng/ml, i.v.) was evaluated in MAP and in SNS activity. Chronic leptin administration was tested on control and HF animals submitted to 7 days of leptin administration. Additionally, the contribution of CB to leptin effects was tested by making the same experiments in animals submitted to carotid sinus nerve resection prior to HF diet. Leptin effects were evaluated on MAP, SNS activity, caloric intake, temperature as well as on insulin sensitivity and glucose tolerance. HF diet increased MAP by 32.87% (control=81.76 mmHg; HF= 108.63mmHg;  $p < 0.05$ ) and SNS activity by 22.54% (control=0.8154; HF=1 burst/sec;  $p < 0.05$ ), compared by Student's t-test. Acute leptin administration increased in a dose-dependent manner MAP in CTR animals (90ng/ml by 2.83% and 360 ng/ml by 27.67) an effect that was blunted in HF animals (180 ng/ml by 0.29% and 260 ng/ml by 2.34%). Also, in CTR group SNS activity increased with leptin administration (90 ng/ml by 6.05%; 180 ng/ml by 23.05%; 270ng /ml by 27.08%, ( $p < 0.05$ ) and 360 ng/ml by 17.30). We can conclude that the effect of leptin in promoting SNS activation and increase in MAP is blunted in obese animals probably via an already developed leptin resistance. Knowing that the CB is involved in the genesis of obesity induced-hypertension

we suggest that leptin action of the CB can be target to ameliorate this pathological feature.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## PCA241

### Agomelatine prevents streptozotocin-induced diabetes and ameliorates diabetic encephalopathy in mice

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Antidepressants are first-line treatments of neuropathic pain. Agomelatine is an antidepressant with a novel mode of action, acting as an MT1/MT2 melatonergic receptor agonist and a 5-HT2C receptor antagonist. Agomelatine has been shown to exert a clear-cut antihypersensitivity effect in streptozotocin (STZ) - induced diabetic neuropathy model. The present study investigated the efficacy of agomelatine in the prevention of diabetes itself and treatment of diabetic encephalopathy with a focus on oxidative stress and inflammatory processes in the brains of streptozotocin (STZ)-induced diabetic mice. Diabetes was induced in mice by intraperitoneal (i.p.) injection of STZ. In the acute experiment, agomelatine was given i.p. 45 minutes before STZ injection. In the chronic experiment, agomelatine treatment was performed six weeks after STZ injection, and following confirmation of development of diabetes, agomelatine was given. Interleukine-1 $\beta$  (IL-1 $\beta$ ) and TACR1 (NK1), which is a tachykinine receptor, were used for the investigation of inflammation in the brain regions including raphe nucleus, periaqueductal gyrus (PAG), amygdala and nucleus accumbens by using real-time polymerase chain reaction (RT-PCR). Agomelatine treatment prevented both hyperglycemia and hypoinsulinemia in STZ-treated mice in a dose-dependent manner. There were no significant changes in plasma triglycerides, total cholesterol and HDL-C levels of diabetic rats treated with agomelatine (5, 10 and 20 mg/kg). However, there was a significant reduction in the LDL-C level in all treated diabetic rats compared to the diabetic control group. Both acute and chronic agomelatine treatments caused changes in IL-1 $\beta$  mRNA and TACR1 mRNA levels in raphe nucleus PAG, amygdala and nucleus accumbens of diabetic mice. Agomelatine increased total antioxidant capacity. In conclusion, our findings suggest agomelatine seems to both prevent development of diabetes and reverse the encephalopathic changes caused by diabetes.

This study was supported by TUBITAK-115S290

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## PCA242

### Effect of Mesenchymal Stem Cells vs. Nigella Sativa Oil on Streptozotocin Induced Type 1 Diabetes in Rats

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Diabetes is a chronic metabolic disorder with no definitive cure. Mesenchymal Stem Cells (MSCs) and Nigella Sativa Oil (NSO) are two promising treatments. This study compares the MSCs vs. NSO on streptozotocin (STZ) induced type 1 diabetes (T1D) in rat model. Adult female albino rats were divided into 7 equal groups of 10: Control group (G1) received saline, Diabetic groups (G2.1 & G2.2) sacrificed after 15 & 30 days of T1D induction respectively, MSCs treated group 1 (G3.1& G3.2) received IV injection of  $1 \times 10^6$  cells/rat 3 days after T1D induction and sacrificed as previous, NSO treated group1 (G4.1 & G4.2) received daily I.P injection of 8 ml/Kg of NSO 3 days after T1D induction and sacrificed as previous. T1D was induced using multiple low doses of STZ (40 mg/Kg). MSCs were isolated from human cord blood samples. Pancreas was extracted and processed for histological and immunohistochemical (IHC) assessment. The mean total islet cell count, percentage of damaged cells, mean number and diameter of islets, and optical density of collagen and brown stained cytoplasm of immunostained sections were calculated using image analysis program. Human male SRY gene sequence in pancreas of female rats was detected by PCR. Values are means  $\pm$  SEM, compared by ANOVA. Results showed low blood glucose levels in both MSCs and NSO groups, which reached near normal levels within 15 days. Histological assessment of control group showed normal architecture, mean number of islets was  $13.6 \pm 3.3$ , mean diameter of islets was  $88.7 \pm 17.2$ , mean total islet cell count of  $2000.70 \pm 90.19$  and mean optical density (MOD) of collagen was  $0.25 \pm 0.07$ . IHC showed strong brown staining of  $\beta$ -cell cytoplasm with MOD of  $0.60 \pm 0.05$ . G2.1 group showed necrotic changes in islet cells, congested dilated blood capillaries with lymphocytic infiltration. Mean number and diameter of islets and mean total islet cell count were lower compared to control ( $10.9 \pm 2.6$ ,  $66.3 \pm 12.3$  &  $1017.90 \pm 76.63$  respectively). Mean percentage of damaged cells was  $52.17 \pm 6.62$ . MOD of collagen was comparable to control, but there was significant decrease in the MOD of the immunoreaction ( $0.30 \pm 0.03$ ). The G2.2 group was worse than G2.1. MSCs group (G3.1) showed marked improvement compared to diabetic groups. Mean number and diameter of islets and mean total islet cell count increased ( $11.0 \pm 1.8$ ,  $71.9 \pm 15.4$  &  $1036.80 \pm 65.44$  respectively), while mean percentage of damaged cells decreased ( $17.16 \pm 3.27$ ). MOD of the immunoreaction significantly increased compared to diabetic group ( $0.52 \pm 0.05$ ). Group G3.2 was better than G3.1. Comparable results were obtained for NSO groups (G4.1). Positive results for SRY gene were detected in MSCs group, indicating homing of stem cells into the pancreas of the female rats. In conclusion, MSCs and NSO exert comparable antidiabetic effects on STZ induced T1D.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCA243

**Effect of L-Arginine and D-ribose L-cysteine supplementation on stress hormones in 4-vinylcyclohexene diepoxide perimenopausal female rats following acute restraint stress**

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Perimenopause involves dynamic neuroendocrine reactions that promote exacerbation of stress responses. L-Arginine and D-ribose have been observed to generally reduce basal levels of stress hormones, but their benefits during perimenopause have not been elucidated.

Immature female Sprague-Dawley rats postnatal day 28 from the colony in the animal facilities of the College of Medicine, University of Lagos, Idi-Araba campus were housed in groups of six per cage and were divided into 3 major groups; (1) Control (injected subcutaneously) with Corn oil (2.5 µl/g BW) for 15 days; (2) VCD, (injected subcutaneously) with 4-vinylcyclohexene diepoxide (160 mg/kg BW) from Sigma-Aldrich, United Kingdom diluted in Corn oil (2.5 µl/g BW) for 15 days; and (3) Aged group was left to age naturally till 150 days. Twelve weeks after VCD/corn oil administrations, and 150 days in Aged group, rats were further divided into 3 sub-groups: L-Arginine (administered orally, 100mg/kg BW) from Now Foods, Bloomingdale, USA., D-ribose (administered orally, 200mg/kg BW) from Max international Salt Lake, USA and a third sub-group received neither for additional 30 days.

At 135 – 145 days of age in Control and VCD groups., 175 – 185 days of age in Aged group on diestrus morning, animals were subjected to acute restraint stress for 30 minutes. The animals were immediately anaesthetized with tribromoethanol (2, 2, 2-tribromoethanol 99%, Sigma Aldrich, 1 mL/100 g body weight of 2.5% solution, intraperitoneal route) humanely sacrificed. Blood samples were drawn before and after stress from the retro-orbital sinus for measurement of serum prolactin, LH, FSH, progesterone, and corticosterone.

In unstressed and stressed animals, L-Arginine and D-ribose significantly increased ( $p < 0.05$ ) progesterone and corticosterone concentrations in Aged and VCD groups compared to Control group. D-ribose significantly increased ( $p < 0.05$ ) FSH, LH, and Prolactin concentrations in Aged and VCD groups compared to Control group. L-Arginine significantly decreased ( $p < 0.05$ ) FSH and LH concentrations in Aged and VCD groups compared to Control group. L-Arginine significantly increased ( $p < 0.05$ ) prolactin concentrations in unstressed animals and also significantly decreased ( $p < 0.05$ ) it in stressed animals in the Aged and VCD groups compared to Control group.

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PCA244

**PROTECTIVE ROLE OF *Allium cepa* Linn JUICE ON MATERNAL DEXAMETHASONE INDUCED ALTERATIONS IN REPRODUCTIVE FUNCTION DURING LACTATION IN MALE OFFSPRING OF WISTAR RATS**

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Maternal exposure to dexamethasone during lactation as been reported to increase testicular oxidative stress in the offspring. *Allium cepa* Linn. (Red Onion) is a known free radical scavenger with antioxidant properties. In this study, the effects of *Allium cepa* Linn on testicular oxidative stress and disruption of reproductive indices in the male offspring following maternal dexamethasone exposure during lactation in Wistar rats were examined.

Twenty (20) lactating dams were assigned into four groups (n=5) and they were treated as follows during lactation: Control (distilled water), dexamethasone (60 µg/kg), *Allium cepa* Linn (5 ml/kg) and dexamethasone (60 µg/kg)+ *Allium cepa* Linn (5 ml/kg). All administration was done orally. Testicular descent, pubertal age were assessed using standard methods. Sperm parameters (Microscopy techniques), hormonal profile (ELISA techniques, Dialab kit Austria), testicular MDA (Buege and Aust, 1978), SOD (Misra and Fridovich, 1972), Catalase (Sinah, 1971) were evaluated. Values (Mean±SEM, n=5) were compared using one way ANOVA.  $P < 0.05$  was significant.

Serum testosterone (1.69±0.175ng/mL), FSH (0.62±0.040 mIU/mL), LH (0.12± 0.006 mIU/mL) were significantly lower ( $P < 0.05$ ) in the Dex only group when compared with the control (6.49±0.171 ng/mL, 1.79±0.098 mIU/mL, 0.16±0.011 mIU/mL respectively). Sperm count (2.18± 0.370 X 10<sup>6</sup>/ml), motility (32±3.742 %), viability (22± 3.742 %) were significantly lower in the Dex only group when compared with the control (6.84± 1.345 10<sup>6</sup>/ml, 66.00± 4.000%, 76.00± 4.000% respectively). Testicular SOD (998.10±16.730 U/L), Catalase (5.86±0.630 µmol/min/mL), GPx (-3.54±0.522 U/L) were significantly lower relative to the control (2124.00±33.680 U/L, 9.93±0.306 µmol/min/mL, 2.28±0.105 U/L respectively). However, there was a significant increase ( $P < 0.05$ ) in days of testes descent (20.20 ± 0.200 days), preputial separation (69.80 ± 0.200 days), testicular MDA (43.40 ± 0.143 µM) with respect to the control (17.60 ± 0.245, days 62.80 ± 0.200 days, 14.30 ± 0.171 µM). Conversely, treatment with *Allium cepa* Linn. juice significantly ( $P < 0.05$ ) raised serum level of testosterone (4.40 ± 0.153 ng/mL), FSH (0.72 ± 0.045 mIU/mL) and LH (0.14 ± 0.006 mIU/mL), sperm motility (70.00 ± 4.472 %), viability (60.00±4.470 %), count (5.62 ± 0.396 X 10<sup>6</sup>/ml), testicular SOD (1707 ± 22.7U/L), Catalase (8.21 ± 1.110 µmol/min/mL) and GPx (4.93 ± 0.422 U/L) of pups in dexamethasone treated rats. Also, *Allium cepa* Linn significantly reduced



( $P < 0.05$ ) testicular MDA level ( $26.46 \pm 0.099 \mu\text{M}$ ) in the pups of dexamethasone treated dams.

The findings from this study suggest that *Allium cepa* Linn could protect against testicular oxidative stress and disruption of the reproductive axis induced by maternal dexamethasone exposure during lactation.

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PCA245

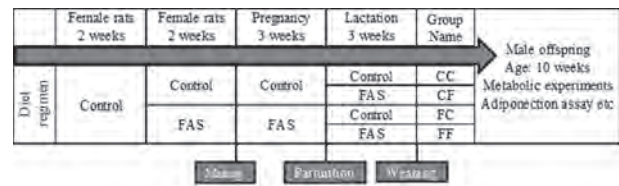
### High-Dose Perinatal Folic-Acid Supplementation Alters Insulin Sensitivity in Sprague-Dawley Rats and Decreases Adiponectin Expression

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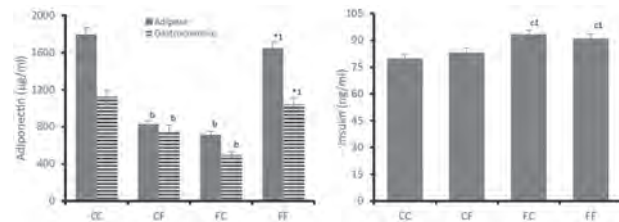
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Altered nutritional environment during foetal and neonatal development leads to detrimental effects in offspring health later in life. Micronutrient supplementation in pregnant women may be a promising strategy for reducing these adverse outcomes. For instance, folic acid supplementation has been recommended prior to and during pregnancy, to reduce the risk of infant neural tube defects. However, the unintended consequences of folic acid supplementation (FAS) during pregnancy, potentially in excess of the recommended upper levels, is a matter of critical importance. This study examined the offspring of female rats exposed to high FAS during pregnancy and/or lactation to determine the effects on glucose tolerance, insulin sensitivity, lipid metabolism, and expression of adiponectin in the offspring. Twenty (20) female Sprague-Dawley rats were divided into four groups ( $n=5$ ). Control (CTR) rats received normal rat chow, while the treatment groups received FAS either during gestation (FC), lactation (CF) or both periods (FF). Food and water intake measured daily. Lipid concentrations were determined with an automatic blood chemical analyzer; insulin and adiponectin levels were measured using ELISA. Glucose tolerance and insulin sensitivity were also determined to evaluate glucose homeostasis. The obtained data was analyzed by GraphPad Prism (v. 5) using One-way analysis of variance (ANOVA). Data expressed as mean  $\pm$  standard error of mean (SEM) and the significant level was set at  $p < 0.05$ . The quantity of postweaning feed and water consumed by FAS rats was significantly ( $p < 0.05$ ) higher than that of the control. In addition, triglyceride (e.g.  $1.30 \pm 0.06$  vs.  $0.73 \pm 0.04$ , FC vs. CTR;  $p < 0.05$ ) and insulin (e.g.  $93.36 \pm 10.27$ ,  $91.0 \pm 8.94$ ; CF & FF respectively) levels were elevated ( $p < 0.05$ ) compared with control ( $79.78 \pm 8.41$ ) but insulin insensitivity was exacerbated (e.g. AUC  $627 \pm 25.08$  vs.  $789 \pm 26.57$ , CF vs. CTR;  $p < 0.05$ ) in adult offspring. The expression of adiponectin in insulin-sensitive gastrocnemius muscles was also significantly decreased (e.g.  $754.93 \pm 99.62$ ,  $492.64 \pm 82.03$  vs.  $1118.6 \pm 108.29$ ; CF, FC vs. CTR) and these were consistent with insulin resistance of FAS offspring. Findings suggest that high-dose perinatal FAS

may promote insulin resistance and dyslipidemia and disrupt glucose metabolism possibly by depressing adiponectin expression. Although this is an animal model and the effects of the diets cannot be directly transposed to humans, this study provides indications of the possible adverse effects of FAS maternal diet on glucose metabolism in the offspring.



Schematic illustration of the study design. FAS = folic acid supplementation.



(A) Adiponectin level in the adipose tissue and skeletal muscle and (B) serum insulin in rat offspring. Data expressed as mean  $\pm$  (SEM). CC = control-control; CF = control-folate; FC = folate control; FF = folate-folate. <sup>b</sup> $p = 0.01$ , <sup>c</sup> $p = 0.001$  vs CC; <sup>1</sup> $p = 0.05$ , <sup>3</sup> $p = 0.001$  vs CF; <sup>x</sup> $p = 0.05$  vs FC

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PCA246

### AT1 receptors in renal FoxD1<sup>+</sup> stromal cells are not essential for normal kidney development

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The importance of ANGII-AT1 receptors for nephrogenesis and renal functions are well known. Specific loss of function mutations or pharmacological inhibition of ANGII-AT1 receptors can lead to severe defects in kidney development in mammals. These renal abnormalities comprise papillary and tubular atrophy, abnormal wall thickening of intrarenal arterioles, renin cell hyperplasia and impairment of urinary concentration ability. However, it is still unclear in which cell types intact ANGII-AT1 receptor signalling is essential for normal kidney development. Stroma progenitor cells which are characterized by the expression of the transcription factor FoxD1 are known to influence nephrogenesis. Furthermore, existing evidence suggests that ANGII-AT1 receptors are expressed by all cells which derive from stroma progenitor cells, such as

renin producing cells, mesangial cells, tubulointerstitial cells and vascular smooth muscle cells.

Therefore, we aimed to investigate the relevance of ANGII-AT1 receptors in FoxD1<sup>+</sup> stroma progenitor cells for renal development and function. For this purpose we generated mice with a constitutive deletion of AT1a receptors in the FoxD1 lineage combined with a global AT1b receptor deletion (FoxD1<sup>+/-Cre</sup> AT1<sup>fl/fl</sup> x AT1b<sup>-/-</sup>).

In wildtype kidneys we found ANGII-AT1a mRNA in vascular smooth muscle cells, renin producing cells, mesangial cells and interstitial cells using in-situ hybridization. In kidneys of FoxD1<sup>+/-Cre</sup> AT1<sup>fl/fl</sup> x AT1b<sup>-/-</sup> animals ANGII-AT1a mRNA could not be detected in these cells. Moreover, the typical vasoconstriction and inhibition of renin secretion after addition of ANG II to isolated perfused kidneys of wildtype kidneys were absent in kidneys of FoxD1<sup>+/-Cre</sup> AT1<sup>fl/fl</sup> x AT1b<sup>-/-</sup> mice. We therefore concluded that the deletion of ANGII-AT1 receptors in FoxD1 stroma progenitor cells was very efficient.

FoxD1<sup>+/-Cre</sup> AT1<sup>fl/fl</sup> x AT1b<sup>-/-</sup> animals, however, showed no signs of structural or functional renal abnormalities compared to control littermates. The papilla, the walls of intrarenal arterioles as well as number and distribution of renin cells appeared normal. Glomerular filtration rates (1204±67.2 vs. 1238±89.1 µl/min per 100 g bodyweight) and systolic blood pressure (114.5±1.73 vs. 112.8±1.55 mmHg) as well as urine osmolality (1779±163.1 vs. 2134±123.9 mOsmol/l) and plasma renin concentrations (64.35±13 vs. 81.65±12 ng<sub>ANGII</sub>/ml·h<sup>-1</sup>) were not significantly different in FoxD1<sup>+/-Cre</sup> AT1<sup>fl/fl</sup> x AT1b<sup>-/-</sup> mice compared to control littermates.

These findings suggest that ANGII-AT1 receptors in renal stromal progenitor cells and their descendants are not required for normal kidney development and renal function. Instead extrarenal ANGII-AT1 receptors in the systemic vasculature could be crucial for kidney development.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA247

### Renal inflammatory response and function in iron overloaded male Wistar rats

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Iron overload has been recognized to be a risk factor for numerous acute and chronic illnesses likely through increased production of reactive oxygen species (Fashola *et al.*, 2013). Within the kidneys it is likely to cause necrotic renal cells as has been observed to occur in bone marrow cells (Yang *et al.*, 2017). This study investigated renal inflammatory response and function in iron-overloaded male Wistar rats.

Thirty-animals were equally distributed into 3 groups and treated with either normal saline (0.2ml; control), iron (as ferrous sulphate) at 15mg/kg and iron at 30mg/kg for 21 days respectively. All treatment was daily administered intraperitoneally. At 21-days post-treatment, blood samples were obtained by cardiac puncture after light intraperitoneal sodium thiopental anesthesia into plain sample bottles. Serum was obtained from these samples and analyzed for iron, ferritin, transferrin, creatinine, urea, albumin, total protein, interleukin-6 (IL-6), prostaglandins E-2 (PGE-2), and tumor necrotic factor-α (TNF-α). Kidney homogenates were also obtained from 5 animals in each group and analysed for superoxide dismutase (SOD), total antioxidant capacity

(TAC), reduced glutathione (GSH), lipid peroxidation (MDA) and nitric oxide levels. Kidney samples were obtained from the remaining 5 animals in each and analysed using H and E and periodic acid Schiff stains. Results are presented as Data were expressed as Mean±SEM and statistical significance was taken at p<0.05 using the Student's T-test.

Graded increase (p<0.05) in serum iron (mg/dL)(84.6±14.4; 90.5±5.5 vs. 58.8±2.1), ferritin (mg/dL)(27.5±5.9; 58.6±5.7 vs. 11.4±1.7), transferrin (mg/dL)(149.4±14.6; 163.3±4.9 vs 64.8±6.6), creatinine (mmol/L)(243.4±33.2; 163.2±14.3 vs. 120.1±18.5), urea (mg/dl)(1.03±0.01; 1.37±0.01 vs. 0.81±0.19), IL-6 (pg/ml)(14.7±1.2; 19.5±3.7), TNF-α (pg/ml) (17.5±1.7; 25.6±1.4 vs. 6.4±1.1) and a reduction (p<0.05) in albumin (g/dL)(2.09±0.02; 1.64±0.16 vs. 3.08±0.03) levels were observed in groups 2 (iron 15mg/kg) and 3 (Iron 30mg/kg) respectively compared to control. Graded reductions in renal SOD (U/ml)(0.16±0.01; 0.12±0.01 vs. 0.34±0.02), GSH (mM) (0.11±0.04; 0.017±0.02 vs. 0.25±0.03) and increases in TAC (mM)(0.81±0.26; 1.86±0.51 vs. 0.23±0.12), MDA (mM) (0.53±0.03; 0.60±0.01 vs. 0.26±0.04) and nitric oxide (mM) (30.7±3.1; 31.4±2.6 vs. 10.2±1.7) levels were observed in groups 2 and 3 compared to control. Histological evaluation of the kidney showed structural and tubular aberrations consistent with renal damage via inflammatory processes in groups 2 and 3 compared to control.

The data obtained from this study suggests that iron-overload causes renal dysfunction by triggering a cascade of systemic and renal inflammatory processes, which then alters renal structure and function.

Fasola *et al.* (2013). Afr J Med Med Sci. 42(3): 231-7.

Yang F, Li Y, Yan G, et al. (2017) Oncotarget. 8(19): 31626-31637.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA248

### Time-Dependent Effects of High Salt Diet on Renal Functions in adult Female Wistar Rats

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Background: High salt diet induces sodium glomerulopathy, with the consequence of impaired renal functions. These renal sodium-dependent effects are thought to the hypertensive effects of high sodium intake, or to increased TGF-β1 levels. However, the duration of high sodium loading may play a role in the development and the severity of such renal effects. Moreover, the high sodium loading could alter the endocrinal renal functions.

Aim: to investigate and compare the effects of high salt diet, for two different durations, on both endocrinal and excretory renal functions in rats.

Materials and Methods: 40 adult female Wistar rats were divided into control group (C, n=20); fed diet containing 0.3% NaCl, 2 week and 12 week-high salt fed groups; fed diet with 8% NaCl for 2 (HS-2W, n=10) and 12 (HS-12W, n=10) weeks respectively. At the end of experimental period, blood pressure was measured. Rats were anaesthetized by i.p. pento-barbitone (40µg/kg B.W.). The separated plasma was used

for determination of urea, creatinine, sodium, potassium, aldosterone, plasma renin activity, erythropoietin and TGF- $\beta$ 1. Left kidney of all rats was assayed for renal tissue nitric oxide (NO) and  $\alpha$ 1 hydroxylase activity. Statistical analysis by One way ANOVA was performed.

Results: High salt diet for 2 weeks caused only a significant rise in plasma levels of urea and TGF- $\beta$ 1 in the HS-2W compared to the C group. HS-12W group exhibited significant rise in plasma levels of urea, creatinine, sodium, potassium, aldosterone, and plasma renin activity, renal tissue NO level, systolic and diastolic pressure values compared to either the C group or the HS-2W group. Plasma erythropoietin level and renal tissue  $\alpha$ 1 hydroxylase were significantly lowered in the HS-12W group compared to either the C or the HS-2W groups. Moreover, plasma TGF- $\beta$ 1 was significantly elevated in HS-12W group compared to the C group only.

Conclusion:

High salt diet for longer duration has more detrimental effects on exocrine and endocrinal kidney functions through elevating TGF- $\beta$ 1.

|                                                                   | Control group<br>(n = 20) | 2 week-High salt Diet<br>Fed group<br>(n = 10) | 12 week-High salt Diet<br>Fed group<br>(n = 10) |
|-------------------------------------------------------------------|---------------------------|------------------------------------------------|-------------------------------------------------|
| Plasma Urea Level<br>(mg/dl)                                      | 34.05<br>$\pm$ 2.35       | 41.55<br>$\pm$ 1.93                            | 75.32<br>$\pm$ 3.34                             |
| P                                                                 |                           | <0.001                                         | <0.001                                          |
| P*                                                                |                           |                                                | <0.001                                          |
| Plasma Creatinine<br>Level (mg/dl)                                | 0.43<br>$\pm$ 0.03        | 0.52<br>$\pm$ 0.05                             | 1.33<br>$\pm$ 0.15                              |
| P                                                                 |                           | NS                                             | <0.001                                          |
| P*                                                                |                           |                                                | <0.001                                          |
| Plasma Na<br>(mEq/L)                                              | 140.55<br>$\pm$ 7.62      | 145.55<br>$\pm$ 4.2                            | 170.45<br>$\pm$ 5.4                             |
| P                                                                 |                           | NS                                             | <0.001                                          |
| P*                                                                |                           |                                                | <0.005                                          |
| Plasma K<br>(mEq/L)                                               | 4.05<br>$\pm$ 0.09        | 4.05<br>$\pm$ 0.14                             | 4.87<br>$\pm$ 0.03                              |
| P                                                                 |                           | NS                                             | <0.01                                           |
| P*                                                                |                           |                                                | <0.01                                           |
| Plasma Aldosterone<br>(ng/ml)                                     | 55.39<br>$\pm$ 5.9        | 30.3<br>$\pm$ 3.16                             | 23.9<br>$\pm$ 2.18                              |
| P                                                                 |                           | NS                                             | <0.05                                           |
| P*                                                                |                           |                                                | <0.005                                          |
| Systolic Blood<br>Pressure (mmHg)                                 | 125.2<br>$\pm$ 2.17       | 129.11<br>$\pm$ 0.95                           | 180.57<br>$\pm$ 3.02                            |
| P                                                                 |                           | NS                                             | <0.001                                          |
| P*                                                                |                           |                                                | <0.001                                          |
| Diastolic Blood<br>Pressure (mmHg)                                | 74.18<br>$\pm$ 3.33       | 75.8<br>$\pm$ 2.39                             | 83.87<br>$\pm$ 3.32                             |
| P                                                                 |                           | NS                                             | <0.001                                          |
| P*                                                                |                           |                                                | <0.001                                          |
| Plasma Erythropoietin Level<br>(mU/ml)                            | 12.8<br>$\pm$ 0.9         | 11.4<br>$\pm$ 1.2                              | 6.4<br>$\pm$ 0.3                                |
| P                                                                 |                           | NS                                             | <0.01                                           |
| P*                                                                |                           |                                                | <0.05                                           |
| Kidney tissue $\alpha$ 1<br>Hydroxylase activity<br>(fmol/mg/min) | 4.94<br>$\pm$ 0.02        | 4.62<br>$\pm$ 0.03                             | 2.32<br>$\pm$ 0.02                              |
| P                                                                 |                           | NS                                             | <0.05                                           |
| P*                                                                |                           |                                                | <0.05                                           |
| Plasma Renin Activity<br>(ng/ml/hr)                               | 1.7<br>$\pm$ 0.1          | 1.8<br>$\pm$ 0.2                               | 43.2<br>$\pm$ 3.8                               |
| P                                                                 |                           | NS                                             | <0.05                                           |
| P*                                                                |                           |                                                | <0.05                                           |
| Plasma TGF- $\beta$ 1<br>(ng/ml)                                  | 1.41<br>$\pm$ 0.7         | 16.02<br>$\pm$ 1.9                             | 20.52<br>$\pm$ 4.55                             |
| P                                                                 |                           | <0.05                                          | <0.005                                          |
| P*                                                                |                           |                                                | NS                                              |
| Kidney tissue NO<br>( $\mu$ M/gm wet tissue)                      | 195.5<br>$\pm$ 27.5       | 211.94<br>$\pm$ 17.5                           | 450.3<br>$\pm$ 57.26                            |
| P                                                                 |                           | NS                                             | <0.01                                           |
| P*                                                                |                           |                                                | <0.01                                           |

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

### Mice with reduced intrarenal angiotensin I-converting enzyme are protected against experimentally induced chronic kidney damage

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**Problem statement:** Mice (C57BL/6J-*tm*<sup>(ACE3/3)</sup> = ACE<sup>-/-</sup>) with reduced intrarenal angiotensin I-converting enzyme (ACE) are protected against angiotensin (ANG) II-induced arterial hypertension. The underlying mechanisms are currently not well understood. As it is known, that the renal renin-angiotensin system (RAS) is involved in the development of chronic kidney damage, we investigated whether ACE<sup>-/-</sup> mice, that have reduced intrarenal ACE but increased intrarenal abundance of ACE2 protein, are protected against experimentally induced chronic kidney damage and whether they show alterations in the renal RAS.

**Methods:** Chronic kidney damage was induced using aristolochic acid I (AAI, 3 mg/kg body weight, i.p., every three days for six weeks followed by six weeks without treatment). At the end of the protocol, animals were anaesthetised with ketamine/xylazine (12,5 mg/ml/2,5 mg/ml, i.p.), the glomerular filtration rate (GFR) was determined using inulin clearance and the kidneys were removed. During the protocol for GFR, ketamine was supplemented as needed. The renal protein abundances of renin, angiotensinogen (AGT), ACE or ACE2 were determined in ACE<sup>-/-</sup> and C57BL/6J control mice by Western blot analyses. Abundances of specific protein species were determined relative to total protein abundance or GAPDH and normalized to controls.

**Results:** GFR was similar in vehicle-treated mice of both strains. Treatment with AAI significantly decreased GFR in controls but not in ACE<sup>-/-</sup> mice. ACE<sup>-/-</sup> mice had 78% lower intrarenal ACE protein abundance than controls and AAI decreased intrarenal ACE protein abundance in both strains. Furthermore, ACE<sup>-/-</sup> mice had significantly higher intrarenal ACE2 protein abundance than controls and AAI decreased intrarenal ACE2 protein abundance in both strains. Intrarenal AGT and renin protein abundances were similar in both strains. AAI increased intrarenal AGT and renin protein abundances in ACE<sup>-/-</sup> mice but not in controls.

**Conclusions:** Mice with reduced intrarenal ACE are protected against experimentally induced chronic kidney damage. It remains to be determined whether the increased intrarenal ACE2 protein abundance helps to protect the ACE<sup>-/-</sup> mice from chronic kidney damage by activating the ACE2/Ang(1-7)/MasR axis.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.



## PCA250

**Different subsets of renal interstitial PDGFR- $\beta$ <sup>+</sup> cells are able to produce Erythropoietin (EPO)**

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Anemia due to insufficient renal EPO production is a typical complication of chronic kidney disease. Therefore a better understanding of the identity of renal EPO producing cells (REPs) is of great clinical relevance. Originally REPs were described as tubulointerstitial CD73<sup>+</sup> fibroblasts located along the border area of inner cortex and outer medulla with recruitment of additional cortical fibroblasts for EPO expression during hypoxic periods. More recent findings suggest that PDGFR- $\beta$ <sup>+</sup> fibroblasts/pericytes are the exclusive renal production sites for EPO and that interstitial PDGFR- $\beta$ <sup>+</sup> cells which are in principle able to produce EPO are widely distributed throughout all kidney zones. Therefore we were interested to know if different subsets of PDGFR- $\beta$ <sup>+</sup> EPO expressing cells exist in the kidney.

For this purpose we investigated the inducibility of EPO expression in three subsets of renal PDGFR- $\beta$ <sup>+</sup> interstitial cells, namely those coexpressing either CD73, Gli-1 or SMMHC together with PDGFR- $\beta$ . Inducibility of EPO expression was tested by cell type specific deletion of the von Hippel-Lindau protein (Vhl) using tamoxifen inducible Cre-loxP systems. Hematocrit (hct) values, plasma EPO concentrations and renal EPO mRNA expression levels were determined as readout of EPO gene expression. Localization and number of EPO expressing cells were determined by EPO mRNA in situ hybridization (ISH) using RNAscope<sup>®</sup>. Values are means  $\pm$  S.E.M.

In Pdgfr- $\beta$ -CreER<sup>T2</sup> Vhl<sup>fl/fl</sup> mice (n=15) 3589 $\pm$ 224 REPs per kidney section (pks) could be detected throughout all kidney zones using EPO mRNA-ISH (controls: 13 $\pm$ 7 REPs pks). Co-ISH for EPO and CD73 revealed that 97% of cortical REPs coexpressed CD73 whereas there was no coexpression in REPs of the outer and inner medulla.

Gli1-CreER<sup>T2</sup> Vhl<sup>fl/fl</sup> mice (n=12) showed 20-fold increased renal EPO mRNA abundance that led to elevated plasma EPO concentrations (1335.0 $\pm$ 95.6pg/ml) and hct values (70.8 $\pm$ 0.8%). Control mice (n=20) had hct values of 49.8 $\pm$ 0.6% and plasma EPO concentrations of 137.9 $\pm$ 18.8 pg/ml. Via RNAscope<sup>®</sup> 188 $\pm$ 38 REPs pks could be detected that were mostly located in the outer stripe of the outer medulla. Only single REPs were localized in the cortex.

EPO mRNA abundance in SMMHC-CreER<sup>T2</sup> Vhl<sup>fl/fl</sup> mice (n=15) was 10-fold elevated (plasma EPO: 1278.0 $\pm$ 93.0pg/ml; hct: 69.2 $\pm$ 1.6%) and 107 $\pm$ 16 REPs pks could be detected on average. These REPs were mostly located in the interstitium of the outer medulla and the proximal part of the inner medulla. Taken together our results show that different subpopulations of interstitial PDGFR- $\beta$ <sup>+</sup> cells exist, which are in principle able to produce EPO. These cells are CD73<sup>+</sup> cells that are mainly localized in the cortical interstitium, Gli-1 expressing interstitial cells mainly localized in the outer medulla and SMMHC-positive pericytes mainly localized in the outer medulla and the proximal part of the inner medulla.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## PCA251

**The importance of Cox-2 in interstitial cells of the renal medulla for kidney function and salt handling**

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Within the kidney cyclooxygenase-2 (Cox-2) is found in the macula densa cells of the cortical distal tubule and in nondefined interstitial cells of the inner medulla. Cox-2 expression at these sites is oppositely regulated by the dietary salt intake. Whilst a mediator role of Cox-2 in macula densa cells for the regulation of renin synthesis and secretion is meanwhile well accepted, the functional relevance of Cox-2 in medullary cells is not that clear. A role of medullary Cox-2 derived prostaglandins for salt dependent blood pressure regulation has been discussed in this context.

It was the aim of this study to characterize medullary Cox-2 expressing cells by identifying distinct cellular markers, using the co-in-situ hybridization RNAscope<sup>®</sup> technique and to investigate the functional role of their Cox-2 expression under a normal and increased salt intake in mice. To evaluate the functional relevance of Cox-2 we assessed basic key parameters of renal function, such as blood pressure, glomerular filtration rate (GFR), urine osmolality and renal electrolyte excretion in mice deficient of Cox-2 in medullary cells. Results are given as means  $\pm$  S.E.M.

Utilizing co-in-situ hybridization, we could identify PDGFR- $\beta$  and Tenascin-C as markers for Cox-2 expressing medullary interstitial cells, whilst, there was only a marginal co-expression of Cox-2 with other renal interstitial markers, such as CD73 and NG2.

For investigating the functional relevance of Cox-2 in interstitial cells of the renal medulla, we generated mice with a tamoxifen inducible deletion of Cox-2 under control of the PDGFR- $\beta$  promoter. After functional assessment under normal salt intake, the mice were fed a high salt diet containing 4% sodium chloride.

With a normal salt intake, the above-mentioned kidney functions remained unchanged after the deletion of Cox-2. In the control animals we could observe a marked increase in the number of Cox-2 expressing cells under a higher salt intake. Furthermore to compensate a higher dietary salt intake the GFR rose in control animals (950,4 $\pm$ 29,2 to 1350 $\pm$ 69,2  $\mu$ l/100g BW, n=9) in line with an increased sodium excretion, while the blood pressure of these animals remained unchanged. In Cox-2 deficient mice, the rise in GFR was attenuated (890,1 $\pm$ 28,8 to 1171 $\pm$ 40,4  $\mu$ l/100g BW, n=15) and the compensatory increase of urine osmolality as seen in control animals was reduced (1629 $\pm$ 40,2 for controls vs. 1363 $\pm$ 82 mOsmol/L, n=11). In addition the Cox-2 deficient animals showed a significant rise in blood pressure (119,2 $\pm$ 1,4 for controls vs. 140,6 $\pm$ 2 mmHg, n=3).

Our results indicate that renal medullary Cox-2 expressing cells belong to the pool of PDGFR- $\beta$ <sup>+</sup> interstitial cells, that also express Tenascin-C. Furthermore, our findings suggest that Cox-2 in medullary cells significantly contributes to salt and blood pressure homeostasis.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCA252

### The effect of prenatal dexamethasone exposure on renal expression of glucose transporters in rat fetuses and offspring

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**Introduction:** Dexamethasone (Dex), a synthetic glucocorticoid (GC), is administered to pregnant mothers at risk of preterm delivery, to induce fetal lung maturation. Offspring exposed to maternal GCs show altered glucose homeostasis such as fasting hyperglycemia, increased hepatic and renal gluconeogenesis, decreased pancreatic  $\beta$  cell number, hyperinsulinemia and decreased glucose tolerance, all of which increase the risk of diabetes development. We hypothesized that the impaired glucose homeostasis can be further exacerbated by the increase in renal proximal glucose reabsorption through the upregulation of sodium-glucose transporters (SGLTs) and glucose transporters (GLUTs), as seen in diabetic patients and animals. Therefore, the aim of this study was to investigate the effect of maternal treatment with Dex on fetal and postnatal renal expression of SGLTs and GLUTs. **Methods:** Pregnant Sprague Dawley dams were randomly divided into 2 groups: saline-treated controls (n: number of dams=6/group) and Dex-treated with 0.2 mg/kg *i.p.* from day 15 of pregnancy till 21 days gestation (dg). Gene expression of SGLT1, SGLT2, GLUT1 and GLUT2 was studied in the kidneys of fetuses at 19 and 21 dg and in 1-month and 4-month-old male and female offspring using real-time PCR. For body and kidney weights, results are expressed as mean  $\pm$  SEM and comparisons were performed using unpaired *t*-test. For PCR, amplification efficiencies and quantitation cycle (Cq) were derived from the raw data using LinReg software. Calculation of fold change and significance were done using REST software. **Results:** Maternal treatment with Dex caused decreases in body weights of fetuses at 21 dg ( $4.56 \pm 0.12$  g vs.  $5.58 \pm 0.16$  g,  $p < 0.001$ ). Dex did not affect body or kidney weights of female adult rats, however at 4 months, males had lower body weights ( $473.8 \pm 9.7$  vs.  $521.6 \pm 11.4$ ,  $p < 0.01$ ) and kidney weight/body weight ( $0.003 \pm 0.0002$  vs.  $0.0024 \pm 0.0001$ ,  $p < 0.05$ ) when compared to controls. Dex treatment had no effect on SGLTs expression in male offspring at any stage, however, in 19 dg females SGLT1 mRNA was increased by 1.78 folds ( $p < 0.05$ ) and in 21 dg females GLUT2 mRNA was increased by 3.59 folds ( $p < 0.001$ ). **Conclusions:** Maternal Dex treatment causes intrauterine growth restriction towards the end of gestation. Despite the decrease in male body and kidney weights, the effect of Dex on glucose transporters' gene expression was only seen in female fetuses. This effect is therefore, not attributed to growth restriction, but rather due to a direct effect of Dex on renal tubules that express GCs receptors. Membrane expression of SGLTs and GLUTs in this model will provide a better understanding of the role of Dex in prenatal programming.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA253

### The effects of acacia senegal (gum arabic) supplementation on inflammatory marker, renal and hematological profile in hemodialysis patients

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Several studies observed that hemodialysis patients have serological confirmation of an activated inflammatory response. Inflammation is one of non-traditional risk factor of cardiovascular disease which is considered as major cause of mortality in end stage renal disease. Causes of inflammation in end stage renal disease are multifactorial, including exposure of blood to dialyzer membranes, infection on vascular access, reduced antioxidants, and increased oxidative stress. Acacia senegal has been claimed to have an anti-inflammatory effects. This study was conducted to investigate the effect of Acacia senegal on inflammatory status in patients on regular hemodialysis. This study was done as phase II clinical trial among patients on regular hemodialysis in Sudan. Forty end stage renal disease patients age (18–80 years) were recruited. Patients took orally 30 g/day GA for 12 weeks. Renal function test (RFT), Complete blood count (CBC) and C reactive protein (CRP) was measured by an enzyme immunoassay method, all of them were measured monthly. Repeated measures analysis of variance (ANOVA) was used to analyze the data. CRP level was significantly decreased ( $P = 0.0016$ ). Acacia senegal significantly decreased both the level of urea and creatinine ( $P = 0.0004$ ) and ( $P = 0.049$ ) respectively. And uric acid level significantly reduced ( $P = 0.0057$ ). Acacia senegal had no effect on serum electrolytes level except the phosphorus which was significantly reduced ( $P = 0.0039$ ). Acacia senegal increased the Hb ( $P = 0.0357$ ) and RBCs count ( $P = 0.0253$ ) significantly without any effect on other hematological parameters. These data suggest that daily supplementation of Acacia senegal (30 g/day) significantly reduced the inflammation in hemodialysis patients. The anti-inflammatory effect of Acacia senegal may have positive impact on other diseases characterized by increase inflammation.

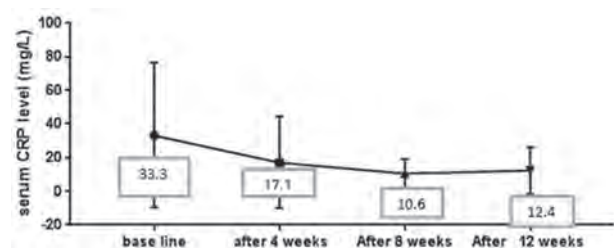


Figure 1: Effect of Acacia Senegal intake on C-reactive protein (CRP) Level ( $P = 0.0016$ )

| Parameter             | Baseline level | After 4 weeks  | After 8 weeks | After 12 weeks | P value  |
|-----------------------|----------------|----------------|---------------|----------------|----------|
| Hb                    | 8.8 ± 2        | 8.2 ± 1.9      | 8.785 ± 1.7   | 9.541 ± 1.97   | 0.0357*  |
| MCV                   | 85.85 ± 5.4    | 83.34 ± 13.6   | 85.55 ± 5.4   | 86.05 ± 5.3    | 0.4405   |
| MCHC                  | 31.89 ± 1.2    | 32 ± 1.4       | 31.6 ± 1.2    | 31.4 ± 1.2     | 0.1598   |
| MCH                   | 27.39 ± 2.1    | 27.34 ± 2.3    | 27.05 ± 2.2   | 27.05 ± 2.11   | 0.8492   |
| WBCs                  | 5.3 ± 1.962    | 5.5 ± 2.26     | 5.2 ± 2.09    | 5.9 ± 2.77     | 0.5916   |
| RBCs                  | 3.206 ± 0.8    | 2.998 ± 0.7    | 3.24 ± 0.7    | 3.5 ± 0.67     | 0.0253*  |
| Platelets             | 181 ± 70.5     | 163 ± 6 ± 58.9 | 187.5 ± 82.3  | 186.4 ± 80.6   | 0.4622   |
| Creatinine (mg/dl)    | 10.03 ± 2.49   | 9.15 ± 2.1     | 9.79 ± 2.38   | 8.73 ± 2.13    | 0.0491*  |
| BUN (mg/dl)           | 165 ± 31.5     | 158 ± 34.8     | 142.9 ± 27.5  | 137.7 ± 31.06  | 0.0004 * |
| Sodium (mEq/L)        | 137.1 ± 2.12   | 136.8 ± 2.96   | 137.9 ± 2.4   | 135.7 ± 15.6   | 0.6960   |
| Potassium (mEq/L)     | 4.85 ± 0.82    | 5.0 ± 0.76     | 4.89 ± 0.81   | 4.80 ± 0.826   | 0.6909   |
| Total Calcium (mg/dl) | 8.5 ± 0.675    | 8.96 ± 0.8     | 8.7 ± 0.88    | 8.5 ± 0.80     | 0.05     |
| Phosphorus (mg/dl)    | 4.95 ± 1.41    | 4.392 ± 1.02   | 5.258 ± 0.94  | 4.708 ± 0.646  | 0.0039*  |
| Uric acid (mg/dl)     | 7.45 ± 1.802   | 7.589 ± 1.5    | 6.66 ± 1.5    | 6.4 ± 1.79     | 0.0057*  |

Table 1 : Effect of Acacia senegal supplementation on blood and renal parameters (predialysis values) (Mean ± SD), (n 40)

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA254

## The nephropathic mycotoxin Ochratoxin A leads to cell cycle arrest in human kidney cell lines

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Filtration, reabsorption and secretion are the three essential processes for renal functions. However, this physiological system turns out to be critical in the elimination process of some harmful substances. The latter include Ochratoxin A (OTA) [1], a mycotoxin produced by *Aspergillus* or *Penicillium* species that is commonly found in foodstuff. Various effects have been attributed to this substance but its main target remains the kidney due to the filtration-reabsorption-secretion system that delays its excretion and leads to its accumulation in tubular cells. The underlying mechanisms by which OTA affects kidney cells remain nonetheless mainly unknown. In the present study, we were therefore interested in identifying putative key drivers in OTA-induced nephropathies. To do so, RNA-sequencing was carried out on human kidney cell lines (HEK-293T and HK-2) that were exposed to low concentrations (nanomolar range) of OTA for 48 hours. Differential expression and pathway enrichment analyses were performed. Moreover, Propidium Iodide (PI)-staining was used for cell cycle analysis by Flow Cytometry and the

level of expression of CDKN1A/p21 was further investigated by RT-qPCR and Western Blot.

Our analysis revealed that the expression of several thousands of genes was influenced by OTA-exposure in human kidney cell lines (with  $|\log_2 \text{Fold Change}| > 1$ , FDR 0.01). These changes in the transcriptome were associated with the regulation of pathways related to DNA damage, protein metabolism and cell cycle regulation. In order to understand the impact of OTA on the latter, cell cycle analysis was additionally performed. The results highlighted a cell cycle arrest in kidney cell lines induced by OTA at physiological concentrations ( $< 100\text{nM}$ ). We, moreover, observed that CDKN1A/p21, a key regulator of the G1/S check-point, was up-regulated under the influence of OTA.

The data obtained for this study thus showed that exposure to OTA leads to global changes in the transcriptome of kidney cell lines, including for genes involved in the regulation of the cell cycle. More precisely, our results suggest that the OTA-induced nephropathies may be due to an arrest of the cell cycle. We propose that the OTA-regulated protein CDKN1A/p21 contributes to this effect.

Rottkord, U., Röhl, C., Ferse, I. et al. *Arch Toxicol* (2017) 91: 1461.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA255

## The ETAR and ETBR in the adult murine kidney as potential factors to regulate synthesis and secretion of renin *in vivo*

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Endothelin-1 is a potent renal vasoconstrictor and has been shown to inhibit renin synthesis and secretion *in vitro*. Among the two endothelin receptor subtypes, ET-1 is thought to mainly act through binding to endothelin-A receptors (ETAR) but has also affinity to bind specifically on endothelin-B receptors (ETBR).

We were interested to examine the relevance of endothelins for the development and physiological regulation of renin secretion and synthesis *in vivo*, in addition if ETs bind on the ETA- and/or ETB-receptors (ETRs) following a  $\text{Ca}^{2+}$ -dependent direct inhibiting effect on renin cells. For this purpose we generated a renin cell-specific ET-receptors knockout mouse on the basis of the Cre/loxP system (ETAR<sup>fl/fl</sup>ETBR<sup>fl/fl</sup>RenCre). The efficacy of ETRs deletion was verified by ETAR immunohistochemistry and ETBR in situ hybridization on kidney sections. In control kidneys ETAR and ETBR immunoreactivity was widely distributed in the kidney including e.g. renin cells, vascular smooth muscle cells and mesangial cells. Exclusively ETBR mRNA signal could be detected on interstitial cells. In ETAR<sup>fl/fl</sup>ETBR<sup>fl/fl</sup>RenCre kidneys both receptors immunoreactivity were lacking on renin cells only. In addition, stroma cell-specific ETRs knockout mice (FOX<sup>D1</sup>Cre/+·ETAR<sup>fl/fl</sup>ETBR<sup>fl/fl</sup>) were generated to monitor potential indirect effects of ETs on renin synthesis and secretion. Therefore immunoreactivity of both receptors was lacking on renin cells, mesangial cells, vascular smooth muscle cells and interstitial cells in FoxD1<sup>Cre/+</sup> animals.

All mentioned ETAR<sup>fl/fl</sup>ETBR<sup>fl/fl</sup>-Cre strains showed normally developed renin cells and had normal renin mRNA abundance, plasma renin concentrations, systolic blood pressure



values, urine osmolarity and osmolytes compared to control littermates.

To further investigate the role of both ETRs on renin secretion and concentration, isolated perfused kidney (ipK) was performed. All ETAR<sup>fl/fl</sup>ETBR<sup>fl/fl</sup>RenCre displayed, similar to controls, an inhibition of renin secretion through ET-1, considering this effect is due to the reduction of blood flow and not because of the decrease of renin concentration. Conversely, ipK results of FOXD1<sup>Cre/+</sup>-ETAR<sup>fl/fl</sup>ETBR<sup>fl/fl</sup> revealed no decline of renin secretion through ET-1 with a nearly constant renal blood flow indicating that these results appeared due to absence of systemic effect in *ex vivo* situation.

Altogether, present findings indicate that ETR isoforms located on renin cell lineage and on stroma derived cells seem to be of less relevance for renin synthesis and secretion *in vivo* whereas not yet addressed tubular effects or possible systemic effects are more responsible for promoting the inhibiting effect through ETs in order to equilibrate general homeostasis. In addition, the function of renal ETRs in terms of renal pathophysiology e.g. renal fibrosis, need to be evaluated prospectively.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

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PCA256

#### **Gene transfer of glyoxalase-1 into the paraventricular nucleus attenuates sympathetic activation and blunt renal dysfunction in diabetic rats**

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Diabetic nephropathy (DN) is a major cause of end-stage renal failure (ESRF) and premature mortality in individuals with chronic Type 1 diabetes mellitus (T1DM). Exaggerated autonomic activity originating in the paraventricular nucleus (PVN) of the hypothalamus is an early step in pathogenesis of DN. Using streptozotocin-induced T1DM rats, we earlier showed that the enhanced autonomic activity in the PVN of T1DM rats arose in part from increased ROS generation, but the underlying reasons for this remain unknown. Recently we discovered supra-physiologic flux of the ROS-generating  $\alpha$ -oxoaldehyde methylglyoxal (MG) in brains of T1DM rats. This study, with approval from the Animal Care and Use Committees, UNMC investigated if the increased MG flux is responsible for the enhanced activity of PVN neurons in T1DM rats. After 8 weeks of T1DM, water intake increased 2.2-fold to 550 ml/24 hrs, urine production increased 3-fold to 150 ml/24 hrs, urine norepinephrine increased 2-fold to  $7.8 \pm 1.2 \mu\text{g}/24 \text{ hrs}$ , serum creatinine increased 2.6 fold to  $2.3 \text{ mg/dL}$ , serum angiotensin II increased by 2.2-fold to  $162 \text{ pg/ml}$ , and blood urea nitrogen increased 5.3-fold to  $100.7 \pm 10.3 \text{ mg/dl}$ , indicative of DN, polydipsia, and polyuria,  $n=10$  animals. In the PVN of T1DM rats, total ROS increased 2.5 fold, the

density of perfused micro-vessels and microvascular leakage assessed using BSA-FITC extravasation were increased 2-fold and reduced 3.2-fold, respectively, activated astrocytes determined from glial fibrillary acidic protein (GFAP) immunoreactivity was increased 3-fold, TNF- $\alpha$  mRNA was increased 2-fold, and the density of vasopressin-positive neurons in the PVN was reduced 3-fold. Expression of the MG-synthesizing enzyme vascular adhesion protein-1 and MG level were also increased 3-fold in the PVN of T1DM rats. In kidneys of T1DM rats, excessive accumulation of BSA-FITC was prominent in the glomeruli and in the proximal tubules, consistent with hyperfiltration. Microinjection of an adeno-associated containing glyoxalase-I (AAV2/9-Glo-I, 100 nL) into the PVN of rats 1 week after the onset of T1DM, blunted increases in water consumption, urine production, urine norepinephrine, serum creatinine, angiotensin II, and blood urea nitrogen. It also blunted loss of micro-vessel perfusion, micro-vessel leakage, astrocytes activation, total ROS, TNF- $\alpha$ , and attenuated loss of vasopressin-immunoreactivity in PVN neurones. Microinjection of AAV2/9-Glo-I blunted VAP-1 and MG upregulation into the PVN and attenuated BSA-FITC accumulation in the glomeruli and proximal tubules. It is concluded that increased MG flux in the PVN is an underlying cause for increased ROS, sympatho-excitation and nephropathy in T1DM animals, and lowering MG flux in the PVN by overexpression Glo-I was sufficient to attenuate sympatho-excitation and blunt DN.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

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PCA257

#### **TRPV4 is expressed in vasopressinergic neurons of the magnocellular region of the rat paraventricular nucleus of the hypothalamus.**

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The paraventricular nucleus of the hypothalamus (PVN) is critical for sensing and responding to changes in plasma osmolality<sup>1</sup>. Increased osmolality activates the PVN to promote arginine vasopressin (AVP) release and sympathetic nerve activity to end organs<sup>2</sup>. The transient receptor potential vanilloid type 4 channel (TRPV4), may be involved in systemic osmoregulation and there is some evidence to support a physiological role for TRPV4 in the PVN osmosensing nuclei<sup>3</sup>. TRPV4 is expressed in the PVN where it is co-localised with AVP containing cells<sup>3</sup>. In the PVN of rat and mouse, TRPV4 and calcium activated potassium ion channels have been shown to couple as osmosensors<sup>4</sup>. Intracerebroventricular administration in mice of hypotonic artificial cerebrospinal fluid decreases blood pressure but not heart rate and inhibition of the TRPV4 ion channel abolishes this effect<sup>5</sup>. These studies demonstrate a role for TRPV4 in osmosensing within the PVN but they do not establish which neurons express the channel. We used retrograde labelling of PVN- spinally projecting preautonomic neurons with immunohistochemistry to determine which neurons express TRPV4 and its relationship with cell groups involved in osmosensing and cardiovascular homeostasis. Experiments were performed on six male Wistar rats in accordance with the Animals (Scientific Procedures) Act, 1986 and the European Commission Directive 86/609/EEC. The rats were anaesthetised intraperitoneally (IP) with medetomidine (0.25ml/100g) and ketamine (0.06ml/100g) prior to spinal cord injection of Fluorogold (FG). Fluorogold (2%)

was pressure injected into the left intermediolateral region at T2. Post-operatively, buprenorphine (0.01ml/100g) was administered and animals allowed to recover for 7-10 days. Following this, animals were humanely killed (pentobarbital 60mg/kg IP), perfuse-fixed (4% PFA) and brain and spinal cord removed. Immunohistochemistry to reveal TRPV4, AVP and oxytocin (OXT) in the PVN was carried out on free floating sections. Tissues was examined using confocal microscopy to determine their relationship with preautonomic PVN neurons. Preautonomic neurons identified by the presence of FG did not express TRPV4. All TRPV4 neurons were found to be vasopressinergic with TRPV4 expressing neurons making up 63% of the total vasopressinergic population. There was no evidence of TRPV4 being expressed on oxytocinergic neurons. A minority of preautonomic neurons were found to express OXT or AVP.

TRPV4 was localised to the vasopressinergic magnosecretory neurons within the PVN. These neurons release AVP to modulate preautonomic neuronal activity in response to plasma osmolality disturbances. Our study indicates an anatomical basis to inform the mechanism by which changes in plasma osmolality alter sympathetic nerve activity.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA258

### Effects of Vitamin B<sub>12</sub> and Ketorolac on pain in Long Evans rats

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Effects of vitamin B<sub>12</sub> on pain have been demonstrated in different animal and human studies. But comparison of these effects with similar effects of ketorolac tromethamine (KT) and their combination have not been established. To assess the effects of vitamin B<sub>12</sub> on pain and also to compare them with those of the combinations of vitamin B<sub>12</sub> with KT in rat models. This experimental study was conducted in the Department of Physiology, Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka, from March 2015 to February 2016. All experiments and animal care were performed

according to the guidelines set in the 'Manual for Care and Use of Laboratory Animals' by the Animal Experimentation Ethics Committee (AEEC) of the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr, b 2002). For this, 20 (twenty) Long Evans rats (215±35 gm) of either sexes were divided into control (A, with 5 ml/kg normal saline)<sup>1</sup> and experimental (B1, with 15 mg/kg B<sub>12</sub>; B2, with 10 mg/kg KT; B3, with B<sub>12</sub>+KT)<sup>2,3</sup> groups with 5 rats in each group. All the drugs and vitamin were administered intraperitoneally<sup>4</sup>, in a single dose just one hour before formalin test. To evaluate the treatments' effect on nociceptive pain, early phase (1<sup>st</sup>-5<sup>th</sup> minutes); on central analgesic system, interphase (6<sup>th</sup>-15<sup>th</sup> minutes); and on inflammatory pain, late phase (16<sup>th</sup>-60<sup>th</sup> minutes) of the formalin test<sup>5</sup> were observed. In all phases, total frequency of jerking and total duration of flexing and licking of the right hind paw were counted after administration of subcutaneous formalin (50 µl, 2.5%)<sup>12</sup> injection. Afterwards, all the rats were euthanized. Statistical analysis was done by ANOVA, followed by Bonferroni post hoc test. In the interpretation of results, p≤0.05 was considered as significant. B<sub>12</sub> lowered only the jerking frequency and KT lowered both jerking frequency and flexing-licking duration significantly (p≤0.001) in the late phase of formalin test. On the other hand, combination of B<sub>12</sub> and KT significantly (p≤0.001) lowered both the study variables in all 3 phases of formalin test. From this study it may be concluded that, vitamin B<sub>12</sub> possess analgesic effects and combination of B<sub>12</sub> with KT is more effective than those of their individual administration.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA259

### Excitatory and inhibitory neurotransmission is imbalanced in an animal disease model of episodic ataxia type 6

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Episodic ataxias (EAs) are rare neurological syndromes characterized by paroxysmal cerebellar incoordination combined with episodes of migraine, epilepsy, or dystonia. A missense point mutation in the gene coding for SLC1A3 (EAAT1) has been identified in a patient suffering from the severe form EA type 6, with seizures and long lasting episodes of movement disorders (Jen et al., 2005). The mutation predicts the substitution of the conserved proline 290 by an arginine (P290R). EAAT1 functions not only as glutamate transporter, but also

as anion channel, and earlier work in heterologous expression systems demonstrated that P290R impairs transport rates and enhances channel activity (Winter et al., 2012; Hotzy et al., 2013). Since genetic ablation of EAAT1 in mice (GLAST<sup>-/-</sup>) did not result in seizures, and promoted only slight ataxias (Watase et al., 1998), we hypothesized that the increase in glial anion fluxes in P290R EAAT1 might be the trigger for the disease rather than reduced glutamate clearance. To study the consequences of an augmented glial Cl<sup>-</sup> conductance on hippocampal neurotransmission, we studied excitatory and inhibitory synaptic transmission by whole-cell patch clamping in acute slices of a knock-in mouse model (*Slc1a3*<sup>P290R/+</sup>). Quantitative receptor autoradiography demonstrated increased AMPA receptor densities in the hippocampus of *Slc1a3*<sup>P290R/+</sup> mice (+31%). We therefore tested AMPA receptor-mediated neurotransmission using whole-cell patch clamping and analysed miniature excitatory postsynaptic currents (mEPSCs) in acute hippocampal slices. Both, the mEPSC amplitudes (WT, 7 ± 0.1 pA; *Slc1a3*<sup>P290R/+</sup>, 9 ± 0.1 pA, ANOVA: p < 0.05) and the mEPSC frequencies (WT, 2.4 ± 0.1 Hz; *Slc1a3*<sup>P290R/+</sup>, 3.0 ± 0.1 Hz, ANOVA: p < 0.05) were significantly increased in dentate gyrus (DG) granule cells from mutant samples. Glial EAATs have been recently shown to be involved in glial Cl<sup>-</sup> homeostasis (Untiet et al., 2015) and an increased anion efflux from glial cells might thus alter [GABA]<sub>ext</sub> by enhancing the electrochemical driving forces acting on glial Na<sup>+</sup>/Cl<sup>-</sup>-dependent GABA transporters (GATs). Using whole-cell patch clamping we measured tonic GABA-mediated Cl<sup>-</sup> currents in DG granule cells, and demonstrated a decrease in tonic inhibitory Cl<sup>-</sup> currents in the samples from *Slc1a3*<sup>P290R/+</sup> mice (WT, 29 ± 6 pA; *Slc1a3*<sup>P290R/+</sup>, 14 ± 3 pA, ANOVA: p < 0.05). These changes in inhibitory and excitatory neurotransmission in the DG could explain the observed seizures by a hyperexcitability of the DG granule cells.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA260

### SIRT2 inhibition suppresses the oxidative stress and apoptotic activity, and increases autophagy of cerebral cortex in the aging process.

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Increased oxidative stress in the elderly is accompanied by apoptosis<sup>1</sup>. Autophagy declines with age<sup>2</sup>. Likewise, an increase in the level of oxidative stress and apoptosis, and a decrease in autophagy are closely related to neurodegenerative diseases<sup>3</sup>. It has been shown that sirtuin 2 (SIRT2) expression in the cerebral cortex increases in the elderly and inhibition of SIRT2 can prevent neurodegenerative diseases<sup>4</sup>. In this study, we aimed to study the role of SIRT 2 expression on oxidative stress, apoptotic activity and autophagy of the cerebral cortex in the young and aged rats.

In the study, 6 groups were formed by using 36 Wistar albino rats (3-month old, n = 18, 22-month old n = 18): Young (injected with PBS), Young-Control (injected with 4% DMSO), Young-AGK-2 (injected with AGK-2 dissolved in 4% DMSO, a specific SIRT 2 inhibitor), Aged, Aged-Control, and Aged-AGK-2. The injections of the control and experimental groups were maintained for 30 days. On the 31st day of the experiment, rats were sacrificed and the cerebral cortex was isolated. Total oxidant status (TOS) and total antioxidant status (TAS) measurements were made using commercial kits and the oxidative stress index (OSI) obtained by the ratio of TOS to TAS. BCL-2, BAX expressions were studied as apoptosis parameters, beclin and ATG-5 as autophagy by Western blotting and real-time PCR. Student's T, Two-way ANOVA and Pearson's r were used for statistical analysis (p < 0.05). Our findings showed that aging decreased the BCL-2, BCL-2 / BAX ratio, beclin and TAS in the cerebral cortex and increased the TOS and OSI. We found that SIRT 2 inhibition by AGK-2 administration reduced cortical TOS and OSI levels, and increased BCL-2, BCL-2 / BAX ratio and beclin in aged animals. As a result, SIRT2 inhibition by AGK-2 may suppress oxidative stress and apoptotic activity, and increases autophagy. SIRT 2 could be a therapeutic target for the prevention of neurodegenerative diseases in the elderly.

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## PCA261

### Age-related changes in the oxidative stress, apoptosis, sirtuin 2 and FoxO3a expression in the hippocampus: protective effects of melatonin

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Aging is closely associated with oxidative stress and apoptosis<sup>1</sup>. Melatonin as a free radical scavenger has anti-apoptotic and anti-aging properties<sup>2</sup>. The pharmacological inhibitors of sirt2 appear to be neuroprotective in the brain<sup>3</sup>. We aimed to demonstrate the effects of melatonin administration on age-related changes in the oxidative stress, apoptosis, sirtuin 2 (SIRT2) and FoxO3a expression in the hippocampus. Twenty four Wistar albino male rats (young: 3 months old, aged: 22 months old) were divided into following groups: (1) Young-control, (2) Young-melatonin, (3) Aged-control, (4) Aged-melatonin. Subcutaneous injections of control (1% ethanol-PBS) and melatonin (10 mg/kg/day) groups were maintained for 21 days. SIRT2, FoxO3a, Bcl-2 and Bax expressions were tested by Western blotting. SIRT2 and FoxO3a protein levels were measured by a sandwich ELISA method. Total oxidant status (TOS) and total antioxidant status (TAS) were measured using commercially available kits. The ratio of TOS to TAS, i.e. the oxidative stress index (OSI), was also



calculated. ANOVA, Pearson's  $r$  were used for statistical analysis ( $p < 0.05$ ).

Aging increased TOS, OSI, SIRT2, FoxO3a and pro-apoptotic Bax, and decreased TAS, anti-apoptotic Bcl-2 and Bcl-2/Bax ratio in the hippocampus, whereas melatonin administration in aged rats entirely reversed this pattern ( $p < 0.05$ ). ELISA and Western blot findings were similar for SIRT2 and FoxO3a ( $p < 0.05$ ). Additionally, OSI was positively correlated with SIRT2, FoxO3a, Bax, and negatively correlated with Bcl-2 and Bcl-2/Bax ratio ( $p < 0.05$ ).

In aging, melatonin administration may prevent oxidative stress and neuronal loss in the hippocampus. Sirt2 and FoxO3a inhibition may be involved in the neuroprotective effects of melatonin.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA262

### Differences in drug response and spontaneous activity of $M_4$ muscarinic receptor knockout mice during day and night time

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Scopolamine and oxotremorine are widely used as drugs for testing the motor activity and temperature in animal models. Cocaine is usually applied with aim to study addiction and effects on motor activity. Mice are nocturnal animals. Surprisingly, the majority of pharmacological studies are performed in the morning, i.e. in the non-active phase of their diurnal cycle.

Therefore, we have compared the effects of scopolamine (0.5 mg/kg s.c.), oxotremorine (0.2 mg/kg s.c.) and cocaine (20 mg/kg s.c.) in inactive (at 9:00) and in active (at 21:00) period of the day on motor activity and temperature using telemetric system. We have used two mouse strains: wild types (C57BL/6NTac) and mice lacking  $M_4$  muscarinic receptors (same genetic background, 12<sup>th</sup> backcrossed generation,  $M_4$  KO), in light/dark 12h/12h conditions, lights on at 6:00. All experiments using drug application were conducted on females ( $n=74$ ) where only differences in biorhythm parameters between  $M_4$  KO and WT were revealed. The telemetric probes were implanted into peritoneal cavity under anaesthesia and mice were left at least for 10 days to recover. The spontaneous motor activity and temperature were analyzed using Chronos-Fit and GraphPad software. The activity in novelty environment was tested using Open field test. We have also compared receptor density (muscarinic, GABA<sub>A</sub>, D<sub>1</sub>-like, D<sub>2</sub>-like dopamine, NMDA and kainite receptors) using autoradiography in several brain areas (motor, somatosensory, visual cortex, striatum, nucleus accumbens, thalamus, hippocampus and CA1, CA3 areas, dentate gyrus, olfactory tubercle, pons and medulla oblongata) in  $M_4$  KO mice and their WT littermates. For comparison of multiple groups we have used one-way ANOVA with post-hoc Tukey's corrections. Values of  $p < 0.05$  were considered as significant.

The effects of drugs depended on the time of application, i.e. if applied in active or inactive phase. After scopolamine and cocaine treatment,  $M_4$  KO mice increased motor activity more profoundly during light (251 % and 348%, respectively) than in dark phase (186.0 % and 198%, respectively) when compared with saline control group. Oxotremorine reduced the temperature more in WT (to 27.3 °C in light and 28.4 °C in dark phase) than in  $M_4$  KO (29.8 °C and 29.3 °C, respectively). Oxotremorine also caused increase of the area under curve of this parameter at 9:00 than at 21:00. There was no difference in the open field between WT and KO when tested at 9:00 AM; however, at 9:00 PM the KO activity was doubled in comparison to WT. At the same time, autoradiography revealed no significant morning vs. evening difference in the number of receptors in the tested brain areas.

Altogether our results indicate the necessity of comparing morning vs. evening drug effects.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA263

### Effect of therapeutic concentration of lithium on live HEK293 cells results in increase of $Na^+/K^+$ -ATPase, change of protein composition and induction of oxidative stress.

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Lithium (Li) represents the standard for the treatment of bipolar disorder. Mechanism of therapeutic Li action at the cellular and molecular level remains not fully elucidated. Among many different hypotheses, induction of oxidative stress (OS) and alteration of expression level and/or activity of membrane ionic channels/transporters received much attention. In this work, we tested whether the long-term exposure of live HEK293 cells to therapeutic Li concentration results in change of plasma membrane (PM) content of  $Na^+/K^+$ -ATPase.  $Na^+/K^+$ -ATPase level was determined in parallel with estimation of general OS and products of lipid peroxidation. HEK293 cells were cultivated in the presence or absence of 1 mM LiCl for 7 (L7 Li-treated, C7 control) or 21 days (L21, C21). Three independent PM and post-nuclear supernatant (PNS) preparations were isolated from all cell types.  $Na^+/K^+$ -ATPase level was determined by [<sup>3</sup>H]ouabain binding and immunoblot assays in PM. General OS was monitored by fluorescent dye 2',7'-dichlorofluorescein diacetate (DCF) in whole cells, PNS and PM. 4-hydroxy-2-nonenal (4-HNE)-protein adducts (immunoblot assay) and malondialdehyde (MDA, HPLC analysis) were determined as products of lipid peroxidation in PNS. Overall protein composition was determined in PNS by 2D electrophoresis followed by MALDI-TOF MS/MS. Values are means ± SEM expressed as % of control values (100%); the significance of difference between control and Li samples was determined by unpaired Student's t-test (triplicate for each sample preparation).  $Na^+/K^+$ -ATPase was increased in PM isolated from cells exposed to Li (L7 153 ± 15%  $p < 0.05$ , L21 115 ± 5%,  $p < 0.05$  immunoblot; L7 121 ± 8%  $p < 0.05$ , L21 120 ± 3%  $p < 0.05$  [<sup>3</sup>H]ouabain binding). In Li-treated cells, the highly increased ( $p < 0.05$ ) DCF fluorescence intensity was noticed

in whole cells, PNS and PM. Accordingly, production of 4-HNE-protein adducts ( $p < 0.05$ ) and MDA ( $p < 0.01$ ) was clearly increased in Li-treated cells. The analysis of CCB-stained gels revealed 16 altered proteins in L7 compared with C7 and 25 in L21 versus C21. The intensity of staining of these protein spots was decreased ( $>2$ -fold). All proteins were functionally related to glycolysis, oxidative stress or calcium homeostasis. Our findings bring the evidence for adaptation of HEK293 cell metabolism to prolonged exposure to therapeutic Li concentration. This adaptation proceeds as up-regulation of  $\text{Na}^+/\text{K}^+$ -ATPase and limited reorganization of cellular protein composition. Metabolism of HEK293 cells growing in culture in the presence of a therapeutic Li concentration may be characterized as the state of discomfort induced by mild OS which does not prevent cells from fast growth.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA264

### Differential regulation of renin transcript levels by glucose depletion, anoxia, proliferation and differentiation in PC12 cells

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The Renin-Angiotensin System (RAS) exerts pro-inflammatory and pro-fibrotic effects thereby promoting cardiovascular diseases [1]. Apart from secretory renin that mediates detrimental effects of the RAS an alternative renin transcript (cyto-renin) has been identified in several species [2]. In contrast to secretory renin, cyto-renin surprisingly protected cardiac cells from necrotic death [3]. We therefore suspect that cyto-renin may play a protective role in the brain. As a first step, we analysed the effects of glucose depletion and anoxia on renin expression in proliferating, stationary and differentiated neuronal cells.

We utilized PC12 cells as a neuronal *in vitro* model system. With BrdU- and propidium iodide staining and Ki-67 qRT-PCR we evaluated the degree of proliferation. Differentiation was induced by nerve growth factor and confirmed by synaptotagmin expression. We quantified renin-mRNAs in proliferating, stationary and differentiated PC12 cells and in PC12 cells subjected to glucose depletion and/or anoxia.

During the first day of culture only cyto-renin was detected in adherent PC12 cells. At this point,  $55.8 \pm 1.8\%$  of PC12 cells was in the proliferating stage. During 5 days of culture, cyto-renin expression increased and secretory renin was induced (cyto-renin – 1 d:  $0.4 \cdot 10^{-7} \pm 0.1 \cdot 10^{-7}$ , 5 d:  $1.7 \cdot 10^{-7} \pm 0.4 \cdot 10^{-7}$ ; secretory renin – 5 d:  $1.3 \cdot 10^{-6} \pm 1.4 \cdot 10^{-6}$ ). By this time  $51.5 \pm 6\%$  of the PC12 cells were in the stationary phase. This was supported by a reduced BrdU-incorporation (1 d:  $5.9 \cdot 10^{-3} \pm 2 \cdot 10^{-3}$  OD, 5 d:  $3.3 \cdot 10^{-3} \pm 4.9 \cdot 10^{-4}$  OD) and a lowered Ki-67-expression (1 d:  $6.6 \cdot 10^{-5} \pm 1.5 \cdot 10^{-5}$ , - 5d:  $3.3 \cdot 10^{-5} \pm 2.1 \cdot 10^{-5}$ ). Thus, the increase of renin expression presumably correlates with PC12 cells entering the stationary phase.

NGF-induced differentiation enhanced cyto- and secretory renin expression in adherent PC12 cells (cyto-renin: control:  $6.3 \cdot 10^{-5} \pm 3.4 \cdot 10^{-6}$ , NGF:  $9.1 \cdot 10^{-5} \pm 9.9 \cdot 10^{-6}$ ; secretory renin: control:  $1.4 \cdot 10^{-4} \pm 6.6 \cdot 10^{-6}$ , NGF:  $4.5 \cdot 10^{-4} \pm 6.3 \cdot 10^{-5}$ ). Thus, renin expression apparently also depends on the extent of differentiation of PC12 cells.

In proliferating adherent PC12 cells glucose depletion with anoxia resulted in a significantly enhanced expression of cyto-renin (control:  $2.9 \cdot 10^{-8} \pm 1.5 \cdot 10^{-8}$ , -Glc/-O<sub>2</sub>:  $7.3 \cdot 10^{-8} \pm 1.1 \cdot 10^{-8}$ ), whereas the secretory renin transcript remained absent.

Our analyses show that cyto- and secretory renin transcript levels are differentially regulated with respect to proliferation, differentiation and ischemia related conditions in PC12 cells. The upregulation of cyto-renin under combined glucose-depletion with anoxia suggests that cyto-renin may play a role during ischemic events. The data indicate that cell cycle as well as the degree of differentiation has to be considered principally for further examination of the regulation and functions of renin transcripts.

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PCA265

### Differential effects of insulin along the hippocampal dorsoventral axis on GABA<sub>A</sub>-activated currents in mouse dentate gyrus granule cells and CA3 pyramidal neurons.

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The hippocampus is an acknowledged center for learning and memory. Besides the well-identified subregions Cornu Ammonis (CA) 1-3 and dentate gyrus (DG) of the hippocampus, increasing evidence suggests the existence of functional segregation along the hippocampal dorsoventral axis [1]. Insulin may influence neuronal transmission throughout the brain and alterations of the insulin signaling pathways may underlie the development of several neurodegenerative diseases [2]. GABA ( $\gamma$ -Aminobutyric acid) is the main neuroinhibitory transmitter and modulates neuronal excitability by binding to synaptic and extrasynaptic GABA<sub>A</sub> receptors (GABA<sub>A</sub>R) that mediate phasic and tonic currents, respectively [3]. Nevertheless, relatively little is still known about the effects of insulin on the GABAergic inhibitory currents in dorsal and ventral hippocampal primary neurons.

GABA<sub>A</sub>R-mediated currents were recorded by the whole-cell patch-clamp technique in DG granule cells and CA3 pyramidal neurons from mouse dorsal (DH) and ventral (VH) hippocampal slices in the absence or presence of insulin (1 nM). Acute hippocampal slices (350  $\mu\text{m}$ ) were prepared from adult (8–12 weeks old) male C57BL/6J mice. Values are indicated as mean  $\pm$  S.E.M., compared by two tails Student's *t*-test. In DG, the frequency of spontaneous inhibitory postsynaptic currents (sIPSCs) recorded in granule cells was significantly higher in VH as compared to DH (DH:  $0.8 \pm 0.12$  Hz,  $n=45$  vs. VH:  $1.7 \pm 0.16$  Hz,  $n=42$ ,  $P < 0.0001$ ). Similarly, under the application of tetrodotoxin (TTX), a voltage-gated sodium channel blocker, the frequency of miniature inhibitory postsynaptic currents (mIPSCs) recorded from granule cells of VH was significantly higher as compared to DH (DH:  $0.49 \pm 0.05$  Hz,

n=20 vs. VH:  $0.85 \pm 0.11$  Hz, n=14,  $P < 0.001$ ). In contrast, the frequency of only the mIPSCs but not the sIPSCs in CA3 neurons was higher in VH as compared to DH (DH:  $1.8 \pm 0.2$  Hz, n=11 vs. VH:  $3.3 \pm 0.2$  Hz, n=10,  $P < 0.001$ ). Application of insulin significantly increased the frequency of GABA<sub>A</sub>-mediated synaptic currents of both sIPSCs and mIPSCs in DG granule cells from both DH and VH, ( $P < 0.05$ ) but insulin did not enhance the tonic current. In contrast, insulin potentiated GABA-mediated tonic current in CA3 neurons from both DH and VH, ( $P < 0.05$ ) but only significantly increased the mIPSCs in DH in these neurons ( $P < 0.05$ ). Thus, the results are consistent with postsynaptic mechanisms of the insulin effects in both DG granule cells and CA3 neurons.

The results demonstrate distinct modes of GABA<sub>A</sub>-mediated inhibition in the DG granule cells and CA3 pyramidal neurons along the dorsoventral axis of the hippocampus which can be differentially modulated by insulin.

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Equally contributed: H.H and O.N;

S.K and Z.J.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA266

## Segmentation and quantitative determination of cellular, neurite- and plasma membrane-motility speed.

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Mobility quantification of single cells, plasma membranes or cellular processes in dense cell cultures is a challenge, because tracking of individual cells is not readily possible. We used the recently developed software “SynoQuant” (designed by AWH; accessible on [www.synosoft.de](http://www.synosoft.de)) to analyze the speed of randomly moving cultured cells, the dynamics of plasma membrane contraction and organelle trafficking. Primary cultures of brain pericytes, hippocampal neurons and chicken telencephalon cells were produced from Sprague Dawley rats<sup>[1]</sup>, Wistar rats<sup>[2]</sup> and chicken embryos<sup>[3]</sup>, respectively. All experiments were carried out in accordance with the guidelines of laboratory animal care in Kuwait University and the protocols were approved by Animal Resource Center. In some cases, cells were exposed to oxygen glucose deprivation (OGD) as described earlier<sup>[1]</sup> or maintained in the presence of drugs that affect cell mobility. Images were collected at various intervals over 15–48h in a temperature-controlled chamber on the phase-contrast microscope. Speed quantifications were obtained by segmenting cellular components and determining their individual velocities separately. Algorithms that use image subtraction (DiffMove), or image correlation analysis (‘COPRAMove’) were used to analyse the motility speed of: 1. Intracellular vesicles in chicken telencephalon glia cells (CTG), 2. Somata and neurites in hippocampal neurons and 3. plasma membrane dynamics in pericytes. Differences between the groups were tested with Student’s t-test or with Wilcoxon-Mann-Whitney U-test. The speed of vesicles in CTGs was measured with both algorithms (Fig.1); the velocity of the

organelles was slowed down by addition of staurosporine, a drug that reduced vesicle movement in the frog neuromuscular junctions<sup>[4]</sup>. Figs. 1B and C visualize vesicle traffic in an overlay image that was composed of two consecutive images, assigned to different color channels. Neurites and somata of hippocampal neurons were measured separately and their motility velocities were compared to the unprocessed series. The visual impression that the neurites moved slower than the somata, was confirmed by the ‘COPRAMove’ quantitative analysis (Fig. 2A, B). The motility of pericytes was examined to quantify membrane ruffling, cell constriction and pseudopodia extrusion. Figs. 2C–F show that the motility of pericytes was consistently and significantly ( $p < 0.01$ ) decreased after 1-hour of OGD. Pericytes showed alternating periods of extension and retraction of pseudopodia under control conditions. In conclusion, this study has revealed that our new software could be used successfully to reconstitute and analyze cellular and subcellular movements in cell cultures.

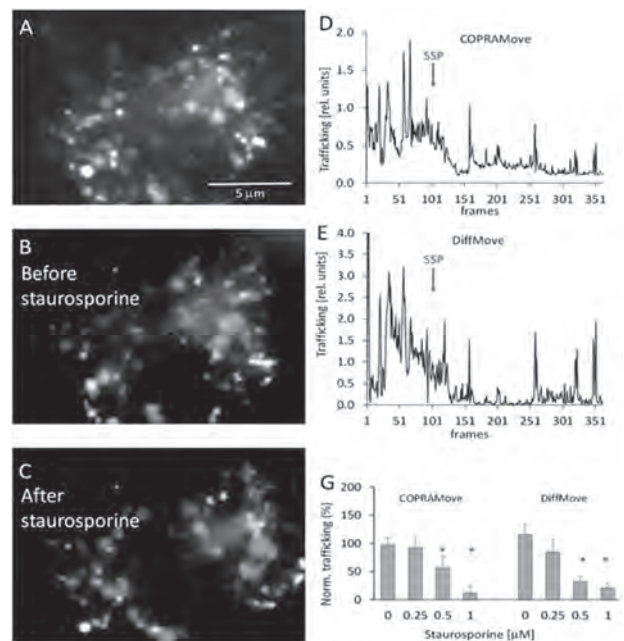


Figure 1. Vesicle motility in chicken telencephalon glia cells. A. Fluorescence image of acidic organelles in a glia cell, labeled with acridine orange; B. overlay image of two consecutive frames, frame 40 (red) and 41 (green) before addition of staurosporine; C. overlay image frame 300 (red) and 301 (green) after addition of staurosporine; D. vesicle trafficking velocity trace, recorded with ‘COPRAMove’ algorithm; E. vesicle trafficking velocity trace, recorded with ‘DiffMove’ algorithm G. Vesicle motility reduction by staurosporine (mean and standard deviation, asterisks indicate significant difference to control,  $p < 0.05$ ).



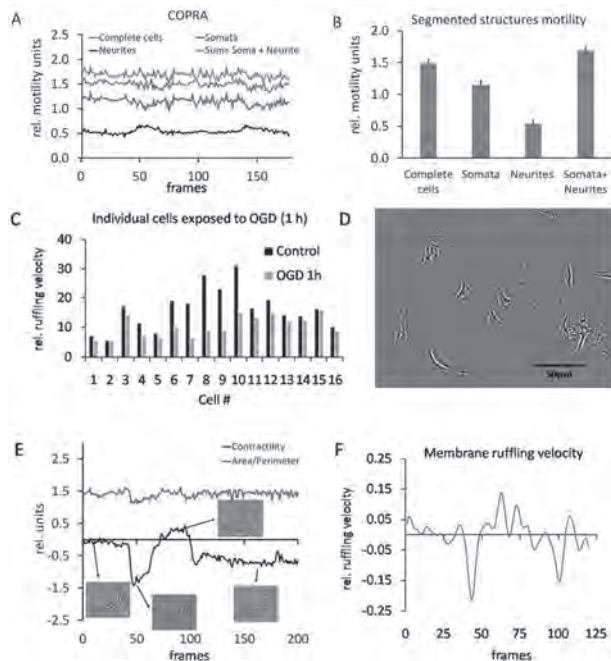


Figure 2. Motility measurements on segmented structures. A. COPRA Move motility traces, obtained from isolated somata, neurites and combination of both. "Complete cells" refers to unsegmented hippocampal cultures, "Somata + Neurites" refers to the summed traces of segmented neurites plus segmented somata; B. Quantification of motility results; C. plasma membrane ruffling velocity of pericytes, measured on isolated cell perimeters; D. Pericyte perimeter outlined by automatic plasma membrane segmentation with the 'Isolate cells from background' (ICB) algorithm; E. contractility and area/perimeter traces show alternation contraction and extension of typical pericyte plasma membrane. The 4 insets show segmented cell perimeters, acquired at corresponding times (arrows) of the experiment; F. moving average of membrane velocity trace, calculated with the 'Trace and Quantify' module. The velocity corresponds to the 1<sup>st</sup> derivative of contractility trace in E.

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PCA267

## The effect of rhein on protein expression and differentiation of SH-SY5Y neuroblastoma cells

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Apart from its connection to the tumorous character, disrupted energy metabolism may be linked to the progression of neurodegenerative diseases. Rhein, a potential candidate for preventing metabolic disorders, has been shown to have various pharmacological properties including anticancer activity and modulatory effects on the bioenergetics. However, its effects on neuronal metabolism are poorly understood. We sought to analyze the impact of rhein treatment on protein expression and the differentiation process of SH-SY5Y neuroblastoma cells. Using two-dimensional gel electrophoresis and MALDI-TOF mass spectrometry we evaluated changes in the proteome of both undifferentiated and retinoic acid-differentiated SH-SY5Y cells after 24-h treatment with rhein. This analysis led to identification of 7 differentially expressed proteins in samples from undifferentiated cells (actin, cytoplasmic 1; tubulin beta chain; tubulin alpha-1A chain; alpha-centractin; protein disulfide-isomerase A3; heterogeneous nuclear ribonucleoprotein A1; 3-ketoacyl-CoA thiolase) and 10 differentially expressed proteins in samples from differentiated SH-SY5Y cells (actin, cytoplasmic 1; tubulin beta chain; tubulin alpha-1B chain; stress-70 protein; heterogeneous nuclear ribonucleoprotein H; thioredoxin-dependent peroxide reductase; peroxiredoxin-6; heterogeneous nuclear ribonucleoprotein L; poly(rC)-binding protein 1; fructose-bisphosphate aldolase A). All of them were downregulated at least 2-fold ( $p < 0.05$ ; Student's t-test) by treatment with rhein. These proteins are involved in cellular metabolism, cytoskeleton organization, transcriptional and translational regulation and antioxidant defence. Validation of selected differentially expressed proteins performed by Western blotting revealed identical trends in the expression of these proteins ( $n=5$ ). Addition of rhein to the cultured cells during differentiation resulted in an altered expression of the following 3 markers: microtubule-associated protein 2, nestin and growth associated protein 43. Relative levels of selected proteins were determined by Western blotting ( $n=5$ ). In parallel, rhein significantly reduced neurite outgrowth ( $136.8 \pm 4.0 \mu\text{m}$  vs  $205.3 \pm 6.7 \mu\text{m}$ ;  $p < 0.001$ ; Student's t-test). Values are expressed as means  $\pm$  S.E.M. Neurite outgrowth was evaluated by tracing neurites ( $n=70$ ) on microscopy images using ImageJ NeuronGrowth plugin. Our results indicate that rhein may strongly interfere with the differentiation process of SH-SY5Y neuroblastoma cells and is capable of inducing marked proteomic changes in these cells. The results also demonstrate that rhein-treated neuroblastoma cells display dysregulation of proteins involved in cytoskeleton organization, protein folding, metabolism and transcription control.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA268

### Sciatic nerve C fibres utilise fructose directly to support conduction whereas A fibre conduction is maintained by Schwann cell fructose-derived lactate

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The ability of fructose to support conduction in the mouse sciatic nerve was investigated for the following reasons: (1) Schwann cells can produce fructose from glucose via the polyol pathway (Champe & Harvey, 2008), which may act as an endogenously created metabolic substrate, (2) in the optic nerve there exist differences between fibre subtypes to effectively use fructose as an energy substrate (Meakin et al. 2007), and (3) in the hippocampus, fructose is supplied to neurons by glial cells in the form of a monocarboxylate e.g. lactate (Izumi & Zorumski, 2009), an intriguing parallel with the ability of Schwann cell glycogen-derived lactate to selectively support A fibres (Brown et al. 2012).

All procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986 under appropriate authority of establishment, project and personal licenses. Adult male CD-1 mice were killed by cervical dislocation and decapitated. Sciatic nerves were dissected, placed in a perfusion chamber, superfused with aerated control aCSF containing 10mM glucose. The stimulus evoked compound action potential (CAP) for A and C fibres were evoked with supra-maximal stimuli (Rich and Brown, 2018).

The ability of the A and C fibre CAPs to recover in the presence of fructose after aglycaemic exposure was assessed. 20mM fructose restored conduction of both A and C fibres, but the onset of A fibre CAP recovery was delayed compared to equivalent experiments with 10mM glucose. C fibre recovery, however, was identical in 10mM glucose or 20mM fructose. To investigate the role of lactate in the utilisation of fructose by the sciatic nerve we carried out recovery experiments in the presence of cinnamate (200µM) to block axonal uptake of lactate. Cinnamate rendered 20mM fructose unable to restore the A fibre CAP but had no detrimental effect on C fibre CAP recovery with either glucose or fructose. The application of lactate biosensors, to measure lactate release from the nerve ( $[lac]_o$ ) developed on these findings further. The stable lactate signal recorded in the presence of 20mM fructose increased with the addition of cinnamate and the delayed A fibre CAP recovery in 20mM fructose was temporally correlated with a delayed increase in  $[lac]_o$  following reperfusion with 20mM fructose, all of which are consistent with axonal uptake of lactate.

It appears that C fibres directly take up and metabolise fructose efficiently whereas A fibre conduction is supported by Schwann cell fructose derived-lactate. Unravelling the metabolic profile of the sciatic nerve and revealing fibre sub-type specific utility of substrates may provide insight into the mechanisms underlying diabetic neuropathy and ultimately help in developing clinically effective neuro-protective strategies.

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PCA269

### Visualization of morphological changes of PSD95 substructures in the living mouse brain by STED microscopy

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Far-field light microscopy is a powerful technique for imaging structures inside living cells, tissue or living animals. However, fine details or substructures of the cell cannot be visualized because of diffraction limited resolution (~200-350 nm). From all novel light microscopy or super-resolution microscopy techniques available presently, STED (STimulated Emission Depletion) nanoscopy stands out for its imaging capabilities in tissue. Here, we used STED nanoscopy to determine short term changes in size and shape of PSD95 in the anesthetized mouse visual cortex. These morphological changes should relate to variations of synaptic strength, and the formation or elimination of synapses, respectively (El-Husseini et al. 2000; Meyer et al. 2014). Adult knock-in mice expressing eGFP fused to the endogenous PSD95 protein were imaged at time points from 1 min to 6 h. All experiments were performed according to ethical rules of the European Union and the guidelines of the national law (Tierschutzgesetz der Bundesrepublik Deutschland, TierSchG) regarding animal protection procedures. General anesthesia was initiated by i.p. injection of 60–80 mg pentobarbital sodium (in 0.9% NaCl) per kg body weight. Once the mouse was anesthetized, the right jugular vein was cannulated, and anesthesia was continued with 75 mg kg<sup>-1</sup> h<sup>-1</sup> methohexital sodium (Brevimyltal®, HIKMA, Gräfelfing, Germany) i.v. throughout the experiment. A tracheotomy was performed to intubate the mouse. The mouse was paralyzed with pancuronium bromide (6 mg kg<sup>-1</sup> h<sup>-1</sup>) and connected to artificial ventilation at 100-120 breaths per minute and a breath volume of 100-140 µl to avoid movements by active respiration. Body temperature was rectally measured and controlled by a heat plate.

Super-resolved large assemblies of PSD95 show different sub-structures; most large assemblies were ring-like, some horseshoe or figure-8 shaped, and shapes were continuous or made up of nanoclusters. The sub-structure appeared stable during the shorter (minute) time points, but after 1 h, more than 50% of the large assemblies showed a change in sub-structure. The morphing of the PSD95 assemblies did not occur continuously; some assemblies were stable over 3 h but then changed after another 3 h, others changed and then regained their original shape. Within our imaging time course of 6 h 90% of the large PSD95 assemblies underwent a morphological change. Overall, these data showed a sub-morphology of large PSD95 assemblies which undergo changes within the 6 hours of observation in the anaesthetized

mouse. At this time we can only speculate on the impact of these changes on the synaptic machinery and therefore did not quantify it further. To further analyze the physiological background of changes it would be necessary to perform experiments with defined stimulation or deprivation as possible triggers for these changes.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCA270

### **Chronic Methamphetamine Alters Neural Oscillations and Synaptic Plasticity in the Hippocampus in Guinea Pigs, *in vivo***

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Methamphetamine (METH) is a highly addictive psychostimulant that affects the brain's memory consolidation processes. Studies show that chronic METH users perform poorly than controls on tasks of verbal and nonverbal memory, recognition, attention, and decision-making. Previous studies by Aduonum and co-workers (2001) found that acute low doses of METH (1 mg/kg IP) facilitated long-term potentiation (LTP, i.e. enduring increase in synaptic efficacy in the hippocampus taken to be the cellular correlate for learning and memory), whereas high doses of METH (10 mg/kg IP) attenuated LTP maintenance. However, the neural mechanism through which METH altered LTP and, therefore, memory consolidation still remain unclear. Within this context, the present study aimed to investigate the effect of chronic high dose METH (10 mg/kg-similar to abused doses) treatment on changes in neural circuit oscillations and synaptic plasticity linked to memory consolidation. We treated twenty male Wistar guinea pigs (3-4 weeks old; weight, 200-250 g) from Charles River Laboratory with 10 mg/kg/day METH (N=12) or phosphate buffered saline (PBS, N= 8) for 7 days. Drugs were administered as a continuous infusion using ALZET mini-pumps (Model 2001). After 7 days of treatment a subset of guinea pigs from each group (METH = 6 and PBS = 4) were anesthetized with urethane (1500 mg/kg) and prepared for electrophysiological recordings. Briefly, small access holes were made in the skull through which bipolar Tungsten electrodes were lowered. Low frequency stimulation (100-120  $\mu$ A, 0.25 ms, at 0.1 Hz) was applied to the left CA<sub>3</sub> and local field potentials and evoked population spikes recorded in ipsilateral CA<sub>1</sub> region, *in vivo*. Subsequently, high frequency stimulation (HFS; 100 Hz, 1 sec, 3 trains) was applied to the CA<sub>3</sub> region to induce LTP in CA<sub>1</sub>. Similar recordings were conducted in the remaining subset of guinea pigs (METH = 6 and PBS = 4) after 7 days of drug washout. All evoked responses were amplified at a gain of 1000x filtered at a bandpass of 100 Hz - 5 kHz and analyzed with Clampfit 10.6. We found that METH-treated guinea pigs exhibited increased spontaneous CA<sub>1</sub> cell discharges and had diminished slow wave oscillations. By contrast, PBS controls exhibited fewer spontaneous discharges and had prominent slow wave oscillations whose peak were inundated with

robust 'ripples', a hallmark of transfer of information from the hippocampus to neocortical structures. We also found that LTP, a cellular correlate of learning and memory was attenuated in the METH-treated guinea pigs when compared to PBS controls. The effects of METH on CA<sub>1</sub> cell discharges, oscillations and LTP were still present even after the 7-day washout. Taken together, our results raise the possibility that METH-induced changes in CA<sub>1</sub> network properties may partly underlie the reported memory impairments associated with the drug.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCA271

### **Comparison between the Functional Restorative Capacity of Adipose Tissue and Bone Marrow Derived Mesenchymal Stem Cells in Cerebellar Ataxic Rat Model**

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Cerebellar ataxias represent a group of disabling disorders of balance and coordination. In absence of definitive therapy so far, stem cells can represent the needed hope. The present study compared the therapeutic efficacy of adipose stem cells (ASCs) and bone marrow mesenchymal stem cells (BM-MSCs) in a rat model of cerebellar ataxia. Forty adult female Albino Wistar rats were equally divided into control, MSG, BM-MSCs and ASCs groups. Six additional adult male rats of the same species were used for isolation of retroperitoneal adipose tissue and bone marrow from femur and tibia. After acclimatization, the latter three groups received intraperitoneal (IP) injection of 4 gm/kg body weight of monosodium glutamate (MSG) once daily for 6 days to induce cerebellar ataxia, whereas the control group received 1 ml saline IP for 6 days. On the 7th day, each of the 2 stem cell groups received one million related cell type dissolved in 250  $\mu$ L PBS, whereas the other 2 groups received only PBS as a single IV injection in tail vein. Motor coordination was assessed weekly afterwards for 4 weeks using rotarod test, open field test and quantitative gait analysis. Then rats were sacrificed, and cerebellum was dissected for histological examination, immunohistochemical analysis of Purkinje cells regeneration and apoptosis using Bax/Bcl-2 ratio, VEGF level, ELISA to detect ILGF-1 and PCR analysis to detect SRY gene expression. Values are means  $\pm$  SD, compared by ANOVA. Physiological assessment showed significant deterioration in MSG group in all tests all over the study period compared to control group, and significant improvements in both stem cell groups with earlier onset in ADS group. Histological study in MSG group revealed widely displaced, distorted and shrunken Purkinje cells with marked decrease in number of Nissl granules per Purkinje cell (0.223 $\pm$ 0.003). In both stem cell groups, there



was significant decrease in the number of distorted Purkinje cells with significant increase in number of Nissl granules;  $0.35 \pm 0.002$  in BM-MSG group and  $0.51 \pm 0.001$  in ASC group. There was significant increase in Bax/Bcl-2 ratio in MSG group ( $2.53 \pm 0.06$ ) as compared to BM-MSG group ( $0.47 \pm 0.05$ ) and to ASC group ( $0.40 \pm 0.03$ ). VEGF immunostaining showed a significant increase in the ASC group ( $46.65 \pm 1.22\%$ ) as compared to BM-MSG group ( $38.70 \pm 0.99\%$ ), which was also significantly higher than MSG group ( $24.44 \pm 2.14\%$ ). Similarly, IGF-1 was significantly higher in ASC group ( $39.77 \pm 2.34$  ng/ml) as compared to BM-MSG group ( $19.32 \pm 0.39$ ) and to MSG group ( $5.93 \pm 0.35$ ). SRY gene could not be detected in the cerebellum of female recipients. In conclusion, our study showed that both stem cell types induced structural, physiological and biochemical improvement of cerebellar ataxia, with significantly better results of ASCs therapy.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA272

### Store-operated calcium entry is profoundly impaired in a mouse model of Alzheimer's disease.

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Although Alzheimer's disease (AD) is the most common form of dementia, its cause is still unclear and a cure has yet to be developed. We have previously shown however that young (P2-P5) neurons derived from transgenic murine models of AD<sup>1,2</sup> exhibit significantly altered endoplasmic reticulum (ER)-mediated  $\text{Ca}^{2+}$  handling compared to controls, suggesting that intracellular calcium homeostasis may be a primary initiator of AD pathogenesis. In addition to excitation-mediated  $\text{Ca}^{2+}$  entry, one of the primary means of loading the neuronal ER with  $\text{Ca}^{2+}$  is via store-operated calcium entry (SOCE). This process is initiated by the depletion of ER  $\text{Ca}^{2+}$  stores and involves the movement of  $\text{Ca}^{2+}$  directly through plasma membrane-located channels into the ER. However, to date, little is known about how this  $\text{Ca}^{2+}$  entry pathway is affected by mutations which predispose to the development of AD. The aims of this study were therefore to, firstly, determine if SOCE was present in mouse primary cultured hippocampal neurons and, if so, to investigate if it was altered in primary cultured hippocampal neurons from the 3xTg mouse model of AD relative to controls.

Hippocampal neurons were cultured from 3-5 day, wild type (non-Tg) and transgenic (3xTg) mice. Neurons were loaded with the  $\text{Ca}^{2+}$ -sensitive dye, fluo-2 AM ( $4\mu\text{M}$ ) and then superfused with standard physiological saline (HBSS) containing TTX ( $1\text{mM}$ ). Intracellular somatic  $\text{Ca}^{2+}$  levels were monitored using Winfluor software. SOCE was evoked by depleting the ER using the SERCA pump inhibitor thapsigargin (TPN) in conjunction with  $\text{Ca}^{2+}$ -free HBSS. The SOCE antagonist gadolinium ( $\text{Gd}^{3+}$ ) was also utilised to determine the relative susceptibility of SOCE in the two different hippocampal genotypes. Data are expressed as mean  $\pm$  SEM and statistical significance was determined using Student's unpaired *t*-test.

Our results demonstrated that SOCE in 3xTg neurons was significantly reduced compared to that evoked in non-Tg neurons (by  $46.3 \pm 9.8\%$ ,  $P < 0.0001$ ; non-Tg,  $n=65$ ; 3xTg,  $n=57$ ). Additionally, the intracellular  $\text{Ca}^{2+}$  elevation evoked by TPN ( $1\mu\text{M}$ ) was significantly reduced in 3xTg neurons relative to non-Tg neurons (by  $84.7 \pm 9.7\%$ ,  $P < 0.001$ ; non-Tg,  $n=25$ ; 3xTg,

$n=11$ ).  $\text{Gd}^{3+}$  ( $50\text{mM}$ ) significantly inhibited SOCE in both non-Tg and 3xTg hippocampal neurons but the degree of SOCE inhibition was significantly greater in non-Tg neurons (by  $21 \pm 4.1\%$ ,  $P < 0.001$ ; non-Tg,  $n=35$ ; 3xTg,  $n=29$ ).

The significantly smaller SOCE displayed by transgenic AD neurons appears to be yet another example of dysregulated calcium handling processes within neurons expressing AD-linked genetic mutations. The reason(s) underlying the disrupted SOCE in the 3xTg neurons remain(s) unclear but may be linked to incomplete emptying of the ER stores due to the particular PS1 mutation expressed within these transgenic mice, which is proposed to inhibit the normal leak of calcium from the ER<sup>3</sup>.

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### Dimerization of sodium channel subtype Nav1.7

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Sodium channels in the peripheral nervous system are known to play a major role in the initiation and transmission of action potentials which can lead to the perception of pain. Mutations in different channel subtypes can lead to either pain syndromes which are associated with increased pain or its total loss. Interestingly, most of the pain-linked mutations occur as a heterozygous mutation, which leads to the simultaneous presence of WT and mutated channels in the expressing cells.

In 2017, Clatot et al showed evidence that Nav1.5 channels form dimers which support a coupled gating mechanism. In our study, we investigate dimerization of Nav1.7 with itself and other sodium channel subtypes, which could enhance or alleviate the effect of pain-linked mutations.

In order to investigate if Nav1.7 might dimerize we used biochemical and electrophysiological approaches. To assess a potential functional coupling, the electrophysiological behavior of the previously reported pain-associated Nav1.7-mutation A1632E (Estacion et al, 2008) was studied via the patch clamp technique in a heterologous expression system using human embryonic kidney cells (HEK cells). HEK cells were transfected with either Nav1.7 alone, Nav1.7 and A1632E combined and the A1632E mutation by itself.

When assessing inactivation gating, we observed a shift for the A1632E mutation compared to WT, and this shift was smaller when WT and A1632E were combined (results of Boltzmann fits:  $V_{1/2}$  for WT =  $81.56\text{mV} \pm 0.94$   $n=20$ , for A1632E =  $68.97\text{mV} \pm 1.00$   $n=33$ , for WT combined with A1632E =  $74\text{mV} \pm 2.68$   $n=19$ ).

We observed a persistent current for the A1632E mutation ( $7\% \pm 0.005$  of the peak inward current,  $n=35$ ) which was absent in our recordings of Nav1.7 WT ( $n=22$ ). In cells which expressed Nav1.7 and A1632, small persistent currents ( $2\%, \pm 0.004$ ,  $n=25$ ) could be measured.

Our results suggest that Nav 1.7 forms dimers. Therefore, we plan to repeat these experiments in the presence of difopein, a 14-3-3 protein inhibitor which was shown to inhibit channel dimerization in Nav1.5 (Clatot et al, 2017; Masters and Fu, 2001) to confirm these findings.

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#### PCA274

##### **The paralogs of the calcium-dependent activator protein for secretion differentially regulate synaptic transmission and peptide secretion in murine dorsal root ganglion neurons**

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The two paralogs of the calcium-dependent activator protein for secretion (CAPS) are priming factors for synaptic vesicles and neuropeptide containing large dense-core vesicles (LDCVs). Yet, it is unclear whether CAPS1 and CAPS2 regulate exocytosis of these two vesicles types differentially in systems such as dorsal root ganglion (DRG) neurons, where synaptic transmission and neuropeptide release are of equal importance. These sensory neurons transfer information from the periphery to the spinal cord, releasing glutamate as the primary neurotransmitter, with co-transmission via neuropeptides in a subset of so called peptidergic neurons. Neuropeptides are key components of the information-processing machinery of pain perception and neuropathic pain generation. Here, we compared the ability of CAPS1 and CAPS2 to support priming of both vesicle types in single and double knock-out mouse (DRG) neurons using a variety of high-resolution live imaging methods. While CAPS1 was localized to synapses of all DRG neurons and promoted synaptic transmission, CAPS2 was found exclusively in peptidergic neurons and mediated LDCV exocytosis. Intriguingly, ectopic expression of CAPS2 empowered non-peptidergic neurons to drive LDCV fusion, thereby identifying CAPS2 as an essential molecular determinant for peptidergic signaling. Our results reveal that these distinct functions of both CAPS paralogs are based on their differential subcellular localization in DRG neurons. Our data imply a major role for CAPS2 in neuropathic pain via control of neuropeptide release.

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#### PCA275

##### **Calcineurin and Reelin Gene Promoter Region DNA Methylation Changes in the Amygdala of Young and Aged Rats**

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DNA methylation, one of the important chromatin modifications of the epigenetic regulation, has been suggested to regulate the expression of genes involved in the formation of memory by preventing transcription. In this study, we aimed to investigate whether there is any age-related changes in DNA methylation and the effect of memory formation and storage on calcineurin (CaN) and reelin (RELN) gene promoter region DNA methylation in the amygdala region of the brain at different time points (1<sup>st</sup> hour, 24<sup>th</sup> hours, 7<sup>th</sup> days, 21<sup>st</sup> days). For this purpose, 123 Sprague-Dawley male rats, 63 for 3-4 months (250-350 g) and 60 for 19-20 months (400-500 g), were used. The contextual fear conditioning model was used to provide the formation of fear memory. The study groups were identified as controls (C), experiments (E) and naïve. The rats were decapitated immediately after freezing behaviour test. The amygdala region of the brain was isolated. DNA methylation changes in the promoter region of CaN and RELN genes were examined by MeDIP RT-PCR analysis method. The methylation level for each gene was determined as fold change relative to the naïve control. Data are compared by Student's *t*-test or non-parametric Mann-Whitney *U* test. The behavioral data show that fear memory formation is significantly observable at all time-points in young rats (C:  $58.10 \pm 18.30\%$ ;  $56.89 \pm 9.33\%$ ;  $52.74 \pm 16.65\%$ ;  $63.08 \pm 15.75\%$ , E:  $84.70 \pm 6.70\%$ ;  $73.22 \pm 6.68\%$ ;  $83.24 \pm 9.49\%$ ;  $81.61 \pm 17.91\%$ ,  $p < 0.05$ , respectively). On the other hand, fear memory formation was observed at the 24<sup>th</sup> hours and on the 21<sup>st</sup> days in the aged rats (C:  $34.81 \pm 10.74\%$ ;  $48.96 \pm 17.29\%$ , E:  $54.11 \pm 16.34\%$ ;  $66.49 \pm 7.42\%$ ,  $p < 0.05$ , respectively), but it was not occurred at the 1<sup>st</sup> hour and on the 7<sup>th</sup> days (C:  $47.47 \pm 12.57\%$ ;  $53.87 \pm 12.86\%$ , E:  $52.55 \pm 25.15\%$ ;  $61.21 \pm 20.65\%$ ,  $p > 0.05$ , respectively).

DNA methylation data indicate that CaN gene methylation decreased on the 7<sup>th</sup> and 21<sup>st</sup> days in the amygdala region of young rats (C:  $1.03 \pm 0.02$ ; E:  $0.97 \pm 0.007$ ,  $p < 0.001$ , C:  $1.00 \pm 0.007$ ; E:  $0.90 \pm 0.01$ ,  $p < 0.001$ , respectively), whereas it increased at the 1<sup>st</sup> and 24<sup>th</sup> hours in aged rats (C:  $0.92 \pm 0.02$ ; E:  $0.97 \pm 0.01$ ,  $p = 0.01$ ; C:  $0.89 \pm 0.05$ ; E:  $1.00 \pm 0.03$ ,  $p = 0.005$ , respectively). RELN gene methylation decreased at the 1<sup>st</sup> hour and on the 7<sup>th</sup> days in the young rats (C:  $1.06 \pm 0.02$ ; E:  $1.01 \pm 0.01$ ,  $p = 0.02$ , C:  $1.06 \pm 0.01$ ; E:  $1.00 \pm 0.03$ ,  $p < 0.001$ ,

respectively) and increased at the 24<sup>th</sup> hours ( $C:0.99\pm0.01$ ;  $E:1.04\pm0.01$ ,  $p=0.002$ ). However, no change was observed in the aged animals ( $p>0.05$ ). As a result, our data show that the methylation levels of *CaN* and *RELN* genes change time dependency and age-related in the evaluated amygdala region during formation and storage of fear memory. These changes may be related with more general cellular regulation rather than a specific involvement with fear memory.

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PCA276

### Autonomic function test in obese among mid-western population of Nepal

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**Purpose:** Obesity is associated with metabolic risk factors such as high blood pressure, blood fat abnormality, and glucose intolerance which may influence the morbidity and mortality of cardiovascular diseases. Beside being a risk factor for cardiovascular disease, certain cancers and type II diabetes, obesity has also been associated with change in autonomic function in human. Although a lot of progress has been achieved in past decade on accessibility and awareness about health, the obesity remains impending and burgeoning health concern in Nepal. With this trend, we can foresee that the Body Mass Index (BMI) one of the commonly used indirect measure of obesity, might potentially turn out to be one of the leading factor of autonomic dysfunction.

**Methods:** 100 healthy subjects were screened and divided into 2 groups- Group I (BMI>30) and Group II (BMI<30). Height was measured by stadiometer with subjects having their shoes removed. Weight was measured with weighing machine in light clothes. BMI was calculated using the formula:  $BMI = \text{weight(kg)} / \text{height(m)}^2$

Resting heart rate (RHR) was recorded with Lead II of ECG. Blood pressure (BP) and Heart Rate (HR) were recorded in supine position and on immediate standing. Cold pressure test: Resting BP was recorded in sitting position. Then the subjects were asked to immerse the hand in cold water, and the BP was measured from other hand. Data was analyzed using SPSS 16 (Statistical Package for Social Science).

**Result:** Our result showed that RHR of Group I ( $79.32 \pm 4.22$ ) was higher than that of Group II ( $74.38 \pm 7.26$ ). However, on student -T test, BP and HR response to immediate standing ( $P=0.34$  &  $P=0.23$  respectively) were non-significant between obese and non-obese person. When the correlation was done for the change in BP in response to cold pressor test in between obese and non obese person it was found to be significant ( $P=0.04$ ).

**Conclusion:** Our data suggests that the BMI can be a predictor of autonomic dysfunction.

**Keywords:** Body Mass Index, Autonomic Function, Resting Heart Rate, Cold pressor test

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA277

### Characterization of an animal model of Lipopolysaccharide-induced Inflammation on cardiovascular and central nervous systems

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Sepsis is a systemic inflammatory state mediated by the innate immune system resulting in an excessive cellular response to severe infection, with high levels of morbidity and mortality (1). Furthermore, patients who survive sepsis, have long-term cognitive and functional impairment (2,3). Animal models are essential to clarify the pathophysiological mechanisms of sepsis. Herein, we characterize an animal model of lipopolysaccharide (LPS)-induced inflammation, evaluating neural and cardiac function, during the initial stages of infection (4). Male Wistar rats (12-20 wks,  $n=24$ ) were injected with LPS (*E. coli* serotype O127:B8; tail vein) and divided into 3 groups: LPS6 (6mg/kg), LPS12 (12mg/kg) and Sham (NaCl 0.9%; 0.1ml/100g). At 6 and 24h after LPS injection, an autonomic evaluation was performed in both conscious animals, with continuous radio-telemetry recording of blood pressure (BP) and heart rate (HR), and in anesthetized animals (induction: sodium pentobarbital, 60 mg/kg, ip; maintenance: 20% solution, v/v) with BP, ECG, HR, tracheal pressure, respiratory frequency (RF) and body temperature continuously monitored. Baro and chemoreflex were evaluated with phenylephrine (0.2ml, 25 µg/ml; iv) and lobeline (0.2ml, 25 µg/ml; iv), respectively (5). Behavioural changes were also evaluated through the elevated-plus maze, open-field and Y-maze tests. Immunohistochemistry and RT-PCR were executed to determine heart and brain inflammatory state. Serum biomarkers levels for organ dysfunction were also measured. Overall our results show a rise in BP and HR, and elevated RF due to increased chemoreflex sensitivity. 24h following LPS injection, all groups have a significantly decreased baroreflex ( $p<0.05$ ), however, 6h post-injection, LPS12 show a statistically significant increase in baroreceptor reflex ( $p<0.05$ ). At both time-points, the two LPS groups present an anxiety-like behavior, associated with less locomotor/exploratory activity and highly significant cognitive impairment ( $p<0.0001$ ). The autonomic evaluation of the anesthetized LPS12 group results in an increase



of the autonomic tone at 6h post-LPS followed by a decrease at 24h. The LPS6 group shows the opposite profile. Interestingly, conscious animals reveal a slightly different profile in both groups, with a continuous increase in autonomic tone for LPS12 and a decrease 24h post-LPS in LPS6 group. The molecular studies show reactive astrogliosis and microgliosis, due to inflammatory processes in the hippocampus, as well as, an upregulation of pro-inflammatory factors in the heart and brain. Serum analysis yielded higher levels of biomarkers for renal and liver dysfunction ( $p < 0.05$ ) and pancreatic and neuromuscular injury ( $p < 0.05$ ), in the LPS12 group. Concluding, LPS administration induces strong alterations in both cardiac and neurological systems and is a good model for further pathophysiological and pharmacological studies related to Sepsis. Hotchkiss RS, Moldawer LL, Opal SM, Reinhart K, Turnbull IR, Vincent JL. Sepsis and septic shock. *Nat Rev Dis Primers*. 2016;2:16045.

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PCA278

### **Role of brain dopamine, histamine and opioid receptors underlying divergent behavioural phenotypes in zebrafish**

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Social animals exhibit dominant subordinate relationships. Dominant animals possess proactive coping styles while subordinate animals possess reactive coping styles. Animals with proactive stress coping styles are considered to be bold, show low attack latency and form routines while animals with reactive stress coping styles are shy, show high attack latency and do not form rigorous routines. Brain dopamine has been reported in aggressive behaviour. However, there is no study available till date that has explored the involvement of opiod and histamine receptors in context of the dominant subordinate behavioural phenotypes. Here, we explored the role of dopamine, histamine and opiod receptors in the brain of zebrafish that have been pre-selected for boldness and then subjected to a dyadic interaction.

In this study we screened 166 zebrafish (101 males and 65 females) and selected them for boldness and shyness based on three different behavioural tests, namely the open field test (OFT), novel object test (NOT) and the shelter test (ST). The fish that showed the highest overall activity in the three tests were taken as bold and the fish that showed the least activity were taken as shy. The fishes that were neither too bold nor too shy were taken as an intermediate group. Then, the bold, shy and intermediate fish were subjected to a dyadic interaction. Briefly, a dyadic interaction consists of a pre-resolution and a post resolution phase (Oliviera et al. 2011). In the pre-resolution phase the prospective dominant and

subordinate fish indulged in frontal display, coming face to face with each other. In the post resolution phase the dominant fish bites and chases away the subordinate fish until it flees away. For this study, after 4 days of staged dyadic contest the brain tissue was collected and analyzed for the expression of dopamine, histamine and opioid receptors.

The results show that class 2 type of dopamine receptors (*drd2b*, *drd3*, *drd4rs*), histamine receptor 3 (*hrh3*) and delta opioid receptor 1b (*oprd1b*) were significantly affected by boldness, bold group having higher expression levels than shy group. Our study shows that boldness and social rank are both associated with specific but different expression pattern of dopamine, histamine and opioid receptors in the brain of zebrafish. We did not see any behavioural differences in the activities of males and females in the three behavioural tests (open field test, shelter test, novel object test) that were conducted. Using zebrafish as a model organism, this is a novel study that adds to our understanding of the roles of dopamine, opoid and histamine receptors in context of divergent behavioral phenotypes.

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PCA279

### **The role of solute carrier transporters in the translocation of the antipsychotic amisulpride at the blood-brain barrier in Alzheimer's disease and in normal ageing**

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Alzheimer's disease (AD) patients with psychosis have an increased sensitivity to antipsychotics, such as amisulpride, and to their side effects. There is evidence that blood-brain barrier (BBB) dysfunction increases with age and with AD and that this may mediate the heightened sensitivity (Reeves et al., 2017). In order to determine the optimal dosage for AD patients, we need to further understand the transport of antipsychotics at the BBB. Previously, solute carrier transporters, such as OCT1 have been implicated in the transport of amisulpride into BBB endothelial cells (Dos Santos Pereira et al., 2014; Sekhar et al., 2018). Thus, we examined the involvement of other organic cation transporters - plasma membrane monoamine transporter (PMAT), and multi-anti-microbial extrusion proteins 1 and 2 (MATE1 and MATE2) in the transport of amisulpride at the BBB.

Accumulation assays were performed in immortalized human cerebral microvessel endothelial cells/D3 (hCMEC/D3) (Sekhar et al., 2017). They were incubated with [<sup>3</sup>H]amisulpride and [<sup>14</sup>C]sucrose with or without inhibitors for PMAT, MATE1, and MATE2. Five different incubation times (5, 20, 30, 60, and 120 minutes) were used to allow accumulation of the radiolabelled solutes in the cells. The results were analysed using two-way

ANOVA and GraphPad Prism Version 5 (GraphPad Software Inc., USA). All data are presented as means  $\pm$  standard error. Inhibition of PMAT for 60 minutes and for 120 minutes led to a significant increase in the cellular accumulation of [ $^3\text{H}$ ] amisulpride by  $40.9 \pm 13.2\%$  ( $p=0.0306$ ) and by  $66.3 \pm 19.4\%$  ( $p<0.0001$ ), respectively ( $n=4$ , passages 30, 31 and 34). Inhibition of MATE1 for 120 minutes resulted in a significant increase in the accumulation of the radiolabelled drug in the cells by  $77.7 \pm 21.1\%$  ( $p<0.0001$ ,  $n=3$ , passages 30, 31 and 34). Whereas, MATE2 inhibition for 5 and for 120 minutes resulted in a significant decrease in the cellular accumulation of [ $^3\text{H}$ ] amisulpride, by  $36.9 \pm 14.1\%$  ( $p=0.0054$ ) and by  $26.4 \pm 11.2\%$  ( $p=0.0093$ ), respectively ( $n=2$ , passages 31 and 34). PMAT, MATE1, and MATE2 may have a role in the translocation of amisulpride at the BBB. Overall, establishing the specific transporters involved in the translocation of amisulpride in good *in vitro* and *in vivo* BBB models would be valuable for improving the risk/benefit profile of existing antipsychotics and for screening of new drugs.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA280

### Insulin-like growth factor 1, Sirtuin 1, Sirtuin 3 and brain injure in mice chronic cerebral hypoperfusion

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Chronic brain hypoperfusion (ChCH) is a factor of risk for central nervous system (CNS) diseases, such as chronic brain ischemia, degenerative diseases, and others. Pathophysiological mechanisms of ChCH are still being investigated. By using complex approach, including genetic and functional methods of investigation, we anticipate to exhibit part of mechanisms underlying ChCH. The present study investigated changes of

IGF1, SIRT1 and SIRT3 expression levels in brain under ChCH induced by unilateral occlusion common carotid artery (CCA). The experiments were carried out on male C57Bl/6j mice 6 weeks old (weight 18-20 g), divided into four experimental groups: (1) control sham group,  $n=10$ ; (2) ChCH group,  $n=10$ ; (3) ChCH+nicotinamide (NAM, 200 mg/kg, 10 d, i.p.) group,  $n=10$ ; (4) ChCH+resveratrol (RV, 10 mg/kg, 10 d, i.p.) group,  $n=10$ . All manipulations were carried out in anesthetized with ketamine (60 mg/kg, i.p.) mice. ChCH was performed using left CCA ligation. In 8 weeks, mice brain were excised; brain tissues from left and right hemispheres immediately studied the features of DNA damage using the single cell gel electrophoresis assay (known as DNA comet assay), frozen hemispheres were stored at  $-80^\circ\text{C}$  until used for real-time PCR to determine IGF1, SIRT1 and SIRT3. The duplex ultrasonography was done in 8 weeks after left CCA ligation for the resistive index (RI) evaluation of right CCA and basilar artery (BA). All data are presented as a mean  $\pm$  standard error of the mean (SEM). One-way analysis of variance was executed to compare means between homogeneous groups accompanied by Bonferroni's post hoc test. The results of the Comet assay showed that ChCH was accompanied by an increase of DNA damage (comets within classes 3 and 4) index of neurons from the unilateral side (the side of CCA ligation) 6.9 times ( $p<0.001$ ), by a decrease level of IGF1, SIRT1, SIRT3 gene expression 11.8, 9.3 and 20.2 times respectively compared with the control,  $p<0.05$ . In modeling of ChCH, from the unilateral side DNA damage index in neurons was reduced by treatment with NAM and RV by 89.6% and by 92.4% respectively compared to control  $p<0.001$ . From the unilateral side NAM and RV was increased the levels of SIRT1 1.7 and 3.5 times, of SIRT3 2.9 and 5.2 times, of IGF1 3.5 and 5.7 times respectively compared with the ChCH,  $p<0.05$ . Under ChCH, RI of the right CCA and BA was increased by 52.26% and by 84.16% respectively compared with control,  $P<0.05$ . NAM and RV was decreased RI of the right CCA by 12.54% and by 23.76% respectively and of BA by 26.36% and by 21.52% respectively compared with ChCH,  $p<0.05$ . Taken together, these data indicate that nicotinamide and resveratrol can detect positive effect on ChCH-induced brain injure by attenuating DNA breaks, which is partly attributable to the upregulation of SIRT1, SIRT3, IGF1-mediated signaling pathway.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA281

### Reduced peripheral brain-derived neurotrophic factor in stroke survivors with cognitive impairment

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Stroke presents a huge burden globally and locally. Very few studies evaluated cognitive impairment following stroke in the Sub-Sahara Africa and none of such studies sought to evaluate the relationship between cognitive impairment and brain-derived neurotrophic factor (BDNF). This study evaluates cognitive impairment in stroke survivors and examines its relationship with peripheral blood levels of BDNF. It is hypothesized that serum BDNF levels significantly correlate with cognitive impairment in a sub-population of the Sub-Saharan

Africa. In this cross-sectional study, cognition was assessed in 47 stroke survivors and 35 normal subjects using the Mini mental state examination (MMSE). Blood sample was taken to determine serum BDNF concentrations using the BDNF ELISA kit. Ethical approval was obtained from the State Health Research Ethics Committee. The data, which were not normally distributed, were expressed as median (10<sup>th</sup>-90<sup>th</sup> percentiles) and analysed using non-parametric tests on SPSS statistics. The age of the stroke survivors and controls were 55.00 (38.8-62.0) and 50.00 (32.8-65.0) years, respectively, while the duration of stroke was 10 (2-45) months. The MMSE scores were 17.00 (6.0-27.2) and 24.00 (21.6-28.0) for the stroke survivors and controls, respectively, and were significantly different ( $U=270.50$ ,  $P=0.001$ ). Serum BDNF values for the stroke survivors and controls were 1.32 (0.43-1.56) ng/l and 2.067 (1.72-2.28) ng/l, respectively, and the difference was statistically significant ( $U=108.00$ ,  $P=0.001$ ). About 78.7 % of the stroke survivors had mild or moderate cognitive impairment which had moderate positive relationship with serum BDNF levels (Kendall's tau  $b=0.58$ ,  $P=0.001$ ). These data suggests that there was very high prevalence of cognitive impairment in the 10 months post-stroke period, which was not associated with socio-demographic factors in the subjects studied. The post-stroke cognitive impairment was associated with reduced brain-derived neurotrophic factor in the peripheral blood of stroke survivors.

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PCA282

### Can Dementia be prevented by mixing Curcumin in Diacetyl containing products?

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Dementia is getting increasingly high with the time and according to National Centre for Social and Economic Modelling (NATSEM) Australia (1), the number of new cases of dementia will increase to 318 people per day by 2025 and more than 650 people by 2056. Despite of all the enormous progress in this field we are not getting any solution to this very serious problem.

One of the reasons could be the presence of synthetic products in our food, for example a flavorant Diacetyl (DA), which is commonly used in the production of processed cheese

and microwave popcorns. DA is structurally similar to MEC (methyl-glucuronic acid, known to induce A $\beta$  aggregation) thus; an investigation into amyloidogenic effects of DA is clearly warranted. DA alone can induce amyloidogenesis at the concentrations as low as 25  $\mu$ M, and even at lower concentrations of 1  $\mu$ M (2). It is capable of permeating through blood brain barrier and is resistant to detoxify by glyoxalase system, thus promoting dicarbonyls formation in the brain (3). Curcumin is well known for its antioxidant, anti-inflammatory, and antitumor properties. Curcumin, due to its lipophilic nature can cross blood brain barrier, inhibit A-beta 40 aggregation, improving behavioral impairment (4).

**Aims and Objectives:**

The aim of this study is to test the anti-dementia effect of curcumin in DA induced animal models as to initiate appropriate strategies for the prevention of dementias such as Alzheimer's disease (AD).

**Method and Material**

We used curcumin as a neuroprotective agent against Diacetyl (DA) induced dementia in male Wistar rats of 3-4 months of age. The rats were divided into 3 groups; two were injected with DA at a dose of 3.0g/kg for 10 consecutive days. Curcumin was given orally to the animals in group 3 at a dose of 80mg/kg. Animals were tested for the behavioral tasks using Rectangular maze test and locomotor activity test.

**Results:**

DA induced dementia like performance was observed using behavioral tasks tests including; Rectangular maze test and locomotor activity test. In DA treated animals, the correct response rate during acquisition and retention period was significantly lower than the control group. Neuroprotective effect of curcumin in DA induced memory loss rats was significantly virtuous and the values of both behavioral tests appear to be similar to that of the control group.

**Conclusion:**

We conclude that curcumin can act as a potential preventive agent for the DA induced dementia and amnesia.

**Key Words:**

Dementia; diacetyl (DA) memory loss rats; curcumin; Rectangular maze test and locomotor activity

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## PCA283

**Erythrocyte deformability in Slovak children with autism spectrum disorder: correlations with clinical features.**

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**Problem statement:**

Erythrocyte deformability represents intrinsic property of erythrocytes to pass through the narrow capillaries in the microcirculation. It is crucial for the oxygen delivery to all tissues in the human body. The data available in medical databases indicate that erythrocytes in autism spectrum disorder (ASD) may be altered. The aim of the present study was to determine erythrocyte deformability in Slovak children with ASD and correlate it with clinical features.

**Methods:**

Our cross-sectional study involved 83 individuals with diagnosed ASD that was determined using Autism Diagnostic Observation Schedule – second edition (ADOS-2), and the Autism Diagnostic Interview-Revised (ADI-R). Erythrocyte deformability was determined by filtration method. Software GraphPad Prism 7 was used for the data analysis. The study was approved by the Ethics Committee of Faculty of Medicine, Comenius University and University Hospital in Bratislava, Slovak Republic. Written informed consent was obtained from all the subjects or their caregivers. The study conformed to the code of ethics stated in the Declaration of Helsinki.

**Results:**

We have found significant negative correlation of erythrocyte deformability with one subdomain of the ADI-R diagnostic tool, specifically: C2 - apparently compulsive adherence to nonfunctional routines or rituals ( $P=0.01$ ). The correlation between erythrocyte deformability and domains A and B, i.e. qualitative abnormalities in reciprocal social interaction and qualitative abnormalities in communication, was not observed.

**Conclusion:**

Abnormalities in erythrocyte deformability may at least partially be involved in pathomechanisms of ASD and contribute to its clinical manifestation. Further research is necessary in order to bring more data and to establish if erythrocyte deformability can serve as prognostic biomarker in ASD.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## PCA284

**RPRFamide interacts with the nonproton ligand sensing domain and slows desensitization by stabilizing the open conformation of Acid-Sensing Ion Channel 3**

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Acid-Sensing Ion Channel 3 (ASIC3) is a member of the Degenerin/Epithelial Na<sup>+</sup> Channel (ENaC) superfamily and primarily conducts Na<sup>+</sup>. It is mainly expressed in the peripheral nervous system and has an important role in pain sensation. ASIC3 conducts rapidly desensitizing currents when activated by protons. RFamides can slow desensitization of ASIC3, but their binding site is yet unknown. In this study we used the most potent RFamide, RPRFamide, to explore the RFamide interaction with ASIC3.

We first showed that the RPRFamide derivative RPR[azido]Famide can be covalently linked to ASIC3 by UV irradiation. When RPR[azido]Famide was covalently linked to ASIC3, desensitization was prevented. We conclude that RPRFamide stabilizes the open state of ASIC3 and has to unbind before desensitization.

Next, we applied in silico docking and identified two potential binding sites: The nonproton ligand sensing domain, which is located in a central extracellular cavity, and the acidic pocket, an extracellular pocket located on the surface of the extracellular domain.

Based on the docking results we created and analysed 40 ASIC3 mutants. These mutants indicate that the nonproton ligand sensing domain has an important role in RPRFamide modulation of ASIC3. In conclusion we show that RPRFamide probably stabilizes the open state of ASIC3 by binding to the nonproton ligand sensing domain.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## PCA285

**Modulation of K<sup>+</sup> channel N-type inactivation by hydrogen sulfide and polysulfides**

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N-type inactivation of voltage-gated K<sup>+</sup> channels is essential for fine-tuning the electrical excitability of neurons. For some types of regulation, Cys residues in the N termini of  $\alpha$  subunits play prominent roles. For example, C13 in K<sub>v</sub>1.4 channels makes the inactivation susceptible to redox state changes [1] and heme binding [2]. Since the endogenously produced gaseous messenger hydrogen sulfide (H<sub>2</sub>S) affects action potential width and frequency [3], we investigated if and

how H<sub>2</sub>S affects N-type inactivation. Rat K<sub>v</sub>1.4 channels were expressed in HEK 293T cells, and currents were measured in the whole-cell patch-clamp configuration at 19–21°C. The H<sub>2</sub>S donor NaHS (1 mM) progressively removed inactivation within about 300 s. Even 200 µM NaHS was effective, and its potency increased with the NaHS solution aging. This suggested that polysulfides, which spontaneously form in NaHS solutions [4], were the active components. In fact, sodium polysulfides (Na<sub>2</sub>S<sub>n</sub>) were about 1000-fold more active than NaHS, with the potency increasing with the number of sulfur atoms; even 100 nM Na<sub>2</sub>S<sub>4</sub> efficiently slowed down inactivation. The reducing agent dithiothreitol (DTT, 1 mM) reversed this effect, suggesting Cys are targets. Consistently, K<sub>v</sub>1.4 mutant C13S was insensitive to 1 mM NaHS and 10 µM Na<sub>2</sub>S<sub>4</sub>. Mass spectrometry analysis of a peptide encompassing the first 61 residues of K<sub>v</sub>1.4, once exposed to NaHS and Na<sub>2</sub>S<sub>4</sub>, showed a mass increase corresponding to one sulfur atom whereas the C13S mutant peptide was unaffected.

Rat K<sub>v</sub>3.4, another mammalian N-type inactivating K<sup>+</sup> channel, exhibited faster inactivation (time constant at 50 mV, mean±SEM: 16.0±1.3 ms, n=8) than K<sub>v</sub>1.4 (84.4±2.8 ms, n=7). Exposure to 1 mM NaHS or 1 µM Na<sub>2</sub>S<sub>4</sub> resulted in a rapid loss of inactivation and a substantial increase of peak current, both of which were reversible with application of 1 mM DTT. Unlike K<sub>v</sub>1.4, the N terminus of K<sub>v</sub>3.4 harbors two Cys (C6, C24). Removal of both Cys was required to eliminate the channel's sensitivity to NaHS and Na<sub>2</sub>S<sub>4</sub>. In the presence of the reagents, inactivation of the single mutants C6S and C24S was slowed but not removed completely. The Cys-specific modifier DTNP (2 µM) and diamide-induced (10 µM) glutathionylation both removed inactivation in K<sub>v</sub>3.4 channels, and both Cys take part in the underlying mechanism. Marked slow-down of inactivation was observed in the whole-cell recording mode where the cytosol is exposed to the potentially oxidizing intracellular pipette solution. Cell-attached recordings after 10 min incubation of the cells with 10 µM Na<sub>2</sub>S<sub>4</sub> or 100 µM diamide yielded similar results. In summary, H<sub>2</sub>S and polysulfides can impair N-type inactivation of K<sub>v</sub>1.4 and K<sub>v</sub>3.4 channels by targeting Cys residues in the N-terminal ball-and-chain domain and are therefore potential modifiers of neuronal electrical excitability.

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PCA286

### Voltage-gated sodium channel activity in Huntington's Disease

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Huntington's Disease (HD) is a fatal neurodegenerative genetic disorder characterized by motor impairments, cognitive decline and psychiatric symptoms. Mutation of the *huntingtin* gene inducing a CAG triplet expansion with more than 36 repeats leads to a pathological phenotype, which in most cases exacerbates when the patient is in his/her 40s. Within the brain, the striatal Medium Spiny Neurons (MSNs)

progressively degenerate. Among other pathological features, MSN intracellular calcium ion levels are increased.

It was recently shown that the beta-4 subunit of the voltage-gated sodium channel (Nav) (encoded by the SCN4B gene) is down-regulated in the striatum of both human *post-mortem* tissue and rodent models of HD (Oyama *et al.*, 2006 ; Miyazaki *et al.*, 2014). Here, we aim to investigate the role of beta-4 in MSN excitability, in their degeneration and thus in the pathophysiology of HD.

HEK cells expressing either hNav1.3, hNav1.6 or rNav1.2 isoform were co-transfected with beta-4, to assess the impact of its presence or absence on sodium channel gating. Beta-4 induces a hyperpolarized shift in the voltage-dependence of activation of both hNav1.3 (-21,04 ± 1,84mV sham vs -27,27 ± 1,77mV with beta-4, p<0.05) and hNav1.6 (-26,73±1,82mV sham vs -30,26 ± 0,75mV with beta-4, p<0.05).

Increased intracellular calcium ion concentration shifted steady-state fast inactivation of Nav1.3 to more hyperpolarized potentials (-63,16 ± 1,19mV without Ca<sup>2+</sup> vs -71,51 ± 2,13mV with Ca<sup>2+</sup>, p<0.05).

To investigate HD-related changes in a more physiological system, we differentiated human induced-pluripotent stem cells (iPSCs) into MSN-like cells according to a published protocol (Stanslowsky *et al.*, 2016). Following 55±3 maturation days, neurons from one HD carrying 72 CAG repeats and two control iPSC lines were recorded using patch clamp, with and without an elevated intracellular calcium ion concentration, to study the electrical activity of Navs in these cells. Preliminary results suggest that Nav activity does not seem to be affected by the HD mutation at this time point. While heterologous expression shows effects of beta-4 expression on sodium channel gating, the iPSC derived MSN-like cells of HD patients may not be old enough yet to show a phenotype. Further adaptation of the differentiation protocol is therefore needed. On the meeting, an immunocytochemical characterization of the MSNs, their sodium channel electrophysiological properties in both voltage-clamp and current-clamp and marker expression will be presented.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCA287

### Characterization of P2X4- and P2X7-receptors in microglia

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Microglia cells are immune-effector cells of the CNS. They are involved in inflammatory processes and nociception. Under pathological conditions, ATP is secreted into the extracellular

space or released by damaged cells and activates purinergic receptors expressed on the microglia cells. Of the purinergic receptors, especially P2X4 and P2X7 receptors (P2X4R and P2X7R), ATP-activated cation channels, have been described to be involved in chronic nociceptive states such as neuropathic pain.

We have investigated the activation of P2X4R- and P2X7R-mediated currents in microglia BV-2 cells by means of the whole cell voltage clamp technique. P2X4Rs were activated at low ATP concentrations ([ATP] = 10  $\mu$ M) and are characterized by desensitizing currents. P2X7Rs were dominantly activated at high [ATP] (0.3 mM) or by Benzoyl-ATP and displayed a slowly increasing current component. The P2X4-receptor-blocker PSB 15417 inhibited the inactivating currents evoked by low [ATP] but was without significant effect at high [ATP]. In addition, the slow activating current component at high [ATP] was inhibited by PSB 15417, which might be explained by the existence of P2X4R/P2X7R heteromers or an additional blocking effect of PSB 15417 on P2X7R. A438079, a P2X7R blocker, especially inhibited the currents evoked at high [ATP]. But A438079 inhibited currents which were activated by low [ATP], too. This either suggests a P2X7R component evocable by low [ATP] or, again, the existence of P2X4R/P2X7R heteromers.

We found an increase of the P2X4R-, but not P2X7R-dependent ion currents after simultaneous incubation with interferon- $\gamma$  (IFN- $\gamma$ ) and lipopolysaccharide (LPS), whereas the exclusive incubation with IFN- $\gamma$ , LPS, dexamethasone or interleukine-4 (IL-4) did not affect the currents significantly. Furthermore, the duration of cultivation significantly influenced the P2X7- and even more the P2X4-receptor-expression, i.e. the ATP-induced currents increased with longer lasting cultivation.

In conclusion, the electrophysiological characteristics of ATP-induced currents did not indicate the existence of P2X4/P2X7 heteromers with a distinct phenotype different from P2X4 or P2X7 homotrimers. The pharmacological characteristics, however, leave open the formation of P2X4/P2X7 heteromers in microglia cells.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA288

### **$\beta$ 1 subunit stabilises sodium channel Nav1.7 against mechanical stress**

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Voltage-gated sodium channels (Navs) are key players in neuronal excitability and pain signaling. Precise gating of these channels is crucial as even small functional alterations can lead to pathological phenotypes such as pain or heart failure. Mechanical stress has been shown to affect sodium channel gating. This suggests that components which stabilize the channel are necessary to ensure precise channel gating in living organisms.

We used whole cell patch clamp of Nav1.7 heterologously expressed in HEK cells in presence and absence of the  $\beta$ 1 subunit to study the impact of the  $\beta$ 1 subunit on mechanical susceptibility of Nav1.7. All groups were measured in the presence and absence of mechanical stress applied by the flow of

extracellular solution on the patched cell via a gravity-driven perfusion system. We show that mechanical shear stress shifts voltage dependence of activation ( $\Delta V_{half}$  = 10.5 mV,  $p$  = 0.0004), and of fast inactivation ( $\Delta V_{half}$  = 6.3 mV,  $p$  = 0.04) to more depolarized potentials. Co-expression of the  $\beta$ 1 subunit, however, protects both voltage dependence of activation and fast inactivation of Nav1.7 against mechanical shear stress.

To study the underlying mechanisms enabling the  $\beta$ 1 subunit to mechanically stabilize Nav1.7, we used molecular dynamics simulations (MD), homology modelling and site-directed mutagenesis. MD simulations were carried out using GROMACS 2016 using the  $\beta$ 1 structure published by Yan et al. 2017. Simulations of WT  $\beta$ 1 and the C43A mutant revealed an intramolecular disulfide bond (Cys21-Cys43) to be crucial for tight binding of the subunit to the channel. Using a homology model of Nav1.7 based on the Nav1.4 $\beta$ 1 Complex (Yan et al. 2017), we show that  $\beta$ 1 binds to segment 6 of domain IV of Nav1.7. To investigate the functional impact of the mutation we generated a C43A  $\beta$ 1 subunit by site-directed mutagenesis. Whole cell patch clamp experiments with the C43A  $\beta$ 1 subunit and Nav1.7 show that the mutant prevents mechanical modulation of voltage dependence of activation, but not of fast inactivation.

Our data reveal a novel feature of  $\beta$  subunit-mediated ion channel modification: the  $\beta$ 1 subunit is able to influence the mechanical susceptibility of Nav1.7. Furthermore our patch data emphasize the unique role of segment 6 of domain IV for sodium channel fast inactivation and confirm previous reports that fast inactivation can be modified by interfering with the extracellular end of segment 6 of domain IV. Our study suggests that physiological gating of Nav1.7 may be protected against mechanical stress in a living organism by assembly with the  $\beta$ 1 subunit.

Yan Z, Zhou Q, Wang L, Wu J, Zhao Y, Huang G, Peng W, Shen H, Lei J & Yan N (2017). Structure of the Nav1.4- $\beta$ 1 complex from electric eel. *Cell* 170, 470–482.e11.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA289

### **Pituitary adenylate cyclase-activating polypeptide activates Na<sup>+</sup>-permeable cation channels in mouse and guinea-pig adrenal medullary cells**

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Pituitary adenylate cyclase-activating polypeptide (PACAP) acts on adrenal medullary (AM) cells as a neurotransmitter of the sympathetic nerve. We have elucidated that PACAP facilitates catecholamine secretion evoked by nAChR or mAChR stimulation and PACAP itself induces a depolarizing inward current in guinea-pig AM cells (Inoue et al., 2000). The ionic mechanism of PACAP-induced currents still remains unclear. Thus, the present experiments are aimed first to investigate the ion channels involved in the PACAP-sensitive current and second to explore whether or not the regulation of ion channels by PACAP is preserved in AM cells of guinea pigs and mice. After the animals were subjected to cervical dislocation, the adrenal gland was taken away and the adrenal cortex was removed from the adrenal medulla. After collagenase treatment, AM cells were mechanically isolated in a bath apparatus. The whole-cell current was recorded at 26–29°C with the



perforated patch clamp technique. Application of 3 nM PACAP and 1 mM muscarine produced inward currents in 6 and 8 of 9 guinea-pigs AM cells, respectively, whereas it induced an enhancement of the muscarinic currents in all the cells examined, respectively. The 1 mM muscarine-induced currents were enhanced by  $122 \pm 44\%$  ( $n = 9$ ) in the presence of 3 nM PACAP, and the extent of enhancement of the muscarinic current did not depend upon the amplitude of PACAP-induced currents. In contrast to guinea pigs, almost all the mouse AM cells (14/15) developed inward currents in response to 3 nM PACAP, whereas only 30% of the mouse cells exhibited inward currents in response to 30 mM muscarine. In guinea-pig and mouse AM cells, 1 mM muscarine-induced currents, which are due to activation of nonselective cation (NS) channels (Inoue et al., 2012), were suppressed by quinine in a dose-dependent manner with an  $IC_{50}$  of 20 mM, whereas PACAP-induced inward currents were not noticeably suppressed by 100 mM quinine. The PACAP current was completely suppressed by the replacement of external  $Na^+$  with N-methyl D-glucamine. Addition of 100 mM  $Zn^{2+}$  to the external solution resulted in the inhibition of PACAP currents by 57%. Similar extents of suppression were noted with a decrease in external pH to 6.4. The present results reveal that PACAP activates  $Na^+$ -permeable cation channels, which are not affected by quinine. The PACAP-regulated cation channel is suppressed by external  $Zn^{2+}$  or a decrease in external pH. These properties of the ion channels are reminiscent of those of TRPM4 or TRPM5. We conclude that PACAP activates  $Na^+$ -permeable cation channels in guinea-pig and mouse AM cells, which differ from NS channels coupled with mAChRs.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCA290

##### **Sphingosine-1-phosphate-induced ATP secretion in microglia is mediated by LRRC8 proteins of volume-regulated anion channels**

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Microglia cells are versatile players coordinating inflammatory and regenerative processes in the central nervous system. We investigated the activation of ion currents and ATP secretion by sphingosine-1-phosphate (S1P) and hypoosmolarity in microglia BV-2 cells. S1P and extracellular hypoosmolar solution evoked an anion conductance with almost linearly time-dependent increasing amplitude. The currents were inhibited by intracellular hypoosmolar solution and by use of the anion channel antagonists NPPB and tamoxifen pointing to the activation of volume-regulated-anion channels (VRAC). The analysis by qPCR indicated the expression of S1P-receptor-subtypes 1, 2, 4 and 5 in the BV-2 cells. The use of the S1PR1-antagonist W123 and the incubation with

pertussis-toxin prevented the S1P-currents showing the involvement of the  $G_i$ -protein-coupled S1PR1 in the activation of VRAC by S1P. According to the progress in the molecular identification of VRAC-channels, we found a high inhibition of S1P- and hypoosmolarity-induced VRAC-currents after LRRC8A-knockdown whereas knockdown of subtypes B-D did not significantly influence the currents. Subtype E was not present in BV-2 cells as revealed by qPCR. Furthermore, S1P and hypoosmolar buffer induced an increase of ATP-levels in the supernatants of BV-2 cells being sensitive to treatment with NPPB, tamoxifen and W123. This indicates that in microglia cells sphingosine-1-phosphate or hypoosmolarity mediate an ATP-secretion via volume-dependent anion channel LRRC8 proteins.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCA291

##### **The roles of TRPV1, TRPA1 and TRPM8 channels in chemical and thermal sensitivity of the mouse oral mucosa**

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##### Motivation:

Spices in food and beverages and compounds in tobacco smoke are supposed to interact with sensory irritant receptors of the transient receptor potential (TRP) cation channel family. TRPV1, TRPA1 and TRPM8 not only elicit action potential signaling through trigeminal nerves, eventually evoking pungent or cooling sensations, but by their calcium conductance they also stimulate the release of calcitonin gene-related peptide (CGRP).

##### Methods:

The immunocytochemical buccal mucosa preparations were evaluated using a confocal laser scanning microscope. CGRP release was measured *ex vivo* using EIA kits (Bertin Pharma, Montigny-le-Bretonneux, France) as an index of neuronal activation to elucidate the chemo- and thermosensory transduction in the isolated superfused mouse buccal mucosa of wildtypes and pertinent knockouts (1).

##### Results:

The lipophilic capsaicin, mustard oil and menthol effectively get access to the nerve endings below the multi-layered squamous epithelium, while cigarette smoke and its gaseous phase were weakly effective releasing CGRP. The hydrophilic nicotine 20mM pH7.4 was ineffective unless applied unprotonated in alkaline (pH9) solution ( $29 \pm 2.4$  pg/mL,  $P=0.0001$ ,  $n=12$ ; ANOVA, LSD test).  $TRPA1^{-/-}$ ,  $TRPV1^{-/-}$  and double knockouts  $TRPA1/TRPV1^{-/-}$  showed a reduction of this nicotine response to a similar degree in any of the three mutant strains ( $F_{3,34}=15.218$ ,  $P<0.001$ ). In WT a bimodal concentration-response relationship was found for AITC with a threshold between 30-100  $\mu$ M, increasing response up to 300  $\mu$ M and much greater CGRP release at 1-10mM ( $F_{4,19}=84.756$ ,  $P<0.001$ ). Also AITC 1mM activated both irritant receptors, all knockout strains showed response deficits, largest the double knockouts ( $F_{3,19}=58.09$ ;  $P<0.001$ ), but only  $TRPA1^{-/-}$  did not respond to 100  $\mu$ M. AITC in combination (1mM) with heat (45°C) showed supraadditive, heat-sensitizing, effects even in double knockouts, suggesting action on an unknown

heat-activated channel and mustard oil receptor. Menthol caused little CGRP release by itself, but in subliminal concentration (2mM) it enabled a robust cold response that was absent in TRPM8<sup>-/-</sup> (P<0.0003, n=4,6) and strongly reduced by TRPM8 inhibitors (P=0.03, n=4,6) but retained in TRPA1<sup>-/-</sup>. Conclusions:

Our results from the oral mucosa show that lipophilic irritants (capsaicin, mustard oil and menthol) contained in foodstuff and drinks easily gain access to trigeminal sensory nerves to activate TRPV1, TRPA1 and TRPM8, respectively, which sensitize to heat or cold, and mediate the release of vasodilatory CGRP. The hydrophilic nicotine requires a strongly alkaline condition to access and weakly activate TRPA1 and TRPV1, while full cigarette smoke (with nicotine) and its filtered gas phase (without nicotine) exert moderate and about equal effects likely through TRPA1.

Kichko TI *et al.* (2018). *Eur J Neurosci* 47, 201-210

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA292

### Neuropathic pain associated with sodium channel Nav1.7 variant R1150W

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Neuropathic pain may be linked to mutations in the voltage gated sodium channel (Nav) subtype Nav1.7 encoded by the SCN9A gene. Interestingly, 13% of the Caucasian population harbor the Nav1.7/R1150W polymorphism, which may render the carrier more prone to develop chronic pain. The impact of this variant on nociceptor excitability is still unclear and only a fraction of carriers with p.R1150W develops severe pain symptoms during their lifetime, such as small fiber neuropathy (SFN). The onset of SFN can be correlated with a reduction of intraepidermal small nerve fibers in subgroups of SFN patients (Cazzato *et al.*, 2017), which seems contra intuitive to the chronic pain syndrome these patients suffer from. The specific genetic background of the expressing cells may play a crucial role and needs further investigation.

Here, we investigated human stem cell derived nociceptors of a SFN patient carrying the Nav1.7/R1150W polymorphism. Skin biopsies showed a decrease of intraepidermal nerve fiber density and an elongation of the dermal nodes of Ranvier. We reprogrammed the patient's fibroblasts carrying Nav1.7/R1150W into induced pluripotent stem cells (iPS cells) and differentiated them into nociceptors using an optimized protocol (Eberhardt *et al.*, 2015 based on Chambers *et al.*, 2012). To investigate the pathophysiological relevance of the patient-specific genetic background, we performed calcium-imaging, patch-clamp analysis and multi-electrode recordings to assess the impact of the genetic variant on cellular excitability of the human nociceptors. With these experiments, we aim to study the pathophysiological basis of this

pain syndrome. Individual *in vitro* testing for the efficacy of analgesic might help to translate these findings back to the patient offering the potential for personalized therapy.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA293

### rArc - Ratiometric genetically-encoded voltage indicators

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The resting membrane potential and membrane potential changes are key to neuronal excitability and many other physiological processes such as cell-cycle progression and transmembrane transport. Thus, measurement of the membrane potential is essential for understanding cellular physiology. All-optical electrophysiology, using light to stimulate and record membrane potential changes, emerges as a new research field. Genetically-encoded fluorescent voltage indicators (GEVIs) are membrane proteins that change their fluorescence upon alteration of the electrical membrane potential. They are less invasive than patch clamp and supposedly less toxic than voltage-sensitive dyes. Currently available GEVIs, however, only provide a relative change in fluorescence as output, i. e., they do not report the absolute membrane potential.

Here we introduce novel ratiometric GEVIs (rArc) based on the membrane-delimited green fluorescent ArcLightQ239, which changes its fluorescence intensity upon membrane potential variation (1). To calibrate the green voltage-sensitive signal of ArcLightQ239, we fused variants of red fluorescent proteins (derivatives of mCherry and mKate2) to the intracellular N terminus of ArcLightQ239. Alternatively, extracellular localization of the red fluorescent protein was achieved via an additional transmembrane helix. While the green signal diminished by about  $44.0 \pm 1.4\%$  (mean  $\pm$  S.E.M.) upon depolarisation from -120 to +60 mV (n = 20, for the mCherry variant rArc2C), the red fluorescence of all rArc variants remained constant in the same voltage range. The kinetics of the green fluorescence change of all rArc variants following a depolarization from -140 mV to 20 mV were similar to that of ArcLightQ239 (double-exponential fit:  $\tau_{\text{au1}} = 20 \pm 2$  ms,  $\tau_{\text{au2}} = 173 \pm 15$  ms,  $\text{Amp1/Amp2} = 0.83 \pm 0.18$ , 23 °C, n = 6). 24 h after transfection in HEK293T cells, red and green fluorescence signals were colocalized. As observed for other red fluorescent proteins (2), intracellular red spots occurred after 48 h leading to a dysbalance of red and green signals. We therefore ranked rArc variants according to the homogeneity of red/green distribution

in cells and the best fluorescence ratio reproducibility when assayed under voltage-clamp conditions.

While the rArc variants, like ArcLightQ239 itself, are too slow for resolving fast action potentials, they promise great potential in characterizing the membrane potentials of resting cells. For example, rArc2C-based fluorescence ratio ( $F_{\text{green}}/F_{\text{red}}$ ) in HEK293T cells significantly (U test,  $p < 0.001$ ) increased from  $1.42 \pm 0.03$  ( $n = 173$ ) to  $1.90 \pm 0.06$  ( $n = 114$ ) when the inwardly rectifying and, hence, hyperpolarizing  $K^+$  channel mKir2.1 (3) was coexpressed. rArc variants will be examined for automated fluorescence recordings and for high-throughput applications such as fluorescence-activated cell sorting (FACS) and fluorescence imaging plate reader (FLIPR) (4) assays.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCA294

##### Implications of maternal obesity on offspring orexigenic neurons development

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A set of evidences supports the hypothesis that adverse *in utero* and early postnatal environments have a significant influence on risk of a myriad of prevalent metabolic diseases. So far, a small number of studies have addressed the role of maternal obesity on the hypothalamic structure/activity of the offspring both short and long terms. The hypothalamus responds to nutrients and hormones as well as integrates the resulting signals in order to balance caloric intake and energy expenditure, which affects whole body energy homeostasis. This study aimed to investigate if maternal obesity could impact on the development of hypothalamic neurons, generating long term abnormalities, and to elucidate potential underlying mechanisms. Here, we used a mouse model of maternal diet-induced obesity. Female C57Bl/6J mice were fed either a control ( $n=9$ ) or a high fat ( $n=6$ ) diet from three weeks prior to mating and during pregnancy and lactation. Male offspring born from control ( $n=6$  for i and ii) and from obese dams ( $n=6$  for i and ii) were evaluated at different time points: i, immediately after weaning; and ii, at 8 weeks of age (both litters weaned onto a balanced Chow diet). Obese dams presented increased body weight during pregnancy (Obese:  $26.53 \pm 1.21$  vs  $22.5456 \pm 0.51$ ,  $p < 0.01$ ) as well as in white (normalized by BW, Obese:  $1.45 \pm 0.24$  vs  $0.47 \pm 0.02$ ,  $p < 0.01$ ) and brown (normalized by BW, Obese:  $0.41 \pm 0.03$  vs  $0.31 \pm 0.006$ ,  $p < 0.05$ ) adipose tissue in the end of lactation. Additionally, obese dams presented hyperglycemia (mg/dL, Obese:  $153.83 \pm 4.21$  vs  $128.11 \pm 8.11$ ,  $p < 0.01$ ) and hyperinsulinemia (ng/mL,

Obese:  $1.47 \pm 0.08$  vs  $0.87 \pm 0.12$ ,  $p < 0.01$ ) at weaning. Male offspring born from obese dams presented decreased body weight at birth (g, Obese offs:  $1.185 \pm 0.03$  vs  $1.399 \pm 0.04$ ,  $p < 0.01$ ). Immunostaining showed that male offspring from obese dams presented increased astrocyte population (qualitative data) in the arcuate nucleus at weaning. In parallel, 8 weeks siblings born from obese dams presented increased gene expression of *Tnf-alpha* ( $p < 0.01$ ), *Interleukin-6* ( $p < 0.05$ ) and the orexigenic neurons, *NPY* ( $p < 0.05$ ) and *AgRP* ( $p < 0.05$ ), in hypothalamus. All values are presented as mean  $\pm$  S.E.M., compared by Student's t-test. The set of results revealed an impaired development of hypothalamic neural circuits modulated by maternal obesity. Our findings suggest that adult offspring from obese dams presented increased levels of important orexigenic and pro-inflammatory components in hypothalamus. Apparently, such outcomes are preceded by an increase in astrocyte population, which control inflammation and neuronal stress, at very premature stage for these animals. As hypothalamus provides many physiological roles involved in feeding and metabolism, the enhanced stress response short term may be favouring the upregulation of *NPY* and *AgRP* neurons population long term.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCA295

##### Common denominators of Hypoxia-inducible Factor activation in brain to regenerate from ischemia/reperfusion

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Ischemic hypoxia results from insufficient blood flow to supply adequate amounts of oxygen and nutrients and causes ATP depletion and rapid cell death. It was long thought that the mammalian brain is a post-mitotic organ and incapable of regeneration following cellular loss, which underpins the devastating impact of cerebral ischemia. Recently, it has become clear that the brain is capable of modest regeneration. Moreover, it was shown that hypoxia and the hypoxia-inducible factor (HIF) are key factors in neural regeneration<sup>1</sup>. Endogenous sensors of hypoxia and their downstream effectors are prime targets for therapeutic manipulation. Key among these targets is the transcription activator HIF, an oxygen-responsive member of the basic helix-loop-helix PAS (PER-ARNT-SIM) family<sup>2</sup>. Especially HIF-2 $\alpha$  is distributed tissue specific and expressed in the developing brain. It modulates gene activity in response to low oxygen and protects neural progenitor cells and neural differentiation processes<sup>3,4</sup>. But in general, the role of HIF-2 $\alpha$  during neural development is not well understood.

To investigate the impact of HIF on neural regeneration, we established a murine neurosphere culture for wildtypic (Wt) and *Hif-2 $\alpha$* -knockout (KO) cells. Neurospheres are heterogeneous cell clusters based on neural stem cells (NSCs). Using this 3D model we were able to mimic basic processes of brain development: neural progenitor cell proliferation, migration, differentiation and apoptosis.

Here, we focused on the signaling pathway of HIF by challenging the proliferating and differentiating neurospheres with up to 4h oxygen/glucose-deprivation (OGD: 0.2% O<sub>2</sub>; glucose free medium). Afterwards, we analyzed the migration



capability by measuring the migration distance of the cells after 24h, 48h and 72h. Before OGD, Wt cells had significantly better abilities to migrate than to the KO. After OGD, neurospheres migrated significantly less than the control and did not differ between Wt and KO ( $n = 3$ ).

Additionally mRNA analysis showed a strong effect on the expression of genes involved in neurogenesis. Here, genes like *Nrg1*, which has a protective function against astrogliosis, or *Grin1*, which is critical for neuronal connectivity and survival, significantly differ between Wt and KO cells after challenging the spheres with OGD ( $n = 5$ ).

These data suggest a restricted capability to regenerate from ischemia without HIF-2 $\alpha$  and decode its role in (re)modeling the CNS.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

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PCA296

### Kainate receptors and neuronal network synchrony in hippocampus

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Neonatal hippocampus in rodents displays synchronous activity, which is essential for the maturation of neuronal network. Kainate type glutamate receptors (KARs) modulate synaptic transmission and neuronal excitability depending on their subunit composition and localization. The expression of different KAR subunits changes through the development. Here we used multichannel recordings to study the role of KAR subunit GluK1 in the spatio-temporal profile of network activity in cultured hippocampal slices from newborn rats. Hippocampal slices were extracted from neonatal rats (P6) and cultured on Multielectrode array probes (MED64; 8x8 50 x 50  $\mu$ m electrodes). Expression of GluK1 in CA3 pyramidal neurons was modified using lentiviral vectors encoding for EGFP or GluK1c together with EGFP. Recordings were acquired by MED64 system. The spatial and temporal profile of recorded activity was analysed using Spike 2 (CED) and custom made Matlab scripts.

Cultured rat hippocampal slices expressing only EGFP ( $n=11$ ) spontaneously generated hypersynchronous patterns with interleaved latent periods after 10 days in culture (DIV10). GluK1c overexpression (OE) in CA3 pyramidal neurons did not alter the duration or spike frequency of the hypersynchronous patterns. However, latent periods were significantly longer ( $p(0,05$ , Student's t-test) in GluK1c OE networks ( $n=8$ ). Hypersynchronous patterns were preceded by short periodic population bursts (duration/100 ms, average incidence 1/s). The

incidence of periodic bursts increased towards the onset of hypersynchronous patterns in control slices. Networks with GluK1c OE presented fewer periodic bursts ( $p(0,001$ , Student's t-test) and the increasing incidence was not observed.

The periodic bursts in latent periods originated and spread over the whole hippocampal network at DIV10 in a recurrent fashion, associated with depolarization from CA1 stratum radiatum propagating to the CA1-CA3 pyramidal cell layer. In GluK1c OE networks, the origin of depolarization was similar, but the propagation was observed only to CA3 pyramidal layer. Cross correlation analyses from periodic burst waveforms revealed that in control slices, coefficients were positive throughout CA1-CA3 pyramidal layer and negative in dendritic layer. With GluK1c OE correlations had more centred positive foci in CA3 pyramidal layer with widely spreading negative coefficients in stratum radiatum.

In summary, the synchrony of neonatal hippocampal network was altered by local modulation of KAR expression, supporting the notion that these receptors might play essential role in the functional integration of neurons in developmental hippocampal circuitries.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

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PCA297

### Quantification of information transfer via gonadotropin-releasing hormone receptors (GnRHR) reveals a marked loss of information through signalling

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Gonadotropin-releasing hormone (GnRH) is a hypothalamic neuropeptide that acts via GnRHR on the pituitary gonadotrope. It is secreted in pulses and acts via GnRHR to activate ERK and Nuclear Factor of Activated T-cells (NFAT). Both of which mediate GnRH effects on the synthesis of reproductive hormones. Using automated fluorescence microscopy, effects of GnRH on cell signalling in individual cells can be quantified revealing marked cell-cell heterogeneity. Information

theoretic approaches, which consider cell-cell heterogeneity and its impact on information transfer, can be used to quantify information transfer via cell signalling pathways. Here we use the mutual information (MI) between GnRH concentration and measured responses ( $I(\text{response}; \text{GnRH})$ ) as a measure of information transfer via GnRHR. MI is measured in Bits, with an MI of 1 Bit indicating a system that can unambiguously distinguish between two inputs. Initially, we did this in fixed LbT2 cells (gonadotrope-derived cells). The cells were stimulated with various concentrations of GnRH ( $0\text{--}10^{-6}\text{M}$ ) before staining for ppERK (immunohistochemistry), and in some experiments, cells were infected with recombinant adenovirus for expression of NFAT-EFP. As  $\text{Ca}^{2+}$  causes NFAT to translocate to the nucleus, the nuclear fraction (NF) provides a readout for elevation of the cytoplasmic  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) by GnRH. Our data, therefore, suggest that most information from the environment is lost through signalling. The  $I(\text{ppERK}; \text{GnRH})$  and  $I(\text{NFAT-NF}; \text{GnRH})$  values were always  $<1\text{Bit}$  despite  $3\text{Bit}$  input. Joint sensing of ppERK and NFAT-NF increased slightly MI values [1,2], suggesting that, by ignoring response dynamics, information transfer is underestimated. Therefore, using live cell measurements and MI calculations taking response trajectory into account, NFAT-EFP translocation was tracked in response to a single pulse of GnRH. The  $I(\text{NFAT-NF}; \text{GnRH})$  values were  $\sim 0.5\text{ Bit}$  at 30 min and remained elevated for 60 min. Taking response trajectories into account increased MI values to  $\sim 0.65\text{ Bit}$ . We also tracked NFAT-EFP translocation responses in cells receiving two pulses of GnRH and found that the information gain from the second pulse was small. Similar experiments were performed using Fluo-4 measurements of  $[\text{Ca}^{2+}]_i$ . The  $I(\text{Ca}^{2+}; \text{GnRH})$  values were  $\sim 0.8\text{ Bit}$ , 24sec after stimulation, reducing to  $\sim 0.66\text{ Bit}$  after 2 min. These values were increased to  $1\text{Bit}$  by consideration of trajectories. Thus, LbT2 cells are unreliable sensors of GnRH concentration because a considerable amount of information is lost through signalling. Although joint sensing, trajectory sensing and sensing repeated pulses increased information transfer, this was typically  $<20\%$  suggesting that most information is lost early in the GnRH signalling cascade, prior to  $\text{Ca}^{2+}$  mobilisation.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

### Selection of effective reference genes for expression studies: RFRP-3 and GPR147 signaling in rat hippocampus

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Real time-polymerase chain reaction (RT-PCR) is widely used for investigation of gene function and interactions at mRNA level in part of functional genomics studies. Numerous factors including sample to PCR profile cause variation and misevaluation of expression levels in quantitative RT-PCR (qPCR) analyses. Housekeeping genes (HKG), that are assumed to be constantly expressed in all cells and tissues, can be thereby used for as an internal control and normalization of variations between samples and analyses. Although several HKGs were available for this purpose, recent research indicated that expressions of some HKGs are biologically affected from various experimental applications, which may cause wrong normalization and evaluation of results. Objective of this study was to select appropriate HKGs to be used in the normalization of gene expression of Rfamide related peptide-3 (RFRP-3) and its receptor (GPR147) signaling on anxiety like behaviors and neurogenesis in rat hippocampus. Osmotic brain minipumps were implanted to 24 rats (male, 300-350 g) which were divided into four groups including control (n=6), RFRP-3 (1 nMol/day, n=6), RF9 (RFRP-3 antagonist; 10 nMol/day, n=6) and RFRP-3 + RF9 (n=6). Drug infusions into lateral ventricles were performed for 15 days. Total RNA isolation and cDNA synthesis were performed from hippocampus tissues. Expressions of 9 HKGs (GAPDH, SDHA, RPL13A, PGK1, HPRT1, 18SrRNA, ACTB, YWHAZ and CycA) at mRNA levels were evaluated using qPCR. Average expression stability values (M) were separately calculated for each groups using BestKeeper, geNorm and NormFinder software. Both results of geNorm and Normfinder algorithms indicated that PGK1, YWHAZ and GAPDH were the most stable genes, respectively. As a conclusion, PGK1 and YWHAZ seems to be the most suitable reference genes for studying RFRP-3 and GPR147 signaling on anxiety like behaviors and neurogenesis in rat hippocampus.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCA299

### Inhibition of mitochondrial fission in the Dorsal Vagal Complex of the Brain prevents Hyperphagia in High Fat Diet Fed Rats

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Worldwide obesity has more than doubled since 1980, with over 600 million cases in 2014. Obesity can lead to many adverse metabolic effects of the cardiovascular and endocrine systems and in the brain. In rodents, the dorsal vagal complex (DVC) of the brain regulates glucose homeostasis and controls food intake through insulin signalling. A 3-day high fat diet (HFD) has shown to induce insulin resistance thus affecting the DVC's ability to regulate glucose metabolism and food intake, though exact mechanistic effects of this are still unknown. HFD feeding is associated with an increase in mitochondrial fission in the DVC. Mitochondrial fission is regulated by dynamin related protein 1 (Drp1), and an increase in Drp1 activity in the DVC has shown to inhibit the insulin signalling pathway. Adenoviruses expressing a constitutively active form of Drp1 (S637A), a dominant negative form of Drp1 (K38A) and a GFP expressing control were injected into the nucleus of the solitary tract (NTS) of the DVC. Our data has shown, rats expressing Drp1-S637A in the DVC, do not decrease food intake in response to an acute insulin treatment, compared to the GFP controls. Confirming that increases in mitochondrial fission results in impaired insulin sensitivity. Furthermore, we found that after 14 days, Drp1-S637A expressing rats were hyperphagic and had an overall increase in body weight and fat accumulation. Following this, we determined if inhibition of Drp1 can restore insulin sensitivity in HFD fed (insulin resistant) rats. Acute insulin treatment, decreased food intake in the HFD fed Drp1-K38A expressing rats, but not in the HFD fed GFP expressing rats, confirming that inhibition of Drp1 can restore insulin sensitivity. In addition, chronic inhibition of Drp1, resulted in a decrease in food intake, body weight and fat accumulation.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA300

### The effect of muscle-specificity and muscle length on the modulation of corticospinal excitability during passive ankle movement in humans

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Modulation in corticospinal excitability has been shown in passive movement of the upper limb, but data on lower limb muscles is scarce. Whilst differences in neuronal behaviour between upper and lower limb muscles have been established, differences also exist between the muscles of the lower limb. For example, neuronal behaviour of the antagonist about the ankle such as soleus (SOL) and tibialis anterior (TA) might differ due to differences in afferent input (1,2) and distribution of corticomotoneuronal projections (3,4). Additionally,

the neuronal behaviour between static position and passive shortening and lengthening may also depend on the muscle length at the point of stimulation (5). Thus, the purpose of this study was to assess corticospinal excitability during static positioning and passive plantar and dorsiflexion in SOL and TA, respectively, with stimulation delivered at short, intermediate and long muscle lengths.

Healthy, young volunteers ( $n = 12$ ; 6 females) were recruited. All testing was performed on the right limb with the subject seated in an isokinetic dynamometer. During passive shortening and lengthening, the ankle was moved through a range-of-motion (ROM) of  $20^\circ$  ( $10^\circ$  of plantarflexion and dorsiflexion;  $0^\circ =$  ankle joint at  $90^\circ$ ) at  $5^\circ \times s^{-1}$ . Transcranial magnetic stimuli (TMS) were delivered with the ankle in a static position and during passive movement at  $0^\circ$ ,  $7.5^\circ$  plantarflexion (long and short muscle length for TA and SOL, respectively) and  $7.5^\circ$  dorsiflexion (short and long muscle length for TA and SOL, respectively) to elicit motor evoked potentials (MEPs). In addition, maximal muscle responses ( $M_{max}$ ) were elicited via supramaximal stimulation of the peripheral nerve in each of the three static positions and were used to normalise MEPs. TMS stimulus intensity was set to  $1.2 \times$  resting motor threshold (separately for TA and SOL) and responses were recorded separately for each muscle.

$MEP/M_{max}$  was not modulated between static positioning, passive lengthening or shortening in SOL ( $0.02 \pm 0.003$  for all;  $p = 0.121$ ). In TA,  $MEP/M_{max}$  was facilitated during passive shortening ( $0.17 \pm 0.02$ ) compared to static positioning ( $0.10 \pm 0.02$ ;  $p = 0.023$ ) and passive lengthening ( $0.09 \pm 0.02$ ;  $p < 0.001$ ). Muscle length at the point of stimulation had no effect on  $MEP/M_{max}$  in TA ( $p = 0.787$ ). These results suggest a differential intrinsic modulation of corticospinal excitability between TA and SOL during passive movement, which is possibly mediated by differences in feedback from spindle receptors causing changes at the supraspinal level, and/or dissimilar, non-uniform distribution of direct corticomotoneuronal projections whereby TA is more susceptible to facilitation (3,4). Alternatively, the fascicle length changes across the ROM might differ for SOL and TA, resulting in different quantity of afferent input.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.



## PCA301

### Exploring the validity of lumbar stimulation for the assessment of lower limb motoneuron excitability in humans

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Mastoid or higher thoracic spinous process electrical stimulation is typically used to assess descending pathway excitability of motoneurons, but is accompanied with high discomfort levels when targeting lower limbs (1). Modelling studies have suggested that electrical stimulation at the lumbar spinous process level (LS) would activate the lateral spinal cord white matter of the L<sub>1</sub> to S<sub>1</sub> spinal segments (2, 3). Thus, the purpose of this study was to assess whether LS activated the same descending pathways to lower limbs as transcranial magnetic stimulation (TMS).

Motor and lumbar evoked potentials (MEPs and LEPs) were recorded in the rectus femoris (RF) and tibialis anterior (TA) of six healthy participants (age: 27 ± 3 years). For LS, cathode and anode electrodes were placed over the L<sub>1</sub> and T<sub>8</sub> spinous processes, respectively. MEP and LEP amplitudes were matched at 10-15% M<sub>max</sub> at rest, with 10 sets of 18 single and paired stimuli delivered at ISIs ranging from -16 ms (TMS before LS) to 14 ms (LS before TMS). The LEP response was graphically subtracted from the paired response at each ISI. The amplitude of the resultant trace was then expressed as a ratio compared to single pulse MEP amplitude, with values of <1 and >1 indicating occlusion and facilitation, respectively (4). Effects of neural drive on MEPs and LEPs were investigated during 90° joint angle isometric contractions at 10, 25, 50, 75, and 100% of maximum voluntary contraction (MVC). For this part of the study, stimulus intensities were set so that MEP and LEP amplitudes matched at 50% M<sub>max</sub> during a 50% MVC contraction. Five respective stimuli were averaged at each contraction intensity.

Stimulation intensity to elicit resting LEPs was 167±63 and 188±95 mA, and TMS intensity was 54±18 and 68±20% for TA and RF, respectively. Significant occlusion was observed for most ISIs ≥ -8 ms ( $p \leq 0.035$ ; Figure 1). For TA, significant facilitation was observed at -14 ms ( $p = 0.039$ ), and for RF at -16 and -14 ms ISIs ( $p \leq 0.048$ ). To elicit a response amplitude of 50% M<sub>max</sub> during a 50% MVC contraction, the LS intensity was 198 ± 31 and 146 ± 61 mA and TMS was 42 ± 11 and 56 ± 24% for TA and RF, respectively. LEP and MEP amplitudes increased with contraction intensity in both muscles ( $p \leq 0.031$ ), with a plateau in responses at 50 and 75% MVC for RF and TA, respectively.

These results suggest that LS was successful in activating the same corticospinal axons as TMS in TA and RF, and is consistent with upper and lower limb data for mastoid stimulation (4, 5). Also, LS intensities are ~70% lower than those reported for eliciting the same amplitude response from mastoid stimulation (5). Thus, LS could be a useful tool for assessing motoneuron pool excitability in the lower limbs at rest and during contraction.

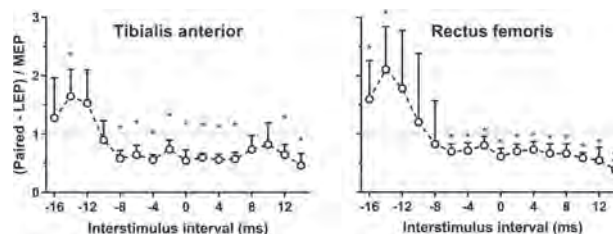


Figure 1. Assessment of occlusion and facilitation in TA and RF. \* indicates significant difference from single-pulse MEP values ( $p \leq 0.05$ ).

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA302

### Effect of cigarette smoking on the nerve conduction study among young adults

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**Introduction:** The effects of cigarette smoking on many organs is well-known. It is recognized as a risk factor for many diseases such as hypertension, ischemic heart diseases, and cancer. However, the effect of cigarette smoking on central and peripheral nervous system is still unclear. The aim of this study was to evaluate the effect of smoking on the nerve conduction study of the median nerve (motor part).

**Methodology:** This study was descriptive institutional based case-control study carried out at AL-Neelain University Faculty of Medicine during 2017. Thirty-one healthy smokers were volunteered to participate and 34 healthy non-smokers were matched and taken as a control. The data were collected using a self-administered questionnaire and the nerve conduction study was measured using power lab serial number G607. The median nerve was stimulated supra-maximally at two points along its course at the wrist and antecubital fossa. Nerve conduction velocity, amplitude, proximal latency and distal latencies were recorded.

The data was analyzed using (SPSS) Version 22 and graph pad prism 7. Comparison of means of continuous data between smokers and non-smokers were tested by independent Student t-test. P-value of <0.05 (two-tailed) was used to establish statistical significance

Results: The result revealed that healthy smokers had a lower amplitude of nerve conduction study compare to healthy non-smokers ( $3.1 \pm 0.08$  and  $4.9 \pm 1.50$  respectively,  $P$  value = 0.000). There was no statistically significant difference in the nerve conduction velocity of smokers and non-smokers ( $P$ -value 0.07).

Also, there was no statistically significant difference in the proximal and distal latencies between smokers and non-smokers ( $P$  value more than 0.05 for both).

Conclusion: Early exposure to cigarette smoking attenuates the amplitude of the median nerve. The changes induced by smoking were not related to the duration of smoking and the number of smoked cigarettes per day.

Keywords: Smoking, Nerve conduction study, Median nerve  
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*-All my thanks to Allah firstly*

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Dr. Humeda suketi Humeda

And

Dr. Lamis Kaddam

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCA303

### **Study of the effects of the intracerebroventricular and the intrathecal administration of kaempferol and GABA<sub>A</sub> antagonists on pain in male rats**

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Kaempferol, a type of flavonoid, has antinociceptive activity. The purpose of this research was to evaluate the interaction between the analgesic effects of kaempferol and GABA<sub>A</sub> receptor in central nervous system in male rat. In this study, we used 220 Wistar male rats. The animals were divided into 22 groups for the intracerebroventricular/ the intrathecal

administration. The groups were for each of the two types of administrations: control, DMSO, sham, bicuculline (1 µg), morphine (10 µg), kaempferol (0.5, 1 and 1.5 mg), bicuculline plus kaempferol (0.5, 1 and 1.5 mg). Subjects were anesthetized by Xylazine (10 mg/kg) and Ketamine (100 mg/kg) and placed in the stereotaxic apparatus (Stoelting, USA) for surgery. Pain assessment test was tail flick. All research and animal care procedures were approved by the Veterinary Ethics Committee of the Hamadan University of Medical Science and were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1985). Totally, in the tail flick test the dosages of 0.5, 1, 1.5 mg of Kaempferol and Bicuculline plus a dosage of 1 mg of Kaempferol showed noteworthy analgesic effects ( $p < 0.001$ ). In the intracerebroventricular injection, pain was significantly decreased with the administration of Bicuculline plus the dosages of 0.5 and 1.5 mg of Kaempferol ( $p < 0.01$ ). However, in the intrathecal administration of Bicuculline plus the dosage of 0.5 mg of Kaempferol attenuated pain of thermal in comparison with groups of control, DMSO, sham, and bicuculline ( $p < 0.01$ ). The results of this examination showed that Kaempferol has analgesic effects. The modulation of GABAergic system by GABA<sub>A</sub> receptor antagonist could be involved in this antinociceptive effects.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCA304

### **Does Colour alter performance on Letter Cancellation tasks?**

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Cognitive function broadly refers to various domains, including memory, language, executive function, processing speed and visuospatial ability. Letter Cancellation Tasks (LCT), a group of cognitive performance tasks are reliable measures of concentration, visual scanning and executive function and useful test for assessment of dementia. Colour is a modality that affects attention, perception and response activation. Ability to discriminate colour has been reported to be altered in Alzheimer's Disease (1). Thus, we hypothesize that addition of colour in LCT could help in detection of cognitive changes in disease like Alzheimer's. As a prelude to the above hypothesis the present study was done to evaluate the effect of colour on performance of LCT in normal individuals.

#### **Materials and Methods:**

The present study was conducted after approval of Institutional Ethics Committee. 22 healthy male subjects in the age group of 18-40 years were recruited for the study after taking written-informed consent.

The subjects with history of visual defects like colour blindness, neuropsychological illness, stroke, or serious mental illness such as depression or schizophrenia; history of drug intake like alcohol, cocaine, heroin etc. and less than 5 years of schooling in English were excluded from the study.

Subjects were asked to cancel out letters in four different colours i.e. Black, Blue, Green and Red versions of letter cancellation tasks in English language administered randomly. Letter cancellation involved One, Two and Three Letter cancellations. Each LCT consisted of 960 letters with 25% as target letters. The font size and RGB values of the letters were kept same

in all versions. The total time taken to complete each test was noted and analysed statistically using SPSS by repeated measures ANOVA. The level of significance was kept at 0.05.

**Results**  
The results are summarised in Table 1:

The time taken to complete LCT showed significant difference ( $p < 0.001$ ) in the four colours (Black, Red, Green and Blue) for each group with the time for Blue being the shortest and for Black being the longest. There was significant difference between the time to complete between the three groups i.e. One LCT, Two LCT and Three LCT for each colour, with the time for Three LCT being the maximum and One LCT being the minimum.

**Conclusions/Implications:**

The present study demonstrated that colour effected the performance on Letter Cancellations Tasks. The changes in time taken reflect the time for processing of signal by the brain which could be at the level of cones or various levels in the visual pathway including the cortex. Disease processes that effect colour processing could thus effect performance on cognitive tests that involve use of different colours.

Table 1: Time taken to Complete LCT (Mean  $\pm$  SD in seconds)

| Colour | One- LCT           | Two- LCT           | Three- LCT         |
|--------|--------------------|--------------------|--------------------|
| Black  | 216.98 $\pm$ 44.88 | 220.30 $\pm$ 31.85 | 251.45 $\pm$ 31.53 |
| Red    | 196.17 $\pm$ 36.21 | 207.63 $\pm$ 31.75 | 232.88 $\pm$ 26.86 |
| Green  | 203.16 $\pm$ 37.89 | 210.41 $\pm$ 24.19 | 242.86 $\pm$ 37.51 |
| Blue   | 183.80 $\pm$ 28.37 | 198.14 $\pm$ 27.12 | 226.64 $\pm$ 26.43 |

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA305

## Electrophysiology of mammalian cochlear hair cells in aging mice.

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Mammalian cochlear hair cells are crucial for detecting sound stimuli and transducing them into electrical signals. The degeneration of hair cells is one of the main pathologies associated with age-related hearing loss (ARHL) but the underlying mechanisms are still largely unknown. To better understand age-related changes in hair cell function, we recorded their biophysical properties from aged mice.

Whole cell patch clamp was performed to record voltage and current responses from apical coil inner hair cells (IHCs) and outer hair cells (OHCs) of C57BL/6N and C57BL/6J mouse strains at 1, 6 and 12 months. These mice are a model for early onset ARHL and have a mutation in *cadherin 23* (*CDH23*), a protein crucial for the function of mechanically sensitive stereociliary bundles of hair cells. To investigate the possible effect of the *CDH23* mutation in C57BL/6N and C57BL/6J mice, we compared them with mice in which the mutation

has been repaired with CRISPR/Cas9 (Repaired mice). We found no obvious deficits in the hair cells from all strains at 1 month. Cell membrane capacitance, which is a measure of their surface area, in OHCs and IHCs decreases with age in all mouse strains. While the size of calcium current in IHCs was similar between strains and ages, the potassium current was smaller in Repaired mice at 12 months.

Our results suggest that the hair cells in ageing mice are smaller in size but retain their characteristic current profile. The *CDH23* mutation may not be the only factor for the changes in hair cells between young and older mice. Ongoing research will provide more evidence on whether the hair cells are normally innervated by the neurons and if they experience oxidative stress with age.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA306

## CD36 polymorphism (rs1761667) and PROP taster status affect the bioelectrical response to oleic acid by electrophysiological recording from the human tongue.

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Oro-sensory perception of dietary fat varies among individuals, thus influencing nutritional status. Several studies showed that fat detection and preferences are associated with common variants in the gene coding for CD36 scavenger receptor which facilitates fatty acid uptake and oxidation. In addition, it is known that non-taster individuals for the oral marker, 6-n-propylthiouracil (PROP), are less responsive to fats and have a lower ability to detect them in foods. However, the role of the PROP phenotype in fat perception is controversial. These divergent conclusions may be due to the fact that sensory analyses are carried out by psychophysical approaches and rely on highly subjective evaluations.

Here we present data about direct measures of the degree of activation of the peripheral gustatory system in response to taste stimulation, obtained by electrophysiological recordings from the tongue, in 35 subjects classified by PROP taster status. All were genotyped for *TAS2R38* gene. The PAV haplotype of *TAS2R38* has a high affinity for PROP, and the G allele of *CD36* polymorphism (rs1761667), is characterized by an increased protein expression. The biopotentials generated by taste stimulation with oleic acid were recorded by means of differential electrophysiological derivations between two silver electrodes, one in contact with the ventral surface of the tongue and one in perfect adhesion with the dorsal surface. The signals (electrotastagrams, ETGs) were recorded by a polygraph for human use, visualized in real time on a PC and analyzed by Clampfit 10.0 software. The amplitude values were determined at 2.5, 5, 10 and 15 s, and the depolarization rate (mV/s) calculated at the same time points.

Oleic acid stimulation evoked positive monophasic potentials characterized by a faster initial rise followed by a slower phase. These deviations, represent a measure of the summated voltage change resulting from the response of stimulated taste cells. In addition, repeated measures ANOVA revealed that the time course of the depolarization amplitude, as well



as depolarization rate, were associated with PROP tasting and CD36. The depolarization amplitude increased during stimulation in PROP tasters and in subjects having at least 1 PAV haplotype in *TAS2R38* or G allele in CD36, but did not change in non-tasters and in subjects homozygous for the non-tasting form in both genes.

In conclusion, we showed that the waveform of bioelectrical signals in response to oleic acid depends on PROP taster status and genotype of *TAS2R38* and *rs1761667* polymorphism of CD36. Our results provide the first objective data on a direct physiological relationship between the oro-sensory perception of fats and variation in these genes.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

#### PCA307

##### **Assessment of pain perception and biomarkers in osteoarthritis patients in South-west, Nigeria**

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Osteoarthritis (OA) is a degenerative joint disease accompanied by pain. The onset and progression of the disease is determined by several factors such as gender, body composition and ethnicity (1-2). There is paucity of information on pain perception and biochemical profile in OA patients in Nigeria. This study therefore, assessed pain perception and some biomarkers in osteoarthritic patients in South-west, Nigeria. A total of sixty (30 healthy and 30 diagnosed with OA) subjects were used for this study. All the subjects underwent the Ischemia-induced pain test and blood samples were taken from them for the determination of Interleukin-6 (IL-6), interleukin-10 (IL-10) and calcitonin gene related peptide (CGRP). The results showed a significantly ( $p < 0.05$ ) higher pain threshold and pain tolerance in healthy individuals compared to OA patients. There was also a significantly ( $p < 0.05$ ) elevated level of IL-6 in the serum of OA patients compared to control ( $13.0 \pm 0.7$  vs  $20.1 \pm 3.2$  pg/dL) and a significantly ( $p < 0.05$ ) lower level of IL-10 in the serum of OA patients compared to control ( $14.3 \pm 3.1$  vs  $4.1 \pm 0.5$  pg/dL). There was no difference in the serum level of CGRP in the control compared with the OA group. In conclusion, OA causes decrease in pain tolerance and threshold which is accompanied by alteration in vital biochemical parameters.

Allen KD.(2010):Curr Opin Rheumatol.22(5):528-532.

Ahn H, Weaver M, Lyon D, Choi E, Fillingim RB. (2017): J Pain. 18(10):1229-1236.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

#### PCA308

##### **Lysophosphatidic acid activates peripheral glial cells**

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Chronic pruritus occurs frequently in hepatobiliary diseases, is hard to control pharmacologically, and can seriously reduce the quality of life. The levels of lysophosphatidic acid (LPA) and its forming enzyme autotaxin were increased in the serum of cholestatic patients with pruritus compared to those without pruritus and correlated with itch severity and response to treatment (Kremer, 2010). We were able to determine by using microfluometry, that LPA 18:1 elevates cytosolic free calcium concentrations in cell cultures from sensory ganglia of C75BL/6 mice. Only 1.6% of the cells activated by LPA18:1 were identified as neurons, the majority were satellite glial cells. An inverse correlation between the LPA-response and the calcium responses to potassium and signature TRP channel agonists could be shown. The involvement of TRP channels was further tested by using transfected HEK293t cells which indicated, that LPA only marginally activates TRPV1/TRPA1. The responses to LPA in glia cells of TRPV1 and TRPA1 knockout mice were not reduced compared to cells from wild type mice. LPA desensitized responses to subsequent stimulation with other pruritogens. Pharmacological assays as well as mRNA levels concordantly suggest the observed responses are mediated by LPA receptor 1. LPA-induced intracellular calcium responses were almost identical in absence or presence of extracellular calcium, but could be depleted by thapsigargin, suggesting a calcium release from the endoplasmic reticulum as downstream signalling. In summary, LPA18:1 is a likely pruritogene in cholestatic pruritus which activates glial cells. This can lead to an altered neuronal function, which poses the question of a glial component in the regulation or even the generation of cholestatic itch.

Kremer AE et al. (2010). Gastroenterology. 139:1008-18

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

#### PCA309

##### **Impaired myogenic tone of skeletal artery feeding atrophic hindlimb and its recovery by exercise training: associated changes in the ion channel currents of the arterial myocytes**

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Altered hemodynamic stimuli to skeletal arteries (SkA) are expected in the atrophic limbs. Here we investigate changes in the myogenic tone (MT) of SkA and the ionic currents of the SkA myocytes. In the rats (Sprague-Dawley rats, 8 weeks old, male) with unilateral sciatic denervation for 5 weeks (Den-5w), video-analysis of pressurized artery revealed impaired MT in the atrophy-side deep femoral arteries (DFA<sub>Atr</sub>) while intact MT in the contralateral side (DFA<sub>CL</sub>). To get tissue samples, the rats were anesthetized with injection of sodium pentobarbital

(60 mg/kg) and were immediately killed by decapitation after confirming a fully anaesthetized state. Whole-cell patch clamp showed that both voltage-operated L-type  $\text{Ca}^{2+}$  current ( $I_{\text{Ca,L}}$ ) and TRPC-like current induced by intracellular  $\text{GTP}\gamma\text{S}$  ( $I_{\text{GTP}\gamma\text{S}}$ ) are lower in the  $\text{DFA}_{\text{Atr}}$  myocytes than  $\text{DFA}_{\text{CL}}$  of Den-5w. Despite the unilateral hindlimb paralysis, the rats could perform treadmill exercise with the intact legs (20 m/min, 30 min, 3 times/wk). In Den-5w, when combined with 4 weeks of the ET exercise training (Den/ET-5w), both the loss of MT and the decay of  $I_{\text{GTP}\gamma\text{S}}$  were prevented while not the decay of  $I_{\text{Ca,L}}$ . The comparison of  $\text{K}^+$  channel currents between  $\text{DFA}_{\text{Atr}}$  and  $\text{DFA}_{\text{CL}}$  myocytes showed complex tendencies. Voltage-gated  $\text{K}^+$  current ( $I_{\text{KV}}$ ) became higher in  $\text{DFA}_{\text{CL}}$  than  $\text{DFA}_{\text{Atr}}$ , and the difference was abolished in Den/ET-5w. In contrast, the inwardly rectifying  $\text{K}^+$  current ( $I_{\text{Kir}}$ ) and paxilline-sensitive  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channel current ( $I_{\text{BKCa}}$ ) were not significantly different between  $\text{DFA}_{\text{CL}}$  and  $\text{DFA}_{\text{Atr}}$ . The recovery of MT and the ionic currents in Den/ET-5w suggest beneficial effects of systemic endurance exercise on vascular physiology in motor nerve injury patients with atrophic skeletal muscles.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCA310

### Epoxyeicosatrienoic acid pathway contributes to sustained hypoxic pulmonary vasoconstriction in mouse intrapulmonary arteries

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Under conditions of generalized alveolar hypoxia caused by a range of lung diseases or at high altitude, exaggerated sustained hypoxic pulmonary vasoconstriction (HPV) contributes to the development of pulmonary hypertension and *cor pulmonale*. The exact mechanisms underlying sustained HPV are not completely understood. The aim of the present study was to investigate the role of cytochrome P450 (CYP) epoxygenase-derived epoxyeicosatrienoic acids (EETs) in sustained HPV. Contractile responses of isolated intrapulmonary arteries (IPAs; 80–200  $\mu\text{m}$  in diameter) of C57BL/6J mice were measured using wire myography. Changes in isometric tension are expressed as percentages of the maximum contraction induced by the exposure to 80 mM KCl (%  $T_K$ ). Values are presented as means  $\pm$  S.E.M. ( $n=8$  for each group except for siRNA experiments, where  $n=5$  for each group), comparisons were performed using two-way ANOVA. Hypoxic exposure (80 min) induced slowly developing sustained contraction in IPAs (to  $13.8\pm 1.3$  vs.  $1.7\pm 1.6$  %  $T_K$  in normoxia at 80 min;  $p<0.001$ ). HPV was suppressed by a CYP epoxygenase inhibitor, MS-PPOH (30  $\mu\text{M}$ ;  $5.1\pm 2.9$  %  $T_K$ ;  $p<0.001$ ), abolished by an EET receptor blocker, 14,15-EEZE (1  $\mu\text{M}$ ;  $-0.3\pm 2.8$  %  $T_K$ ;  $p<0.001$ ), and potentiated by TPPU (3  $\mu\text{M}$ ), an inhibitor of the EET-degrading enzyme, soluble epoxide hydrolase ( $32.2\pm 4.6$  %  $T_K$ ;  $p<0.001$ ). All these agents did not affect IPA tone in normoxia ( $1.9\pm 1.9$ ,  $-0.3\pm 2.8$ , and  $5.4\pm 5.1$  %  $T_K$ ,

respectively). Knockdown of the soluble epoxide hydrolase gene, *Ephx2*, in isolated arteries using siRNA also augmented HPV compared to non-targeting control ( $82.4\pm 5.6$  vs.  $37.7\pm 4.5$  %  $T_K$ ;  $p<0.001$ ). No changes in tone of the transfected IPAs were observed in normoxia ( $2.8\pm 6.4$  and  $5.5\pm 2.6$  %  $T_K$  in the siRNA and control group, respectively). Applications of exogenous EETs (5,6-, 8,9-, 11,12- and 14,15-EET; 1.5  $\mu\text{M}$ ) were without effect in non-precontracted IPAs ( $0.7\pm 1.1$ ,  $0.9\pm 0.3$ ,  $-0.7\pm 0.3$  and  $-0.2\pm 1.1$  %  $T_K$ , respectively, vs.  $0.3\pm 0.9$  %  $T_K$  in control). By contrast, in IPAs precontracted with KCl, 5,6-EET, unlike other regioisomers, induced pronounced contraction ( $20.1\pm 3.2$ ,  $-3.2\pm 0.4$ ,  $-1.9\pm 1.3$  and  $-1.0\pm 1.5$  vs.  $-0.2\pm 0.7$  %  $T_K$  in control;  $p<0.001$  for 5,6-EET vs. control). In patch-clamp experiments, hypoxia caused depolarization in isolated pulmonary artery smooth muscle cells to  $-25.4\pm 2.1$  mV (from  $-36.8\pm 1.7$  mV in normoxia;  $p<0.01$ ), and 5,6-EET evoked significant inward whole-cell currents at a holding potential close to this value ( $-55.86\pm 15.0$  pA at  $-20$  mV vs.  $16.0\pm 16.3$  pA at  $-40$  mV;  $p<0.01$ ). We speculate that hypoxia activates EET synthesis in IPAs. The resulting release of 5,6-EET, presumably in conjunction with hypoxia-induced depolarization in pulmonary artery smooth muscle cells, elicits vasoconstriction. In summary, the obtained results indicate that EET pathway contributes to sustained HPV in mouse IPA.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCA311

### Ferulic acid alleviates haemodynamic alterations and aortic stiffness in rats fed a high-carbohydrate, high-fat diet

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Metabolic syndrome (MetS) is a cluster of metabolic disorders including obesity, hypertension, hyperglycemia and dyslipidemia. Chronic intake of high-carbohydrate and high-fat (HCHF) diet causes MetS and increases risk of cardiovascular disease (CVD). Increased central arterial stiffening is a marker for increased CVD risk. Compounds that could prevent MetS and improve haemodynamic status and arterial stiffening could be beneficial for health promotion. Ferulic acid (FA) is a major phenolic compound found in rice bran, cereals and various types of fruits and vegetables. This study was designed to test whether FA supplementation could restore haemodynamic status and aortic stiffness in rats fed a HCHF diet. Male Sprague-Dawley rats (200–250 g) were divided into 4 groups of 6 rats each. Group 1 was fed a standard chow diet with tap water as drinking water, and propylene glycol (PG) was administered orally as a vehicle for FA. Group 2–4 were fed a HCHF diet with 15% fructose supplemented drinking water, and administered orally with PG or FA (30 or 60 mg/kg body weight/day, respectively). Rats were given free access to the specified diet and drinking water for 16 weeks. At week 10, PG or FA was administered to rats in the assigned groups for the last 6 weeks of the experimental period. At the end of the

experiment, rats were anaesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg). The arterial blood pressure and aortic pulse wave velocity (PWV) were determined in vivo as previously described (Fitch *et al.*, 2001). Subsequently, hindlimb blood flow (HBF) was measured by placing an electromagnetic flow probe around the abdominal aorta. Hindlimb vascular resistance (HVR) was calculated from the mean arterial pressure (MAP) divided by HBF. Rats were euthanized with pentobarbital sodium (120 mg/kg). Blood samples were collected for biochemical assays. Values are means  $\pm$  S.E.M., compared by ANOVA. FA significantly alleviated the signs of MetS by decreasing fasting blood glucose, plasma cholesterol and triglycerides compared to HCHF controls ( $p<0.05$ ). FA (30 and 60 mg/kg) significantly improved haemodynamic status by reducing MAP ( $116\pm 2.0$ ,  $108\pm 1.9$  vs.  $124\pm 4.1$  mmHg HCHF-fed rats,  $p<0.05$ ) and HVR ( $23\pm 0.9$ ,  $17\pm 1.1$  vs.  $37\pm 1.6$  mmHg/mL/min/100 g tissue HCHF-fed rats,  $p<0.05$ ). Aortic PWV of HCHF diet fed rats significantly increased when compared to standard chow-fed rats ( $p<0.05$ ). FA (30 and 60 mg/kg) reduced aortic PWV ( $7.2\pm 0.2$ ,  $6.3\pm 0.3$  vs.  $8.2\pm 0.4$  m/s HCHF-fed rats,  $p<0.05$ ). These data suggest the potential of FA in preventing the progression of MetS and improvement of haemodynamic status and large artery compliance.

Fitch RM *et al.* (2001). Nitric oxide synthase inhibition increases aortic stiffness measured by pulse wave velocity in rats. *Cardiovasc Res* 51, 351-358.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA312

#### **Antihypertensive and antioxidative effects of whole grain essential oil in nitric oxide deficient hypertensive rats**

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Hypertension is one of the major risk factors for cardiovascular disease (CVD). Nitric oxide (NO) plays an important role in the regulation of cardiovascular function. Inhibition of NO synthesis results in development of hypertension. Oxidative stress is associated with the pathogenesis of hypertension. Consumption of healthy diet that contains natural antioxidants appears to reduce blood pressure and may decrease risk for CVD. Whole grain essential oil (WEO) extracted from brown rice contains high levels of dietary antioxidants which may protect against diseases related to oxidative stress. Therefore, this study aimed to investigate the antihypertensive effect of WEO in NO deficient hypertensive rats. Hypertension was induced in male Sprague-Dawley rats (200-230 g,  $n=6$ /group) by receiving N<sup>ω</sup>-nitro-L-arginine methyl ester (L-NAME), a NO synthase inhibitor, at dose of 50 mg/kg/day in drinking water for 3 weeks. Simultaneously, L-NAME-treated rats were intragastrically administered daily with deionized water (DI) for untreated control group or WEO (1 or 2 ml/kg)

for treated groups. Rats received tap water and intragastrically administered with DI were served as normotensive controls. At the end of experiment, rats were anaesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg). The left femoral artery and femoral vein were cannulated for continuously monitoring arterial blood pressure and for infusion of vasoactive agents, respectively. Rats were sacrificed by overdose of anaesthetic drug. Blood samples and thoracic aorta were collected for assay of oxidative stress markers. Data were expressed as means  $\pm$  S.E.M., compared by ANOVA. Results showed that WEO significantly decreased arterial blood pressure of L-NAME-treated rats compared to normotensive controls ( $138.9 \pm 5.8$ ,  $118.1 \pm 3.7$  vs.  $160.4 \pm 3.6$  mmHg L-NAME control  $p<0.05$ ). The vascular response to vasoconstrictor, phenylephrine ( $0.1 \mu\text{M}$ ) was augmented while the response to endothelial-dependent vasodilator, acetylcholine ( $30 \text{ nM}$ ), was blunted in L-NAME-treated rats. WEO significantly improved the vascular responsiveness of L-NAME hypertensive rats ( $p<0.05$ ). The antihypertensive effect of WEO was associated with a reduction in vascular superoxide production, plasma malondialdehyde and protein carbonyl ( $p<0.05$ ). Moreover, WEO also increased eNOS expression and suppressed p47<sup>phox</sup> NADPH oxidase in the vascular tissues ( $p<0.05$ ). The present results provide evidence for the antihypertensive and antioxidative properties of WEO and suggest that WEO might be useful as a dietary supplement against hypertension.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA313

#### **Rice bran hydrolysates lower blood pressure and improve vascular function in L-NAME-induced hypertensive rats**

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Hypertension is an important public health problem worldwide. Numerous experimental models of hypertension have been developed and N<sup>ω</sup>-nitro-L-arginine methyl ester (L-NAME)-induced hypertension is a well established model. Inhibition of nitric oxide (NO) synthesis with L-NAME induces a marked increase of blood pressure, endothelial dysfunction and oxidative stress. This model is useful for studying the influences of oxidative stress, NO deficiency and also evaluating antihypertensive agents. Tubtim Chum Phae rice, a new breed of red pericarp rice, is a hybrids of mixed Hom Mali rice and Sang Yhod rice. Rice bran hydrolysates extracted from Tubtim Chum Phae defatted rice bran (TCRH) consist of bioactive compounds with high antioxidant activities. This study aimed



to investigate whether TCRH could reduce blood pressure, ameliorate endothelial dysfunction and oxidative stress in L-NAME-induced hypertensive rats. Male Sprague-Dawley rat (180-220 g, n=30) were randomly divided into 5 groups (n=6): control+deionized water (DI), control+TCRH 500 mg/kg, L-NAME+DI, L-NAME+TCRH 250 mg/kg, and L-NAME+TCRH 500 mg/kg. The control group received tap water and the L-NAME-treated group received L-NAME (50 mg/kg/day) in drinking water for 3 weeks. TCRH (250 or 500 mg/kg/day) or DI, as a vehicle was fed to rats simultaneously with L-NAME. At the end of experiment, rats were anesthetized with pentobarbital sodium (60 mg/kg, i.p.). The left femoral artery was cannulated for monitoring arterial blood pressure. Hindlimb blood flow (HBF) was measured by placing electromagnetic flow probes around the abdominal aorta and hindlimb vascular resistance (HVR) was calculated from the mean arterial pressure (MAP) and HBF. Vascular reactivity was evaluated by infusing acetylcholine (10 nmol/kg) through an additional catheter in the femoral vein. Blood samples and aortic tissues were collected for biochemical and protein expression analyses. Values are means  $\pm$  S.E.M., compared by ANOVA. TCRH at both concentrations significantly reduced arterial blood pressure ( $174 \pm 1.4$ ,  $156 \pm 2.9$  vs.  $194 \pm 3.9$  mmHg L-NAME controls,  $p < 0.05$ ) and vascular resistance. The vascular responses to acetylcholine of L-NAME rats-treated with TCRH were significantly increased ( $40 \pm 0.8$ ,  $49 \pm 0.1$  vs.  $31 \pm 0.9$  % L-NAME controls,  $p < 0.05$ ). The reduction in blood pressure was associated with a decrease in plasma angiotensin-converting enzyme activity and an increase in plasma nitrate/nitrite and eNOS protein expression. TCRH also reduced plasma malonaldehyde and increased blood glutathione level. The overall findings suggest that TCRH exhibits an antihypertensive effect via its antioxidant capacity and vasodilatory property.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA314

#### Identification of Kv7 channels in rat mesenteric artery endothelium and role in endothelial derived relaxations.

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**Introduction:** Kv7 channels have been identified as key regulators of arterial diameter and receptor mediated dilatation in many arteries (1). However, no study has considered expression of Kv7 channels in the endothelium and the possible impact of channels localised therein.

**Objective.** The goal of the study was to; ascertain whether Kv7 were present in the endothelium of rat mesenteric arteries and to determine if Kv7 blockers modulate endothelium-dependent relaxations.

**Methods and materials.** Male Wistar rats (175-225g) were killed by methods in accordance with UK Animals Scientific Procedures Act and Australian legislation. 2<sup>nd</sup>-4<sup>th</sup> order mesenteric arteries were dissected, cleaned of fat and then either mounted in a wire myograph (DMT, Aarhus) for isometric tension recording or perfusion fixed for intact artery

immunocytochemistry using Kv7.4 and 7.5 primary (Abcam, ab65797, lot GR94754; Millipore; ABN1372, lot Q2476155, respectively) and Alexa 594 secondary antibody. In myograph studies endothelial integrity was tested with the addition of 10  $\mu$ M carbachol producing greater than 90% relaxation of arteries precontracted with 3  $\mu$ M methoxamine or 1  $\mu$ M U46619. Where necessary endothelium was removed by gentle mechanical abrasion.

**Results.**

Confocal imaging (n=3-5) showed Kv7.4 as low diffuse in endothelium and absence at internal elastic lamina (IEL) holes; with 7.5 being diffuse and punctate in endothelium and at a proportion of IEL holes. In isometric tension studies carbachol mediated relaxations were reduced by incubation with either the NO synthase inhibitor L-NAME (100 mM) or prostacyclin IP receptor antagonist CAY-10471 (10  $\mu$ M) and impaired markedly by apamin (10 nM) and TRAM-34 (1  $\mu$ M) combination. Incubation with the pan Kv7 blocker linopiridine (10 mM) reduced the relaxation produced by 300 nM carbachol from  $45.9 \pm 4$  % to  $75.1 \pm 7$  % (n=6). Incubation with Kv7.1 specific antagonist HMR 1556 attenuated relaxation produced by 300nM CCh from  $45.9 \pm 4$  % to  $66.4 \pm 7$  % (n=7). A response that could not be emulated by the use of non-specific potassium channel inhibitors tetraethyl ammonium (1 mM) and 4-aminopyridine (1 mM). Illoprost mediated relaxations were abrogated by CAY-10471 and attenuated by linopiridine (10  $\mu$ M, n=5). Relaxations produced by the Kv7.2-7.5 enhancers ML213 and acrylamide S1, but not Kv7.1, were enhanced in arteries with intact endothelium compared to arteries without endothelium.

**Conclusions.**

This study has revealed that Kv7.4/5 are present in the endothelium of rat mesenteric arteries, as well as in the smooth muscle. Moreover, we show that Kv7 isoforms contribute to endothelial derived receptor mediated vasorelaxant responses. This study also shows the presence of endothelium enhanced responses only to Kv7.2-5 activators in rat mesenteric arteries.

Barrese V, Stott JB & Greenwood IA. (2018). KCNQ encoded potassium channels as therapeutic targets. *Annual Reviews of Pharmacology & Toxicology*. 58:625-648

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA315

#### Cytochrome P450 reductase contributes to NADPH-dependent signal in membrane assays used for measuring Nox activity

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The molecular basis of cardiovascular diseases (CVD) is complex and oxidative stress is a strong candidate contributor towards its development. The Nox NADPH oxidase family of enzymes uses NADPH as substrate to produce ROS (reactive

oxygen species) and have been considered important sources of oxidative stress occurring in atherosclerosis, hypertension and heart failure. Such conclusions were mainly based on NADPH-stimulated assays in membrane fractions which were thought to measure NADPH oxidase (Nox) activity. These assays were often used to overcome the limitations of the available ROS probes. However, the contribution of Nox enzymes to the highly detected signal was not validated at the time they emerged. We have generated a triple Nox knockout mouse (Nox1-Nox2-Nox4<sup>-/-</sup>) which exhibited attenuated endothelial dysfunction, hypertension and hypertrophic response to angiotensin II (AngII). However, the knockout of the three main Nox enzymes had no impact on chemiluminescence signals in aorta, heart and kidney homogenates. In HEK cell overexpressing Nox4 and Nox5 (as sources of H<sub>2</sub>O<sub>2</sub> and superoxide, respectively) the high ROS production of intact cells could not be recapitulated in NADPH-stimulated membrane assays with lucigenin. Thus, the signal has to derive from a source other than NADPH oxidases. Using a combination of native protein electrophoresis, NADPH-stimulated assays and mass spectrometry, cytochrome P450 reductase (POR) was identified as the possible source of the assay signal. Microsomes overexpressing POR, cytochromes b5 and P450 generated a NADPH-dependent signal in assays utilizing lucigenin, L-012 and dihydroethidium (DHE). Knockout of POR by CRISPR/Cas9 technology (POR<sup>-/-</sup>) in HEK293 cells overexpressing Nox4 or Nox5 did not interfere with ROS production in intact cells. However, POR knockout abolished the signal in NADPH-stimulated assays using membrane fractions from the very same cells. Moreover, membranes of rat smooth muscle cells (SMC) treated with angiotensin II showed an increased NADPH-dependent signal with lucigenin which was abolished by the knockout of POR but not by knockout of p22phox. POR shares structural and biological similarities with Nox enzymes as both contain flavin domains and consume NADPH to produce ROS. Interestingly, loss of POR and Nox activity also had similar physiological consequences: Cell proliferation was reduced to a similar extent as was agonist-induced phosphorylation of p38MAP kinase. Thus, we show that POR contributes to NADPH-dependent signal in membrane assays and shares biological effects attributed originally to Nox enzymes.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCA316

### **Sex differences in the circulatory responses to an isocapnic cold pressor test**

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The cold pressor test (CPT) elicits a transient increase in sympathetic nervous activity, minute ventilation ( $V_E$ ), mean arterial pressure (MAP), and common carotid artery (CCA) diameter. The extent of dilation of the CCA in response to the CPT is used as a clinical indicator of cardiovascular health status, however, the potential sex differences have yet to be explored. In response to an isocapnic (i.e. controlled CO<sub>2</sub>) CPT, we hypothesized that  $V_E$ , MAP and dilation of the CCA would be reduced in females compared to males. In 20 healthy young participants (10 female and 10 male), we measured the respiratory, cardiovascular, and CCA response during

an isocapnic CPT, which consisted of a three-minute right foot immersion into 0-1 degrees Celsius water. The partial pressure of end-tidal CO<sub>2</sub> and O<sub>2</sub> ( $P_{ET}CO_2$  and  $P_{ETO_2}$ , respectively; via respiratory gas analyzer), blood pressure (via finger photoplethysmography), heart rate (via electrocardiogram), and CCA diameter and velocity (via Duplex ultrasound) were simultaneously recorded immediately prior to, and during the isocapnic CPT. During the isocapnic CPT,  $P_{ET}CO_2$  and  $P_{ETO_2}$  were controlled to baseline values using a dynamic end-tidal forcing system. The main findings during the isocapnic CPT were the following: 1) no sex differences were present in absolute or relative  $V_E$  ( $P=0.801$  and  $P=0.179$ , respectively); 2) the absolute MAP response remained unchanged between males and females ( $P=0.905$ ), but the relative MAP response was reduced in females by 51% ( $P=0.008$ ); and 3) the absolute CCA diameter response remained unchanged between males and females ( $P=0.667$ ), but the relative CCA diameter response was reduced in females by 55% ( $P=0.029$ ). Furthermore, the relative CCA diameter response was significantly correlated with the relative MAP response in males ( $r=0.42$ ,  $P=0.019$ ), females ( $r=0.43$ ,  $P=0.019$ ), and in males and females combined ( $r=0.55$ ,  $P<0.001$ ), indicating that this response was in part due to the increase in MAP during the CPT. Since the CPT is used as a clinical tool to assess cardiovascular health status, sex differences should be considered in future studies.

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### **Epithelial Na<sup>+</sup> channel's ability to mediate arterial shear force responsiveness depends on glycosylated asparagines and the extracellular matrix**

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Healthy arteries sense and respond to shear force and impairment of this response is a hallmark of vascular dysfunction. The mechanisms how arteries sense shear force are poorly understood. The epithelial Na<sup>+</sup> channel (ENaC) formed by  $\alpha$ ,  $\beta$  and  $\gamma$  subunits is an emerging candidate for this role. ENaC's open probability is regulated by shear force (1) and the expression and function was reported in endothelial cells (2). Aim of our research is to reveal how ENaC senses shear force to regulate arterial blood pressure (BP) regulation. We hypothesise that ENaC N-glycans attached to asparagines interact with the extracellular matrix (ECM) to mediate shear force responsiveness.

Whole cell currents of human  $\alpha$ ,  $\beta$ ,  $\gamma$  ENaC in response to shear force were recorded in *Xenopus* oocytes. Extracellular asparagines of  $\alpha$  ENaC were replaced against alanines by site directed mutagenesis. Pressure myography on freshly isolated carotid arteries from C57BL/6 mice was performed to characterise ENaC-mediated effects on arterial diameter in response to shear force. In oocytes and carotid arteries ECM components

(hyaluronic acid/heparan sulfate) were enzymatically degraded by hyaluronidase or heparanase. Further, endothelial specific adenoviral transduction of wildtype (n=14), mutated  $\alpha$  ENaC (n=14) or empty vector (n=15) was performed in C57BL/6 mice. Mean BP of these mice was measured by the tail-cuff method before and after viral transduction.

Two glycosylated asparagines at position 312 and 511 in the extracellular domain of  $\alpha$  ENaC contribute to shear force sensing. Replacement of these asparagines individually decreased the shear force currents in *Xenopus* oocytes by approx. 40% (n $\geq$ 15, P $\leq$ 0.01). Degradation the ECM of hyaluronidase or heparanase impaired shear force mediated effects of expressed ENaC in oocytes and ENaC-mediated effects in carotid arteries. In mice overexpressing wild type  $\alpha$  ENaC caused an increase in BP from 115 $\pm$ 2 to 157 $\pm$ 5 mmHg (n=14, P<0.001). Transduction of  $\alpha$  ENaC with replaced asparagines showed a markedly decreased rise in BP (114 $\pm$ 2 to 124 $\pm$ 2 mmHg; n=14, P<0.01). No change in BP was observed in mice transduced with an empty vector (before 112 $\pm$ 2; after 115 $\pm$ 1 mmHg, n=15, P=0.9).

The ability of ENaC to sense shear force depends on glycosylated asparagine of  $\alpha$  ENaC and an intact ECM. Replacement of glycosylated asparagines in the finger and palm domain of ENaC impair ENaCs ability to response to shear force and prevent an increase of arterial BP. These observations support our hypothesis that the N-glycans attached to the asparagines provide a connection to the ECM to form a shear force sensitive complex that contributes to BP regulation.

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### Elastin derived peptides augments abdominal aortic aneurysm formation

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Abdominal aortic aneurysm AAA is a chronic dilatation of the abdominal aorta. It is a potentially life threatening disease as it develops asymptomatic and is often first discovered at the time of rupture. One key element in AAA formation is degradation of the elastin fibers in the aortic wall. When elastin is degraded small bioactive elastin derived peptides (EDPs) are released to the circulation. These EDPs affect vascular smooth muscle cells in the aortic wall and attract monocytes to the aortic wall leading to further degradation of elastin.

We hypothesize that the level of circulating elastin derived peptides (EDPs) augments AAA expansion and that inhibition of circulating EDPs will inhibit AAA expansion.

Nine weeks old C57bl/6J male mice were anesthetized i.p. with a mixture of ketamine (100 mg/kg) and xylazine (10mg/kg) and AAA was induced by intraluminal elastase infusion in the infrarenal aorta at the day of surgery and every third day, mice were given synthetic EDPs or scrambles peptides

(SC) i.v. until termination at day 14. In addition, 10-12 week old male ApoE<sup>-/-</sup> mice were anesthetized with isoflurane gas and osmotic mini-pumps filled to release angiotensin II (1 $\mu$ g/kg/min) were implanted subcutaneously to induce AAA formation over a period of 28 days. Mice were given anti-elastin neutralizing antibody or control IgG i.p. once a week during the experimental period. AAA size was determined as by subtracting baseline outer aortic diameter from maximal abdominal aortic diameter at the time of termination. At termination, mice were fixed by systemic perfusion of 4% paraformaldehyde and aneurysms were embedded in paraffin for morphological analyses.

Fourteen days after elastase infusion the maximal abdominal aortic diameter was significantly increased in the EDP treated group when compared to the SC treated group (1.33 $\pm$ 0.07 vs. 1.13 $\pm$ 0.06 mm, n=17-19, p<0.05). Inhibition of circulating EDPs by EDP neutralizing antibodies when compared to IgG controls showed a clear trend toward decreased AAA expansion based on maximal outer aortic diameter (1.4  $\pm$ 0.1 vs. 2.2  $\pm$ 0.4 mm, n=8-9, p=0.059) and wet weights of the abdominal aorta (median: 9.2 vs. 22.1 mg, n=7-9, p=0.07) 28 days after angiotensin II infusion in ApoE<sup>-/-</sup> mice. In the aneurysmal wall of the elastin neutralizing antibody treated group elastin was more preserved when compared to the IgG treated group (elastin degradation score (1-4): 2.0  $\pm$  0.4 vs. 3.4  $\pm$  0.4, n= 8-9, p<0.05). The number of CD45 positive leukocytes in the aneurysmal wall was significantly higher in the IgG treated mice when compared to the elastin neutralizing antibody treated group, while no obvious differences were detected in the number of CD206 positive anti-inflammatory M2 macrophages between groups.

In conclusion, EDPs augment abdominal aortic aneurysms. Thus, inhibition of circulating EDPs shows great potential against AAA expansion.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

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### Differential effects of Integrin-linked kinase knockdown and vascular endothelial growth factor receptor blockade on melanoma cell growth and angiogenesis

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Angiogenesis is the process by which new blood vessels are formed from a pre-existing microvasculature [1]. It is also a key feature in the growth and progression of several cancers. Studies have identified vascular endothelial factor (VEGF) as an important regulator of angiogenesis in both the physiological and pathological settings [1,2]. In the context of tumours, VEGF signalling was shown to be impaired in several neoplasms. This discovery led to the development of therapies against VEGF [1-3]. While antiangiogenic VEGF-targeted therapy has resulted in increased cancer patient survival, the improvement has been marginal partly due to the development of resistance [2,3]. Therefore there is a need for the identification of alternative or complimentary therapeutic strategies. Integrin-linked kinase (ILK) is an effector of integrin-mediated cell adhesion which is involved in the regulation of the PI3k/Akt pathway [4]. The kinase has also been linked to the promotion of tumour-associated neovascularisation. The purpose of the present study was to study the effects of a multi-targeted therapeutic strategy comprising



ILK-knockdown and the blockade of VEGF receptors -1,-2 or VEGFR-3 in human melanoma cells and endothelial cells. To evaluate the effects of ILK knockdown the human vein endothelial cells and melanoma cells, purchased from the American Type Culture Collection, were used. The effects of inhibiting ILK using siRNA alone and in combination with a broad spectrum tyrosine kinase inhibitor, Sunitinib, or in combination with a VEGFR-3 inhibitor MAZ-51, were evaluated on cell growth using the crystal violet nuclear staining assay. The effects of treatments on cell migration and invasion were studied using the xCELLigence Real Time Cell Analysis (RTCA) system. The expression of proangiogenic and antiangiogenic proteins were determined using protein array analysis. Protein expression was further studied using Western blotting. The combination of Sunitinib and Integrin linked kinase knock-down resulted in a more severe decrease in melanoma migration and invasion as well as angiogenesis when compared to the individual treatments or to ILK knockdown + VEGFR-3 blockade. Both combination treatments inhibited cell growth with comparable potency. Also, results from western blot and protein array studies revealed a decrease in the expression of several proangiogenic factors including platelet derived growth factor (PDGF) and basic fibroblast growth factor (bFGF), while a number of endogenous angiogenesis inhibitors such as Platelet Factor 4 (PF4) were upregulated. The combination approach of ILK-knockdown and Sunitinib may be useful in the elaboration of antiangiogenic therapy in melanoma, further studies are warranted.

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### **Anandamide Modulates Epigenetic Regulation by Nuclear Receptor Mechanism**

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**Objective** – The endocannabinoid system regulates stress, food intake and emotional behavior. Endocannabinoids are also beneficial in the cardiovascular system as they reduce blood pressure and infarct size. Whether endocannabinoids, however, also have anti-inflammatory functions is controversial. We therefore set out to identify the impact of endocannabinoids on inflammatory signaling in vascular cells.

**Results** – Anandamide (AEA), the ethanolamide of arachidonic acid, is one of the best-characterized endocannabinoids. We therefore focused on this compound. Pretreatment of murine and human aortic smooth muscle cells (hSMCs) with AEA decreases the Interleukin-1 $\beta$  and Tumor necrosis factor

$\alpha$ -induced upregulation of inflammatory genes (e.g. CCL2) on mRNA and protein level. These effects were not mediated by peroxisome proliferator-activated receptors (PPARs) or the classic cannabinoid receptors CB1 or CB2 and are therefore potentially mediated by intracellular cannabinoid receptors. RNA sequencing data revealed a massive upregulation of the nuclear receptor family NR4A in response to AEA. Knock-down of NR4A by lentiviral CRISPR/ Cas9 in SMC blocked the anti-inflammatory effect of AEA. Similarly, overexpression of NR4A in HEK cells could promote the effect anti-inflammatory effect of the endocannabinoid. In line with this, reporter gene assay carrying the NR4A binding motifs demonstrated that AEA promoted NR4A recruitment to the construct and this resulted in inhibition of transcription. Thus, we suggest that AEA treatment activates NR4A receptors and promotes their interaction with nuclear corepressors. Indeed, chromatin immunoprecipitation experiments in vascular cells show that the endocannabinoid treatment results in increased formation of heterochromatin at the promoter of the important inflammatory cytokine CCL2 promoter. Such a formation of heterochromatin is often a consequence of the recruitment of corepressors.

**Conclusions** – AEA reduces the inflammatory response of vascular SMC by epigenetic silencing of inflammatory genes. This is mediated by activation of NR4A and their recruitment of nuclear corepressors.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

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### **Lifelong maladaptation to terrestrial high-altitude is associated with inadequate dietary antioxidant vitamin intake and accelerated cognitive decline**

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**Background, aims**

Cognition declines with age<sup>1</sup>. Additionally, chronic exposure to lack of oxygen as seen in highlander dwellers leads to cognitive changes<sup>2</sup>. Exaggerated systemic oxidative stress has been reported in patients with Chronic Mountain Sickness (CMS+) a maladaptive syndrome characterised by cognitive and neurological deficits<sup>4</sup>. Diet may attenuate the rate of cognitive decline, particularly consumption of the primary water and fat-soluble chain-breaking antioxidants (vitamins C & E), given their collective capacity to attenuate oxidative stress<sup>5</sup>. The aim of this study was to determine if a link exists between inadequate dietary antioxidant vitamin intake in CMS+ patients and accelerated cognitive decline/early dementia.

## Methods

Twenty two male highlanders with CMS (CMS+; age 56±11 years), 14 male highlanders without CMS (CMS-; age 52±12 years) and 28 male aged matched lowlander controls (LL; age 67±6 years) consented to the study. Participants were interviewed to collect a 24-hour structured dietary recall and data were analysed using NetWISP dietary analysis software (V4.0, Tinuvial Software; UK). Montreal Cognitive Assessment (MoCA) was used to assess cognition according to recommended procedures. Data were tested for normality using Shapiro-Wilks tests. Kruskal-Wallis, Mann-Whitney and Chi-Square tests were performed to compare the groups. Significance level was established at  $P<0.05$  and data are expressed as mean ± standard deviation (SD).

## Results

Dietary vitamin C consumption was shown to be deficient in CMS+ relative to CMS- and lowlanders (Table). Dietary vitamin E consumption was also deficient in both CMS+ and CMS- relative to lowlanders ( $P<0.05$ ). The CMS+ patients were also characterised by a lower MoCA score compared to CMS- and lowlanders (Table).

## Conclusion

The findings demonstrate that patients who fail to adapt to the terrestrial extremes of high-altitude are characterised by an inadequate intake of dietary antioxidant vitamins. Given that these antioxidants can selectively “repair” aqueous superoxide, peroxy, and alkoxyl radicals, any functional deficiency in dietary intake may contribute to cerebrovascular endothelial dysfunction, attenuated cerebral perfusion and explain the accelerated cognitive decline reported herein.

## Daily Antioxidant Intake & MoCA

| Antioxidants & Montreal Cognitive Assessment (MoCA) | Aged lowlanders (LL) (n = 28) | Highlanders (n = 36) |               |
|-----------------------------------------------------|-------------------------------|----------------------|---------------|
|                                                     |                               | CMS- (n = 14)        | CMS+ (n = 22) |
| Ascorbate (mg)                                      | 101 ± 53                      | 66 ± 33              | 47 ± 35a b    |
| α-Tocopherol (mg)                                   | 8 ± 2                         | 5 ± 4                | 4 ± 2b        |
| MoCA < 26*                                          | 12                            | 7                    | 19c           |
| MoCA > 26*                                          | 16                            | 7                    | 3c            |

a Borderline difference vs CMS- ( $P<0.08$ ); b Significant difference vs LL ( $P<0.05$ ); c Chi-Square=10.321, df=2,  $P<0.05$  (significant difference vs CMS- and LL); \*Cognitive decline <26/30 (education corrected)

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PCA322

## *Parastrephia quadrangularis* (Meyen) Cabrera exerts a hypotensive effect on rats through a negative inotropic effect and endothelium-dependent vasodilation

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*Parastrephia quadrangularis* (Pq) is an endemic species of the Andes Cordillera, popularly known as “Tola”. It has a wide use in folk medicine to treat altitude sickness. The purpose of the study was to evaluate the cardiovascular effects and the vascular response of the hydroalcoholic extract of *P. quadrangularis* (HAE Pq) and its pure isolated compounds. **Methods:** Several metabolites were isolated from the *P. quadrangularis*, and their structures were characterized by nuclear magnetic resonance (NMR), and mass spectrometry. Male Sprague-Dawley rats (6–8 weeks of age, 130–180g) were used. The animals were anesthetized with ketamine (42 mg/Kg, i.p.) and xylazine (5 mg/Kg, i.p.). Blood pressures recordings were recorded from the carotid artery of rats receiving HAE Pq (40 mg/Kg) through gavage for 10 days. The isolated right atrium and isolated heart of rat (Langendorff) were used to study the cardiac functions. The vascular response to HAE Pq and its pure compounds were evaluated in rat aorta. Values are means ± S.E.M., compared by ANOVA. The experiments of this study were conducted following the ARRIVE guidelines. **Results:** The oral treatment with HAE Pq (40 mg/Kg b.w.) significantly reduced the mean arterial blood pressure (MAP; 87±5 mmHg control vs. 66±1 mmHg with 40 mg/Kg HAE Pq;  $p<0.05$ , n=4) and the diastolic blood pressure (DBP; 75±6 mmHg control vs. 49±1 mmHg with 40 mg/Kg HAE Pq;  $p<0.01$ ) in normotensive rats. HAE Pq did not cause a significant decrease in the heart rate or the beating frequency of rat right atrium. The decrease of the MAP could be a result of a negative inotropic effect (Left ventricular pressure 75±4 mmHg basal vs. 51±1 mmHg with 100 mg/mL HAE Pq;  $p<0.05$ , n=5), and an enhancement of the vascular endothelial relaxation (29±9 % in endothelium-denuded aorta vs. 58±7 % in intact aorta; 100 mg/mL HAE Pq;  $p<0.001$ , n=5). Seven metabolites were isolate from HAE Pq, but four metabolites (10<sup>-4</sup> M) showed similar vasodilation to extract in intact rat aorta (111±4% with 100 mg/mL HAE Pq; n=5): 5,3',4'-trihydroxy-7-methoxyflavanone (99±4%), 5-hydroxy-7,4',3'-trimethoxyflavanone (79±11%), 3,5,4'-trihydroxy-7,8,3'-trimethoxyflavone (93±9%) and 5,4'-dihydroxy-3,7,8,3'-tetramethoxyflavone (119±11%). In conclusion, oral administration of Pq decreases the blood pressure in normotensive animals, in part, by decreasing of cardiac contractility and increasing vasodilation response. These findings support the use of Pq in folk medicine.



*Parastrephia quadrangularis* (Meyen) Cabrera

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA323

### Cancer cells “hijack” the services of platelets by phagocytic uptake

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#### Background:

It is well known that platelets support cancer cells in nearly every step of metastasis [1]. During tumor cell-induced platelet-activation platelets release a plethora of mediators, platelet-derived microparticles (PMPs) and exosomes that regulate cancer cell activities. Furthermore, activated platelets bind to cancer cells and form a platelet cloak around cancer cells as a physical barrier to protect the cancer cell from immune surveillance and facilitate cancer cells sustained adhesion to the vessel wall, extravasation and metastasis. Among these different ways platelets unfold their protumorigenic and prometastatic effect another way of transfer between platelets and cancer cells remained almost unnoticed: The phagocytic uptake of platelets.

#### Methods:

We used human non-small cell lung cancer cells (A549) and freshly isolated human platelets to study their interaction in suspension. A549 cells were incubated for different time intervals with fluorescently labelled platelets on a tilting table and under laminar flow conditions. Samples were analysed by confocal laser scanning microscopy (CLSM) and fluorescence-based flow cytometry (FACS).

#### Results:

Current research indicates a cloak-formation of platelets around circulating cancer cells. We never observed such cloak formation, instead, all experiments showed an uptake of platelets by A549 cells. The half-maximal uptake was observed after 38 min. This platelet uptake was completely abolished by the Dynamin inhibitor Dyngo4a. This, together with CLSM images indicate a phagocytic uptake of platelets by A549 cancer cells. Immunofluorescence staining of the platelet specific proteins CD42a (GP IX) and GPIIb/IIIa after A549-platelet incubation revealed an appearance of these proteins in the plasma membrane of A549 cells. CLSM scans of the FACS samples proved that there was no platelet attached to A549 cells. Staining of platelets with a FITC-labeled anti-CD42a

antibody prior to incubation and time-laps CLSM indicated an endosomal escape of CD42a.

We performed platelet uptake experiments with another highly metastatic cell line, the melanoma derived MV3 cells and with the non-cancerous, SV40 large T-antigen transformed, lung epithelial cell line (16HBE14o). Incubation with PKH67 stained platelets for 1h revealed an uptake of platelets in both cancerous cell lines but not in the immortalized cells. This preliminary data indicate that the uptake of platelets represents a more general mechanism by which cancer cells hijack the service of platelets

#### Conclusion:

Cancer cells phagocytose platelets to utilize platelet proteins through phagocytic recycling to their cell surface. This was shown for the cell adhesion proteins CD42a and GPIIb/IIIa. This novel insights in platelet-cancer cell interactions will allow further mechanistic elucidation of platelet-assisted metastasis and thus may contribute to the optimization of cancer therapies.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA324

### Tinzaparin and Tirofiban reduce tumor cell-platelet interaction: a single cell force spectroscopy analysis of two promising drugs

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Circulating tumor cells get in contact with blood components including a bidirectional interaction with platelets. Platelets can be activated by tumor cells in order to protect them from the immune surveillance and to facilitate adherence to the vessel wall. Thus, impeding the tumor cell-platelet interaction should reduce metastasis. In this study, we use single cell force spectroscopy, an AFM-based technique, to quantify and characterize the effect of two drugs in a tumor cell-platelet interaction model: Tinzaparin (TZP) and Tirofiban (TRF). Tinzaparin is a low molecular weight heparin, consisting of polysaccharides with chain lengths up to 6kDa. Tirofiban is an inhibitor of the main platelet receptor glycoprotein IIb/IIIa (GPIIb/IIIa) used in some cases of myocardial infarction. The anticoagulant drug heparin was shown to reduce metastasis in animal models and clinical studies without clear knowledge about its mode of action. Tirofiban has never been evaluated in the context of metastasis before.

Single cell force spectroscopy was performed with human non-small cell lung cancer cells (A549) and a dense layer of freshly isolated platelets. Force-distance curves were analyzed regarding maximum adhesion force (FA), total detachment work (WD), elasticity and tethering events. Tinzaparin (innohep®, Leo Pharma) and Tirofiban (Tirofiban Hexal®) were used in different concentrations to obtain dose-response-curves.

Tinzaparin as well as Tirofiban reduced the tumor cell-platelet interaction significantly in a dose dependent manner: The maximal reduction of FA and WD was -33.1% and -41.6% for TZP (N=2-4 cells, n=42-111) and -28.6% and -48.2% for TRF (N=2-4, n=50-89) respectively. The sigmoid fit of the dose-response relations reveals an  $IC_{50}$  of 0.01-0.04 U/mL for TZP and an  $IC_{50}$  of 0.002-0.25 ng/mL for TRF. P-selectin was identified



as target for TZP in paired experiments with TZP and anti P-selectin antibodies (N=6). Combined treatment with both drugs showed no additive effect on FA and WD (N=3). While both drugs do not alter significantly the elasticity of the involved cells, they show a decrease of membrane tether frequency ( $p < 0.001$ , Mann Whitney test). Membrane tether analysis revealed that TZP and TRF caused largely identical tether distributions. These results indicate that both drugs target the same mechanism. We assume that the effect of TZP is attributed to the inhibition of P-selectin-induced outside-in signaling which prevents activation of GPIIb/IIIa. Our study quantified the inhibitory effect of TZP and TRF on the tumor cell-platelet interaction at the single cell level for the first time. Since the contact of tumor cells with platelets is thought to facilitate metastasis, our results suggest that Tinzaparin and Tirofiban have the potential to serve as anti-metastatic drugs in low (subclinical) concentrations.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA325

### The Englerin A-sensing three charged residues for TRPC5 channel activation

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The classical transient receptor potential channel 5 (TRPC5), known as a nonselective cation channel (NSCC), has a crucial role in calcium influx. TRPC5 has been reported to be activated by muscarinic receptor activation and extracellular pH change, and inhibited by protein kinase C (PKC) pathway. Recent studies have also suggested that TRPC5 is extracellularly activated by englerin A (EA), but the mechanism remains unclear. The purpose of this study is to identify the EA-interaction sites in TRPC5 and thereby clarify the mechanism of TRPC5 activation. TRPC5 channels are over-expressed in human embryonic kidney (HEK293) cells. TRPC5 mutants were generated by site-directed mutagenesis. The whole-cell patch-clamp configuration was used to record TRPC5 currents. Western analysis was also performed to see the expression of TRPC5 mutants. To identify the EA-interaction site in TRPC5, we first generated pore mutants. When screening the mutants with EA, we observed the EA-induced current increases of TRPC5 abolished in K554N, H594N, and E598Q mutants. The current increases of other mutants were reduced in different levels. We also examined the functional intactness of the mutants that had no effect by EA with TRPC5 agonists such as carbachol (CCh) or GTP $\gamma$ S.

Conclusion: Our results suggest that the three residues, Lys-554, His-594, and Glu-598, in TRPC5 are responsible for direct interaction with EA inducing the channel activation. We also suggest that although other pore residues are not critical, they could partly contribute to the EA-induced channel activation.

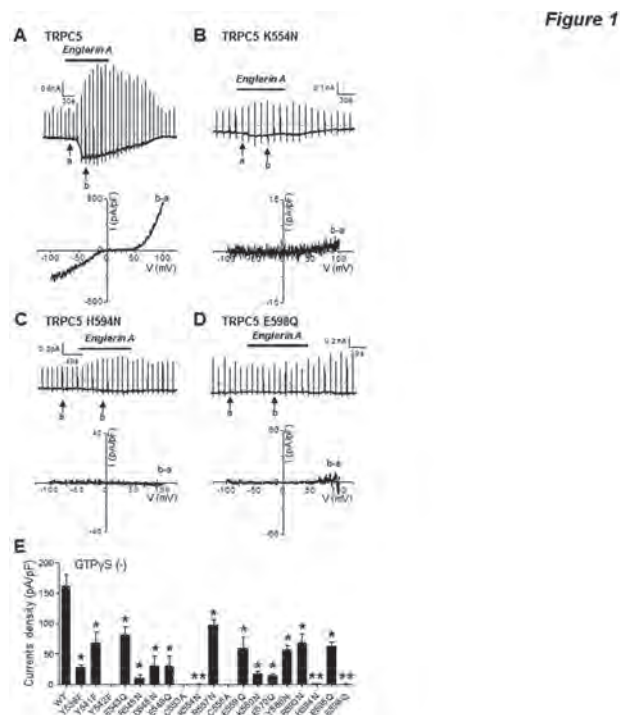


Figure 1

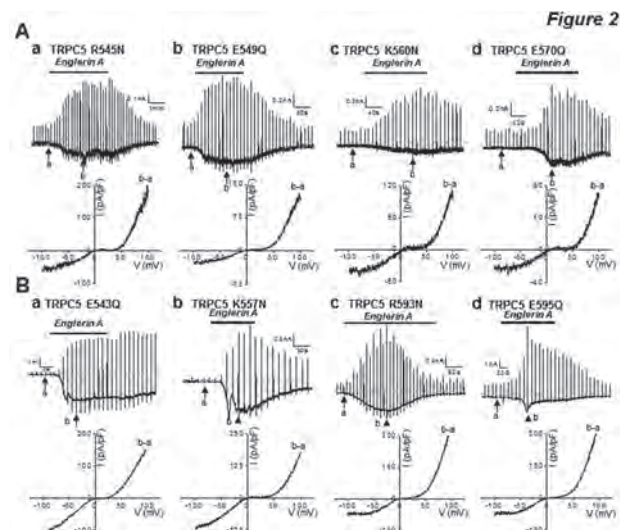


Figure 2

This study was supported by grants from the National Research Foundation of Korea, which is funded by the Ministry of Science, Information and Communication Technology (ICT), and Future Planning (MSIP) of the Korean government (grant number: 2015R1A2A1A05001756), and by the Education and Research Encouragement Fund of Seoul National University Hospital (I. So). S.J, J.K. and E.P. were supported by the BK21 plus program from the MSIP.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA327

### Increased contribution of the inward-rectifying K<sup>+</sup> channels to K<sup>+</sup>-induced dilation of cerebral arteries in Familial hemiplegic migraine type 2.

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Regulation of cerebral blood flow is of great importance for normal brain function. Neurovascular coupling is responsible for matching blood flow and neuronal activity. It has been suggested that release of K<sup>+</sup> from astrocytic endfeet, mediating neuronal signaling, is sensed by the adjacent arterioles and is a component of neurovascular signaling. Increased interstitial [K<sup>+</sup>] hyperpolarizes smooth muscle cells (SMC), dilate arterioles and increase blood flow to meet the metabolic demand of active neurons.

Familial hemiplegic migraine type 2 (FHM2) is a classical migraine with aura. FHM2 is associated with single-point mutations in the Na<sup>+</sup>,K<sup>+</sup>-ATPase  $\alpha 2$  isoform gene including G301R mutation. Dilation of the middle cerebral artery (MCA) in the affected hemisphere was shown in FHM2 patients during a migraine attack.

We hypothesize that this is due to abnormal K<sup>+</sup> signaling between astrocytes and SMC leading to changes in neurovascular coupling.

A mouse model bearing the G301R mutation phenocopying several FHM2-relevant disease traits was used in this study. MCA from both wild type and FHM2 mice were compared in-vitro in isometric myographs. Pre-constricted MCA were relaxed by increasing bath concentrations of K<sup>+</sup>, [K<sup>+</sup>]<sub>out</sub>. This protocol was repeated with 10  $\mu$ M ouabain, a Na<sup>+</sup>,K<sup>+</sup>-ATPase inhibitor, 30  $\mu$ M BaCl<sub>2</sub>, a K<sup>+</sup>-inward rectifying channel (K<sub>ir</sub>) inhibitor and after denudation of endothelium. In some experiments, membrane potential was measured simultaneously with isometric force recordings. Expression of K<sub>ir</sub> was semi-quantified immunohistochemically in whole-mount preparations and in Western Blots (WB).

Arteries from FHM2 mice had stronger K<sup>+</sup>-induced relaxation in comparison with WT at [K<sup>+</sup>]<sub>out</sub> in the range of 7 - 18 mM. There was no difference in the relaxation between the sexes of same genotype. Ouabain had no effect on the K<sup>+</sup>-induced relaxation of FHM2 arteries and only slightly inhibited the relaxation of WT. BaCl<sub>2</sub> suppressed the K<sup>+</sup>-induced relaxation almost completely in both groups. Accordingly, FHM2 arteries hyperpolarize stronger at 9 mM [K<sup>+</sup>]<sub>out</sub> than WT, and this difference was abolished by BaCl<sub>2</sub>. This was not due to changes in the K<sub>ir</sub> expression in SMC, as is evident from whole-mount staining and WB analyses. Removal of the endothelium suppressed the relaxation primarily in the FHM2 group and abolished the difference between the two groups.

FHM2-associated mutation in the Na<sup>+</sup>,K<sup>+</sup>-ATPase  $\alpha 2$  isoform leads to abnormal sensitivity of MCA to interstitial K<sup>+</sup> possibly due to increased contribution of endothelial K<sub>ir</sub>. This might be the mechanism behind hyperperfusion associated with the headache stage of migraine attacks.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA328

### Adenosine and L-arginine transport are modulated by an alkaline intracellular pH in human umbilical vein endothelial cells from gestational diabetes mellitus

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Human umbilical vein endothelial cells (HUVECs) from gestational diabetes mellitus (GDM) pregnancies show reduced transport of the endogenous vasodilator nucleoside adenosine via the human equilibrative nucleoside transporters 1 and 2 (hENT1/2) (1) but increased transport of the amino acid L-arginine via the human cationic amino acid transporter 1 (hCAT-1) and activation of the endothelial nitric oxide (NO) synthase (2). Increased NO generation is seen in an alkaline medium and elevated NO reduced the hENT1/2 expression and activity in HUVECs (3). Thus, we evaluated whether HUVECs from GDM show an altered intracellular pH (pHi) and its consequences on the hENT1/2 and hCAT-1 transport activity. HUVECs were isolated (collagenase digestion) from full term normal (n = 22) or GDM (n = 22) pregnancies collected at the Clinical Hospital CHRISTUS-UC (Chile). The investigation conforms to the principles outlined in the Declaration of Helsinki. HUVECs were cultured in medium 199 supplemented with sera (20%) and antibiotics up to passage 2 under standard conditions (37 °C, 5% O<sub>2</sub>, 5% CO<sub>2</sub>). The pHi was measured in cells loaded (10 min) with the fluorescent pH-sensitive probe 2,7-bicarboxyethyl-5,6-carboxyfluorescein acetoxymethyl ester (BCECF-AM, 12 mmol/L) and exposed to NH<sub>4</sub>Cl (0.1 – 20 mmol/L) (i.e., acid pulse). Basal and pHi recovery rate (dpHi/dt) were estimated (up to 360 s) in cells exposed to 5  $\mu$ mol/L 5-N,N-hexamethylene amiloride (HMA, Na<sup>+</sup>/H<sup>+</sup> exchangers general inhibitor), 0.1  $\mu$ mol/L zoniporide (NHE1 inhibitor), 0.1  $\mu$ mol/L concanamycin A (V-ATPases inhibitor), or 10  $\mu$ mol/L Schering (H<sup>+</sup>/K<sup>+</sup>-ATPase inhibitor). HUVECs from GDM show higher basal pHi compared with cells from normal pregnancies (pHi = 7.6  $\pm$  0.1 vs. 7.2  $\pm$  0.1, respectively) (values are mean  $\pm$  S.E.M., compared by unpaired ANOVA, P<0.04) in the absence of inhibitors. Zoniporide and HMA reduced the basal pHi (pHi = 7.3  $\pm$  0.2 and 7.2  $\pm$  0.3, respectively) in GDM to those in normal pregnancies. The dpHi/dt in GDM was higher (2.1  $\pm$  0.3 fold) than in normal pregnancies. Zoniporide and HMA reversed this increase in dpHi/dt to values in normal pregnancies. The GDM-reduced hENT1/2-mediated adenosine maximal transport capacity at basal pHi was reversed by NH<sub>4</sub>Cl-induced intracellular acidification (EC50 = 1.3  $\pm$  0.2 mmol/L NH<sub>4</sub>Cl). The GDM-increased hCAT-1-mediated L-arginine maximal transport capacity was reversed by zoniporide- and HMA-induced intracellular acidification to values normal pregnancies. In conclusion, HUVECs from GDM pregnancies show an alkaline pHi which results in inactivation of hENT1/2 but increased hCAT-1 activity.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA329

### Hematopoietic Hypoxia-inducible factor 2 $\alpha$ deficiency ameliorates pathological retinal neovascularization via modulation of endothelial cell apoptosis.

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A hallmark of proliferative retinopathies, such as retinopathy of prematurity (ROP), is pathological neovascularization triggered by hypoxia. Synergisms between hypoxia, angiogenesis and the innate immune response have been suggested, which could be integrated at the level of the hypoxia-inducible factors (HIFs). Interestingly, HIF2 $\alpha$  is highly expressed in endothelial and hematopoietic cells, e.g. macrophages and microglia.

Here we studied the role of HIF2 $\alpha$  in hematopoietic cells for pathological retina neovascularization in the murine model of ROP (oxygen-induced retinal neovascularization). Briefly, seven-days old mice were exposed to 75% O<sub>2</sub> for 5 days, and then returned to normoxic conditions. Eyes were collected at postnatal day 17 (P17) and neovascularization was quantified by counting of epiretinal nuclei in PAS stained histological sections (Smith et al., 1994). In order to study hematopoietic specific deletion of Hif2 $\alpha$ , Vav-cre (Stadtfeld et al., 2005) mice were crossed with Hif2 $\alpha^{fl/fl}$  (Gruber et al., 2007) mice. Littermates from Vav-Cre- Hif2 $\alpha^{fl/fl}$  (Hif2 $\alpha$ -HWT) and hematopoietic Hif2 $\alpha$  deficient Vav-Cre+ Hif2 $\alpha^{fl/fl}$  (Hif2 $\alpha$ -HKO) mice were compared in the animal experiments (approved by the Landesdirektion Sachsen, Germany).

We found that hematopoietic-specific deficiency of HIF2 $\alpha$  ameliorated the pathological neovascularization in the ROP-model (n=21-26; HWT 37.57 $\pm$ 3.21, Hif2 $\alpha$ -HKO 23.7 $\pm$ 3.74%; P<0.01; 2-tailed t-test), which was associated with enhanced endothelial cell apoptosis. Enhanced apoptosis was concluded from increased cleaved caspase-3 staining in the pathological endothelial cells in retinal whole mounts (n=8; HWT 100 $\pm$ 15.18%, Hif2 $\alpha$ -HKO 245.7 $\pm$ 23.17%; P<0.001; 2-tailed t-test). The latter finding resulted from an up-regulation of the apoptosis inducer FasL in Hif2 $\alpha$ -deficient microglia (assessed by qPCR and Western blot). Consistently, pharmacological inhibition of FasL by an intravitreal injection of a FasL-blocking antibody (at P14 of the ROP: 1  $\mu$ g in the right eye and isotype control antibody in the left eye; mice anaesthetized with 0.03 mg/g BW Ketamin and 1  $\mu$ g/g BW Dormitor) reversed the reduced pathological neovascularization due to hematopoietic-specific Hif2 $\alpha$  deficiency (n=6-12 mice; Hif2 $\alpha$ -HWT vs. Hif2 $\alpha$ -HKO plus FasL-blocking antibody not significant; 2 tailed t-test).

Taken together, our experiments identified that hematopoietic cell HIF2 $\alpha$  contributes to pathological retina angiogenesis. Our findings not only provide novel insights regarding the complex interplay between immune cells and endothelial cells in hypoxia-driven retina neovascularization but may also have implications for novel therapeutic approaches in proliferative retinopathies.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA330

### Vascular Scrib Limits Atherosclerosis by Maintaining Arterial Integrity

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The polarity protein Scrib contributes to planar cell polarity. Because endothelial Scrib facilitates endothelial inflammatory signaling, we hypothesized that Scrib contributes to atherosclerosis development.

Spontaneous atherosclerosis development was determined in tamoxifen-inducible Scrib conditional knockout (KO) mice, crossed into ApoEKO mice, under high fat diet (HFD). Accelerated atherosclerosis was determined in the partial carotid artery ligation model during HFD treatment. Deletion of Scrib resulted in increased vascular lumen loss in the partial ligation model. Similarly, spontaneous atherosclerosis-development was accelerated in the brachiocephalic trunk of Scrib ApoE double KO animals compared to Cre negative littermates. Mechanistically, Scrib KO decreased acetylcholine- as well as flow-induced vascular relaxation of isolated arteries and flow-induced AKT-phosphorylation of carotid endothelial cells (CEC). As determined by next generation sequencing of CEC, knockout of Scrib reduced the expression of eNOS, shear stress-responsive genes and of cell adhesion molecules. In line with altered cell adhesion, basal lung permeability to Evans blue was increased in endothelial specific Scrib deficient mice. Immune cell extravasation into the lung was elevated after endothelial specific Scrib KO (2h LPS treatment).

Conclusion: Scrib maintains arterial integrity and has anti-atherosclerotic functions.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA331

### The NADPH oxidase Nox4 promotes endothelial differentiation from murine induced-pluripotent stem cells

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Nox4 is a constitutively active NADPH oxidase that produces H<sub>2</sub>O<sub>2</sub>. It is expressed in endothelial cells, and has been shown to contribute to differentiation of mesenchymal cells. Therefore, we hypothesize that Nox4 promotes endothelial differentiation.



MEFs from WT and Nox4<sup>-/-</sup> mice were reprogramed into iPSCs and then differentiated into endothelial cells by VEGF and BMP4 stimulation. In the course of differentiation, Nox4 expression increased in WT cells. Nox4 knockout results in a prolonged expression of pluripotency markers and in a diminished expression of endothelial markers, such as Flk-1 and eNOS. Differentiated iPSC derived endothelial cells of Nox4<sup>-/-</sup> mice showed lower tube formation and sprouting capacity. A matrigel plug assay revealed lower capacity of Nox4<sup>-/-</sup> iPSC-ECs to integrate into a newly formed vascular network. As a potential mechanism, we identified an increased K27 triple methylation at histone H3. Demethylation of H3 is mediated by Jmjd3 and methylation by Ezh2, both of which were not differentially expressed in Nox4<sup>-/-</sup> vs. WT cells. Interestingly a BIAM switch assay identified Jmjd3 to be less oxidized in Nox4<sup>-/-</sup> cells. We therefore conclude Nox4 oxidizes and thereby activates Jmjd3. This then results in decreased levels of H3K27me3 and promotes endothelial differentiation.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

### PCA332

#### **Laser Doppler Perfusion Imaging can be used for *in vivo* continuous perfusion assessment**

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The *in vivo* assessment of the microcirculatory status is becoming increasingly important for studying cardiovascular function. Among several technologies available for the noninvasive perfusion quantification, Doppler-based flowmetry (LDF) and perfusion imaging (LDPI) techniques are often employed. LDPI is almost exclusively used for the perfusion assessment over large tissue areas, however, it can also be used in a continuous mode, recording flow in a much smaller area over time, similar to LDF. Despite the proximity between these techniques, these signals have not been compared this way. Our objective was to explore the LDPI 'continuous mode' from the peripheral circulation of anesthetized mice and compare the obtained values with LDF. We used a group of C56/BL6 male mice (n=5, 7 w.o., 25.0 ± 0.8g) anesthetized with a ketamine (137.5 mg/kg) and xylazine (11.0 mg/kg) mixture (i.p.). All procedures involving animal experimentation were performed in accordance with the current ethical guidelines for the protection of animals used for scientific purposes in the EU. Twenty minutes after induction, LDF and LDPI signals were acquired for 30 minutes on the plantar aspect of the lower limb. The acquisition sampling rate of LDPI (left limb) was 40 Hz, and 128 Hz (later downsampled to 40 Hz) for LDF (right limb). The wavelet transform (WT) was applied to both signals to obtain their spectral decomposition. Both LDF and LDPI signals showed five spectral bands in similar spectral regions, which are compatible with the cardiorespiratory (LDF: 4.7-1.8Hz; LDPI: 4.7-2.8Hz), myogenic (LDF: 0.1-0.048Hz; LDPI: 0.1-0.054Hz), sympathetic (LDF: 0.048-0.026Hz; LDPI: 0.054-0.028Hz), endothelial NO-dependent (LDF: 0.013-0.0071Hz LDPI: 0.013-0.0070Hz) and NO-independent (LDF:

0.0067-0.0047Hz; LDPI: 0.0070-0.0050Hz) components. Therefore, each component amplitude was comparable for both techniques. Since both signals were acquired under the same wavelength and band width parameters, we conclude that the observed differences in the spectral bands' width are explained by the differences in the paths taken by the emitted light. With LDPI, light travels through room air before crossing the skin, while with LDF it directly crosses the skin. These similarities between these techniques suggest that LDPI is a suitable alternative to LDF for continuous perfusion measurements.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

### PCA333

#### **Application of Texture Analysis to the spectral decomposition of Photoplethysmography vascular signals**

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Texture analysis (TA) allows the characterization of features in image patterns as mathematical descriptors. One of TA's most relevant descriptors is entropy, defined as the randomness or "disorder" of an image, showing a "coarse" or "rough" appearance, with irregular patterns. Entropy, applied to physiological signal processing, refers to the randomness of a signal over time, e.g. the competence of a biological system. Physiological signals are often complex and multiscaled, and for appropriate analysis are often decomposed into their main spectral components with mathematical tools such as the wavelet transform (WT). One of the outputs of this analysis is the 2D scalogram, a time-scale representation where the different components of the signal appear as bands of different textural patterns. Our objective was to apply texture analysis functions to assess the textural entropy (TE) of WT-derived photoplethysmography (PPG) scalograms, and compare this with the complexity index (CI), a measure of signal entropy calculated with Multiscale Entropy Analysis (MSE). PPG signals were collected from both feet of twelve healthy subjects (both sexes, 26.0 ± 5 y.o.) lying supine while evoking a standard venoarteriolar reflex (VAR) test lying supine, in three phases – 10 min baseline, 10 min with one foot (test) lowered, and 10 min recovery in the initial position. All procedures complied with the Helsinki Declaration and subsequent amendments. The entropy of each PPG spectral component was quantified by two different approaches (i) as the complexity index (CI) of each component's frequency interval, and (ii) from the signal's 2D scalograms, obtained from TA. A p<0.05 was adopted. All components had significantly different CI between feet during VAR, and all CI decreased significantly in both feet, except for the cardiac component in the control foot. TA revealed a decrease in the TE of most components in both feet, but only the changes in the respiratory, NOd and NOi components were significant. All components, except the sympathetic, revealed significantly different TE between feet. MSE suggests that VAR decreases the randomness of most PPG signal components. TA seems to suggest the same, further reinforcing the conclusion that VAR induces a change in the competence of vascular regulation mechanisms. Therefore,

TA seems to be an adequate instrument to further explore the oscillatory properties of vascular signals.

Study supported by FCT-IP, project UID/DTP/04567/2016

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

### PCA334

#### **Hypoxic stabilized p22phox modulates vascularization in vitro and in vivo and promotes pulmonary hypertension**

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**Background:** Hypoxia and reactive oxygen species (ROS) have been shown to play a role in the pathogenesis of pulmonary hypertension, a potentially fatal disorder characterized by pulmonary vascular remodeling, elevated pulmonary arterial pressure and right ventricular hypertrophy. The multiprotein enzymes of the NADPH oxidase family are an important source of cardiovascular ROS. However, how they are linked in the context of hypoxia-induced pulmonary hypertension is not completely understood. **Aims:** We therefore investigated the role of p22phox, which is required for ROS production of the majority of NADPH oxidases, in the response to hypoxia in vitro and in vivo.

**Results:** We found that hypoxia decreased ubiquitinylation and proteasomal degradation of p22phox which was dependent on prolyl hydroxylases and the E3 ubiquitin ligase pVHL, and resulted in p22phox stabilization and accumulation. Hypoxic induction of p22phox contributed to ROS generation after reoxygenation, and promoted endothelial cell proliferation, migration and angiogenesis under hypoxia. On the other hand, in mice harboring a point mutation (Y121H) in the p22phox gene which resulted in decreased p22phox stability and subsequent loss of this protein, sprouting of pulmonary vessels and in vivo angiogenesis were reduced. Mechanistically, p22phox promoted HIF activity and stabilization of HIF1 $\alpha$ , the major cellular regulator of hypoxia adaptation. This response was mediated by reduction of ascorbate and Fe(II) levels which are required for activity of prolyl hydroxylases. Increased levels of p22phox, HIF1 $\alpha$  and ATF4 – a mediator of the integrated stress response – were also detected in lungs and hearts from wild-type mice with hypoxia-induced pulmonary hypertension. On the contrary, p22phox deficient mice were protected against the development of hypoxia-induced pulmonary hypertension. Concomitantly, HIF1 $\alpha$ , and ATF4 – levels were decreased, while the levels of miR-140, a tumor suppressor miRNA which protects against pulmonary hypertension, were increased in lungs and hearts from hypoxic p22phox-deficient mice.

**Conclusion:** These data identify p22phox as important regulator of the hypoxia response in vitro and in vivo. Inhibition of p22phox-dependent NADPH oxidases might be a promising therapeutic strategy against pulmonary hypertension induced by hypoxia and possibly other conditions.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

### PCA335

#### **Influence of high-density-lipoproteins on the endothelial glycocalyx**

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The endothelial glycocalyx (eGC) is a negatively charged, carbohydrate-rich layer that lines the luminal side of the vascular endothelium. As the primary physical barrier between blood and the vessel, the eGC serves as a firewall against endothelial hyperpermeability and leukocyte recruitment in systemic inflammatory diseases. High-density lipoproteins (HDL) are known to be atheroprotective by amongst others reducing endothelial inflammation through inhibiting cell apoptosis and enhancing availability of nitric oxide. If HDL exerts eGC-protecting effects as well is currently unknown. Here we hypothesize that HDL or specific HDL-bound factors such like Sphingosin-1-phosphate (S1P) may improve eGC integrity.

To study the influence of HDL on eGC integrity, we determined thickness and stiffness of the eGC in endothelial cells (EA.hy926) using live-cell atomic force microscopy (AFM). Additionally, immunofluorescence intensities of main constituents of the eGC such as heparan sulphate were measured with confocal microscopy. To study the potential eGC-protective effect of HDL and S1P, endothelial cells (EA.hy926) were incubated with physiological concentration of HDL (0.5 mg/ml) or S1P (1  $\mu$ M). HDL used for *in-vitro* incubation experiments was isolated from serum of apparently healthy volunteers with ultra-centrifugation. To identify potentially involved S1P-receptors we coincubated EA.hy926 with S1P and the specific S1P1 and -3 receptor (S1PR) inhibitor (VPC, 10  $\mu$ M).

Incubation of endothelial cells with HDL or S1P alone is sufficient to maintain eGC integrity compared to control conditions (serum-free buffer) (Figure 1). Accordingly, immunofluorescence intensity of heparan sulphate is preserved by HDL or S1P, whereas immunofluorescence was markedly decreased by control conditions. However, the protective effect of HDL or S1P was completely abolished by addition of W146.

These results demonstrate that HDL from healthy patients is sufficient to maintain the eGC. eGC-strengthening effects may be mediated through HDL-bound S1P. Our data suggest that S1P mediates its eGC-enhancing effects via S1P1 or -3R. S1P concentration is known to be reduced in systemic inflammatory diseases like diabetes mellitus. The eGC-protective role of S1P needs to be addressed in further studies.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA336

### Pregestational maternal obesity-associated human umbilical vein endothelial dysfunction results from endoplasmic reticulum stress

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Pregestational maternal obesity (PGMO) is associated with an adverse cardio-metabolic outcome in the offspring (1). A key step in the development of cardio-metabolic alterations is the endothelial dysfunction (2). Endothelial dysfunction associates with endoplasmic reticulum (ER) stress (3), a condition seen in human umbilical vein endothelial cells (HUVECs) from PGMO (4). However, whether HUVECs from PGMO show ER stress-dependent endothelial dysfunction is unknown. The aim of this study was to determine whether HUVECs from women with PGMO show endothelial dysfunction due to ER stress. HUVECs were isolated (collagenase digestion) from full term normal (n = 10) or PGMO (n = 12) pregnancies collected at the Clinical Hospital UC-CHRISTUS (Chile) after informed consent approved by the UC-CHRISTUS ethics committee. The investigation conforms to the principles outlined in the Declaration of Helsinki. Cells (passage 3) were incubated (8 h) without or with tunicamycin (5 µmol/L, ER stress inducer), tauroursodeoxycholic acid (TUDCA, 100 µmol/L, ER stress reducer), or both. L-Arginine transport kinetics (0-1000 mmol/L L-arginine, 3 µCi/mL L-[<sup>3</sup>H]arginine, 1 min incubation, 37°C), the protein abundance of the human cationic amino acids transporter-1 (hCAT-1), total endothelial nitric oxide synthase (eNOS), eNOS phosphorylated in serine<sup>1177</sup> (pSer<sup>1177</sup>-eNOS) and threonine<sup>495</sup> (pThr<sup>495</sup>-eNOS) protein abundance was determined by western blot. NOS activity was estimated in cells preloaded with 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM) in the absence or presence of N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME, 100 µmol/L, NOS inhibitor). Values are mean ± S.E.M., compared by unpaired ANOVA, P<0.05. Cells from PGMO showed higher maximal L-arginine transport capacity (V<sub>max</sub>/K<sub>m</sub>) (1.5 ± 0.3 fold), hCAT-1 protein abundance (1.6 ± 0.3 fold), and pThr<sup>495</sup>-eNOS (1.7 ± 0.3 fold) compared with cells from normal pregnancies. However, pSer<sup>1177</sup>-eNOS and NO generation were reduced (42 ± 12 and 42 ± 15%, respectively) in HUVECs from PGMO compared with normal pregnancies. TUDCA blocked the effects of PGMO. Incubation of cells from normal pregnancies with tunicamycin increased the V<sub>max</sub>/K<sub>m</sub> (2.2 ± 0.4 fold) and the hCAT-1 protein abundance (1.5 ± 0.2 fold) but reduced the NO generation (37 ± 20%). In conclusion, HUVECs from women with PGMO show endothelial dysfunction due to PGMO-induced ER stress.

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FONDECYT 1150377, CONICYT (RV-L, MS, LSi), Vicerectorate of Research (RV-L, MS, LSi) and Faculty of Medicine, PUC (LSi) PhD fellowships (Chile), UMCG, U Groningen (LSi) PhD fellowship (The Netherlands).

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PCA337

### DIFFERENT ESTROGEN RECEPTORS MEDIATE miRNA REGULATION BY ESTRADIOL IN HUMAN ENDOTHELIAL CELLS

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Estrogen vascular, and mostly endothelial, effects are mediated through signalling pathways involving both classical (ERα and ERβ) and G-protein mediated (GPER) estrogen receptors (ER). Recently, microRNA (miRNA) have emerged as key regulators of endothelial function. Since there are few studies focused on the mechanisms involving estrogen-regulation of miRNA expression in endothelial cells, we aimed to discover the role of the different estrogen receptors (ERα, ERβ and GPER) in the endothelial regulation of miRNA expression by estradiol (E2).

Studies were performed in primary human umbilical vein endothelial cells (HUVEC) exposed to a physiological concentration of E2 (1 nmol/L) for 24 hours. Changes in global miRNA expression were determined by miRNA microarrays and validated by qRT-PCR. The role of different ER on the E2-modified miRNA was also analysed by qRT-PCR by using specific ER agonists (PPT for ERα, DPN for ERβ, G1 for GPER) and antagonists (ICI182780 as non-selective antagonist for ERα and ERβ, MPP for ERα and G15 for GPER).

E2 modified the expression of 114 miRNA (p < 0.05): 70 miRNA were up-regulated and 44 down-regulated. To validate microarray findings, qRT-PCR was performed using another set of RNA samples. The most differentially expressed miRNA obtained in microarrays were selected and analysed by qRT-PCR: miR-30b, miR-487a, miR-4710 and miR-501 were upregulated whereas miR-378h and miR-1244 were downregulated. When studied the contribution of ER, results demonstrated ERα played a central role in the E2-modulation of miR-30b-5p, miR-378h and miR-487a-5p. In addition, E2 induction of miR-4710 was mediated through GPER, whereas miR-1244 expression depends on the three ER. E2-induced miR-501-3p expression was reverted by ICI, but not by MPP or G15, suggesting a possible role of ERβ. Unpredictably, miR-501-3p expression was not changed after isolated exposition with the different agonists.

In conclusion, our results indicate that miRNA may act as crucial epigenetic regulators of gene expression in E2-treated human endothelial cells and make an important contribution on the role of ER in vascular actions mediated by E2. Moreover, they also could provide insight into the physiological mechanisms by which sex hormones levels can contribute to sex-differences in vascular function.

This work was supported by the Spanish Ministerio de Economía y Competitividad, Instituto de Salud Carlos III – FEDER-ERDF (grant FIS PI16/00229). D.P-C is founded by a Generalitat Valenciana/FSE (Fondo Social Europeo) fellowship (grant number APOSTD/2018/A/144).

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.



PCA338

### Modulation of endothelial chromatin remodelling complexes by long non-coding RNAs

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Long non-coding RNAs modulate chromatin remodelling complexes and thereby regulate the epigenetic landscape and gene expression. Complexes are recruited to their genomic targets where they either covalently modify histones or alter nucleosomal structure by utilising ATP<sup>1</sup>. However, the mechanisms governing the modulation of chromatin remodelling complexes and their recruitment to gene-specific promoters are largely unknown. We hypothesise that complexes, through a particular cell-type- and condition-specific subunit composition, are modulated by an even more specific network of lncRNAs; as is the case for the notable Polycomb Repressive Complex 2 (PRC2), whose main functions require lncRNAs such as Xist, HOTAIR and Kcnq1ot1<sup>2</sup>. We recently identified the lncRNA MANTIS as a crucial component of the endothelial SWI/SNF complex. MANTIS was found to stabilise the interaction between the core ATP-ase, BRG1, and BRG1-associated factor 155 (BAF155). The scaffolding role of MANTIS maintained the ATP-ase activity of the complex, its targeting to angiogenesis-associated genes and, ultimately, vascular function<sup>3</sup>. This highlights the importance of lncRNAs in the normal functioning of a chromatin remodelling complex. Subsequently, a spheroid outgrowth assay after siRNA against BRG1 clearly demonstrated a fundamental role for BRG1 in endothelial function. Additional protein subunits of endothelial SWI/SNF were identified by mass spectrometry and one of them, Double PHD Fingers 2 (DPF2), was also found to uphold endothelial function. RNA-IP-sequencing was employed in order to identify novel RNA interaction partners of different chromatin remodelling proteins such as EZH2, BRG1, BRM, SMARCA5 and BAF170. We identified lncRNAs including EPHA1-AS1, CACNA1G-AS1, MALAT1, NEAT1 and BARX1-AS1 that appear to have significant implications in the endothelium. Importantly, there was an overlap of lncRNA profile for the SWI/SNF complex members BAF170, BRM and BRG1, but not with the PRC2 member, EZH2. Since lncRNAs are already known to modulate chromatin remodelling complexes we will investigate this relationship further in the context of the vascular system to dissect specific endothelial gene programs under physiological and pathophysiological conditions.

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PCA339

### Effect of angiotensin II type 1 receptor antagonist losartan on expression and activity of antioxidative enzymes, cell adhesion molecules and proinflammatory cytokines in cerebral blood vessels of Sprague-Dawley rats

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**INTRODUCTION:** Present study has assessed the effects of losartan (angiotensin II type 1 (AT-1R) receptor antagonist) on gene expression of antioxidative enzymes (Cu/Zn SOD, Mn-SOD, EC-SOD, GPx1, GPx4, CAT) and NOX1, gene expression of endothelial cells adhesion molecules (ICAM-1, VCAM-1, E-selectin, endocan), pro-inflammatory cytokines (IL-1, IL-6, TNF- $\alpha$ ) and chemokine GRO- $\alpha$  (CXCL1) in cerebral vessels, and on systemic oxidative stress.

**METHODS:** Healthy male Sprague-Dawley rats were fed low salt diet for 7 days (0.4% NaCl; LS group) or LS diet+losartan (40 mg/day in water ad libitum). Following dietary protocol, rats were anesthetized with ketamine (75 mg/kg) and midazolam (2.5 mg/kg) and sacrificed by decapitation. Oxidative stress was assessed by measurement of 8-iso prostaglandin concentration and antioxidative enzymes activity assay in serum samples by spectrophotometry. mRNA levels were determined by quantitative real-time PCR in surface cerebral vessels. Data were analysed by t-test, presented as mean  $\pm$  SD. All experimental procedures are conformed to the European Guidelines for the Care and Use of Laboratory Animals (directive 86/609) and were approved by the local and national Ethical Committee (No. 525-10/0255-15-6).

**RESULTS:** 8-iso prostaglandin concentration was significantly increased in LS+losartan group (0.31  $\pm$  0.08 ng/ml) compared to LS group (0.23  $\pm$  0.08 mg/ml). Expression of GPx1 and Cu/Zn SOD was significantly decreased in LS+losartan group (GPx1 0.63  $\pm$  0.24, Cu/Zn SOD 0.73  $\pm$  0.20) vs. LS group (GPx1 1.23  $\pm$  0.38, Cu/Zn SOD 1.09  $\pm$  0.18). Expression of MnSOD, EC-SOD, CAT and NOX1 was significantly increased in LS+losartan group (MnSOD 2.53  $\pm$  0.66, EC-SOD 1.22  $\pm$  0.34, CAT 4.89  $\pm$  1.55, NOX1 0.006  $\pm$  0.003) compared to LS (MnSOD 0.96  $\pm$  0.21, EC-SOD 0.61  $\pm$  0.20, CAT 0.87  $\pm$  0.11, NOX1 0.003  $\pm$  0.003). GPx4 expression in vessels and serum GPx activity was not different between groups. Serum enzymes' activity (U/mgP) of SOD (1.97  $\pm$  0.53 vs. 1.57  $\pm$  0.24) and CAT (0.85  $\pm$  0.25 vs. 0.55  $\pm$  0.32) was significantly decreased in LS+losartan group. Expression of chemokine GRO- $\alpha$  was significantly increased in LS+losartan group (1.29  $\pm$  0.53 vs. 3.69  $\pm$  1.14) without significant change in expression of other cytokines ( $p > 0.05$ ). Expression of VCAM-1 was significantly increased in LS+losartan group (1.91  $\pm$  0.58) vs. LS group (1.12  $\pm$  0.34).

**CONCLUSIONS:** Blockade of AT-1 receptor causes increased vascular and systemic oxidative stress, possible due to increased expression of NOX1 (a source of superoxide), and decreased activity of antioxidative enzymes despite their increased expression in vessels. In addition, blockade of AT1 receptor leads to endothelial cell activation as their phenotype changes to neutrophil-attractive (increased expression of VCAM-1 and GRO- $\alpha$ ).

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PCA340

**Isometric flexor muscle activity alters microcirculatory perfusion in the human lower limb**M.M. Florindo<sup>1,3</sup>, H. Silva<sup>1,2</sup>, S. Nuno<sup>1</sup> and L. Monteiro Rodrigues<sup>1,2</sup><sup>1</sup>CBIOS (Research Center for Biosciences and Health Technologies), U Lusófona, School of Health Sc & Technologies, Lisboa, Portugal, <sup>2</sup>Pharmacol. Sciences Departm., U Lisboa, Faculty of Pharmacy, Lisboa, Portugal and <sup>3</sup>Portuguese Red Cross Health School, Avenida de Ceuta, Edifício Urbiceuta, 1300-125 Lisboa, Portugal, Lisboa, Portugal

The 'muscle pump' is a known determinant of vascular physiology, particularly in regard to venous return. Only a few studies to date have addressed the impact of the muscle pump on microcirculation. Our objective was to compare the effect of the isometric activity of two antagonistic muscle groups (plantar flexors and dorsal flexors) on the lower limb microcirculation. Six healthy volunteers ( $31 \pm 9$  years old) both sexes (three female and three male) were selected after informed written consent. All procedures complied with the Helsinki Declaration and subsequent amendments. Two sequential postural changes in the standing position were requested, plantar flexion (PF) and dorsal flexion (DF), with a 10 minutes washout period in between, each consisting of three phases - 5 minutes baseline (Phase 1), 1 minute isometric muscle activity (Phase 2) and 5 minutes recovery at the initial position (Phase 3). Blood perfusion was assessed in both feet in the medial arch by photoplethysmography (PPG) and laser Doppler flowmetry (LDF). Pulse rate (PR) was also obtained from the PPG signal. Descriptive and comparative statistics were applied ( $p < 0.05$ ). PPG and LDF registered opposite perfusion responses during Phase 2 of both protocols. The LDF signal increased significantly ( $p = 0.028$  for both DF and PF), while PPG showed a significant decrease ( $p = 0.028$  for DF and  $p = 0.027$  for PF) for the same movements. PR did not change significantly in any of the posture changes. Additionally, the PPG signal was significantly higher in PF ( $p = 0.002$ ) during Phase 2 but no differences could be observed with LDF and PR. These opposite responses obtained with LDF and PPG, which share the same optical principle, suggest that they detect different perfusion phenomena, likely because they operate at different depths. Therefore, the selection of the appropriate technology for these purposes is a critical experimental issue. Our results also suggest that, in the present experimental conditions, both PF and DF impact microvascular perfusion, pointing to the role of muscular activity in local vascular regulation during motion.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCA341

**Endothelial VEGFR2 KO during CFA induced inflammation is detrimental to murine tibiotalar cartilage integrity**E.D. Vittersø<sup>1,2</sup>, N. Beazley-Long<sup>1,2</sup> and L. Donaldson<sup>1,2</sup><sup>1</sup>Life Sciences, University of Nottingham, Nottingham, UK and <sup>2</sup>Arthritis Research UK Pain Centre, Nottingham, UK**Introduction**

The progressive rheumatic disease inflammatory arthritis affects up to 1.1% of the population. The pathophysiology of the disease includes chronic pain, joint inflammation, synovial hyperplasia, degraded bone and cartilage, and dysregulated angiogenesis (Biniecka et al. 2014; Ursum et al. 2013). We hypothesised that endothelial knockout (KO) of vascular endothelial growth factor receptor 2 (VEGFR2) would affect cartilage pathology in Freund's complete adjuvant (CFA) induced inflammation in mice.

**Methods**

Tie2CreER<sup>T2</sup>xVEGFR2<sup>fl/fl</sup> or wildtype mice were dosed with 5mg tamoxifen or vehicle (intra-peritoneal, in sunflower oil, 1mg per day over 5 days) to induce knockout of endothelial VEGFR2. One week later CFA (160mg in 80mL mineral oil) was injected subcutaneously around the ankle joint in KO or control mice under brief isoflurane anaesthesia (2-3% in O<sub>2</sub>). On day 14 post injection animals were killed, and ankle joints removed and decalcified. Ankles were sectioned, and treated with Toluidine blue, staining nuclei blue and chondrocytes purple. Severity of cartilage loss in the presence of chondrocytes (bleaching), cartilage surface smoothness, and cartilage thickness was graded out of 4 (as described by Pettit et al., 2001), and analysed with one-way ANOVA and Tukey's multiple comparisons test. All animal procedures were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986/Amendment Regulations 2012 and with University of Nottingham Animal Welfare and Ethical Review Group approval.

**Results**

An overall significantly exacerbated cartilage pathology was found in CFA injected Cre<sup>+</sup>Tamoxifen<sup>+</sup>KO mice ( $n = 5$ , mean  $\pm$  SEM =  $2.9 \pm 0.4$ ) compared with sham injected KO controls ( $n = 4$ ,  $0.6 \pm 0.4$ ;  $p < 0.0001$ ). After further investigation this was found to be caused by significantly exacerbated bleaching in the CFA injected mice ( $3.5 \pm 0.4$ ) compared with sham controls ( $0.4 \pm 0.4$ ;  $p < 0.0001$ ), in addition to a reduced cartilage thickness in CFA injected mice ( $3.0 \pm 0.4$ ) compared with sham controls ( $0.4 \pm 0.4$ ;  $p = 0.001$ ). No significant differences were found for surface smoothness.

**Conclusions**

Endothelial VEGFR2 KO is detrimental to tibiotalar cartilage integrity during CFA induced inflammation.

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PCA342

**Histone deacetylases are differentially oxidized by NADPH oxidases**T. Schader<sup>1</sup>, O. Löwe<sup>1</sup>, J. Backs<sup>2</sup> and K. Schröder<sup>1</sup><sup>1</sup>*Institut für Kardiovaskuläre Physiologie, Frankfurt/Main, Germany* and <sup>2</sup>*Molecular Cardiology and Epigenetics, Heidelberg, Germany*

NADPH oxidases produce reactive oxygen species differing in localization, type and concentration. Only Nox4 directly produces H<sub>2</sub>O<sub>2</sub> while the others such as Nox5 produce superoxide anions. In contrast to superoxide, H<sub>2</sub>O<sub>2</sub> can directly oxidize cysteine residues, a post-translational modification that influences activity, stability, localization and protein-protein interactions of the affected protein. Importantly, knock down of Nox4, but not of other Noxes, reduces angiogenesis after hindlimb ischemia in mice<sup>1</sup>. Moreover, class IIa histone deacetylases (HDACs) also regulate angiogenesis through deacetylation and repression of target proteins<sup>2,3</sup>. We hypothesize that Nox4 derived H<sub>2</sub>O<sub>2</sub> specifically oxidizes HDACs which disrupts repressor complexes. Thereby, Nox4 positively influences endothelial function and angiogenesis. Here we aim to analyze if HDACs can be differentially oxidized by Nox4. As an artificial system inducible Nox4 and Nox5 overexpressing HEK cells were generated. Nox4 expression and activity is achieved in a tetracycline dependent manner, while Nox5 is stably overexpressed and acutely activated by PMA. As target proteins we analyzed class IIa HDACs 4 and 5. BIAM switch assays revealed enhanced oxidation of HDAC4 and 5 upon Nox4 upregulation in a DPI sensitive manner. In contrast, Nox5 derived superoxide did not affect HDAC oxidation. Moreover, tube formation assays with HUVECs were performed to assess the angiogenic function of HDAC4. Overexpression of HDAC4 reduced the tube formation ability which was restored using a redox dead HDAC4 mutant. We conclude that Nox4 oxidizes class IIa HDACs which potentially leads to activation of repressed pro-angiogenic factors.

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PCA343

**CASEIN KINASE 1, A POTENTIAL REGULATOR OF THE MINERALOCORTICOID RECEPTOR**

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The mineralocorticoid receptor (MR) is a ligand-dependent transcription factor. Upon binding of aldosterone the MR translocates into the nucleus, binds to GRE elements and promotes the transcription of its target genes.

Clinical trials have identified the MR as a mediator of cardiovascular disease (CVD). The mechanisms that trigger the switch of the MR from a regulator of water-electrolyte homeostasis to a mediator of pathological changes remain unclear. As the shift towards pathological MR effects is not caused by elevated blood pressure or aldosterone excess, an inappropriate activation of the MR by factors in the micro-milieu

has been suggested. There are indications that post-translational modifications of the receptor can modulate its activity. A potential kinase for the MR is the ubiquitously expressed casein kinase 1 (CK1). Our aim is to investigate the interaction between CK1 and MR as a mechanism for mediating pathophysiological MR effects.

To examine if CK1 affects genomic MR activity, GRE reporter assays were performed in HEK cells transfected with MR after incubation with the pharmacological CK1-inhibitor D4476 (10 µM). We found that CK1 inhibition leads to a reduction in basal and in aldosterone-induced genomic MR-activity (41% and 35% reduction N=4, n=8-12; p<0.05). This effect was confirmed with another CK1-inhibitor PF670462 (5µM) (41.7% and 56.8% reduction ; N=3, n=7-9; p<0.05). To test if CK1 modulates MR function through direct phosphorylation we performed peptide microarrays and correlated the results with in silico predictions using NetPhos 2.0.

Altogether 5 potentially CK1-dependently modified amino acids (S170, S468; S501; T800; S936) were identified and analyzed further. To narrow down the search for relevant sites, we examined the effects of D4476 on a truncated MR construct missing the regulatory N-terminal domain (CDEF-MR). We observed a clear reduction in reporter activity in stimulated as well as in unstimulated cells (19.4% and 45.7% reduction; N=3, n=7-9; p<0.05). Furthermore we performed reporter assays overexpressing the glucocorticoid receptor, a close MR relative. Under D4476-treatment the GR shows the same tendencies as the MR with reduced basal as well as dexamethasone-induced genomic activity (45% and 36% reduction; N=2, n=4-6; p<0.05 respectively).

Our results implicate, that CK1 can modulate the genomic activity of the MR and GR and that this interaction does not require the N-terminal A/B domain. Results from in silico prediction and peptide array experiments suggest that CK1 can phosphorylate the MR directly. Whether the main regulatory effects are exerted by direct phosphorylation of the receptors and/or by phosphorylation of e.g. the translocation machinery remains a subject of future investigations.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA344

**A new GUI (graphical user interface) to facilitate photoplethysmography signal processing**H. Silva<sup>1,2</sup>, F. Viana<sup>3</sup>, H. Vieira<sup>2</sup>, R. Francisco<sup>2</sup>, H.A. Ferreira<sup>4</sup> and L. Monteiro Rodrigues<sup>1,2</sup>

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The analysis of physiological signals is a complex process, from the preprocessing phase, in which artifacts are removed from the raw signals, to the application of specific analytical algorithms allowing the extraction of useful description parameters. Without the use of dedicated software that often accompanies the signal acquisition hardware, signal analysis can be particularly cumbersome. Photoplethysmography (PPG) is a noninvasive optical technique, commonly used in the clinical setting for the estimation of pulse oximetry (SpO<sub>2</sub>)



and for the quantification of skin perfusion. The oscillatory nature of this class of signals, based on the contribution of different components (cardiac, respiratory, neurohumoral) allows the quantification of several parameters through proper instrumentation. However, PPG signals often carry artifacts that hinder a reliable and more detailed analysis. Here we describe a new MATLAB-based graphical user interface (GUI) to facilitate the processing of PPG signals acquired with Blood Volume Pressure sensors (PLUX Wireless Biosignals, Portugal). The GUI performance was tested with a sample data of PPG signals collected from the toes of 20 young healthy subjects ( $21.5 \pm 2.1$  y.o.) while sitting upright for 10 minutes. The procedures complied with the Helsinki declaration and following amendments and subjects gave their informed written consent prior to their participation. The GUI provides three main features: (1) a Finite Impulse Response filter with a Bohman window, allowing the selection of filter order and cutoff frequencies; (2) a fast Fourier transform frequency spectrum, serving as a control of the filter process; (3) a peak detection feature that operates by adjusting an amplitude threshold. The peak detection feature allows the detection of local maxima and minima, from which the pulse rate and the pulse reflection delay (the time difference between the pulse arrival peak and the reflected peak) may be calculated. Different filter parameters for a clear identification of parameters are proposed. This dedicated GUI is easy to use and speeds up PPG signal processing.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

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PCA345

### Short-term high salt diet induces changes in CD11a expression of peripheral blood granulocytes and monocytes in young healthy women

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It has been proposed that inflammation has an important role in the mechanisms underlying endothelial dysfunction induced by high-salt (HS) intake, and that the leukocyte-endothelial cell interactions are important for this process. This study aimed to assess the effect of 7-days salt intake modulation (low salt – LS and HS diet) on leukocytes activation defined by CD11a expression (part of LFA-1/Leukocyte Function Antigen 1, which is ligand of ICAM-1/5/Intercellular Adhesion Molecules 1-5) using flow cytometry in young healthy women. 15 young healthy women participated in this study. After initial 7-days LS diet (<3.2 g salt/day) all participants took 7-days HS diet (~14 g salt/day). Blood pressure (BP) was measured, and 24h urine samples were analyzed for sodium, potassium, urea and creatinine levels before and after diet protocols. Also, before and after corresponding diet protocols, flow cytometry measurement of CD11a expression on peripheral blood leukocytes was performed. Cells were stained and incubated with a mixture of antibodies: CD45-PerCP, CD14 FITC, CD11a/LFA-1 PE-Cy7 and CD16 APC. Biotinylated antibody was labeled with

streptavidin PE Cy7 (BD Bioscience) on room temperature for 30 min in the dark and washed. Following, erythrocytes were lysed by BD Lysing Solution for 10 min at 37°C. After last washing process, cells were fixated with 1 % formaldehyde. At least 10,000 live leukocytes were collected by a BD FACS Canto II cytometer and analyzed using the FlowLogic software (Inivai Technologies). Results of CD11a expression are presented as mean fluorescence intensity. This study was approved by the Ethical Committee of the Faculty of Medicine University of Osijek (Class: 602-04/17-08/12, No: 2158-61-07-17-42). Changes in 24h urinary sodium confirmed that subjects conformed to the diet protocol. BP was reduced after LS diet, but not changed after HS diet protocol. HS diet protocol significantly decreased CD11a expression on CD16<sup>high</sup> granulocyte (median expression of CD11a+ CD16<sup>high</sup> granulocytes LS diet  $1834 \pm 214$  vs. HS diet  $1280 \pm 323$ ,  $P < 0.001$ ), monocytes (median expression of CD11a+ monocytes LS diet  $1191 \pm 232$  vs. HS diet  $1024 \pm 260$ ,  $P = 0.0242$ ) and lymphocytes (median expression of CD11a+ lymphocytes LS diet  $705 \pm 148$  vs. HS diet  $501 \pm 124$ ,  $P = 0.001$ ) in peripheral blood, compared to LS diet measurement. The present study demonstrated that one week of HS diet significantly decreased expression of CD11a on CD16<sup>high</sup> granulocytes, monocytes and lymphocytes when compared to LS diet in young healthy women. It is possible that such decrease in activated leukocytes expressing high-level of CD11a provoked by short-term HS loading occurs due to activated leukocytes adhesion and migration through endothelial layer at the place of vascular injury.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

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PCA346

### Acute hypoxia decreases microvascular endothelium-dependent function in healthy men

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Previous studies have shown that hypoxia (whether acute or chronic) impairs endothelium-dependent and endothelium-independent macrovascular function. However, at present the effects of hypoxia on the microvasculature in humans are unknown. In disease states microvascular dysfunction often precedes macrovascular dysfunction, suggesting a causative role. Assessment of microvascular endothelial function is therefore important to help uncover the complex mechanisms of endothelial dysfunction. Thus, we aimed to examine whether hypoxia differentially affects endothelium-dependent and endothelium-independent microvascular function in healthy men. We hypothesized that acute hypoxia would decrease microvascular function. Eleven healthy men (Age: 23 (2) years, Height: 184 (5) cm, Body Mass: 82 (14) kg,  $VO_{2max}$ : 53 (8) ml/kg/min) completed this randomised, double-blind, crossover study. Participants were exposed to 30-min normoxia ( $F_{O_2} = 0.21$ , sea level) or 30-min hypoxia ( $F_{O_2} = 0.12$ ; equivalent ~5000m) before undergoing assessment of microvascular endothelial function using laser doppler imaging with iontophoresis of

acetylcholine (ACh – endothelium-dependent) and sodium nitroprusside (SNP – endothelium-independent). Microvascular endothelial function was determined by calculating the percentage change in perfusion relative to baseline perfusion. Statistical difference between normoxia and hypoxia was determined by a combination of Wilcoxon signed rank test and Hodges-Lehman 95% confidence intervals. The hypoxic exposure induced moderate hypoxaemia (SpO<sub>2</sub> mean (SD): 79 (4) %). Baseline perfusion was not different between conditions ( $p = 0.9$ ). Thirty minutes of hypoxia significantly impaired endothelium-dependent microvascular function compared to normoxia (% change in perfusion after ACh administration (median [IQR]): normoxia = 600 [324] %, hypoxia = 191 [295] %;  $p = 0.04$ ; 95% CI [-437, -24]; Fig 1A), but did not alter endothelium-independent microvascular function (% change in perfusion after SNP administration: normoxia = 626 [602] %, hypoxia = 405 [630] %;  $p = 0.7$ ; 95% CI [-256, 265]; Fig 1B). In conclusion, acute hypoxia impaired endothelium-dependent microvascular function in healthy men but did not affect endothelium-independent microvascular function. These findings suggest that in clinical conditions characterised by hypoxia (i.e. obstructive sleep apnoea), loss of nitric oxide-mediated dilatation may contribute to impairment in microvascular health and excess cardiovascular disease risk. Further studies examining the chronic effects of hypoxia on microvascular endothelial function are warranted.

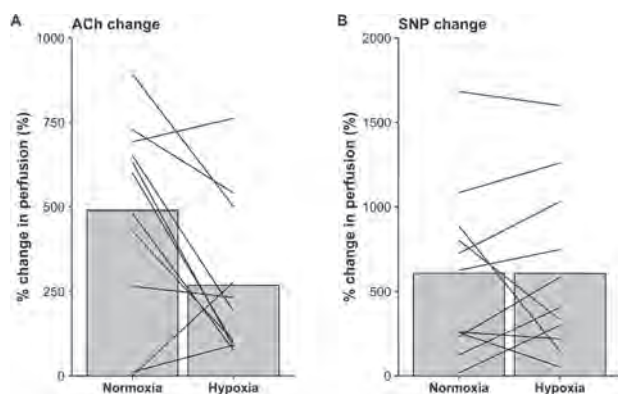


Fig 1. Microvascular function following 30-min exposure to normoxia and hypoxia. (A) PEndothelium-dependent microvascular function determined by percentage change in perfusion in response to iontophoresis of acetylcholine (ACh;  $p = 0.04$ ). (B) Endothelium-independent microvascular function determined by percentage change in perfusion in response to iontophoresis of sodium nitroprusside (SNP;  $p = 0.7$ ).

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA347

### The role of beta arrestins and PDEs in smooth muscle tone regulation of vessels and airways

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The regulation of smooth muscle tone in vessels and airways is essential to optimize blood and airflow. Its dysregulation can result in high blood pressure or obstructive lung disease. In order to develop alternative therapeutic strategies novel molecular targets involved in smooth muscle tone regulation need to be identified. Beta arrestins are promising candidates, because of their claimed key role in the modulation

of beta adrenergic receptors (ARs) and further direct effects on cAMP signaling. Earlier reports have proposed that beta arrestins upon beta2 AR activation can recruit phosphodiesterase 4 (PDE4) to the plasma membrane in HEK 293 cells, thereby modulating local cAMP levels. Due to the relevance of cAMP for smooth muscle tone this finding suggests that beta arrestins may affect vascular tone via PDE signaling.

We have investigated this aspect by performing isometric force measurements in a wire myograph in pulmonary arteries (PAs), aortas and tracheas from WT, beta arrestin 1-/- and beta arrestin 2-/- mice. After pre-constriction with the alpha1 AR agonist phenylephrine (10<sup>-7</sup> M) dose response curves applying the beta AR agonist isoprenaline (ISO) (10<sup>-9</sup> -10<sup>-4</sup> M) were recorded with or without pre-treatment with the PDE 4 inhibitor roflumilast (10<sup>-5</sup> M). In addition, qPCR experiments were performed to determine the expression levels of different PDE subtypes.

Isometric force measurements revealed that ISO induced a dose-dependent relaxation of PAs, aortas and tracheas after phenylephrine-dependent pre-constriction. PAs from beta arrestin 1-/- animals show a reduced ISO-dependent relaxation compared to WT and beta arrestin 2-/- mice. In contrast, EC50 values of ISO dose response curves in aortas and tracheas of all genotypes were similar. When tissues were pre-treated with roflumilast ISO dose-response curves in PAs were shifted to the left in WT and beta arrestin 1-/-, but not in beta arrestin 2-/- animals. However, the lower potency of ISO in beta arrestin 1-/- compared to WT PAs remained unchanged. In the aortas roflumilast did not affect ISO dose response curves of all genotypes investigated, whereas it shifted all ISO dose-response curves to the left in tracheas; this was especially pronounced in beta arrestin 2-/- tracheas. In qRT-PCR experiments no difference in the expression of PDE isoforms was detected in PAs of the different genotypes.

Our results demonstrate that beta arrestin 1 and 2 affect vascular tone regulation in PAs and airways; this appears to be unrelated to the expression levels of PDEs. PDE4 inhibition unmasks enhanced cAMP signaling in tracheas, especially in beta arrestin 2-/. This could be due to the previously reported reduction of beta2 AR internalization in beta arrestin 2 deficiency. Thus, we are planning to further explore the role of beta arrestins for tone regulation in vessels and airways in future studies.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA348

### Evidence of a physiological perfusion balance between human limb pairs

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A particular perfusion dynamic, responsible for a circulatory steady state between both hind limbs has been described in human, regarded as a long-term vascular adaptation to ischemia in patients with peripheral arterial occlusive disease. More recently, similar observations were described in the human foot as a consequence of postural vasoconstriction, and in the mouse during hyperoxia following a hindlimb

ischemia. In the present study we approach the effect of massage, a well known technique, over this apparent balance. A group of 29 young healthy subjects ( $19.9 \pm 1.56$  y.o.), both sexes (16 female, 13 male), body mass index (BMI)  $< 24.9$  kg/m<sup>2</sup> and ankle-brachial index (ABI) of 1.06, were selected from previously outlined inclusion and non-inclusion criteria. All procedures complied with the Helsinki Declaration principles and subsequent amendments. Subjects were submitted to a massage protocol consisting of three phases - a 10 minute stabilisation (Phase I), a 5 minute massage in one randomly selected limb (Phase II) and 10 minute recovery (Phase III). The contralateral limb served as control. Perfusion was assessed in both limbs by laser Doppler flowmetry (LDF) and photoplethysmography (PPG), and signals decomposed by the wavelet transform (WT) into their main spectral bands (cardiac, respiratory, myogenic, sympathetic, endothelial NO dependent and endothelial NO independent). Respiratory rate (RR), pulse rate (PR) and blood pressure (BP) were also quantified. Descriptive and comparative statistics were ( $p < 0.05$ ). A perfusion increase was detected in both limbs by LDF and PPG during massage, statistically significantly in the massaged limb. The WT from both technologies showed a decrease in the cardiac, sympathetic and in the endothelial components, as well as an increase in the respiratory and myogenic components. The observed perfusion increase might then involve an increase in myogenic and a decrease in sympathetic activities, prevailing over the observed decrease of endothelial activities. Therefore, WT analysis seems to be useful to further explore complex signals such as LDF and PPG, but more importantly, the confirmed increase in the contralateral foot induced by single limb massage confirms the referred particular dynamics between human hind limbs, likely intended to preserve the lower limb circulatory homeostasis.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

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PCA349

### **Angiotensin II type 1 receptor antagonist losartan enhances vascular reactive oxygen species production in middle cerebral arteries of Sprague-Dawley male rats**

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Numerous studies provided the data that the renin-angiotensin system (RAS) is critical for maintaining microvascular structure and vascular reactivity. The angiotensin II (ANG II) is the active end product of RAS that mediates its signal transduction and exerts its functions via ANGII receptors. Blockade of angiotensin II type 1 receptor (AT1R) signaling contributes to impairment of vascular reactivity to various vasodilator stimuli; however, the mechanism is not clear (1-3). Suppression of ANG II levels with high salt diet leads to increased vascular oxidative stress (4, 5). Present study aimed to determine the effects of AT1R antagonist losartan on vascular reactive oxygen species production (ROS) and level of vascular oxidative stress in middle cerebral arteries of rat.

11 weeks old healthy male Sprague-Dawley rats (n=17) were divided to a control (LS, n=6) and LS+Losartan group (n=11), both fed with standard rat chow with 0.4% NaCl, while rats

from LS+Losartan group received 40 mg of Losartan (daily dose) in drinking water for 7 days. On the 8<sup>th</sup> day, prior to decapitation with a guillotine, rats were weighed and anesthetized with intraperitoneal injection of ketamine (75 mg/kg) and midazolam (2.5 mg/kg). Middle cerebral arteries were isolated and cannulated on pressure myograph, under flow ( $\Delta 80$  mmHg) or no flow conditions, and in the absence / presence of TEMPOL (superoxide scavenger, 100  $\mu$ M final concentration) *in vitro*. ROS production was determined by DHE (dihydroethidine) fluorescence measurements on Zeiss Axioskop MOT2 microscope with Olympus DP70 camera using a Zeiss filter set 15 with 546-nm wavelength for excitation and a 590-nm wavelength for emission with beam splitter at 580nm. Images were processed and analyzed with ImageJ software. All experimental procedures conformed to the European Guidelines for the Care and Use of Laboratory Animals (directive 86/609) and were approved by institutional Ethical Committee.

Flow-induced vascular ROS production was significantly increased in LS+Losartan group compared to LS group ( $p=0.0023$ ), while in the absence of flow, ROS productions were similar between experimental groups. TEMPOL *in vitro* in LS+Losartan group significantly decreased vascular ROS production compared to LS+Losartan in the absence of TEMPOL [flow ( $p=0.004$ ) and no flow ( $p=0.0012$ ) conditions], and restored ROS to basal values compared to LS group in flow conditions.

AT1R antagonist losartan enhances flow-induced vascular ROS production in the middle cerebral arteries of Sprague-Dawley rats. Since superoxide scavenging by TEMPOL restores reactive oxygen species production to control levels, results suggest increased level of vascular oxidative stress due to AT-1 receptor blocking.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.



## PCA350

**Perivascular Cells Mediate Penile Erection**

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The penis presents a specialized vascular bed, which in addition to endothelial and smooth muscle cells contains a large number of perivascular cells of unknown function. Erection is dependent on the cross talk among these cells, leading to increased blood inflow and reduced outflow due to a veno-occlusive system and resulting in expansion of the corpora cavernosa. We discovered that the mouse penis contains a specific, proliferative, subpopulation of perivascular cells that express the sodium-dependent glutamate/aspartate transporter Slc1a3. Experimentally induced increase in the number of Slc1a3<sup>+</sup> cells, by cell specific deletion of the transcription factor Rbp-Jk, resulted in non-sexually stimulated long-lasting erections. Laser speckle contrast imaging of blood flow showed that expansion in the number of Slc1a3<sup>+</sup> cells lead to exacerbated penile blood flow response to vasodilators such as acetylcholine and reduced response to the vasoconstrictor phenylephrine, a selective  $\alpha_1$  adrenergic receptor agonist. Cell specific light-induced optogenetic stimulation of Slc1a3<sup>+</sup> cells increased penile blood flow, which was prevented by pre-treatment with nioxetine, an inhibitor of the norepinephrine transporter Slc6a2. In aged or diabetic mice, both conditions associated with dysfunctional penile erection, a reduced number of Slc1a3<sup>+</sup> perivascular cells and altered penile blood perfusion were observed. In conclusion, the mouse penis contains a Slc1a3<sup>+</sup> perivascular cell population that mediates erection through modulation of vasodilation.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

Weanling SD Rats were fed a high salt diet (SR) containing 8% NaCl for 6 weeks while control rats (NR) had normal rat chow containing 0.3% NaCl. At the end of the feeding period, the rats were euthanized by cervical dislocation, the abdomen was opened and 2mm rings from the abdominal aorta were isolated and mounted in perfusion chamber containing Physiological Salt Solution (PSS, Sofola, 2002), at 37°C and gassed with 95%O<sub>2</sub>-5% CO<sub>2</sub> gas mixture.

After pre-contraction of the rings with 10<sup>-7</sup>M phenylephrine, relaxation responses to Ach, from 10<sup>-9</sup> to 10<sup>-4</sup>M were determined, in the absence or presence of L-NNA as well as response to sodium nitroprusside (SNP). Percent relaxation (Mean±SEM) was obtained, plotted as cumulative response curves and analysed by ANOVA and SNK postHoc test with significance at P<0.05. Total RNAs were isolated from homogenates of abdominal aorta from SR and NR using Aurum<sup>TM</sup> Total RNA Mini Kit and cDNA was synthesized from total RNA using Omniscript RT Kit. The eNOS mRNA amplicons obtained were separated by electrophoresis on 1.5% agarose gel and visualized by ethidium bromide staining under UV light.

Results show that relaxation response curves to Ach in the absence of L-NNA were similar in both NR (n=5) and SR (n=5) (P>0.5). However in the presence of L-NNA, relaxation response to Ach was significantly attenuated in NR (P<0.05, ANOVA) but was virtually unchanged in SR (P>0.05). Specifically, at Ach dose of 10<sup>-4</sup>M, percent (%) relaxation was reduced in NR from 86.4±7.0 to 57.5±8.7 (P<0.001) but not in SR, 78.3±8.9 to 73.0±6.3; (P>0.05). In both groups the percent relaxation to 10<sup>-5</sup> M SNP was the same at 100%. The expression of eNOS mRNA in the abdominal aorta of salt-loaded rat was much reduced when compared with that of the control group. These results indicate that, following the earlier suggestion of signaling switch for relaxation from EDRF-NO to EDHF in high salt fed rats (Sofola et al, 2002), the present results show the reduction of eNOS expression in SR which will explain the switch in signaling mechanism.

Sofola OS, Knill A, Hainsworth R, Drinkhill MJ (2002). *J Physiol* 543, 255 - 260

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA351

**High salt diet in Sprague Dawley rats alters relaxation response of abdominal aortic ring preparation and reduces eNOS mRNA expression.**

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In the pressurized mesenteric artery preparation of Sprague Dawley (SD) rats, high salt diet produced identical relaxation response to acetylcholine (Ach) as in control rats. However following blockade with L-NAME, the Ach induced relaxation was not affected in high salt rats but was attenuated in control rats and the lack of response was reversed by Apamin and Charybdotoxin, suggesting that EDHF rather than EDRF-NO might play a more significant role in the response in salt loaded rats (Sofola et al, 2002). In view of the lack of effectiveness of L-NAME in high salt fed rats, the present experiments were designed to evaluate the relaxation responses to Ach and also determine the expression of EDRF-NO in the two groups of rats.

## PCA352

**The effects of ageing on the biomechanical properties of the rat middle cerebral artery**

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Ageing has a well characterised impact on systemic arteries, including remodelling and increasing arterial stiffness. However, cerebral vasculature has a number of unique properties making it structurally and functionally distinct from systemic arteries. With these in mind, it is likely that cerebral arteries have a distinctive ageing process. While the age-related properties of large systemic elastic arteries have been well characterised, the effects of ageing on the biomechanical properties of resistance-sized cerebral arteries has been relatively overlooked. These biomechanical properties have important implications for cerebral autoregulation, as well as the perivascular drainage of extracellular solutes. The aim of this study was to assess the effects of ageing on the structure and biomechanical properties of the rat middle cerebral artery.

Male Wistar rats aged between 14-16 weeks (young) and 56-58 weeks (old) were euthanised by CO<sub>2</sub> inhalation and cervical dislocation. Middle cerebral arteries were dissected and mounted on a wire myograph, bathed in PSS at 37°C and continually gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub> (n=5 per group). Passive properties were assessed by incremental stretches of 0.05 mm every 2 minutes. For each incremental stretch, the peak force and plateau force were measured. In a subgroup, additional segments were fixed in formalin and stained using Miller's method for elastin and collagen (n=4 per group). Wall thickness and composition were analysed using ImageJ software. Biomechanical relationships were assessed by two-way ANOVA, while histological data was assessed by independent sample t-test with significance accepted as p<0.05.

Arteries from old rats showed a significant reduction in the tension generated in response to stretch compared with arteries from young rats (p<0.05). This was true whether measured at the peak or plateau of the tension-length curve. This equated to a reduced Young's modulus in the arteries from old rats (p<0.01). However, there was no significant difference in arterial compliance or internal circumference measured at 100 mmHg (p>0.05). Histological analysis showed that wall thickness and elastin: collagen ratio were similar between the two age groups (p>0.05).

Large systemic arteries have been shown to demonstrate an age-related increase in arterial stiffness, as well as changes in arterial structure and composition. However, this data suggests that this is not the case for cerebral arteries. Instead, cerebral arteries become less stiff with age and produce no obvious change in composition or structure. These findings have important implications for the autoregulatory maintenance of cerebral blood flow in the aged brain. Moreover, this data may influence our current understanding of many cerebrovascular conditions, including Alzheimer's disease and aneurysm.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA353

# **The mechanisms underlying cooling induced contraction in detrusor smooth muscle involves neither TRPA1 nor TRPM8.**

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Treatment of underactive bladder (UAB) is not established. Cooling induced contraction (CIC) of detrusor smooth muscle had been reported (Seham, *et al*, 1999), however the precise mechanism is not conclusively understood. The present study aimed to elucidate the mechanism of CIC, and examined using TRPA1 and TRPM8 knockout mice.

Materials: Isolated intact detrusor preparations were obtained from human and TRPA1/TRPM8 (These channels are activated by low temperature.) knockout mice bladder. The procedures of human tissue have been approved by ethical committee at Kyushu University medical school. The urinary bladder were obtained from the patients who underwent radical cystectomy after approval of informed consent. The study protocol of mice was approved by the Animal Care and Committee of Kyushu University. The mice were sacrificed with deep

anesthesia (>5% Isoflurane) and urinary bladder were immediately isolated.

Tension Force Measurement: The detrusor smooth muscle was dissected into small strips measuring approximately 1 mm in width, and 3-4 mm in length for preparation. These strips of detrusor smooth muscle were mounted to transducer for isometric force recording.

Ca<sup>2+</sup>-imaging study: Human detrusor smooth muscle cells were cultured on coverslips and incubated with fluo-4/AM. Fluo-4 fluorescence images were obtained using a confocal-scanning microscope.

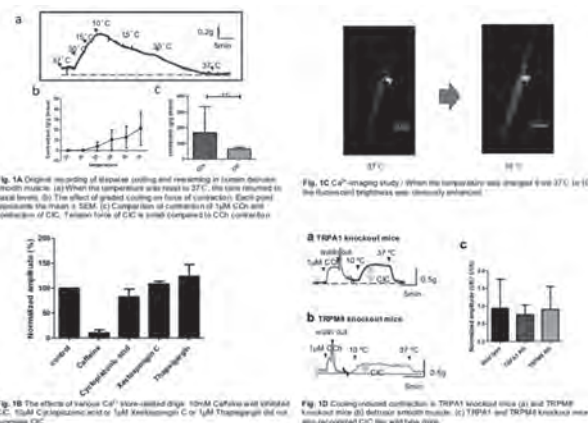
Values are means ± S.E.M., compared by student's t-test.

## **Results**

[Results] The graded extracellular temperature change from 37 °C to 10 °C inversely induced a sustained contraction, while reversed by returning temperature (Fig. 1A). Even in the presence of tetrodotoxin and atropine, these responses were also reproducible. Ca<sup>2+</sup>-free extracellular solution (0.003 ± 0.002 fold, n = 4) or nifedipine (0.08 ± 0.03 fold, n=3) or Caffeine (0.01 ± 0.004 fold, n = 4) well inhibited CIC. Cyclopiazonic acid (0.83 ± 0.08 fold, n = 4) or Xestospongine C (1.09 ± 0.03 fold, n = 4) or Thapsigargin (1.23 ± 0.13 fold, n = 4) did not suppress CIC (Fig. 1B). Rise of intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) induced by cooling and failure of [Ca<sup>2+</sup>]<sub>i</sub> increase by removing extracellular Ca<sup>2+</sup> were confirmed (Fig. 1C). TRPA1 and TRPM8, activate by cold, knock out mice were also occurred to CIC as well as wild type mice (wild: 0.94±0.27 g/g n=9, TRPA1KO: 0.75±0.05 g/g n=11, TRPM8KO: 0.91±0.33 g/g n=4) (Fig. 1D).

## **Conclusion**

The present study indicated that CIC appeared to result from myogenic origin whether or not neural control. Extracellular Ca<sup>2+</sup> is essential for the generation of CIC, suggesting that mechanism underlying CIC requires the transmembrane Ca<sup>2+</sup> entry or intracellular Ca<sup>2+</sup> metabolic process. This CIC was not provoked by the activation of representative cooling induced activation ion channel, TRPA1 and TRPM8. If this mechanism is further understood, it might lead to the development new treatment of UAB.



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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA354

**The effects of Coenzyme Q-10 on gestational diabetes myometrium**

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Coenzyme Q-10 (CoQ-10 or Ubiquinone) is a naturally occurring quinone that is found in most aerobic organisms from bacteria to mammals. CoQ-10 is believed to be an important cellular antioxidant defense, higher levels of CoQ-10 in gestational diabetes patients may be a compensatory mechanism, in response to an activated oxidative stress, probably associated to hyperglycaemia and insulin resistance (Giannubilo et al., 2011). CoQ-10 has undergone extensive research, but not in the area of smooth muscle physiology. We therefore studied the effects of CoQ-10 on gestational diabetes myometrium. Pregnant rats were randomly distributed into 2 groups: non-gestational diabetic rats and gestational diabetic rats. Induction of diabetes were performed by a single dose of streptozotocin 60 mg/kg body weight with intraperitoneal injection on day 5 of gestation. Blood glucose levels were monitored 2 days after diabetic induction and blood glucose levels equal or higher than 200-300 mg/dL was considered as diabetes. Animals were sacrificed by CO<sub>2</sub> asphyxia around 19-21 days of gestation. The uterine horn was removed and longitudinal muscle strips were separated. The strips were attached to a transducer in the organ bath apparatus and changes in isometric force responded to CoQ-10 were measured. CoQ-10 significantly increased myometrial contractile activity ( $p < 0.05$ ). Thus, the integral area under the contraction was increased to  $128.29 \pm 20.94$  in non-gestational diabetic rats and  $114.16 \pm 15.91$  in gestational diabetic rats compared to spontaneous control 100% ( $n = 5$  for each). Although the underlying mechanism whereby CoQ-10 affected the spontaneous uterine contractility in both non-gestational and gestational diabetic rats is not yet clarified, the safety of use as supplementation is concerned. Giannubilo SR et al. (2011) Diabetes Res Clin Pract 94(2), 230-235.

The authors would like to thank Suranaree University of Technology for financial support.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA355

**Fasudil modulates hypoxia-induced contraction in the isolated bovine digital vein: preliminary results**

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Hypoxia-induced contraction in the pulmonary vasculature is well described and calcium-sensitization, mediated by Rho kinase, is known to be a contributing factor (1). However, the effect of hypoxia in superficial veins has not been fully investigated. The bovine digital veins (BDVs) are superficial veins located in the subcutaneous tissue and drain blood from the claw. Isolated BDVs display hypoxia-induced contraction that is resistant to endothelium removal and extracellular calcium concentration depletion (2). The aim of the current study was to determine the effect of fasudil, a selective Rho kinase inhibitor, on isolated BDVs submitted to hypoxic conditions. BDVs were obtained from six cattle (*Bos indicus*) at a local abattoir and submitted to *in vitro* isometric contraction studies as previously described (2-3). Four parallel BDVs were used from the same animal and contracted to a similar degree with phenylephrine (0.6-1.0  $\mu$ M) under hyperoxic conditions (95% O<sub>2</sub>-5% CO<sub>2</sub>). After a submaximal contraction was achieved, four experimental groups were established: group 1-BDVs received no further treatment; group 2-BDVs were exposed to hypoxic conditions (95% N<sub>2</sub> - 5% CO<sub>2</sub>) for 15 min; groups 3-BDVs & 4-BDVs were also exposed to hypoxic conditions but before adding phenylephrine, they were incubated with 1  $\mu$ M fasudil and 10  $\mu$ M fasudil respectively for 30 min. Responses were registered as: contraction (g/mg), percentage (%) of change over a basal submaximal contraction and area under the curve (AUC: arbitrary units). Negative and positive values outline isometric force below and above the basal contraction respectively. Values are presented as means  $\pm$  S.E.M or 95% C.I. (lower - upper), compared either by one-way or two-way ANOVA. The initial phenylephrine-induced contraction (g/mg) was similar ( $p > 0.05$ ) in all groups (group-1:  $0.56 \pm 0.1$ ; group-2:  $0.60 \pm 0.1$ ; group-3:  $0.51 \pm 0.03$ ; group-4:  $0.43 \pm 0.07$ ). Hypoxia caused an enhanced contractility ( $p < 0.05$ ) characterized by a peak contraction above the basal level at 5 min (Figure 1; group-1: 0.10% (-9.00 - 40.0); group-2: 24.0% (2.60 - 40.0); group-3: 20.7% (13.3 - 60.0); group-4: 5.01% (-23.0 - 64.5)). The total contractile response as AUC confirmed a significant ( $p < 0.05$ ) concentration dependent effect of fasudil on the hypoxia-induced contraction (group-1: -4387 (-13430 - 4657); group-2: 1164 (-6667 - 8994); group-3: 550 (-7770 - 8870); group-4: -8002 (-16248 - 243.8)). Hypoxia-induced contraction in BDVs was modulated by fasudil. Therefore, the Rho-kinase pathway could contribute to the observed hypoxia-induced contraction as was suggested in other veins under *in vitro* stressful conditions, such as reduction of temperature (3). The potential use of fasudil in clinical scenarios related to hypoxia in superficial veins of the limbs remains to be established.



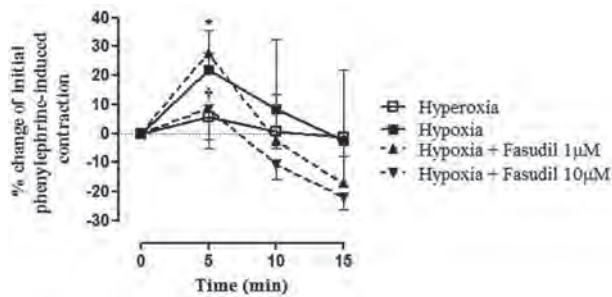


Figure 1. Effect of fasudil (1  $\mu$ M-10  $\mu$ M) on the hypoxia-induced contraction in phenylephrine-contracted bovine digital veins. Mean  $\pm$  S.E.M; n = 6. \* p<0.05 significant difference between hyperoxia versus hypoxia; and † p<0.05 significant difference between fasudil 10  $\mu$ M versus hypoxia.

Ward L & McMurtry I (2009). *Curr Opin Pharmacol* 9, 287–296.  
 Risso A et al. (2012). *Research in Veterinary Science* 92, 299–305.  
 Zerp H et al. (2010). *Journal of Applied Physiology* 109, 11–18.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA356

## Effects of pomegranate extract on postpartum sow reproduction

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Pomegranate, *Punica granatum* L. (Punicaceae), is a nutrient dense fruit rich in phytochemical compounds especially phytoestrogens (Miguel et al., 2010). Extracts of all parts of the pomegranate fruit exhibit therapeutic properties (Lansky and Newman, 2007) and target a range of diseases including infertility. The effects pomegranate extract on female reproduction has never been investigated in the domestic animals. The aim of this work was therefore, to examine the effects of pomegranate extract on sow reproduction. The effects on vaginal cornification and estrous cycle were particularly investigated. To do so, pomegranate fruits were collected, aired dried, and extracted with 95% ethyl alcohol. The extracts were evaporated to dryness under reduced pressure by rotary evaporator, lyophilized by freeze dryer and stored at -20°C until use. Postpartum sows were randomly divided into 3 groups each group contained 4 animals. The animals were orally administrated with distilled water 2 cc/day (group 1), pomegranate extract 0.5 g in 2 cc distilled water/day (group 2), or pomegranate extract 1.0 g in 2 cc distilled water/day (group 3). The administration was started from day 1 after parturition (day 0) until estrus returns (24-27 days). Vaginal smears were checked every week for cornified cells and the day estrus return recorded. The results showed that pomegranate extract helped to shorten the length of estrous cycle in sows. It was shown that the estrous cycle length after parturition was significantly reduced in the groups that received pomegranate extract when compared with the control group (p<0.05). The estrous cycle length was 26.75 $\pm$ 0.5, 25.5 $\pm$ 0.57, and 25 $\pm$ 0.81 in group 1, 2, and 3, respectively. It should be noted that changes in estrus return was consistent with the significant increases in vaginal cornification (p<0.05). The percentage of cornified cells was 80.00 $\pm$ 4.08, 82.50 $\pm$ 4.78, and 87.50 $\pm$ 2.50 in group 1, 2, and 3, respectively. In conclusion, pomegranate extract may help to promote uterine involution

leading to shorten the length of estrous cycle in postpartum sows.

Miguel et al. (2010) *J Med Plants Res* 4(25), 2836–2847

Lansky EP & Newman RA (2007) *J Ethnopharmacol* 109(2), 177–206

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PCA357

## Efficient ablation of renin cells in adult kidney using inducible transgenic mouse models, evaluated by a novel automatic histological quantification (FACS on a slide)

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In previous experiments, we demonstrated that an inducible knockout of *Gsα* in renin-producing cells (RPCs) leads to reduced renin production and kidney damage (1). It remained unknown to what extent the RPCs and renin *per se* are necessary for the maintenance of adult kidney function and morphology.

To differentiate between renin deficiency and absence of RPCs, we generated two transgenic mouse models. We crossed our inducible RPC tdTomato-reporter strain (mRen-rtTAM2-LC1-tdT; wildtype controls, WT) and with *Gsα*-floxed mice (mRen-rtTAM2-LC1-tdT-*Gsα*; reduced renin production) and DTA mice (mRen-rtTAM2-LC1-tdT-DTA; RPC ablation upon inducible expression of diphtheria toxin A). Adult mice received continuously doxycycline to induce recombination (water: 2mg/ml doxycycline, 10 mg/kg enalapril, 5% saccharose for 18 days; afterwards chow: 625mg/kg doxycycline). Four and twelve weeks after start of induction kidneys were harvested (uninephrectomy/ end biopsy, inhalation narcosis: 2-3% isoflurane) for immunohistochemistry or FACS analysis to evaluate the induction efficiency. Automatic histological analysis of whole kidney sections (up to 100 000 cells) was performed with new algorithms based on Fiji and R software. FACS revealed reduced number of renin positive cells in both transgenic strains compared to WT mice (four weeks: WT 0.15 $\pm$ 0.01%, *Gsα* 0.08 $\pm$ 0.03%, DTA 0.05 $\pm$ 0.01%; twelve weeks: WT 0.13 $\pm$ 0.02%, *Gsα* 0.03 $\pm$ 0.01%, DTA 0.04 $\pm$ 0.004%; p<0.05). Evaluation of the knockout efficiency by tdT-positive cells displayed comparable decrease in DTA mice, but no change at the later time point in *Gsα* mice (four weeks: WT 0.17 $\pm$ 0.03%, *Gsα* 0.09 $\pm$ 0.03%, DTA 0.02 $\pm$ 0.004%; twelve weeks: WT 0.13 $\pm$ 0.02%, *Gsα* 0.13 $\pm$ 0.03%, DTA 0.01 $\pm$ 0.01%; p<0.05). This discrepancy was expected because tdT-positive renin cells are continuously recruited in both strains, while cells in which recombination already occurred, persist in *Gsα* mice (as tdT-positive renin-negative cells) but are ablated in DTA mice. Automatic histological analysis showed similar results for renin (four weeks WT 0.09 $\pm$ 0.01%, *Gsα* 0.02 $\pm$ 0.01%, DTA 0.03 $\pm$ 0.01%; twelve weeks WT 0.10 $\pm$ 0.01%, *Gsα* 0.02 $\pm$ 0.01%, DTA 0.02 $\pm$ 0.004%; p<0.001) and tdT (four weeks WT 0.31 $\pm$ 0.03%, *Gsα* 0.08 $\pm$ 0.03%, DTA 0.02 $\pm$ 0.01%; twelve weeks WT 0.22 $\pm$ 0.03%, *Gsα* 0.27 $\pm$ 0.09%, DTA 0.05 $\pm$ 0.02%; p<0.05). Data are means $\pm$ SEM, n=5-9. Statistical test: 2-way ANOVA. Using two independent methods and two different readouts we confirmed the high efficiency of our inducible mouse model for targeting renin cells (71 $\pm$ 8% in *Gsα* and 88 $\pm$ 4% in

DTA mice). The novel automatic histological analysis is equally reliable to FACS and has the advantage to require less tissue for quantification.

Lachmann P et al. (2017). *J Am Soc Nephrol*, 28(12):3479-3489

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA358

### Activation of the lactate receptor, GPR81, alters renal vascular function by direct and indirect mechanisms

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G protein-coupled receptor 81 (GPR81) is highly expressed in adipocytes and activation by the endogenous ligand lactate or by the specific agonist AZ'5538<sup>1</sup> inhibits lipolysis. The receptor is also found in non-adipose tissue<sup>2</sup> but the function here is not well understood; recent studies show that GPR81 activation increases blood pressure<sup>1</sup> (BP). Here, we screened for GPR81 expression in the kidney and vasculature and used AZ'5538 to assess the effect of GPR81 activation on BP and renal haemodynamics *in vivo*.

End point PCR confirmed expression of GPR81 in kidney, aorta, mesenteric, renal and interlobar arteries taken from adult male C57BL/6J mice (n=4) culled by cervical dislocation. Other mice (n=6) were anaesthetised with Inactin (120mg/kg IP), the jugular vein cannulated for IV infusion (0.2ml/h/10g) of a) 5% mannitol vehicle and b) AZ'5538 (250µM over 15 mins). BP was recorded via the carotid artery. Data are mean±SD. AZ'5538 increased SBP by 14.9±6.5mmHg, whereas the vehicle had no significant effect (0.7±3.1mmHg). The pressor effect of AZ'5538 was absent in GPR81 null mice (n=4), confirming on-target action. In separate experiments, renal blood flow was measured by a Doppler ultrasound probe placed around the left renal artery or by pulse-wave Doppler ultrasound in anaesthetised mice (n=4). Renal blood flow was decreased compared to vehicle control in both studies (-0.33±0.23ml/min vs vehicle 0.04±0.02ml/min, P=0.027 and -185±25mm/s vs vehicle -0.4±20mm/s, P<0.01, respectively). Next, activation of GPR81 was assessed *ex vivo* by wire myography. Vessel segments were incubated with 30µM AZ'5538 for 15 minutes (n=8 for all). Renal arteries showed significant vasoconstriction (19.7±13.9% of maximal response to high potassium salt solution, P<0.01 against vehicle by two-way ANOVA); mesenteric arteries and the aorta showed no changes in tone. In all vessels incubated with GPR81 agonist, the response to the endothelium dependent vasodilator acetylcholine was blunted but responses to the nitric oxide donor sodium nitroprusside were not (Table 1). This shows that GPR81 activation influences vascular function and renal haemodynamics by both direct and indirect mechanisms. The sensitivity of the renal circulation to GPR81 activation may become physiologically significant when lactate concentration rises, as can occur in renal disease.

|               | Aorta       |               |         | Mesenteric artery |               |         | Renal artery |               |         |
|---------------|-------------|---------------|---------|-------------------|---------------|---------|--------------|---------------|---------|
|               | Vehicle     | GPR81 agonist | P value | Vehicle           | GPR81 agonist | P value | Vehicle      | GPR81 agonist | P value |
| ACH -log(ESR) | 6.67 ± 0.31 | 5.31 ± 0.31   | 0.05    | 6.70 ± 0.08       | 5.52 ± 0.47   | <0.01   | 7.41 ± 0.17  | 6.23 ± 0.10   | 0.03    |
| SNP -log(ESR) | 7.57 ± 0.21 | 7.57 ± 0.21   | 0.87    | 7.28 ± 0.09       | 7.61 ± 0.10   | 0.25    | 7.45 ± 0.12  | 7.09 ± 0.14   | 0.09    |

Table 1: EC<sub>50</sub> values from non-linear dose response curves in isolated vessel segments from C57BL/6J mice. Vessels were treated with either vehicle or 30µM GPR81 agonist, AZ'5538 prior to precontraction with phenylephrine. Cumulative dose response curves for acetylcholine (ACH) and sodium nitroprusside (SNP) are shown as mean±SD (n=8 for all). Two-way ANOVA was performed; significant P values are highlighted in red.

Wallenius K et al. (2017). *JCI Insight* 2, E92564

Liu C et al. (2009). *J Biol Chem* 284, 2811-2822

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA360

### Investigation Effects of Metformin in rats urinary bladder

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Metformin is currently considered as first medication for the pharmacological management of patients with type 2 diabetes mellitus (T2DM) according to the American Diabetes Association/European Association for Study of Diabetes guidelines. Metformin acts by lowering intestinal glucose absorption, developing peripheral glucose uptake, decreasing fasting plasma insulin levels and boosting insulin sensitivity. Metformin has few adverse side effects, the most widespread is gastrointestinal symptoms, but effects of metformin on urinary bladder is hardly known. In this study, we investigated effects of metformin on rats urinary bladder. Adult female Sprague-Dawley rats (n=12) were used in this study. For examination of the functional role of metformin in bladder, the bladder was quickly removed and placed in Krebs solution and it was formed a flat sheet. Then 12 longitudinal strips were formed from sheet, measuring 2-4 × 6-12 mm and anchored in (5, 10 and 20 mL) organ baths containing Krebs solution and bubbled with 95% O<sub>2</sub>/ 5 % CO<sub>2</sub> at 37°C. The tension formed by the tissues was measured using transducers MP150 instrument (Biopac Systems, Inc., U.S.A.). All tissues was then left to equilibrate for 60 min with rinsing every 30 min under a resting tension of 1.5 g before starting experiment. After equilibration, acetylcholine (10<sup>-4</sup> M) was given to the all baths. After one hour rinsing, metformin (2 mM) was given and incubated 30 min. After 30 min, again acetylcholine was given same dose without rinsing and then these part was repeated with metformin 10 mM and 20 mM doses. Control group values were accepted as 100 %, and change calculated as percent (%) for all treatment groups for peak to peak amplitude, frequency and area values. Data were given as means ± SE and statistical analysis of the results was implemented by one way ANOVA. In all conditions, differences

were considered significant when  $p < 0.05$ . 2 mM dose has no significant effects compared to control at frequency and area values, but it has significant increase at peak to peak amplitude ( $127.65 \pm 4.24$ , change %,  $p < 0.01$ ). Also, peak to peak amplitude has significantly increased in 10 mM and 20 mM ( $163.86 \pm 8.57$  and  $162.58 \pm 6.76$ , respectively, change %,  $p < 0.001$ ) compared to control. Frequency has significantly decreased in 10 mM ( $37.75 \pm 4.71$ , change %,  $p < 0.001$ ) and 20 mM ( $16.29 \pm 2.41$  change %,  $p < 0.001$ ). In addition, area has significantly increased at only 20 mM dose ( $3767.64 \pm 733.70$  change %,  $p < 0.001$ ) compared to control. In conclusion, metformin increased urinary bladder contraction in dose-dependent manner.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

### PCA361

#### **Interleukin-18 promotes osteo-/chondrogenic transdifferentiation of vascular smooth muscle cells through SGK1**

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**Background:** Increased interleukin-18 (IL-18) levels are associated with vascular smooth muscle cell (VSMC) osteo-/chondrogenic transdifferentiation and subsequent vascular calcification, but the underlying mechanisms are, however, still elusive. The present study explored the possible involvement of the serum- and glucocorticoid-inducible kinase 1 (SGK1), a key regulator of osteo-/chondrogenic transdifferentiation of VSMCs, in IL-18-induced vascular calcification.

**Methods:** Experiments were performed in primary human aortic smooth muscle cells (HAoSMCs) following treatment with recombinant human IL-18 protein in the presence or absence of  $\beta$ -glycerophosphate, following SGK1 knockdown by silencing or pharmacological inhibition of the PI3K. The mRNA expression was analyzed by using quantitative RT-PCR, ALPL activity by a colorimetric method and calcification by Alizarin Red staining and calcium assay.

**Results:** SGK1 mRNA expression was up-regulated in HAoSMCs by IL-18 treatment in a concentration-dependent manner, effects reaching statistical significance at 10 ng/ml IL-18 concentration. These effects were paralleled by a dose-dependent increase in osteogenic markers *MSX2*, *CBFA1* and *ALPL* mRNA expression in IL-18 treated HAoSMCs. Moreover, phosphate-induced SGK1 and osteogenic markers mRNA expression and, thus, osteo-/chondrogenic transdifferentiation of HAoSMCs were further augmented in the presence of IL-18 in the cell culture medium. In accordance, IL-18 aggravated phosphate-induced calcification of HAoSMCs. Silencing of SGK1 blunted IL-18-induced expression of osteogenic markers *MSX2*, *CBFA1* and *ALPL* mRNA in HAoSMCs as compared to negative control silenced HAoSMCs. The effects of IL-18 on SGK1 expression were suppressed in the presence of PI3K inhibitors LY294002 or wortmannin.

**Conclusions:** IL-18 up-regulates SGK1 in VSMCs and SGK1 participates in the intracellular signaling of IL-18-induced osteo-/chondrogenic transdifferentiation of VSMCs.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

### PCA362

#### **Fibulin-3 ameliorates phosphate-induced vascular smooth muscle cell calcification through inhibition of oxidative stress**

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**Background:** Medial vascular calcification induced by hyperphosphatemia is associated with cardiovascular events and mortality in chronic kidney disease (CKD) patients. This process is actively promoted by osteo-/chondrogenic transdifferentiation of vascular smooth muscle cells (VSMCs) and involves cellular oxidative stress. Fibulin-3, an extracellular matrix protein, inhibits vascular oxidative stress in hypertension. Thus, this study investigated the effects of Fibulin-3 on phosphate-induced VSMC calcification and the possible underlying mechanisms. **Methods:** Primary human aortic smooth muscle cells (HAoSMCs) were treated with control or  $\beta$ -glycerophosphate without or with recombinant human Fibulin-3 protein and hydrogen peroxide as an exogenous source of oxidative stress. Calcification was analyzed by quantification of calcium content and Alizarin Red staining, the mRNA and protein expression by quantitative RT-PCR and Western blotting, respectively and the antioxidant capacity and ALPL activity by colorimetric methods.

**Results:** Phosphate treatment increased mineral deposition, tissue-nonspecific alkaline phosphatase (ALPL) activity and mRNA expression of osteogenic and chondrogenic markers *MSX2*, *CBFA1*, *SOX9* and *ALPL* in HAoSMCs. All these effects were blunted by additional treatment with Fibulin-3, suggesting that Fibulin-3 is able to interfere with osteo-/chondrogenic transdifferentiation and calcification of HAoSMCs. Furthermore, Fibulin-3 reversed the decrease in total antioxidant capacity and up-regulation of oxidative stress markers *NOX4* and *CYBA* mRNA expression in HAoSMCs during high phosphate conditions. Accordingly, Fibulin-3 suppressed the expression of oxidative stress downstream effectors *PAI-1* and matrix metalloproteinases *MMP2* and *MMP9* as well as apoptosis marker *BAX/BCL2* mRNA expression ratio in phosphate treated HAoSMCs. The inhibitory effects of Fibulin-3 on phosphate-induced osteo-/chondrogenic transdifferentiation of HAoSMCs were suppressed by additional treatment with hydrogen peroxide.

**Conclusions:** Fibulin-3 inhibits endogenous oxidative stress in VSMCs during hyperphosphatemia and, thus, interferes with phosphate-induced osteo-/chondrogenic transdifferentiation and calcification of VSMCs. Up-regulation or supplementation of Fibulin-3 may be beneficial in reducing the progression of vascular calcification during hyperphosphatemic conditions such as CKD.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.



PCA363

### Role of cytosolic serine hydroxymethyl transferase 1 (SHMT1) in osteo-/chondrogenic transdifferentiation and calcification of vascular smooth muscle cells

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**Background:** Polymorphisms in the cytosolic serine hydroxymethyl transferase 1 (SHMT1) gene, a key enzyme in one carbon (methyl) metabolism, are associated with increased risk of cardiovascular events. However, the physiological roles of SHMT1 in vascular smooth muscle cell (VSMC) function are still elusive. Medial vascular calcification is a risk factor for cardiovascular events and mortality in chronic kidney disease (CKD) patients. Thus, the aim of the present study was to examine the possible involvement of SHMT1 in phosphate-induced osteo-/chondrogenic transdifferentiation and calcification of VSMCs.

**Methods:** Experiments were performed in primary human aortic smooth muscle cells (HAoSMCs) following silencing of the SHMT1 gene by small interfering RNA and additional treatment with control or high phosphate and/or antioxidants TIRON and TEMPOL. The mRNA expression was determined by qRT-PCR, total antioxidant capacity and tissue-nonspecific alkaline phosphatase (ALPL) activity by colorimetric assays and calcification by quantification of calcium content and Alizarin Red staining.

**Results:** Phosphate treatment up-regulated SHMT1 mRNA expression in HAoSMCs. Silencing of endogenous SHMT1 expression in HAoSMCs significantly increased osteogenic transcription factors *MSX2* and *CBFA1* mRNA expression as well as *ALPL* mRNA expression and activity as compared to negative control silenced HAoSMCs, effects suggesting an increased osteo-/chondrogenic transdifferentiation. In addition, calcification analysis revealed higher mineral deposition in SHMT1 siRNA silenced HAoSMCs than in negative control siRNA silenced HAoSMCs. Silencing of SHMT1 decreased total antioxidant capacity and up-regulated superoxide-generating NADH/NADPH oxidase system components *NOX4* and *CYBA* mRNA expression in HAoSMCs and, thus, SHMT1 knockdown induced cellular oxidative stress. In accordance, silencing of SHMT1 promoted oxidative stress-downstream osteogenic signaling, which includes increased mRNA expression of matrix metalloproteinase *MMP2* as well as apoptosis marker *BAX/BCL2* expression ratio in HAoSMCs. Additional treatment with antioxidants TIRON or TEMPOL was able to blunt the increased osteogenic markers mRNA expression in SHMT1 silenced HAoSMCs. **Conclusions:** Silencing of SHMT1 promotes osteo-/chondrogenic signaling in VSMCs, at least partly, by inducing cellular oxidative stress and aggravates calcification of VSMCs during high phosphate conditions. The present observations advocate for a regulatory role of SHMT1 in VSMC calcification during pathological conditions such as CKD.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCA364

### C-reactive protein induces osteo-/chondrogenic transdifferentiation of vascular smooth muscle cells via oxidative stress

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**Background:** Medial vascular calcification is a key risk factor associated with cardiovascular events and the excessive mortality in chronic kidney disease patients. Vascular calcification is actively promoted by osteo-/chondrogenic transdifferentiation of vascular smooth muscle cells (VSMCs) and involves pro-inflammatory processes. Increased C-reactive protein (CRP) levels are associated with vascular calcification in hemodialysis patients. The present study explored whether CRP directly contributes to the progression of vascular calcification and the potential mechanisms.

**Methods:** In-vitro experiments were performed in primary human aortic smooth muscle cells (HAoSMCs) treated with recombinant human CRP protein with or without high phosphate conditions, in the presence of antioxidants TEMPOL and TIRON or following knockdown of FCGR2A by silencing. Mineralization was determined by quantification of calcium deposition, total antioxidant capacity and ALPL activity by colorimetric methods and mRNA expression by quantitative RT-PCR.

**Results:** Treatment with calcification medium increased calcium deposition in HAoSMCs, an effect further augmented by CRP treatment. Addition of CRP alone to the cell culture medium up-regulated osteogenic markers *CBFA1* and *ALPL* mRNA expression in HAoSMCs, suggesting that CRP is directly inducing osteo-/chondrogenic transdifferentiation. Accordingly, ALPL activity was also increased by CRP treatment. The mRNA levels of the markers of oxidative stress and critical components of the superoxide-generating NADPH oxidase system *NOX4* and *CYBA* were up-regulated while total antioxidant capacity of HAoSMCs was decreased after CRP treatment. Treatment of HAoSMCs with antioxidants prior to CRP treatment suppressed osteogenic markers *CBFA1* and *ALPL* mRNA expression, and thus, the CRP-induced osteo-/chondrogenic transdifferentiation of HAoSMCs. Moreover, silencing of the FCGR2A gene encoding the Fc fragment of IgG receptor IIA was able to suppress CRP-induced oxidative stress as well as osteogenic markers mRNA expression in HAoSMCs. **Conclusions:** CRP may directly induce osteo-/chondrogenic transdifferentiation of VSMCs by promoting FCGR2A-dependent cellular oxidative stress. Thus, CRP may actively contribute to the progression of vascular calcification in patients suffering from chronic kidney disease.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCA365

**The genetic deletion of Kv $\beta$ 2 results in increased MYH1 expression and a protective role in muscle atrophy**

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Skeletal muscle accounts for over 40% of an individual's mass and therefore plays a central role in metabolism, energy expenditure, and physical strength. Muscular atrophy or the wasting of muscle tissue can occur from numerous pathophysiological states including muscle denervation injury, disuse and/or unloading and, even excessive glucocorticoid usage. These pathological states can often result in different forms of atrophy ultimately affecting different muscle fiber types. Some states of atrophy such as those caused by denervation or disuse tends to significantly alter the type I or oxidative muscle fibers. Because muscle atrophy can affect fiber types differently a greater understanding of fiber type differentiation and/or switching may provide insight into novel drug targets and gene treatments.

Earlier literature identified the Kv $\beta$  subunits; a voltage-gated potassium channel subunit highly expressed within excitable tissue including skeletal muscle including Kv $\beta$ 2 was associated with myogenesis and myotube formation. Recently our lab demonstrated that Kv $\beta$ 2 knockout (KO) mice present with significantly decreased muscle weights in the Gastrocnemius, Soleus, and Biceps femoris. In addition, Gastrocnemius and Soleus muscles demonstrated significant increases in MYH1 expression indicative of type I muscle fibers when compared with wildtype control. This molecular data was further corroborated with physical assessments demonstrating a significant increase in duration and endurance during the grip hanging test in Kv $\beta$ 2 KO mice (n=10 mice 600sec vs. 370sec). Therefore, based upon previous literature and current findings we hypothesize that Kv $\beta$ 2 KO mice are protected from muscle fiber type I atrophy. Indeed, 14-day sciatic denervation models resulted in a delayed response of muscle atrophy in Kv $\beta$ 2 KO mice.

The genetic deletion of Kv $\beta$ 2 results in a significant shift in type I muscle fiber differentiation as evident by increased MYH1 expression and succinate dehydrogenase positive fibers. This unique phenotype may provide significant protection from muscle atrophy caused from denervation injury resulting in significant decreases in type I muscle fibers. Identifying the Kv $\beta$ 2 subunit as a novel regulator of differentiating muscle fiber types may elucidate novel targets in similar muscle atrophy states including disuse and excessive glucocorticoid usage.

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## PCA366

**NFAT5/TonEBP - a mechanoresponsive transcription factor that controls arterial remodeling**M. Zappe<sup>1</sup>, A. Feldner<sup>1</sup>, C. Arnold<sup>1</sup>, D. Komljenovic<sup>2</sup>, C. Sticht<sup>1</sup>, C. De La Torre<sup>1</sup>, M. Hecker<sup>1</sup>, W. Neuhofer<sup>1</sup> and T. Korff<sup>1</sup><sup>1</sup>Heidelberg University, Heidelberg, Germany and <sup>2</sup>DKFZ, Heidelberg, Germany

Cells are continuously exposed to mechanical stress that may wreak havoc if exceeding physiological levels. Consequently,

cell type-specific mechanisms strengthening their integrity or evading harmful stressors are indispensable. In arteries, vascular smooth muscle cells (VSMCs) respond to an increase in wall stress by reorganizing the arterial architecture which - in the long run - causes detrimental arterial hypertrophy and stiffening. In this context, transcriptional determinants controlling cellular stress responses have been insufficiently studied. We identified the transcription factor 'nuclear factor of activated T-cells 5' (NFAT5) as a regulatory element of mechanical stress responses and intended to further characterize its impact on the VSMC phenotype *in vitro* and *in vivo*. Biomechanical stress downregulated NFAT5 isoform A in VSMCs (3-fold, p<0.05, n=3) while isoform C was upregulated (1.8-fold, p<0.05, n=3). Analyses of corresponding Flag-tagged NFAT5 proteins revealed that isoform C but not A enters the nucleus of mechanoactivated VSMCs (10.3-fold vs. isoform A, p<0.001, n=3). On the functional level, overexpression of NFAT5c stimulated VSMC migration and proliferation. The relevance of these observations for biomechanically evoked arterial remodeling was investigated in mice allowing for induction of SMC-specific genetic ablation of Nfat5. As evidenced by transcriptome studies, loss of Nfat5 in mouse VSMCs impairs expression of gene products controlling cell cycle, cytoskeletal dynamics and cellular adhesion. SMC-specific Nfat5 deficiency did not alter the general vascular architecture and blood pressure levels under baseline conditions or DOCA/salt-induced hypertension. However, proliferation of VSMCs and thickening of the arterial wall was attenuated during hypertension-mediated arterial remodeling (2.4-fold, p<0.05, n=5). These findings identify NFAT5 as physiologically relevant mechanoresponsive transcriptional determinant regulating the activation of VSMCs in the biomechanically stressed arterial wall during pressure-mediated arterial remodeling.

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## PCA367

**Renal resistance arteries are sensitized to L-type Ca<sup>2+</sup> channel activation in response to renal denervation**S. Pfannkuch<sup>1</sup>, J. Witte<sup>1</sup>, R. Rettig<sup>1</sup>, R. Schubert<sup>2</sup> and O. Grisk<sup>1</sup><sup>1</sup>Physiology, University of Greifswald, Karlsburg, Germany and<sup>2</sup>Cardiovascular Physiology, Medical Faculty of Mannheim, Mannheim, Germany

Perivascular innervation is a determinant of vascular smooth muscle cell (VSMC) phenotype. We tested if local renal denervation in young adult rats sensitizes renal resistance arteries to L-type Ca<sup>2+</sup> channel activation and if increased sensitivity to L-type Ca<sup>2+</sup> channel activation or noradrenaline (NA) supersensitivity can be abolished by K<sub>ATP</sub> channel activation. We further investigated the effects of L-type Ca<sup>2+</sup> channel activation on basal vascular tone and of K<sub>ATP</sub> channel activation on NA-induced vascular tone in human renal resistance arteries. Eight-week-old rats underwent bilateral renal denervation or sham denervation under ketamine/xylazine anesthesia (100/10 mg\*kg<sup>-1</sup>, i.p.). At the age of 12 weeks, kidneys were removed under pentobarbital anesthesia (60 mg/kg, i.p.) and third generation renal artery branches were dissected for myography and mRNA expression studies. Tissue samples from human kidneys were obtained from patients who underwent nephrectomy.

L-type  $\text{Ca}^{2+}$  channel activator (S(-)-BayK8644)-induced tension was higher in arteries from denervated rat kidneys than in arteries from sham-denervated kidneys (60 vs. 30% of  $\text{K}^{+}$ -induced tension;  $p < 0.01$ ). The  $\text{K}_{\text{ATP}}$  channel activator levcromakalim blocked S(-)-BayK8644-induced vasoconstriction in arteries from denervated and sham-denervated rats. S(-)-BayK8644 increased vascular tension to 100% of  $\text{K}^{+}$ -induced tension in human renal resistance arteries which was blocked by levcromakalim. Arteries from denervated kidneys showed higher NA sensitivity than arteries from sham-denervated kidneys ( $\log\text{EC}_{50}$  -6.62 vs. -6.12 mol/l;  $p < 0.01$ ). In both groups, levcromakalim similarly shifted the NA concentration response curves to the right. Levcromakalim also shifted the NA concentration-response curves to the right in human renal resistance arteries.  $\text{K}_{\text{ATP}}$  channel blockade did not affect basal or NA-induced vascular tone in rat and human intrarenal arteries. Transcriptome analyses did not reveal statistically significant effects of renal denervation on  $\text{K}^{+}$  channel mRNA abundances. PCR analyses showed that mRNAs of all  $\text{K}_{\text{ATP}}$  channel constituents are present in human renal resistance arteries.

Local renal denervation sensitizes renal resistance arteries to L-type  $\text{Ca}^{2+}$  channel activation suggesting that VSMC membrane potential (MP) is depolarized compared to the VSMC MP of innervated intrarenal arteries. A depolarized MP does not contribute to NA supersensitivity in denervated renal resistance arteries.  $\text{K}_{\text{ATP}}$  channels are functional in rat and human renal resistance arteries. They do not contribute to basal or NA-induced vascular tone.

S. Pfannkuch was supported by a Gerhard Domagk scholarship, University of Greifswald.

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PCA368

### How Does Extracellular Acidification Increase Mouse Uterine Contractions? How much do we know?

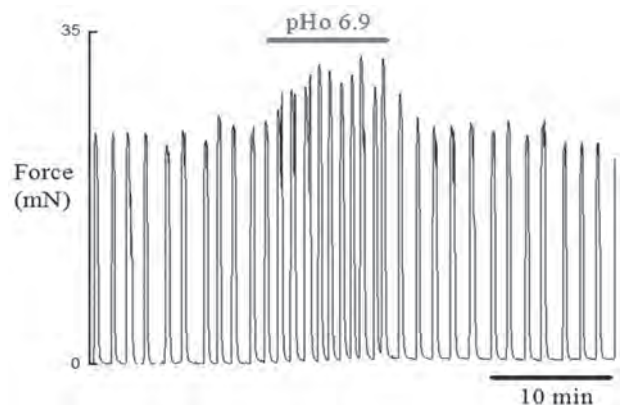
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Adequate uterine contraction is crucial to safe delivery of the foetus. Acidity can play an important role in affecting myometrial contraction but the effects, especially concerning external acidification, are unclear. We have found that extracellular acidification ( $\text{pH}_o$ ) consistently increases the contractility of mouse myometrium. Here we are investigating the possible mechanism/s behind the stimulatory effect of acidic  $\text{pH}_o$ . In uterine tissue from pregnant mouse, a decrease of  $\text{pH}_o$  stimulated contraction, particularly by increasing their amplitude and frequency. These data are consistent with the large increases in force found in non-pregnant guinea-pig myometrium (Naderali and Wray, 1999), and an increased frequency (but not amplitude) in pregnant human myometrium (Pierce et al., 2003). Ion channels sensitive to external acid and conducting inward current (ASICs) have been reported in nervous tissues. Although our western blot data showed expression of ASICs in the mouse myometrium, inhibition of the ASICs by Amiloride did not affect the stimulatory effect of the acidic  $\text{pH}_o$ . These data suggest either that the ASICs are non-functional or that they were not stimulated by an external pH of 6.9. As the simultaneous measurement of the  $\text{pH}_i$  showed a drop in the pH inside the cell in response

to the change in the  $\text{pH}_o$ , these data showed the changes of force more closely followed the change of external pH than intracellular pH. Membrane depolarization opens the L-type calcium channels (LVCC) resulting in calcium influx and initiation of uterine contractions. Blocking LVCC decreased the force of contraction but still  $\text{pH}_o$  increased the force. This suggests that external acidification is acting on another type of channels that assists calcium entry. The relation between extracellular acidification and cell membrane depolarization was tested. Extracellular acidification, under high KCl, caused initial increase in the force. These findings also suggest other depolarizing channels which are activated by extracellular acidification and allow more calcium influx.  $\text{P}_{2\text{X}}$  receptors are cation-selective channels, located on the plasma membrane and they are mainly gated by extracellular ATP, and mediate the influx of extracellular cations ( $\text{Ca}^{2+}$  and  $\text{Na}^{+}$ ) into the cell, which subsequently depolarize the membrane and open voltage-gated CCs. However,  $\text{P}_{2\text{X}_7}$  purinoceptors blockade by hydrochloride hydrate didn't affect the stimulatory effect of acidic  $\text{pH}_o$ .

We therefore conclude that external acidification within the physiological range, increases force by a mechanism that is not via ASICs, purinoceptors or directly on L-type Ca channels. Work is ongoing to test other possible mechanisms.



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To Princess Nourah bint Abdulrahman University for sponsoring my PhD

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PCA369

### Organotypic expression of vascular smooth muscle Kv7.1 channels – potential for renoprotection?

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Introduction: Blood flow regulation depends on the contractile properties of vascular smooth muscle. The latter is controlled largely by voltage-gated potassium (Kv) channels, especially Kv7 channels. Vascular smooth muscle expresses



mainly Kv7.1, Kv7.4 and Kv7.5 channels. Kv7.1 channels are expressed only in a few vessels, in particular in renal arteries. Therefore, this study addressed the hypothesis that Kv7.1 channels contribute to the regulation of the contractility of renal arteries.

**Methods:** Renal segmental arteries of Wistar rats were studied using real-time qPCR and isometric myography. Intact kidneys were explored using constant flow perfusion.

**Results:** In renal segmental arteries, mRNA expression of Kv7.1 channels was larger than that of Kv7.4 and Kv7.5 channels. The Kv7.1 channel opener R-L3 (L-364,373) reduced methoxamine-induced contractions of isolated renal segmental arteries in a concentration dependent manner. At moderate concentrations of R-L3, its effect was abolished completely by HMR1556, a selective Kv7.1 channel blocker. HMR1556 alone was without effect on methoxamine-induced contraction. Further, HMR1556 did not affect the anticontractile effect of the cGMP-coupled vasodilator ANP and the cAMP-coupled vasodilator urocortin. Notably, the effect of ANP and urocortin were the same in the absence and the presence of R-L3. In addition, R-L3 reduced methoxamine-induced perfusion pressure in intact perfused kidneys. This effect was attenuated considerably by HMR1556. HMR1556 alone was without effect on methoxamine-induced perfusion pressure. Urocortin reduced methoxamine-induced perfusion pressure. This, effect was the same in the absence and the presence of R-L3.

**Conclusions:** The results show that Kv7.1 channels are expressed in rat renal segmental arteries, but do not contribute to MX-induced contraction or ANP- and urocortin-induced relaxation. After pharmacological activation, Kv7.1 channels reduce basal tone and basal perfusion pressure, but leave MX-induced contraction as well as ANP- and urocortin-induced relaxation intact. Thus, Kv7.1 channel activation improves renal perfusion without altering vasoconstrictor- or vasodilator-evoked regulation suggesting that these channels may serve as targets for renoprotection.

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PCA370

### Organization of vascular smooth muscle cells as multilayered organoids triggers their resting state

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In vitro propagation of cells is usually based on culture media supplemented with growth factors and/or fetal calf serum (FCS) to promote cellular growth and activation. However, for vascular smooth muscle cells (VSMCs) these conditions attenuate the quiescent/contractile phenotype that predominates in vivo and is characterized by low proliferation as well as increased abundance of proteins of the contractile apparatus. Thus, delineation of environmental parameters which support the resting/contractile state of cultured VSMCs as in vivo remains an important challenge.

In this context, our study intended to define culture conditions promoting the contractile phenotype of easy accessible human umbilical artery smooth muscle cells (HUASMCs) by i) exposing them to serum-free medium, ii) organizing them as 3D multilayered organoids and iii) enabling communication with human umbilical vein endothelial cells (HUVECs) in 3D co-culture organoids.

While HUASMCs are relative undemanding cells and were grown in DMEM supplemented with 15% FCS (standard conditions), they nevertheless showed robust expression of VSMC markers such as smooth muscle myosin heavy chain or  $\alpha$ -smooth muscle actin ( $\alpha$ SMA). Serum-free medium did not affect the marker expression but decreased proliferation (Ki67 positive cells (mean  $\pm$  S.D.); standard vs. serum-free:  $33.48 \pm 12.60$  % vs.  $10.08 \pm 6.60$  %,  $p < 0.05$ ,  $n = 3$ ). However, organization of HUASMCs as 3D organoids promoted a contractile phenotype as evidenced by increased VSMC marker abundance ( $\alpha$ SMA; fold change vs. control:  $1.84 \pm 0.59$ ,  $p < 0.01$ ,  $n = 8$ ) and decreased proliferation (Ki67 positive cells; control vs. organoids:  $13.65 \pm 0.82$  % vs.  $0.01 \pm 0.01$  %,  $p < 0.001$ ,  $n = 3$ ). This finding was further supported by microarray-based gene set enrichment analysis (log2-fold change vs. control: e.g. cell cycle:  $-2.51$ ,  $p < 0.01$ ,  $n = 3$ ). In addition, co-culture of HUASMCs with HUVECs further enhanced the acquisition of a contractile phenotype shown by an increase in the abundance of the VSMC marker calponin (fold change vs. control:  $2.12 \pm 0.48$ ,  $p < 0.01$ ,  $n = 5$ ). To investigate whether VSMCs cultured as organoids can reenter an activated state, they were seeded in 3D collagen gels supplemented with 15% FCS. Under these conditions, HUASMCs increase their proteolytic capacity to invade the extracellular matrix which was inhibited by treatment with the MMP-Inhibitor GM6001 (cumulative sprout length after 24h [ $\mu$ m]; control vs. inhibitor:  $1523 \pm 121$  vs.  $479 \pm 94$ ,  $p < 0.0001$ ,  $n = 4$ ).

In conclusion, culture of VSMCs in 3D organoids rather than exposure to serum-free medium promoted a resting phenotype thus displaying a superior culture technique for enabling organotypic in vivo-like differentiation of VSMCs. Since organoid VSMCs can reenter an activated state, this method could serve as an excellent basis for tissue engineering purposes or functional analyses of compounds targeting VSMCs.

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PCB001

### The influence of haemoglobin concentration, arterial oxygen content and blood viscosity on hypoxic pulmonary vasoconstriction in acute and chronic hypoxemia.

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During acclimatisation to high altitude, haemoconcentration occurs that may exacerbate hypoxic pulmonary vasoconstriction (HPV) due to the increase in blood viscosity (4). Independent to the frictional force of viscosity, erythrocytes can augment HPV via nitric oxide scavenging by oxyhaemoglobin and reactive oxygen species generation by deoxyhaemoglobin (2), or attenuate HPV via the generation of nitric oxide (1) and S-nitrosothiol (3). The balance between these regulatory processes is thought to vary depending on the duration of hypoxia (5). Moreover, for the same arterial partial pressure of oxygen ( $\text{PaO}_2$ ), haemoconcentration will increase arterial oxygen content ( $\text{CaO}_2$ ), and recent evidence suggests this

may decrease the HPV response. Therefore, we sought to determine the role of erythrocyte-dependent modulation of HPV in humans by performing two sequential studies. Pulmonary artery systolic pressure (PASP; echocardiography) was assessed during (i) an isovolemic haemodilution at sea level ( $n=10$ ) in normoxia and during acute isocapnic hypoxia ( $\text{PaO}_2$   $40 \pm 2$  mmHg); and (ii) before and after a hypervolemic haemodilution ( $n=11$ ) to normalise haemoglobin concentration (Hb) to sea level values following  $6 \pm 2$  days of acclimatization to 5050 m above sea level, and during a further decrease in  $\text{PaO}_2$  via acute pokilocapnic hypoxia ( $\text{FiO}_2=0.15$ ). Blood viscosity was measured using a cone and plate viscometer at a shear rate of  $225 \text{ s}^{-1}$  and  $\text{CaO}_2$  was determined from Hb and  $\text{PaO}_2$  via sampling from a radial artery catheter. In the sea level trial, haemodilution decreased Hb concentration from  $14.2 \pm 0.9$  to  $11.4 \pm 0.5 \text{ g dl}^{-1}$ . Despite a decrease in viscosity ( $3.5 \pm 0.3$  vs.  $2.8 \pm 0.3 \text{ cP}$ ,  $P<0.001$ ), haemodilution increased PASP in both normoxia ( $1.6 \pm 1.5$  mmHg,  $P=0.008$ ) and hypoxia ( $4.5 \pm 2.4$  mmHg,  $P<0.001$ ). PASP was elevated with acute hypoxia to a greater extent following haemodilution ( $4.9 \pm 5.3$  vs.  $7.8 \pm 5.3$  mmHg; interaction  $P=0.005$ ), as reflected in a greater PASP/ $\text{CaO}_2$  slope post haemodilution ( $-1.14 \pm 1.2$  vs.  $-2.16 \pm 1.3$  mmHg/ml  $\text{dl}^{-1}$ ,  $P<0.001$ ). In chronic hypoxia, Hb concentration ( $16.1 \pm 0.9$  vs.  $14.1 \pm 1.0 \text{ g dl}^{-1}$ ,  $P<0.001$ ) blood viscosity ( $4.5 \pm 0.6$  vs.  $3.7 \pm 0.4 \text{ cP}$ ,  $P<0.001$ ) and  $\text{CaO}_2$  ( $18.8 \pm 1.4$  vs.  $16.7 \pm 1.3 \text{ ml dl}^{-1}$ ,  $P<0.001$ ) were all reduced following haemodilution. However, PASP remained unchanged ( $22.7 \pm 5.2$  vs.  $24.5 \pm 5.2$  mmHg,  $P=0.14$ ). In contrast, acute pokilocapnic hypoxia increased PASP both pre ( $2.4 \pm 2.1$  mmHg,  $P=0.004$ ) and to a greater extent post haemodilution ( $5.1 \pm 4.2$  mmHg,  $P=0.002$ ). Collectively, these findings demonstrate that haemodilution augments the HPV response to acute but not chronic hypoxia, and the increase in PASP appears to be independent to mechanical viscosity with moderate changes in haemoglobin concentration.

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PCB002

# **Low-pressure baroreflex control of renal sympathetic nerve activity in rats exposed to chronic intermittent hypoxia: Effect of TRPV1 channel blockade.**

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Ischemic kidney is associated with sympathetic over-activity and impaired baroreflex control of blood pressure. Kidney hypoxia is observed in the chronic intermittent hypoxia (CIH)-induced hypertension model. This study investigated the baroreceptor reflex in a CIH rat model and whether renal transient receptor potential vanilloid 1 (TRPV1) contributes to the modulation of low-pressure baroreceptor reflex regulation of renal sympathetic nerve activity (RSNA). Wistar rats (225-272g) were exposed to CIH (90s exposure to 5%  $\text{O}_2$  followed by 210s exposure to 21%  $\text{O}_2$ , 8 hr/day,  $n=4$ ) or to normoxia (sham, 21%  $\text{O}_2$ ,  $n=4$ ) for 14 days. On day 15, the rat was anaesthetized (induction: urethane,  $\alpha$ -chloralose and sodium pentobarbitone mixture i.p. (416, 27 and 33mg/kg), maintenance: urethane and  $\alpha$ -chloralose mixture (62 and 4mg/kg i.p.). Cannulae were inserted into the femoral artery to measure mean arterial pressure (MAP) and, heart rate (HR), and femoral vein to infuse saline (3ml/hr). The right kidney was exposed, renal nerves were dissected and held on bi-polar electrodes to record RSNA. After a 90min stabilization, the low-pressure baroreflex was studied by two consecutive volume expansion (VE) trials. In control conditions, rats received intrarenal infusion of saline (1ml/hr) 30min before and during VE (0.25ml saline/100g body weight/min for 30min i.v.). During test conditions, a TRPV1 blocker, capsazepine (CPZ) was infused (5 $\mu$ g/ml/hr) 30min before and during a repeat VE trial. RSNA sympatho-inhibitory response curves to VE were obtained from control and test conditions in sham and CIH rats. Rats were euthanized and the left kidney was harvested for histological assessment using hematoxylin and eosin stain. Values are mean $\pm$ S.D. No major histological changes were revealed. Glomerular tuft area was equivalent between sham ( $7332 \pm 1628 \mu\text{m}^2$ ,  $n=3$ ) and CIH rats ( $7492 \pm 1531 \mu\text{m}^2$ ,  $n=5$ ;  $p=0.533$ , Mann Whitney test) when normalized to body weight. Basal MAP ( $102 \pm 13$  vs.  $99 \pm 7$  mmHg) and RSNA ( $0.73 \pm 0.18$  vs.  $0.93 \pm 0.47 \mu\text{V.s}$ ) were similar in sham and CIH groups, but HR ( $414 \pm 6$  vs.  $471 \pm 36$  bpm,  $p<0.05$ ) was higher in CIH rats. VE decreased RSNA over the 30min period from baseline in sham rats and to a lesser extent in CIH rats ( $p=0.055$ , repeated measures two way ANOVA). In addition, area under the curve (AUC) for the sympatho-inhibition during VE was smaller in the CIH compared with the sham group ( $498 \pm 284$  vs.  $1308 \pm 819$ ,  $p=0.05$ , one tailed independent t-test). CPZ had no effect on the sympatho-inhibitory response to VE in sham and CIH rats (AUC, Sham: Saline vs. CPZ,  $1308 \pm 819$  vs.  $1526 \pm 642$ ; CIH:  $498 \pm 284$  vs.  $690 \pm 637$ ). Preliminary findings suggest that moderate exposure to CIH does not induce pathological changes in the kidney but blunts low-pressure baroreflex control of RSNA through a mechanism that might not involve renal afferent TRPV1.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB003

**Long-term recording of renal and lumbar sympathetic nerve activity in response to myocardial infarction in conscious rats**

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Sympathetic nerve activity (SNA) has been implicated in the morbidity and mortality associated with acute myocardial infarction (MI). However, little is known regarding the time course and degree of changes in SNA caused by MI. This study was designed to continuously measure renal sympathetic nerve activity (RSNA) and lumbar sympathetic nerve activity (LSNA) in a longitudinal manner for 28 days in response to MI by ligation of the left anterior descending artery (LAD). Male Wistar rats were randomly allocated to LAD ligation (MI group) or sham surgery. The rats were anaesthetized with isoflurane and pentobarbital sodium and chronically implanted with a Data Sciences International telemetry device and a bipolar renal and lumbar nerve electrode for continuous measurement of the mean arterial pressure (MAP), heart rate (HR), RSNA, and LSNA. After 7 days of surgical recovery, the MAP, HR, RSNA, and LSNA were monitored continuously for 4 days (control period; Days -4 to -1). Four days later, the animals were anaesthetized by inhalation of isoflurane (4%) and intubated, and the heart was exposed via a left thoracotomy. The LAD was ligated with a 6-0 suture near its origin. Another group of rats underwent sham ligation. Transthoracic echocardiography was performed before (Day -4) and after (Day 24) the experiment. After the experiment, the heart was excised and stained with Masson trichrome to confirm the infarct size. The rats in the MI group were divided into two subgroups: those that died within 3 days after LAD ligation (MI-3Days group) and those that survived for 24 days (MI-Survivor group). On Day 1 in the MI-3Days group, LSNA increased by  $200\% \pm 40\%$  and MAP decreased by  $25 \pm 5$  mmHg while RSNA and HR did not change significantly relative to the pre-MI values. On Day 1 in the MI-Survivor group, LSNA tended to increase while RSNA and HR did not change significantly relative to the pre-MI values. In the MI-Survivor group, RSNA increased gradually from Day 9 and reached a statistically significant level by  $20\% \pm 3\%$  on Day 24, while LSNA and MAP remained unchanged compared with the sham group. The major differences in the response of SNA between the MI-3Days and MI-Survivor groups were the massive increase in LSNA and sustained reduction of MAP observed on Day 1. Why LSNA increased in a regionally specific manner in the MI-3Days group remains unknown; however, LSNA could serve as a marker for predicting a poor prognosis after MI. On Day 24 in the MI-Survivor group, RSNA was significantly higher than that in the sham group; however, there were no differences in MAP, HR, or LSNA. These findings suggest that RSNA is likely to play a significant role in compensating for the malfunction of cardiac performance caused by MI. This study shows that LSNA and RSNA respond to MI differently over time and in a region-specific manner after MI.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## PCB004

**Preventing Hypertension after Ischemic Stroke in the Spontaneously Hypertensive Rat**P. Thakkar<sup>1</sup>, C. Barrett<sup>1</sup>, A. McGregor<sup>3</sup>, A. Barber<sup>2</sup>, J.F. Paton<sup>1,4</sup> and F. McBryde<sup>1</sup>

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Over 80% of patients show a transient increase in blood pressure (BP) after ischemic stroke, regardless of their previous blood pressure level. However, whether this increased pressure is necessary to enhance or maintain oxygenation of penumbra tissues remains uncertain. We aimed to determine the impact of preventing post-stroke hypertension in the spontaneously hypertensive (SH) rat model of ischemic stroke.

Male SH rats ( $374 \pm 9$  g) were instrumented under isoflurane anaesthesia (2-3% in 100% oxygen) to allow the long-term telemetry recording of BP, intracranial pressure (ICP) and brain tissue oxygen in the penumbra ( $pO_2$ ). After recording a 3-day baseline, ischemic stroke was induced on Day 0 via a two-hour occlusion of the middle cerebral artery (MCAo), under anaesthesia as above. The increase in BP after stroke was either Untreated ( $n=6$ ), or Treated ( $n=6$ ) and controlled to baseline levels with the clinically-indicated adrenergic antagonist, labetalol ( $0.25\text{mg/kg/hr}$  sc via osmotic pump). Neurological recovery (SHIRPA score) was assessed on Days 1, 3, 7 and 10 to evaluate the functional recovery from stroke over time, and post-mortem stroke infarct size assessed by histology on Day 10.

Following stroke, BP increased rapidly in Untreated SH rats, reaching a peak of  $35 \pm 3$  mmHg above baseline within 24 hours. Treatment with labetalol significantly reduced BP on D0 and D1 ( $p < 0.05$ ), which in combination with a small increase in ICP ( $+3 \pm 0.8$  mmHg in Untreated;  $+0.4 \pm 0.3$  mmHg in Treated;  $p = 0.047$ ) on D0 resulted in a lower cerebral perfusion pressure ( $\text{CPP} = \text{MAP} - \text{ICP}$ ) in Treated animals. Penumbra tissue oxygen levels were not reduced with labetalol treatment, with a small but significant elevation in  $pO_2$  seen in the treated animals in the 24 hours after stroke. Treated and Untreated rats showed a similar impairment after stroke in terms of neurological score, functional outcome and infarct volume ( $274 \pm 26\text{mm}^3$  vs Untreated  $256 \pm 11\text{mm}^3$ ).

Treating post-stroke hypertension does not appear to have a major impact on the functional recovery from stroke in rats with pre-existing hypertension. Future studies will examine the impact of blood pressure lowering (1) in SH rats already receiving treatment for pre-existing hypertension, and (2) treatment to normotensive BP levels after stroke in SH rats, to mimic the clinical situation of undiagnosed pre-existing hypertension.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

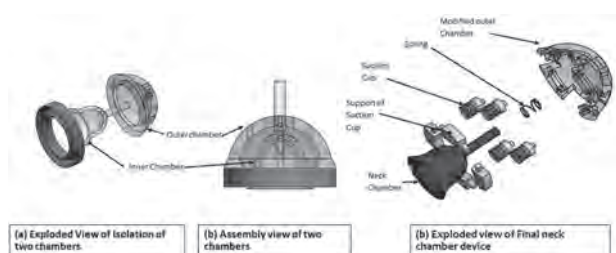


PCB005

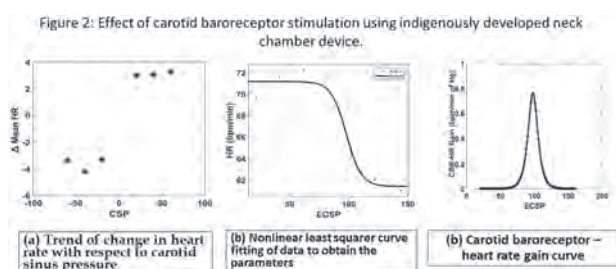
### Designing, Development and Testing of Indigenous Paired Neck Chamber Device for Evaluation of Baroreflex Sensitivity.

P.H. Kamble<sup>1</sup>, R. Sharma<sup>1</sup>, P. Paliwal<sup>2</sup>, K. Desai<sup>2</sup> and A. Singhal<sup>1</sup><sup>1</sup>Physiology, All India Institute of Medical Sciences, Jodhpur, Jodhpur, India and <sup>2</sup>Mechanical Engineering, Indian Institute of Technology, Jodhpur, Jodhpur, India

Neck collar device is widely practiced method of autonomic function assessment in sophisticated autonomic function research laboratories. But there are several limitations of neck collar device such as it creates discomfort to the patient due to a tight seal around the neck, unilateral testing of carotid baroreceptors is not possible, there are chances of stimulation of neck receptors other than carotid baroreceptors and it is expensive. To overcome these limitations, we designed and indigenously developed a neck chamber device which consisted of cup shaped outer and inner chamber. (Figure 1) Outer chamber consisted of four suction cups to hold the device over the neck, while the inner cup was connected to pump which can create controlled positive and negative pressure inside the chamber. After obtaining Institutional Ethics clearance, 30 young healthy volunteers within age 20-25 years were recruited for testing. Neck chamber stimulation was given in steps of -20, -40, -60, 0, +20, +40 and +60 mm of Hg with the gap of 60 seconds between each stimulation. The changes in RR interval, HR and BP were recorded. Then saturation point, threshold point and optimal gain was calculated using logistic equation derivative and gain curve plot. (Figure 2) The mean optimal gain or baroreceptor sensitivity was  $-0.579 \pm 0.045$ , while the saturation point and threshold point were  $0.028 \pm 0.0018$  and  $-0.028 \pm 0.0018$  respectively. The results of the present study were consistent with the previous studies using neck collar. Thus, we have indigenously developed a low cost, strap less, unilateral and bilateral baroreceptor stimulating neck chamber device. In future, we have planned to test the validity and reliability of present device as to replace the mechanical functioning with the automated electronic features.



Design of neck chamber device



Baroreceptor Gain curve

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB007

### Acute changes of natriuretic peptides during pulmonary hyperinflation

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of Copenhagen, Denmark, Copenhagen, Denmark

Pulmonary hyperinflation attained by glossopharyngeal insufflation (GPI) challenges the circulation by compressing the heart and pulmonary vasculature and leads to ~50% decrease in pulmonary and central blood volume (CBV)<sup>1</sup>. Atrial natriuretic peptide (ANP) is considered an important element for acute and chronic regulation of body fluid homeostasis. Cardiac muscle cells release ANP into circulation under high blood volume and pressure<sup>2</sup>. With decreased CBV during pulmonary hyperinflation, we expected an inhibition of ANP to maintain homeostasis. The present study evaluated how changes in venous plasma ANP reflect the changes in lung and atria volumes during GPI. Seven healthy experienced breath-hold divers performed three breath-holds with different attained lung volume (end tidal expiration, roughly half the amount of maximal GPI and maximal GPI). Cardiac output, pulmonary and atria volumes were assessed by magnetic resonance imaging. Heart rate and beat-to-beat blood pressure were determined by finger photoplethysmography. Venous plasma ANP was determined by radioimmunoassay. During GPI, the lung volume increased by  $1.9 \pm 1.2$  L ( $26 \pm 19\%$ ,  $p < 0.001$ ) above the total lung capacity ( $7.6 \pm 1.4$  L). Atria decreased in volume by  $11.2 \pm 5.2$  mL ( $30 \pm 11\%$ ,  $p < 0.001$ ). ANP did not change with GPI ( $p = 0.14$ ). There was no correlation between venous ANP and changes in pulmonary or atria volume. Venous plasma ANP is not a marker of acute CBV changes which occur as a result of compressed atria and pulmonary vasculature during pulmonary hyperinflation.

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Stein, B. C. and R. I. Levin (1998). "Natriuretic peptides: physiology, therapeutic potential, and risk stratification in ischemic heart disease." *Am Heart J* 135(5 Pt 1): 914-923.

Per Lav Madsen was funded by the Danish Heart Association. Tanja Mijacika, Zeljko Dujic, Ivan Drvis and Otto Barak were funded by Croatian Science Foundation (grant no IP-2014-09-1937).

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB008

### Increased DNA damage with comet assay in patients with pulmonary hypertension

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Pathologic lesions involve increased oxidative stress and inflammatory processes in pulmonary hypertension (PH) (1). The purpose of our study was to investigate the potential contribution of DNA damage in PH. The study comprised 28 untreated PH patients (59.93±11.19 years) and 28 age- and sex-matched (59.86±11.92 years) healthy controls. All participants underwent a right-heart catheterization to confirm the diagnosis and to assess hemodynamics. Fifty cells per slide/sample were scored to evaluate DNA damage using the comet assay (2). Several parameters including head length, tail length, head intensity, tail intensity, tail moment, tail migration were evaluated. Continuous data were reported as mean ± standard deviation. Mann Whitney U test was used for independent groups. For categorical variables, Chi-square test was used. SPSS, 24.0 was used for statistical analyses and p value less than 0.05 was considered statistically significant. The average pulmonary vascular resistance was 5.64±2.99 woods, mean right atrial pressure 9.70±5.38 mmHg, cardiac index 3.12±1.18 l/min/m<sup>2</sup>, mixed venous O<sub>2</sub> saturation was 64.77±13.33. In our study, PH patients had significantly higher tail length, tail moment and tail migration values compared to the control group (Table 1). Higher tail length values are known as the signs of increased DNA breaks, however tail moment, which is derived from the tail length and % DNA in the tail, shows more stable predicting performance for DNA damage according to most researchers. These results suggest that patients with PH could have increased DNA damage and this damage could be contributing to the pathophysiology of the disease

DNA damage assay parameters of the study groups

| Comet assay values  | Pulmonary hypertension (PH) (n:28) | Control group (n:28) | p values |
|---------------------|------------------------------------|----------------------|----------|
| Head length (µm)    | 29.22 ± 9.53                       | 27.13 ± 4.63         | 0.33     |
| Tail length (µm)    | 24.02 ± 11.34                      | 16.88 ± 3.55         | 0.0001*  |
| Head intensity (%)  | 84.98 ± 13.24                      | 90.86 ± 7.91         | 0.123    |
| Tail intensity (%)  | 15.02 ± 13.24                      | 9.14 ± 7.91          | 0.123    |
| Tail moment (µm)    | 1.93 ± 2.36                        | 0.87 ± 1.03          | 0.013*   |
| Tail Migration (µm) | 10.3 ± 12.24                       | 3.62 ± 2.75          | 0.03*    |

Ranchoux B, Meloche J, Paulin R, Boucherat O, Provencher S, Bonnet S. DNA damage and pulmonary hypertension. *Int J Mol Sci.* 2016;17:17.

Collins A.R. Measuring oxidative damage to DNA and its repair with the comet assay. *Biochim Biophys Acta.* 1840 (2) (2014), pp. 794-800.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB009

### Comparison of nonlinear and linear heart rate variability parameters in diagnosis of cardiac autonomic neuropathy in patients of multiple system atrophy

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Background:

The purpose of this study was to compare sensitivity and specificity of Nonlinear and Linear heart rate variability parameters (HRV) in diagnosing cardiac autonomic neuropathy (CAN) in multiple system atrophy (MSA) patients.

Methods:

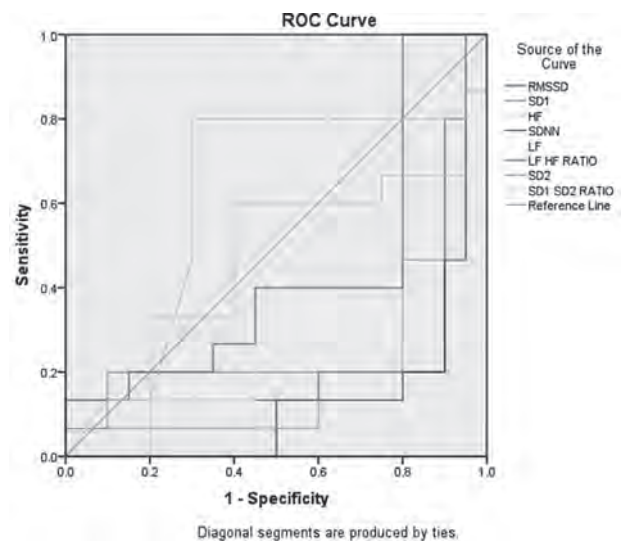
Nonlinear (Poincare analysis) and Linear (Time and Frequency domain analysis) HRV parameters were investigated among 35 MSA patients. ROC curve was plotted to compare sensitivity and specificity of individual tests. Ewings Scoring was used as the gold standard for diagnosis of CAN.

Results:

The area under curve for SD1/SD2, HF and LF/HF ratio is 0.620, 0.453 and 0.424 with 95% confidence interval of 0.417-0.823, 0.248-0.658 and 0.222-0.631 respectively. Time domain parameters had smaller area under curve as compared to Poincare and Frequency domain parameters.

Conclusions:

This suggests that Nonlinear (Poincare) parameters specially SD1/SD2 has better sensitivity and specificity as compared to the Linear parameters of HRV in diagnosing CAN in MSA patients.



We would like to acknowledge Autonomic Function Lab, AIIMS Jodhpur and AFT technician Mr. Shiv Kumar.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB010

**To evaluate the diagnostic efficacy of parasympathetic autonomic response latency in type 2 diabetes mellitus**

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<sup>1</sup>Physiology, All India Institute of Medical Sciences, Jodhpur, India and <sup>2</sup>Physiology, RKGMC, Hamirpur, India

**Introduction:**

The clinical complications of diabetes induced cardiac autonomic neuropathy (CAN) include resting tachycardia, orthostatic hypotension, exercise intolerance and myocardial infarction with increased risk of mortality. To identify early stages of CAN tests heart rate variability (HRV) and autonomic reactivity based on Ewing score are helpful. Latency parameters are routinely used in most of the usual evoked potential studies. It has been found to have diagnostic and prognostic value for many disorders. Sharma et al in 2004 had identified this parameter in cardiovascular autonomic reactivity testing and addressed it in normal healthy young adults who underwent a short term physical training. Thus latency, i.e. time taken by the system to manifest peak change in the cardiovascular response to various stimuli which perturb the hemodynamic status should also be measured and quantified. This would provide an additional dimension to the already routinely quantifiable cardiovascular autonomic response parameters. To the best of our knowledge, no study has been undertaken to study these parameters in diabetic patients.

**Methods:**

So the authors evaluated the diagnostic efficacy of Tachycardia and Bradycardia latencies (TL and BL) in routine parasympathetic reactivity tests which included Lying to standing and Valsalva manoeuvre. A total of 52 diagnosed Type 2 DM subjects were included in this study. The participants were excluded if they had any other systemic or endocrine disorder other than diabetes. The statistical analysis of the data was done by using ROC curve and its standard parameters.

**Results:**

The area under curve for tachycardia latency and bradycardia latency during lying to standing test was 0.624 and 0.632 with 95% confidence interval of 0.393-0.441 and 0.441-0.824 respectively. Similarly, the area under curve for tachycardia latency and bradycardia latency during Valsalva maneuver was 0.499 and 0.590 with 95% confidence interval of 0.288-0.710 and 0.384-0.796 respectively. While E:I ratio, 30:15 ratio, Valsalva ratio and Delta HR during DBT had smaller area as compared to latency parameters.

**Conclusion:**

This suggests that latency parameters have better sensitivity and specificity as compared to individual parasympathetic reactivity tests.

We would like to acknowledge Autonomic Function Lab, AIIMS Jodhpur and AFT technician Mr. Shiv Kumar.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## PCB011

**Middle cerebral artery blood flow velocity regulation during short-term normobaric isocapnic hypoxia**

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University of Birmingham, Birmingham, UK

Hypoxia elicits ventilatory and cerebrovascular adjustments in order to defend against potential reductions in brain oxygenation. Hypoxia-mediated increases in cerebral blood flow can occur via local mechanism(s), but a secondary contribution may come from the elevated ventilation (e.g., augmented respiratory muscle pump and neurovascular coupling). We sought to isolate the contribution of hypoxia *per se*, from the possible secondary effect of increased ventilation, on increases in cerebral perfusion during short-term normobaric isocapnic hypoxia. In 11 young, healthy men ( $25 \pm 3$  yr, mean  $\pm$  SD), middle cerebral artery mean blood flow velocity ( $MCA_V$ ; transcranial Doppler), mean arterial pressure (MAP; Finometer) and the partial pressure of end-tidal carbon dioxide ( $P_{ET}CO_2$ ) and oxygen ( $P_{ET}O_2$ ) were recorded. Cerebral vascular conductance index (CVCi) was calculated as  $MCA_V / MAP$ . After a 10 min baseline, participants undertook four 10 min experimental trials during which  $P_{ET}O_2$  and  $P_{ET}CO_2$  were controlled using an end-tidal forcing system. The trials were; 1) normoxia [ $P_{ET}O_2 = 100$  mmHg], 2) hypoxia [ $P_{ET}O_2 = 50$  mmHg], 3) hypoxia with breathing frequency and tidal volume volitionally controlled at target levels measured during normoxia [ $P_{ET}O_2 = 50$  mmHg], and 4) normoxia with breathing voluntarily matched to target levels observed during hypoxia [ $P_{ET}O_2 = 100$  mmHg]. During all experimental trials,  $P_{ET}CO_2$  was held constantly to a baseline +1 mmHg level. Trials were separated by a rest period of  $\sim 20$  min. Compared to normoxia (trial 1), during hypoxia (trial 2) increases were observed in ventilation (by  $\sim 65\%$ ),  $MCA_V$  ( $45 \pm 2$  vs.  $48 \pm 3$   $cm \cdot s^{-1}$ , mean  $\pm$  SE) and CVCi ( $0.84 \pm 0.05$  vs.  $0.95 \pm 0.05$  mmHg/ $cm \cdot s^{-1}$ ) ( $P < 0.05$ ). Hypoxia with controlled breathing (trial 3) also increased  $MCA_V$  ( $52 \pm 3$   $cm \cdot s^{-1}$ ) and CVCi ( $0.88 \pm 0.04$  mmHg/ $cm \cdot s^{-1}$ ) ( $P < 0.05$  vs. trial 1), while ventilation was not different to normoxic levels ( $P > 0.05$  vs. trial 1). During normoxia with controlled breathing (trial 4) ventilation was elevated as during hypoxia with spontaneous breathing ( $P < 0.05$  vs. trial 1), but  $MCA_V$  ( $44 \pm 3$   $cm \cdot s^{-1}$ ) and CVCi ( $0.81 \pm 0.05$  mmHg/ $cm \cdot s^{-1}$ ) were not different to that observed in normoxia with spontaneous breathing ( $P > 0.05$  vs. trial 1). In summary, at the level of isocapnic normobaric hypoxia studied, changes in blood oxygen concentration, and not a secondary effect of an increase in ventilation, make the predominate contribution to increases in cerebral perfusion.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## PCB012

**Chronic exposure to chlorpyrifos changes cardiac morphometry in rats.**

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Previous studies from our group showed that acute exposure to chlorpyrifos (CPF), an organophosphorus (OP) compound,



impairs cardiovascular reflexes in rats (Cunha *et al.*, 2018). Some evidence suggests that low level chronic exposure to OP compounds induces cardiac hypertrophy (Calore *et al.*, 2007). However, whether long term exposure to CPF affects cardiac morphometry in rats remains unexplored. Male Wistar rats were intraperitoneally injected with CPF for 1 month (3 times/week), at the following doses: 7 mg/kg (CPF 7; n=25) and 10 mg/kg (CPF 10, n=19). A control group received saline (NaCl, 0.9%) for the same period (SAL, n=21). The study followed the recommendations of the Brazilian National Council for Animal Experimentation and was approved (approval number 20/2016) by the University Committee for Animals Ethical Use (CEUA-UFES). After treatment, animals were euthanized by decapitation, and hearts and blood samples collected for measurement of plasma butyrylcholinesterase (BuChE) activity to confirm OP exposure. As evidence has shown that anaesthesia may interfere with BuChE activity (Nana *et al.*, 1977), decapitation was conducted by a well experienced-trained researcher, without the use of anaesthetics. After removing the hearts, left ventricles (LV) were separated and both wet and dry weight documented (SAL, n=11, CPF 7, n=15 and CPF 10, n=11) and ventricular mass normalized to body weight (LV/BW). Some ventricular tissue (SAL, n= 10, CPF 7, n=10 and CPF 10, n=8) was sectioned and stained with hematoxylin–eosin or picosirius red to determine the smaller diameter and collagen content, respectively. Images were captured with a video camera (AxioCam ERc 5s, Carl Zeiss) coupled to an optical microscope (Olympus AX70, Olympus Corporation) under 400X magnification. All histologic analysis was performed using ImageJ software (v.1.43u, National Institutes of Health). Data were analysed by ANOVA followed by Dunnett's *post hoc* test and expressed as mean  $\pm$  error standard of mean (EPM). No statistical difference was observed for wet ( $F_{(2,32)} = 0.87$ ;  $P = 0.43$ ) or dry ( $F_{(2,32)} = 0.55$ ;  $P = 0.58$ ) left ventricular mass when standardises with body weight, among all groups tested. On the other hand, myocytes smaller diameter and percentage of collagen content was significantly increased in CPF 10 group compared to SAL animals ( $P < 0.05$ ). BuChE activity was significantly decreased in all CPF treated animals when compared with SAL group ( $P < 0.05$ ). Our data shows that despite the absence of changes in LV/BW ratio, CPF treatment altered cardiac morphometry in the animals exposed to OP. These results may have important clinical implications for laborers, such as farmers, frequently exposed to these compounds.

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Cunha AF *et al.* (2018). *Toxicology* 398-399: 13-22.

Nana A *et al.* (1977). *Acta Anaesthesiol Belg.*; 28(3):183-7.

Financial Support: Fapes Foundation.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB013

### Possible Effects Of Agomelatine On Human Heart Muscle Contractility: An In Vitro Experimental Study

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Turkey and <sup>3</sup>Physiology, Hitit University Faculty of Medicine, Corum, Turkey

#### AIM

The novel antidepressant agomelatine is an agonist of melatonin receptors (MT1 and MT2). This drug has also serotonin 2c receptor antagonism properties. The effect of agomelatine on cardiovascular system is not clear so far. The aim of the present study is to investigate the effect of agomelatine on human atrium contraction.

**METHODS:** Human atrium tissues (n=30) were taken from patients who underwent coronary bypass surgery. The patients' ages were between 47 to 72. All tissues were placed in organ baths containing Krebs solution, thermoregulated at 37 °C and aerated (95 % O<sub>2</sub> and 5% CO<sub>2</sub>). Changes in isometric tensions were recorded using a four channel force displacement transducer. All tissues washed for 3 hours in order to diminish the effects of anaesthetic agents. Adrenaline (10<sup>-1</sup> M) was administered in tissue cabs to induce isometric contractions. Contraction with measurements were used as contraction parameters. Cumulative agomelatine (10<sup>-9</sup> to 10<sup>-4</sup>) doses were added in organ baths. The contractions were recorded accordingly. Friedman and Kruskal Wallis tests were used for statistical evaluation.

**RESULTS AND CONCLUSIONS:** Inhibition of contraction was statistically significant in the last cumulative dose of agomelatine (10<sup>-4</sup> M) ( $p < 0.05$ ) following the administration of adrenaline. Results of this study showed that agomelatine may cause negative inotropic effects at certain high doses on human atrium muscle. Further studies are needed to clarify the mechanism(s) of effect of agomelatine. These results suggest that agomelatine may have beneficial effects on treatment of cardiovascular diseases.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB014

### A high salt diet results in augmented osmolal gradients in rat skin

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Excessive salt (NaCl) ingestion or retention in the body is linked with hypertension. Although the kidney is the major regulator for body Na<sup>+</sup>, it has also been shown that the skin is involved in Na<sup>+</sup> homeostasis and can be an important reservoir in case of Na<sup>+</sup> retention. Little is known about the local distribution of sodium during high salt conditions. In previous experiments in rats we found that a high salt diet gave a significant increase in blood pressure, and resulted in increased Na<sup>+</sup> accumulation in the skin along with an increased skin osmolality when compared with rats given low salt diet (Nikpey *et al.*, Hypertension, 69, 660-668, 2017). The plasma osmolality

did not differ significantly between the groups. These studies suggested that excessive  $\text{Na}^+$  accumulation in the skin creates a hypertonic microenvironment, but also that there might be gradients within the skin that may have consequences for the local handling and storage of osmolytes, including  $\text{Na}^+$ . We wanted to explore this gradient in further detail because of its potential physiological importance for skin electrolyte homeostasis. Male Sprague Dawley rats received either high-salt diet; HSD (chow having 8% salt and 1% saline) or low salt-diet (control); LSD (chow having <0.1% salt and tap water) for 14 days. Upon termination of the feeding period, back skin was harvested and frozen. Contiguous sections of skin parallel to the surface from epidermis (40mm) through upper dermis to lower hypodermis (all 100mm) were prepared with a cryomicrotome, and were eluted in distilled (milli-Q) water for 24 h and analyzed for  $\text{Na}^+/\text{K}^+$  in the eluate by HPLC. Assuming that there is an interstitial fluid volume of 40% in all layers, we found that there is a  $\text{Na}^+$  gradient from epidermis to hypodermis in both HSD (epidermis; 147mM – dermis; 173mM,  $n=1$ ) and LSD (epidermis; 143mM – dermis; 99mM,  $n=1$ ) rats. Simultaneously, the  $\text{Na}^+$  content in each layer of HSD skin was higher compared with LSD skin. The  $\text{Na}^+$  gradient was significantly higher in the skin layers of the HSD rat (149mM,  $n=1$ ) compared to the LSD rat (90mM,  $n=1$ ) ( $p<0.05$ , ANOVA). Because urea might also be a contributor to skin osmolality and a potential gradient, we assessed urea using an assay kit (Urea colorimetric assay kit, Biovision) in eluted skin sections from HSD and LSD rats. Urea concentration was higher in the superficial than in the deeper layers in both HSD and LSD skin. Thus urea concentration averaged 10 and 8.3 mmol/ml/mg dry weight in epidermis–dermis and deeper dermis, respectively, in HSD ( $n=2$ ) with corresponding values of 10 and 1.7 mmol/ml/mg dry weight in LSD ( $n=2$ ). Our preliminary experiments suggest that urea as well as  $\text{Na}^+$  may contribute to the observed osmolal gradient and supports the notion of countercurrent exchange in the skin.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

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PCB015

#### Proarrhythmic effects of $\beta$ -adrenergic stimulation in a pharmacological model of LQT3 in the absence of action potential prolongation.

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Long QT syndrome type 3 (LQT3) is a congenital cardiac channelopathy associated with sudden death. Dysfunction in the cardiac sodium channel results in delayed repolarisation of the action potential.  $\beta$ -blockers are the first line of treatment, although their efficacy has been questioned and the role of  $\beta$ -adrenergic stimulation in cardiac arrhythmias occurring in LQT3 is not fully understood.

We aimed to evaluate the effects of  $\beta$ -adrenergic stimulation alongside the influence of pacing rate on proarrhythmic parameters in the ventricular action potential.

Experiments were conducted with local ethical approval and in accordance with UK Home Office and European Parliament Directive 2010/63/EU guidelines on the use of animals in research. Firstly, monophasic ventricular action potentials were recorded from Langendorff perfused Wistar rat hearts,

stimulated for 60 s at 6, 9 and 12 Hz consecutively. The pacing protocol was performed in the absence or presence of 100 nM isoproterenol (ISO), before and 15 min after the addition of 50ng/l anisoprolol (AP-A). Control hearts underwent a time-matched protocol in the absence of any drugs/toxin. Owing to the high incidence of non-recoverable arrhythmia in AP-A+ISO treated hearts, an additional group was studied with 5 min between pacing protocols. Statistics were performed using ANOVA or Student t-tests. Data is expressed as means  $\pm$  SEM.

Secondly an *in silico* experiment was made using LabHEART - a computational model of the rabbit ventricular myocyte. As a surrogate for LQT3, background sodium conductance was increased from 114  $\mu\text{S}/\mu\text{F}$  to 300  $\mu\text{S}/\mu\text{F}$  at pacing rates of 1, 2 and 3 Hz.

Increasing the pacing rate from 6 to 12 Hz did not significantly alter action potential duration at 90% repolarisation ( $\text{APD}_{90}$ ) in control hearts ( $36.61 \pm 12.54$  to  $32.34 \pm 15.97$  ms,  $n = 6$ , N.S.). AP-A did not cause prolongation of  $\text{APD}_{90}$  at any of the pacing frequencies measured ( $33.40 \pm 13.25$  to  $27.64 \pm 11.30$  ms at 6 Hz,  $32.37 \pm 11.25$  to  $17.35 \pm 10.92$  ms at 12 Hz,  $n = 6$ , N.S.). The addition of ISO did not have a significant effect on  $\text{APD}_{90}$  in either control or AP-A treated hearts (time matched data: Con  $31.96 \pm 23.21$  ms,  $n = 6$ , AP-A  $27.64 \pm 13.25$  ms,  $n = 6$ , ISO  $35.83 \pm 11.7$  ms,  $n=4$  N.S.). Despite an absence of APD prolongation, only 50% hearts treated with AP-A alone and 17% AP-A+ISO did not go into irreversible fibrillation before completion of pacing protocols, compared to 100% control hearts.

In labHEART,  $\text{APD}_{90}$  increased from 218 ms to 226 ms at 1Hz and from 225 ms to 257 ms at 3 Hz. At the higher sodium conductance, simulation of a supramaximal dose of ISO altered the  $\text{APD}_{90}$  to 216 ms and 189 ms at 1 and 3 Hz, respectively. These results show that 50 ng/l AP-A does not cause action potential prolongation in isolated rat hearts. Despite this, ISO in addition to AP-A does increase arrhythmogenic risk. This suggests that  $\beta$ -stimulation in LQT3 may have proarrhythmic consequences by mechanisms independent of APD prolongation.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

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PCB016

#### Novel quantitative tool for the blood flow imaging

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Blood flow imaging became an essential part of biomedical research, particularly in the vascular and neurovascular physiology. It includes various imaging modalities, such as laser Doppler, diffusive correlate spectroscopy, functional ultrasound, laser speckle contrast imaging (LSCI), optical coherence tomography and two-photon microscopy. This techniques cover different resolution, complexity and price levels. Laser speckle contrast imaging is one of the most extensively used tools for the wide-field blood flow characterization (1), as it provides great scalability and high frequency, non-scanning, imaging. It, however, has several drawbacks, with calibration difficulty arguably being the most crucial of them. Comparing contrast values between different experiments, even within the same protocol, proved to be unreliable, thus limiting LSCI

to the relative flow change evaluation and response imaging. Use of multi-exposure speckle imaging (MESI) solves some of this issues (2), but at the expense of system and analysis complexity, which is not always acceptable.

We have developed a new dynamic laser speckle imaging (DLSI) tool that allows quantitative wide-field blood flow characterization as well as diffusive and static scattering components estimation while keeping the system and analysis simple compared to MESI. DLSI allows quantitative estimation of the correlation time  $\tau_c$  in the units of time (see Fig 1, A), which can then be used to calculate the flow speed. Using a mice stroke model we demonstrate that DLSI provides a precise flow change measurement and allows estimation of the static scattering, mapping the tissue swelling in the core of the stroke (see Fig 1, B). We show that DLSI, which is informative but requires processing of high volumes of data, can be used to calibrate LSCI, thus solving contrast imaging problems and providing a lightweight, affordable quantitative tool for the blood flow imaging.

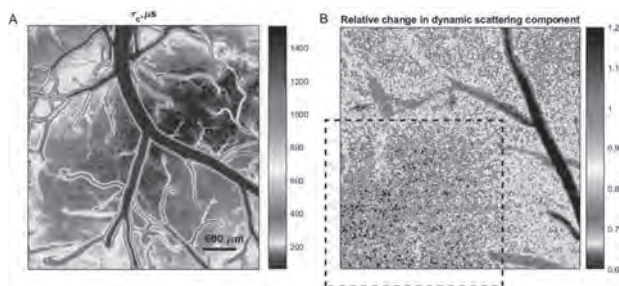


Figure 1. A - correlation time ( $\tau_c$ ) map of the mice cortex. A 2.5x2.5 mm<sup>2</sup> region of the cranial window is shown. Shorter correlation time corresponds to the faster blood flow. B - relative change of the dynamic scattering of the cortex tissue during the stroke. A 1 mm<sup>2</sup> region of the cranial window is shown. Reduction of the dynamic scattering (corresponds to increase of static scattering) implies tissue swelling and maps the core of the stroke (highlighted by the black rectangle).

Briers D, Duncan DD, Hirst ER, Kirkpatrick SJ, Larsson M, Steenbergen W, Stromberg T, Thompson OB. Laser speckle contrast imaging: theoretical and practical limitations. *Journal of biomedical optics*. 2013 Jun;18(6):066018

Parthasarathy AB, Tom WJ, Gopal A, Zhang X, Dunn AK. Robust flow measurement with multi-exposure speckle imaging. *Optics express*. 2008 Feb 4;16(3):1975-89.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB017

### Non-invasive evaluation of the systolic left ventricular load

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Impaired vascular-ventricular coupling might be an additional underlying factor for the development of imbalance between myocardial oxygen supply and oxygen demands (1, 2). The objective of our study was to assess non-invasively left ventricular load and its dependency on central arterial hemodynamics.

Sixty-five healthy and gender-matched individuals were divided in two groups according to their age: 20y/o and 50y/o. Applanation tonometry was performed using the Sphygmocor device (AtCor Medical). Central pressures and

the major indices of pulse wave analysis were computed using Sphygmocor software. Data are presented as means  $\pm$  SEM. The general characteristics and the relevant central hemodynamic parameters of the studied subjects are presented in Table 1.

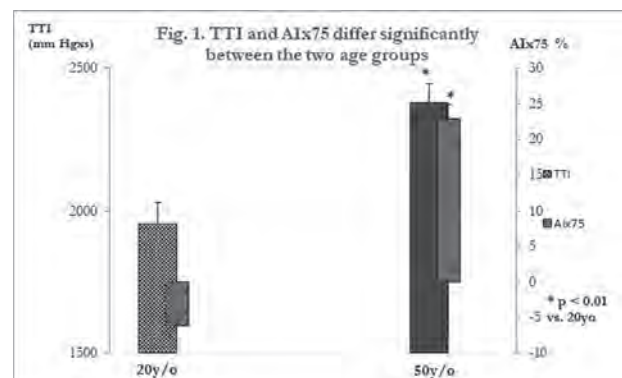
Central systolic and pulse pressures as well as the augmentation index (Alx75), standardized for heart rate 75 beats per minute (bpm), were significantly higher in the 50y/o group as compared to the young individuals. All these parameters are relevant markers of arterial stiffness (3, 4) and evidenced the development of morphological and functional alterations of the central arterial wall in the middle aged group. The time-tension index (TTI) computed from the systolic pressure area under the pulse waveform is presumed to represent the left ventricular load and myocardial oxygen demand. TTI was significantly higher in the 50y/o subjects as compared to the 20y/o group (Fig. 1). Moreover, we have shown the presence of significant correlation between TTI and Alx75 ( $p < 0.01$ ) in both age groups. This finding confirmed the impact of arterial stiffness on the development of impaired vascular-ventricular coupling. In addition, negative correlation was shown between Alx75 and the subendocardial viability ratio (SEVR), a parameter associated with pressure and time for coronary perfusion.

In conclusion, applanation tonometry might be utilized for non-invasive evaluation of the left ventricular load, which is an important parameter of cardiovascular risk.

Table 1

| Groups             | Middle aged individuals (50y/o, n = 33) | Young individuals (20y/o, n = 32) |
|--------------------|-----------------------------------------|-----------------------------------|
| Age (years)        | 53 $\pm$ 2                              | 21 $\pm$ 0.7                      |
| Heart rate (bpm)   | 70 $\pm$ 1                              | 68 $\pm$ 2                        |
| Aortic SAP (mm Hg) | 120 $\pm$ 3#                            | 98 $\pm$ 2                        |
| Aortic PAP (mm Hg) | 43 $\pm$ 3#                             | 29 $\pm$ 1                        |
| Alx75 (%)          | 23 $\pm$ 3#                             | -6 $\pm$ 2                        |
| TTI (mmHg x s)     | 2378 $\pm$ 66#                          | 1954 $\pm$ 73                     |

#  $p < 0.01$  vs. 20yo, Mann-Whitney test



Time tension index (TTI) and augmentation index corrected for heart rate 75 bpm (Alx75) differ significantly between the two age groups.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.



## PCB018

**Investigation of Glyphosate Toxicity and its Mechanisms using Zebrafish (*Danio rerio*) Vertebrate Animal Model**

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Human population is constantly exposed to health risks by exposure to chemicals belonging to various classes such as pesticides, pollutants and chemicals used in food industries etc. Unlimited and uncontrolled use of such pesticides can have serious consequences for human health. Glyphosate is one such chemical which is a non-selective, broad-spectrum herbicide, used to prevent the growth of weeds together with genetically-modified soy, corn, canola and cotton as well as in home gardens and parks. Few studies have reported a number of health issues that can arise as a result of chronic exposure to glyphosate as a pollutant.

The overall objective of this study was to investigate the glyphosate induced pathology in zebrafish (*Danio rerio*) animal model including developmental abnormalities and cardiotoxicity. Our results indicate that glyphosate induced morphological and developmental abnormalities in developing zebrafish embryos. LC<sub>50</sub> (Lethal concentration 50) was found to be approximately 66 µg/ml. To investigate cardiotoxicity, we chose heartbeat as a cardiac function assessment parameter. To assess heartbeat reliably and reproducibly, used our own developed ZebraPace (Gaur et al., 2018) method. Glyphosate-induced cardiotoxicity in zebrafish embryos as indicated by a significant decrease in the heartbeat in embryos exposed to 50 µg/ml (Roy et al., 2016) and 100 µg/ml of glyphosate for 48 and 72 hours. We attribute that to the selective down-regulation of L-type calcium channel (encoded by *cacna1c*) accompanied by a selective upregulation of heat shock protein (encoded by *hspb11*) without any observed changes in ryanodine receptor (encoded by *ryr2a*) expression studied by the real-time qPCR assay. *hspb11* has been used as a marker for changes in cytosolic calcium concentration. Therefore, we speculate that glyphosate may be inducing cardiotoxicity by changes in calcium signaling. In addition, in our experiments, glyphosate reduced the expression of nitric oxide (NO) as detected by fluorescence imaging using DAF-FM. NO is cardioprotective therefore any change in NO expression also indicates cardiotoxicity.

Overall, our results indicate that glyphosate can induce cardiotoxicity and suggests caution in its continued and extended use especially when bioaccumulation of glyphosate has been reported.

Gaur, H., Pullaguri, N., Nema, S., Purushothaman, S., Bhargava, Y., and Bhargava, A. (2018). ZebraPace: An Open-Source Method for Cardiac-Rhythm Estimation in Untethered Zebrafish Larvae. *Zebrafish*.

Roy, N.M., Ochs, J., Zambrzycka, E., and Anderson, A. (2016). Glyphosate induces cardiovascular toxicity in *Danio rerio*. *Environ Toxicol Pharmacol* 46, 292-300.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## PCB019

**Effect of Nifedipine on Heart Glucose 6-Phosphate Dehydrogenase of Ischemia / Reperfusion Injured Rat**A. Tanyeli<sup>1</sup>, E. Senturk<sup>2</sup>, E. Eraslan<sup>3</sup>, M. Güler<sup>4</sup> and M. Sentürk<sup>5</sup>

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**AIM:** The purpose of this study is to investigate the effect of nifedipine drug on rat heart tissue glucose 6-phosphate dehydrogenase (G6PD) enzyme in ischemia / reperfusion (I / R) and I / R injured rats.

**METHODS:** Control, sham, I / R (1:24 hours) and nifedipine + I / R are as follows: A total of 24 male Wistar albino rats weighing 250-300 g were equally and randomly divided into four groups. In the last group, nifedipine was administered at the 4 mg/kg dose before intraperitoneal ischemia period. Heart tissues were removed after bilateral I/R process. Tissue levels of enzyme activity of glucose 6-phosphate dehydrogenase (G6PD) were measured. Enzymatic activity was measured by using Beutler's method by using a spectrophotometer. The assay system contained 0.1 mM Tris-HCl (pH 8.5) with 0.5 mM EDTA, 10 mM MgCl<sub>2</sub>, 0.2 mM NADP<sup>+</sup> and 0.6 mM (G6P) in a total volume of 1 ml.

**RESULTS:** Specific activity values were determined for G6PD enzyme at four different experimental groups. Groups were determined as follows: Control group 0.109 EU/mg protein, sham group 0.082 EU/mg protein, I/R group 0.114 EU/mg protein, and nifedipine + I/R group 0.079 EU/mg protein.

**CONCLUSION:** As a result, the activity of the G6PD enzyme was determined to be mostly inhibited in nifedipine + I/R group among all applications. Activity value of nifedipine + I/R group decreased by about 28% compared to controls was observed that  $p \leq 0.05$  is as meaningful.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## PCB020

**Investigation of Intraperitoneally Apelin-13 Infusion on Lung and Kidney Tissues Antioxidant Properties in Experimental Obesity Model in Rats**

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Obesity is a chronic disorder with increasing prevalence worldwide and occurs when energy intake is greater than energy expenditure. Obesity continues to be a public health problem in the general population, and also significantly increases oxidative stress in cell of tissue. Oxidative stress is one of the factors causing obesity, and emerges with the imbalance between the reactive oxygen types and the antioxidant defense system of the cell. The reactive oxygen types that increase in obesity cause apoptosis through the oxidation of DNA, protein and lipids. On the other hand, the weight loss that occurs due to calorie limitation decreases oxidative stress. Apelin is an adipokine excreted from the fat tissue. Apelin-13, is the most active form of apelin. The antioxidant effects of apelin-13 have been reported in literature. This

study was conducted to determine the effects of apelin-13 infusion on the oxidative stress that occurs due to obesity formed in rats.

40 Wistar Albino male rats were used in the study. The rats were separated into 4 groups as the control, sham, 10 ug/kg/day apelin-13, and 100 ug/kg/day apelin infusion (n=10). The rats were fed with high-fat diet feed (Research diet, D12451) as of the 21th day of birth for 12 weeks. After 12 weeks, it was determined that obesity occurred in the rats by scoring according to the Lee Index. Then, the rats were anesthetized (except for the control group), and osmotic mini pumps (Alzet 2ML2) were placed in the peritoneal areas. With the help of the osmotic mini pumps, infusion of normal saline (solvent) was performed to the sham group at a 120 µl volume (5 µl/hour) daily; and the infusion of 10 ug/kg apelin-13 and 100 ug/kg were performed to the Study Groups. After 14 days, the rats were decapitated, and the lung and kidney tissue samples were collected. The SOD and CAT analyses were performed on the tissues collected. Depending on the apelin-13 infusion in the lung and kidney tissues, it was determined that there were increases in the SOD and CAT enzyme levels when compared with the control and sham groups ( $p < 0.05$ ). The results of the present study show that the apelin-13 plays active roles in the reduction of the oxidative damage in obese rats.

This study was supported by Inonu University BAP (Project no: TCD-2017-647).

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB021

# Different Intrinsic Heart Rate Resetting Responses and the Associated Changes in Cardiac mRNA Expression with Warm Acclimation in Rainbow Trout

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Intrinsic heart rate (IHR) resetting is hypothesised to maintain cardiac scope with warm temperature acclimation in rainbow trout. However, little is understood about the timescale of this process, and the mechanisms causing this change.

The original aim was to follow the timescale of IHR resetting of rainbow trout. Two groups of rainbow trout, acclimated to 4°C, were transferred 12°C to follow the timescale of IHR resetting (measured *in vitro*). While one group reset IHR within 1 h at 12°C (Figure 1); the second group did not. In fact, they showed no IHR difference after 10 weeks of acclimation to either 4°C or 12°C (Figure 2). This variation in intrinsic heart rate resetting was unexpected. However, this serendipitous discovery created an opportunity to better link changes in mRNA expression with IHR resetting responses, rather than more general warm acclimation responses, in different cardiac tissues.

Therefore, a Fluidigm qRT-PCR system was used to compare mRNA expression of 30 cardiac function proteins, after 3 weeks of warm acclimation, in three cardiac tissues (sinoatrial node [SAN], atrium and ventricle), for fish with, and without, IHR resetting, and 1 h after acclimation with IHR resetting (n=7). Under control conditions at 4°C, higher HCN1,  $Ca_v1.3$  and  $col1\alpha1$  expression was unique to the SAN; while higher RYR3 and  $NKA\alpha3$  expression was unique to the ventricle. Warm acclimation to 12°C for > 3 weeks, both with

and without IHR resetting, was associated with downregulation of RYR3 in the atrium and HCN2a2 in the SAN, and upregulation of HCN3 in the ventricle. Downregulation of  $NKA\alpha1c$  in all three tissue types and S100 in the SAN, and upregulation HCN1,  $Na_v1.6$ , and  $NKA\alpha3$  in only the ventricle were unique to the fish that reset IHR. Downregulation of HCN2a1 in the atrium and HCN1 in the ventricle, and upregulation of HCN3 in the SAN, and TGF- $\beta1$  and  $col1\alpha1$  in the atrium were unique to fish that didn't reset IHR. However, no mRNA expression changes were observed after 1 h of 12°C acclimation.

While the initial IHR resetting does not involve changes in mRNA expression, changes in mRNA expression are apparent over the longer term. Moreover, these results suggest that while IHR resetting can be fast, it cannot be assumed to always occur in rainbow trout.

Statistical significance =  $p < 0.05$ .

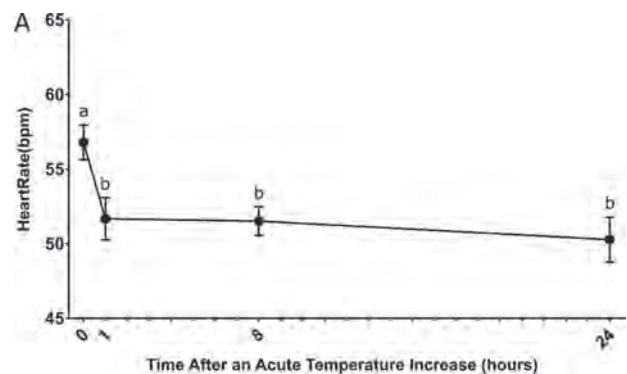


Figure 1 – IHR at 12°C, over time, in response to an acute temperature increase from 4°C to 12°C, in fish with an IHR resetting response (mean  $\pm$  SEM (n=6-9)). Dissimilar letters indicate statistically significant differences between time points (One-way ANOVA and Tukey *post hoc*).

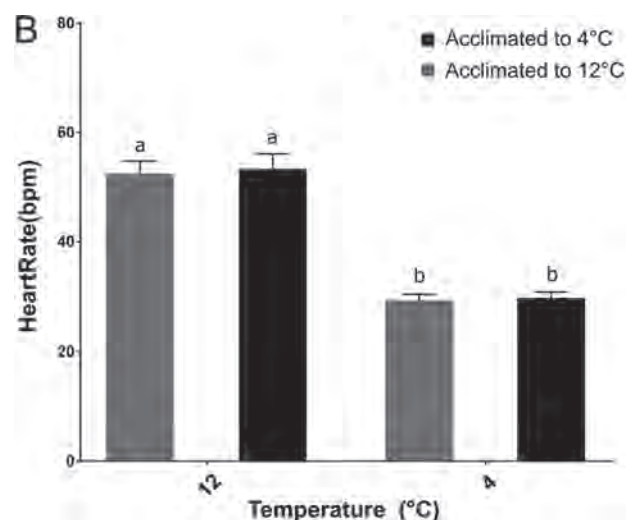


Figure 2 – IHR after 10 weeks acclimation to either 4°C or 12°C, measured at both 4°C and 12°C, in fish without an IHR resetting response (mean  $\pm$  SEM (n=7-8)). Dissimilar letters indicate statistically significant differences between groups (Two-way ANOVA and Tukey *post hoc*).

Funded by NSERC Canada

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB022

**Clinical ECG-gated MRI investigation of intracardiac shunting physiology in the red footed tortoise (*Chelonoidis carbonarius*).**

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Modern imaging techniques enable non-invasive blood flow measurement, providing opportunities for sophisticated investigation of intracardiac shunting in reptiles. In chelonians, flow is under autonomic control; increased parasympathetic tone causes pulmonary vasculature constriction and increased resistance, consequently increasing systemic flow (Q<sub>sys</sub>) via intracardiac shunting in the undivided chelonian ventricle<sup>1</sup>. Flow determination previously required surgical implantation of probes<sup>2</sup>. Here, a clinical 1.5 Tesla MRI system with ECG-gating allowed non-invasive visualisation of the entire cardiac cycle and repeatable cross-sectional flow measurements in the major systemic and pulmonary (Q<sub>pul</sub>) arteries of five anaesthetised red-footed tortoises (isoflurane 5% in oxygen). The waveforms of flow resemble measurements in chelonians via Transonic probes, showing early peaks in Q<sub>pul</sub> related to the pulmonary circulation's greater distensibility and lower diastolic pressures. Flow measurements (mean±SD) are used to address changing shunt pattern via application of 1 mg/kg atropine in a crossover design. Shunt direction reversed from 0.24 ± 0.07 ((Q<sub>pul</sub>/Q<sub>sys</sub>) saline) to 1.25 ± 0.38 (atropine), as total Q<sub>pul</sub> quintupled to 2.98 ± 0.35 ml/sec (atropine vs saline Student's T test p<0.01), while total Q<sub>sys</sub> was unchanged (2.58 ± 0.58 ml/sec saline, 2.51 ± 0.54 ml/sec atropine). Left and right Q<sub>pul</sub> did not significantly differ from one another under saline or atropine conditions (Student t test: p=0.25, 0.45 respectively) and while variable right aortic branching hampered determination, Q<sub>sys</sub> was 3.32 ± 0.27 times left aortic flow under saline, 2.97 ± 0.65 after atropine. This MRI application holds potential for further understanding the complex cardiovascular systems of ectothermic vertebrates.

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Heidi Meldgaard Jensen for Animal Care.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## PCB023

**Derivation of heart rate variability (HRV) indicators in older pet cats from stethoscopes: A Deep Learning neural network (AI) approach**

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Stethoscope are still a frontline tool in first opinion practice and give the clinician useful diagnostic data. We believe that artificial intelligence (AI) could increase further the clinical potential of this technique. To investigate this we recorded feline heart rate (HR) with Bluetooth stethoscopes and analysed the data *post hoc*.

**Aims:** To test (i) whether AI could be used to extract inter-beat intervals from stethoscope data and facilitate heart rate variability analysis (HRV) and (ii) whether age and/or gender influence short range HRV parameters of healthy cats.

**Methods:** Data were collected by Bluetooth stethoscope. Several hundred clearly audible records (from 48 cats) were manually labelled with MIDI software creating labels for each S1 heart sound. Training data were then augmented by 3 methods; (i) scaling and inverting, (ii) superposition of random amplitude, phase and frequency noise (in the range 50Hz- 18kHz) and (iii) superposition of randomly scaled sets of ambient noise. This created, in effect, 10,000+ labelled training records. Training sufficiency was calculated as selectivity and sensitivity of the network to detect beats in a 20% random sample of labelled data kept separate from the training process itself. HRV analysis (Matlab) included SDNN/RR (SDNN normalised by respiration rate) and Lomb-Scargle high frequency, low frequency (LF) and very LF powers. We included 117 patients; 52 female, 65 male age range 7 to 10. **Results:** Initial training reached 92% sensitivity and 88% selectivity to detect previously unseen, but "labelled" S1 sounds. The overall correlation between expert (veterinarian) estimation of mean HR (by ear) and CNN predicted mean HR gave Spearman's Rho of 0.63, p<0.005. We found no statistically significant correlation between age and any of our selected HR or HRV parameters, but gender was significantly associated with both time domain (SDNN/RR) and frequency domain (LF) components of HRV (higher in female).

**Conclusion:** The Deep Learning AI approach showed great promise; we found significant trends in HRV between genders, but not with age. In terms of age, there was a very limited age range included and we will in future extend this range by many years. Estimation of HR, by ear, when HR exceeds 180 bpm is extremely challenging and so the relatively weak correlation between AI and human does not necessarily imply inadequacies of our numerical methods. Whilst conducting this study we observed that the major weakness of these methods could be the underlying training data rather than the AI architecture itself. Close inspection of prediction outputs suggested that where the network makes "errors", subjectively, it appeared that such "errors" were in the labelling itself rather than the prediction. Full exploitation of this technology will require the generation of more accurate training sets.

Thank you to the BBSRC, Royal Canin

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*



## PCB024

**Loss of barrier integrity in alveolar epithelial cells downregulates ENaC expression and activity via  $\text{Ca}^{2+}$  and TRPV4 activation**A. Dagenais<sup>1,2</sup> and Y. Berthiaume<sup>1,3</sup><sup>1</sup>Cellular and molecular biology of the lung, Institut de recherches cliniques de Montreal, Montreal, QC, Canada, <sup>2</sup>Quebec Respiratory Health Network, Quebec, QC, Canada and <sup>3</sup>Université de Montréal, Montreal, QC, Canada

The epithelial Na channel (ENaC) plays an essential role in lung physiology by modulating the amount of liquid lining the respiratory epithelium. Here we tested the effect of breaking alveolar epithelial cell barrier integrity on ENaC expression and function. We found that either mechanical wounding by scratching the monolayer or disruption of tight junction with EDTA induced a ~50% decrease of  $\alpha$ ,  $\beta$  and  $\gamma$  ENaC mRNA expression and an 80% reduction of ENaC short-circuit current ( $I_{sc}$ ) at 6h. Scratching the cell monolayer generated a  $\text{Ca}^{2+}$  wave that spread from the margin of the scratch to distant cells. Pre-treatment with BAPTA-AM, an intracellular  $\text{Ca}^{2+}$  chelator, abolished the effect of mechanical wounding and EDTA on  $\alpha$  ENaC mRNA expression, suggesting that  $[\text{Ca}^{2+}]_i$  is important for this modulation. We tested the hypothesis that a mechanosensitive channel such as TRPV4, a cationic channel known to increase  $[\text{Ca}^{2+}]_i$ , could mediate this effect. Activation of the channel with the TRPV4 specific agonist GSK-1016790A (GSK) decreased  $\alpha$  ENaC mRNA expression and almost completely abolished ENaC  $I_{sc}$ . Pre-treatment of alveolar epithelial cells with HC-067047 (HC0), a specific TRPV4 antagonist, reduced the extent of  $\alpha$  ENaC mRNA downregulation by mechanical wounding and EDTA. Altogether our results suggest that mechanical stress induced by wounding or TRPV4-mediated loss of tight junction increases  $[\text{Ca}^{2+}]_i$  and elicits a  $\text{Ca}^{2+}$  wave that affects ENaC expression and function away from the site of injury. These data are important to better understand how  $\text{Ca}^{2+}$  signalling affects lung liquid clearance in injured lungs.

This work was funded by the IRCM Gosselin-Lamarre chair in clinical research

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB025

**The ACE-inhibitory effects of Isoleucine-Tryptophan after oral intake in man.**M. Martin<sup>1</sup>, D. Hagemann<sup>2</sup>, T. Henle<sup>2</sup> and A. Deussen<sup>1</sup><sup>1</sup>Institute of Physiology, TU Dresden, Dresden, Germany and <sup>2</sup>Institute of Food Chemistry, TU Dresden, Dresden, Germany

Angiotensin-Converting Enzyme (ACE)-inhibitors are treatment of choice in hypertensive patients. Some natural peptides from food proteins have structural similarities with pharmaceutical ACE-inhibitors and show ACE-inhibiting action. Because hypertension is a public health problem worldwide, such peptides may be of interest for early treatment or disease delay for mild hypertensives. We revealed the ACE-inhibition and antihypertensive effect of the natural dipeptide isoleucine-tryptophan (IW) and its effect on cardiac remodelling and coronary flow reserve in spontaneously hypertensive rats. However, especially with using IW for preventive purposes,

the effectiveness has to be proven in man. Thus, aims of this project were to determine the ACE-inhibiting effect and bioavailability of IW after 1) oral intake of whey hydrolysate with high content of IW (MPH) in man, and 2) combined intake of MPH with food ingredients and 3) effects on blood pressure after intake of MPH. Normotensive volunteers received MPH in different doses (n=10), intact whey protein (n=2) or water (n=8). MPH was applied alone or combined with intake of oats, protein, carbohydrates or fat; n=8). Blood samples were taken at fixed time points for analysis of ACE activity (in vitro, using the substrate Hip-His-Leu) and assessment of IW concentration in plasma by LC-MS/MS method. Blood pressure was assessed in a randomized double-blinded manner. Values are mean $\pm$ SD, compared by ANOVA. Intake of 10g MPH (equivalent to 50mg IW) induced a decrease in plasma ACE activity to 82 $\pm$ 4% (p<0.05) of baseline ACE activity and an increase in plasma IW concentrations (max. 20 $\pm$ 5nM, p<0.05). The maximum of ACE inhibition coincided with the peak IW concentration. 50g MPH increased plasma IW concentrations to 99 $\pm$ 58nM (p<0.05). In parallel, ACE activity was decreased to 75 $\pm$ 7% (p<0.05). Intake of non-hydrolysed whey protein increased IW concentration only to 4 $\pm$ 1nM and decreased ACE activity to 90 $\pm$ 3%. No changes in IW plasma concentration and ACE activities were observed following water application. Combined intake with food ingredients extended the IW rise of plasma concentration and ACE inhibition. With carbohydrates the ACE inhibition was diminished. No change in systolic and diastolic blood pressure was found after intake of 10g MPH or water. Thus, in healthy volunteers oral intake of IW alone or with other food ingredients results in an increase of IW plasma concentration and ACE inhibition in normotensives without unwanted hypotensive effects. However, the ACE inhibition cannot be explained by the peptide concentration achieved in plasma considering the in vitro  $\text{IC}_{50}$  (0.7 $\mu$ M). Further studies are mandatory to clarify the underlying cause-effect relationship for peptide ACE inhibitors in order to further developing innovative food additives with preventive effects on ACE activation and related cardiovascular diseases.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB026

**Effects of ESI-05 induced Epac inhibition on action potential duration are modified by intracellular  $\text{Ca}^{2+}$  buffering and abolished by pyruvate in isolated rat ventricular myocytes**

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We have shown previously that the Epac2 inhibitor ESI-05 can induce arrhythmias *in vivo* and prolong the action potential (AP) in ventricular myocytes via a mechanism that involves increased mitochondrial reactive oxygen species (ROS) production and ranolazine sensitive activation of the late sodium current ( $\text{I}_{\text{Na,late}}$ ) (1). Here we describe further work showing that aspects of the experimental conditions can markedly influence the effects of ESI-05 in rat ventricular myocytes. In current clamp mode, AP's were elicited by 10 ms depolarizing pulses every 10 s. Patch electrodes contained (mM) KCl 130, NaCl 10, EGTA 5 or 0.1, HEPES 5, MgATP 5,  $\text{CaCl}_2$  1, and  $\text{MgCl}_2$  1; pH 7.2. The perfusate contained (mM) NaCl 120, KCl 5,  $\text{CaCl}_2$  1,  $\text{MgCl}_2$  1, HEPES 10, glucose 10 and pyruvate 0 or 5; pH 7.4. Results are presented as mean  $\pm$  S.E.M. and statistical analysis was performed using Student's t-tests where

$p < 0.05$  was considered statistically significant. One aspect that commonly varies among studies is the level of EGTA within the patch pipette. While millimolar EGTA will abolish  $\text{Ca}^{2+}$  cycling, effects that persist under these conditions can be assumed to be  $\text{Ca}^{2+}$  independent, providing useful mechanistic information. In the present study, the  $\text{APD}_{90}$  was more than doubled (to  $221\% \pm 21$ ,  $n=10$ ,  $p<0.001$ ) by ESI-05 with 5 mM EGTA in the pipette. This effect was blocked entirely when 5 mM pyruvate was present in the perfusate ( $n=13$ ,  $p>0.05$ ), which is sometimes included due to its beneficial effect on cell viability. Pyruvate has antioxidant properties and its ability to block the effects of ESI-05 is consistent with our previous finding that antioxidants prevent ESI-05 induced activation of  $\text{I}_{\text{Na}_{\text{late}}}$  and AP prolongation (1). These experiments were repeated after reducing the level of EGTA to 0.1 mM, which is sufficient to allow  $\text{Ca}^{2+}$  cycling and contraction. Under these conditions, the mean increase in  $\text{APD}_{90}$  induced by ESI-05 was similar ( $261\% \pm 83$ ,  $n=6$ ). However, the response was more variable and some cells exhibited a much greater prolongation, leading to early afterdepolarisations (EADs). As with 5 mM EGTA, this effect was inhibited by pyruvate, suggesting a ROS dependent mechanism. However, ranolazine failed to prevent AP prolongation ( $n=8$ ) or EADs, suggesting that effects on  $\text{I}_{\text{Na}_{\text{late}}}$  may not dominate when  $\text{Ca}^{2+}$  buffering more closely approximates to the physiological state.

Yang Z, Kirton HM, Al-Owais M, Thireau J, Richard S, Peers C. et al. Epac2-Rap1 signaling regulates reactive oxygen species production and susceptibility to cardiac arrhythmias. *Antioxidants & Redox Signaling*. 2017 27:117-132.

Funding from the British Heart foundation is acknowledged.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB027

### Stretch promotes oxidation of elastic I-band titin but not inextensible A-band titin in heart and skeletal muscles

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**Background.** The mechanical function of the giant muscle protein titin is modulated by reversible oxidation. In vitro, titin oxidation is promoted by unfolding of immunoglobulin-like (Ig) domains from titin's spring region. Ig unfolding exposes previously hidden cysteines, which can then be S-glutathionylated (1). This modification hinders Ig-domain refolding, thus altering titin-based stiffness. The unfolded Ig domains risk aggregation causing increased myocardial stiffness, e.g., in failing hearts. However, it is unknown whether titin oxidation occurs *in vivo* and whether there is differential oxidation of sarcomeric I-band (extensible) and A-band (inextensible) titin. Therefore, we measured *in vivo* titin oxidation as a function of stretch and oxidative stress.

**Methods and Results.** Titin oxidation was studied in 3 models. Mouse hearts were perfused in a Langendorff apparatus with physiological buffer containing 0.1 mM  $\text{H}_2\text{O}_2$  or 1 mM DTT. Mouse skeletal (leg) muscles were stretched *ex vivo* in presence of 2 mM GSH/0.5 mM diamide (oxidant) and compared

to non-stretched muscle. The third model was the aorto-caval shunt mouse heart, which is under chronic volume overload (preload-increase) and develops oxidative stress (2); sham-operated animals served as controls. Titin oxidation was quantified by *isotope-coded affinity tag* labelling followed by mass spectrometry. In all models, oxidative stress significantly increased the ratio of oxidized (GSSG) to reduced (GSH) glutathione. Several hundred cysteines in titin became more oxidized under oxidative stress. I-band and A-band titin sites showed similar increases in oxidation level under non-stretch conditions. However, stretch or preload-increase under oxidative stress increased the proportion of oxidized cysteines more in I-band titin than in A-band titin, in both shunt:sham hearts and stretched:non-stretched leg muscles. This differential oxidation was not observed in hearts perfused in the Langendorff apparatus, probably because of insufficient stretching. Several Ig domains from elastic titin found to be preferentially oxidized were recombinantly expressed. Thermal unfolding of these Ig domains, followed by oxidation, consistently resulted in increased aggregation. Interestingly, the aggregation could be prevented by the presence of chaperone  $\alpha\text{B}$ -crystallin, which is known to bind to titin Ig domains (3).

**Conclusion.** Titin oxidation occurs *in vivo* and elastic titin becomes relatively more oxidized than A-band titin following a stretch. This effect is a consequence of the increased Ig-domain unfolding in the stretched state. Unfolded, oxidized titin is prone to aggregation, which can be prevented by  $\alpha\text{B}$ -crystallin. Understanding the mechanisms of oxidative stress-induced titin modifications may help design strategies for the treatment of heart failure patients with overly stiff hearts.

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(2) Mohamed BA, Schnelle M, Khadjeh S, Lbik D, Herwig M, Linke WA, Hasenfuss G, Toischer K. Molecular and structural transition mechanisms in long-term volume overload. *Eur J Heart Fail* 18:362-71, 2016.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB028

### Label-free analysis of the $\text{Na}^+/\text{Ca}^{2+}$ -exchanger (NCX) isolated from iPSC-derived cardiomyocytes

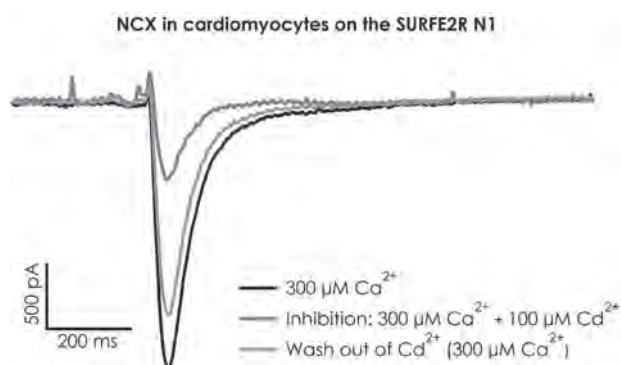
M. Barthmes, A. Bazzone, U. Thomas, A. Brüggemann, M. George, N. Fertig and A.R. Obergrussberger

Nanion Technologies GmbH, Munich, Germany

The Sodium-Calcium Exchangers (NCX) play an important role in the cellular calcium homeostasis under physiological and pathological conditions. NCX has been of interest as a pharmacological target for many years, in particular because clinical trials involving inhibitors of the sodium-proton exchanger, NHE, have delivered mixed results. Inhibition of the reversed mode of NCX is thought to be beneficial in ischemia/reperfusion injury by reducing cardiac, neuronal and renal infarct areas. Moreover, inhibition of NCX is has been proposed to exhibit an anti-arrhythmic effect and therefore, may provide a novel target for the treatment of a variety of arrhythmic pathologies. So far, a number of studies have shown promising results but investigations are limited by the currently

available NCX inhibitors such as KB-R7943, SEA-0400 and SN-6 which are only partially specific.

To drive the progress in pharmacological NCX research, new methods to measure NCX function are needed. At the current time, functional investigation of NCX range from patch-clamp, calcium flux assays, Langendorff-perfused hearts to studies in whole animals. We have developed an electrophysiological method to investigate NCX function which is based on the solid supported membrane (SSM) technique. HEK cells overexpressing NCX were used on an automated SSM device recording from either 1 or 96 wells simultaneously. This vastly increases throughput which is a critical factor in screening of pharmacological agents for NCX activity. In addition, human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) were used on a single-well SSM electrophysiology device and NCX was recorded from these cells. NCX was activated using  $\text{Ca}^{2+}$  in the buffer and inhibited by  $\text{Cd}^{2+}$ , and this inhibition was reversed upon washout of  $\text{Cd}^{2+}$ . NCX was blocked by KB-R7943 with an  $\text{IC}_{50}$  of 10.7  $\mu\text{M}$ . As expected, NCX recorded from hiPSC-CMs was blocked by the pro-arrhythmic compound, bepridil with an  $\text{IC}_{50}$  of 13  $\mu\text{M}$ . Furthermore, the pro-arrhythmic effects of bepridil were investigated using electrical impedance and extracellular field potential (EFP) measurements in order to find out the effects of bepridil on contractility and electrophysiology of hiPSC-CMs in a beating monolayer.



Activation of NCX by  $\text{Ca}^{2+}$ -containing buffer and subsequent block by  $\text{Cd}^{2+}$  recorded on the SURFE2R N1. The effect of  $\text{Cd}^{2+}$  was reversible upon wash-out.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB029

## Does collagen drive transverse tubule remodelling in human heart failure?

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In heart failure remodelling of the transverse (t) tubules leads to disrupted  $\text{Ca}^{2+}$  release and contraction. In the failing human heart the t-tubules have elevated levels of glycosylation as demonstrated by labelling with the lectin wheat germ agglutinin (WGA). Notably, WGA can bind to the dystrophin-associated glycoprotein complex leading us to postulate that increases in this complex may contribute to the increased WGA labelling and play a role in t-tubule remodelling. In this work we aimed to identify the glycosylated proteins responsible for increased WGA labelling of t-tubules. Samples of left ventricle (LV) were obtained from age and sex matched healthy human donors and patients with idiopathic dilated

cardiomyopathy in end-stage heart failure. All human tissue was obtained with written and informed consent following national guide- lines. Western blot was used to probe LV homogenates. This analysis identified 1.7 fold increase in total WGA positive proteins in heart failure samples ( $0.64 \pm 0.05 \text{ AU}$  vs  $0.37 \pm 0.02$   $n = 11$  failing, and  $n = 8$  donor hearts,  $P=0.001^{***}$ ). In particular, 140 kDa WGA positive band was elevated 5.7 fold in heart failure ( $0.61 \pm 0.06 \text{ AU}$  vs  $0.11 \pm 0.04 \text{ AU}$   $n$  as above,  $P=0.001^{***}$ ). Mass spectrometry indicated the 140 kDa band was collagen VI. Interestingly mutations in this protein are associated with Ulrich congenital muscular dystrophy and Bethlam myopathy suggesting an important role in muscle physiology. Subsequent west- erns confirmed the 140 kDa band was collagen VI with 2.4 fold increase in expression in heart failure ( $0.55 \pm 0.08 \text{ AU}$  vs  $0.23 \pm 0.02 \text{ AU}$   $n$  as above,  $P=0.01^{**}$ ). Calculation of WGA / protein ratio indicated a 3.2 fold increase in glycosylation of collagen VI in heart failure ( $0.45 \pm 0.07 \text{ AU}$  vs  $0.14 \pm 0.04 \text{ AU}$   $n$  as above,  $P=0.001^{***}$ ). Notably, glycosylation of collagen VI is essential for fibril formation. Confocal microscopy of LV tissue sections showed that collagen VI was located at the t-tubules and that label diameter was increased in heart failure ( $328 \pm 11 \text{ nm}$  vs  $272 \pm 15 \text{ nm}$ ,  $n=7$  failing hearts,  $n=5$  donor hearts,  $P = 0.014^{*}$ ). Super-resolution microscopy demon- strated collagen VI was located within the t-tubule lumen and that in heart failure its diameter increased ( $374 \pm 43 \text{ nm}$  vs  $231 \pm 18 \text{ nm}$ ,  $n=5$  failing hearts,  $n=5$  donor hearts,  $*P=0.018$ ) alongside a displacement in the cytoskeletal protein dystro- phin (Fig1). Subsequent confocal and super-resolution imaging of Collagens I, III and IV showed that these proteins were also located within the t-tubule lumen and diameters increased in sections from the failing human heart. Conclusion: Increased expression and labelling of collagens suggest that fibrosis contributes to t-tubule remodelling in human heart failure. For further methodological details, including statistical design, refer to the study publication (1).

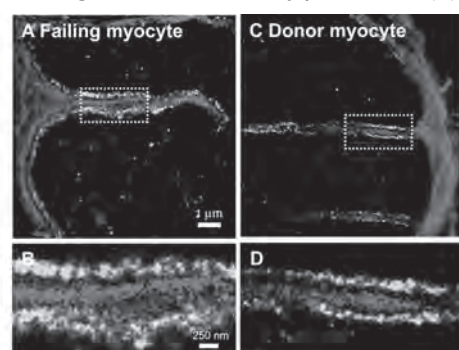


Figure 1. Super-resolution microscopy demonstrates increased collagen VI within the t-tubules of myocytes from failing human hearts. (A) T-tubule in failing myocyte. (B) Enlargement of box in 'A'. (C) T-tubule from normal donor myocyte. (D) Enlargement of box in 'C'. Red = collagen VI labelling, green = dystrophin labelling.

Crossman DDJ, Shen X, Jüllig M, Munro M, Hou Y, Middleditch M, Shrestha D, Li A, Lal S, Remedios CGC dos, Baddeley D, Ruygrok PNP, Soeller C. Increased collagen within the transverse tubules in human heart failure. Cardiovascular Research 2017;113:879–891.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.



## PCB030

**The Transverse Tubular System is Preserved by Activation of the Glucocorticoid Receptor in Cultured Rat Cardiomyocytes**

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The transverse tubular system (t-system) of ventricular cardiomyocytes is essential for excitation-contraction coupling. It is remodeled in cardiac disease, contributing to impaired cardiac contractility. However, mechanisms leading to t-system remodeling are incompletely understood. Because previous studies suggest upregulation of excitation-contraction coupling by glucocorticoid receptor (GR) activation, we investigated effects of GR activation on t-system structure. Ventricular cardiomyocytes were isolated from adult female Wistar rats and kept in culture for 3 days in either M199 + vehicle or M199 + corticosteroid agonist/antagonist. Freshly after isolation and after 3 days in culture, membranes in living cells were stained and imaged by confocal microscopy. T-system distance, volume fraction and orientation were analyzed. Additionally, cells were fixed and stained for JPH2, RyR2 and L-type  $\text{Ca}^{2+}$  channels (LTCC). Cell capacitance and LTCC currents were measured by whole-cell voltage clamping. JPH2 and BIN1 mRNA expression was quantified by RT-PCR. In tissue from cardiac-specific GR knockout mice, we measured RyR2-LTCC distances by confocal and STED microscopy. T-system density was reduced after 3 days in culture, as indicated by increased intracellular t-system distance ( $0.53 \pm 0.02 \mu\text{m}$  vs.  $0.66 \pm 0.02 \mu\text{m}$ ,  $n \geq 67$ ,  $p < 0.01$ ) and reduced volume fraction ( $8.7 \pm 0.2\%$  vs.  $7.7 \pm 0.2\%$ ,  $p < 0.05$ ). The fraction of longitudinally oriented components was increased ( $14.5 \pm 0.9\%$  vs.  $21.7 \pm 0.7\%$ ,  $p < 0.01$ ). Both dexamethasone (DEX,  $1 \mu\text{M}$ ) and corticosterone ( $1 \mu\text{M}$ ) treatment prevented the changes in t-system density. Blocking the GR by mifepristone ( $10 \mu\text{M}$ ) abrogated the effects of DEX, whereas spironolactone ( $10 \mu\text{M}$ ), an inhibitor of the mineralocorticoid receptor, did not. Cell capacitance, which is proportional to cell membrane area including the t-system, was higher in myocytes treated with DEX than in vehicle after 3 days ( $83.3 \pm 4.1 \text{ pF}$  vs.  $115.5 \pm 4.5 \text{ pF}$ ,  $n \geq 25$ ,  $p < 0.001$ ), confirming results obtained from microscopic imaging. Furthermore, LTCC current density was substantially larger in DEX-treated cells ( $7.8 \pm 0.6 \text{ pA/pF}$  vs.  $14.3 \pm 1.0 \text{ pA/pF}$ ,  $n \geq 25$ ,  $p < 0.001$ ). Fluorescent staining revealed increased relative amounts of JPH2 near the surface sarcolemma after 3 days in culture ( $11.7 \pm 2.6\%$  vs.  $19.7 \pm 2.3\%$ ,  $p < 0.01$ ), which was not prevented by DEX. However, DEX treatment decreased RyR2-LTCC distance ( $0.58 \pm 0.02$  vs.  $0.71 \pm 0.02$ ,  $p < 0.01$ ), and GR knockout mice showed greater RyR2-LTCC distance than control mice. Surprisingly, JPH2 and BIN1 mRNA levels were downregulated by DEX in cultured rat myocytes ( $p < 0.01$ ). Dexamethasone preserves t-system density in primary culture of adult rat left-ventricular cardiomyocytes, most likely via activation of the GR. GR knockout mice exhibited reduced RyR2-LTCC coupling. We suggest that glucocorticoid signaling is involved in t-system regulation.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## PCB031

**High fat-load induces cardioprotection in hearts from obese mice**

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An ischemic insult is associated with increase circulating fatty acids (FA) due to an adrenergic activation of adipose tissue lipolysis. Therefore, hearts will not only be challenged by hypoxia, but also by an acute FA-load, which have been shown to induce adverse cardiac effects such as mitochondrial dysfunction, oxidative stress, oxygen wasting and inefficiency. Although obesity is a contributing factor to the development of type 2 diabetes and heart failure it remains unclear if and how the obesity-associated chronic hyperlipidemia affects the cardiac response to an acute FA-load. Thus, we have examined the effect of high FA on hearts from a murine model of obesity. Diet-induced obesity (DIO) was obtained by feeding 5-wk old male C57BL/6J mice obesogenic diet for 20 wks. Age-matched chow-fed mice were included as controls (CON). *Ex vivo* left ventricular (LV) function (working heart perfusions,  $n=8-11$ ) and ischemic susceptibility (LV post-ischemic functional recovery and infarct size, Langendorff perfusions,  $n=12-15$ ) were examined in hearts exposed to normal ( $0.35 \text{ mM}$ ) or high ( $1.8 \text{ mM}$ ) palmitate levels. We also assessed myocardial  $\text{O}_2$  consumption ( $\text{MVO}_2$ ), FA oxidation and mechanical efficiency ( $n=12-15$ ), as well as myocardial ROS content (DHE tissue staining) and mitochondrial respiration (high-resolution respirometry,  $n=6-8$ ).

DIO mice were obese with elevated plasma FA levels ( $0.37 \pm 0.03$  vs.  $0.58 \pm 0.04 \text{ mM}$ ,  $p < 0.01$ ) and insulin resistance (4.4 fold higher HOMA-IR). They also developed diastolic dysfunction with only a mild systolic dysfunction. High FA perfusion did not alter LV function in neither CON nor DIO hearts. However, elevated FA decreased mechanical efficiency (due to increased  $\text{MVO}_2$ ,  $28 \pm 2$  vs.  $37 \pm 2 \mu\text{mol/min/g}$ ,  $p < 0.01$ ), induced oxidative stress and reduced mitochondrial OXPHOS rate and coupling (RCR) in CON hearts. These FA-mediated changes were not found in DIO hearts. Furthermore, in contrast to CON hearts (where ischemic tolerance was not altered by the FA-load), DIO hearts exposed to high FA levels showed increased functional recovery ( $53 \pm 5$  vs.  $36 \pm 5\%$  Rate-Pressure-Product,  $p < 0.01$ ) and decreased infarct size ( $47 \pm 2$  vs.  $62 \pm 5\%$ ,  $p < 0.02$ ). This cardioprotective effect was corroborated in hearts from *db/db* mice which is a model of obesity and type 2 diabetes ( $54 \pm 6$  vs.  $36 \pm 5\%$  recovery of RPP,  $p < 0.05$ , and  $55 \pm 5$  vs.  $72 \pm 2\%$  infarction,  $p < 0.01$ , respectively).

This study show that obese/diabetic hearts are resistant to the adverse effects a high FA-load. We therefore suggest that, although chronic dyslipidemia may play a role in the development of diabetic/obesity-mediated heart failure, these hearts undergo adaptive changes that counteract adverse effects of high FA and where elevated FA levels exert cardioprotection.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

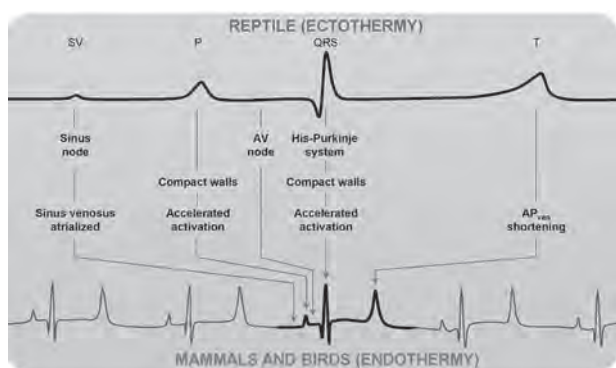
PCB032

### The electrocardiogram of vertebrates: evolutionary changes from ectothermy to endothermy

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The electrocardiogram (ECG) reveals that heart chamber activation and repolarization are much faster processes in mammals and birds compared to ectothermic vertebrates of similar size. Temperature, however, affects electrophysiology of the heart and most data from ectotherms are determined at body temperatures below those of mammals and birds. We reviewed the effects of temperature on intervals in the ECG of ectothermic and endothermic vertebrates. Data gaps were covered by novel data on the ECG of reptiles that were raised to mammalian body temperatures (*Iguana iguana*, N=4; *Python regius*, N=5; *Alligator mississippiensis*, N=4). We compared animals of approximately 1kg to minimize the influence of heart mass. Compared to mammals and birds, the reptiles at 35-37 °C had 4 times lower heart rates, 2 times slower atrial and ventricular conduction (longer P- and QRS-wave duration), and 4 times longer PR interval (atrio-ventricular delay) and QT intervals (total ventricular repolarization). In this study, we conclude that the faster chamber activation in endotherms cannot be explained by temperature alone. Based on histology, we show that endotherms have a more compact tissue architecture. In mammals, disorganization of the compact wall by fibrosis associates with conduction slowing and we suggest the compact tissue architecture allows for faster chamber activation. The short cardiac cycle and high heart rates that characterizes mammals and birds, however, is predominantly accommodated by shortening of the atrioventricular delay and the QT interval, which is so long in a 1kg iguana that it compares to that of an elephant.



Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB033

### Optogenetic stimulation of $G_q$ signaling in cardiomyocytes using Neuropsin (OPN5)

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In the heart, chronic  $G_q$  stimulation can result in pathological modulation of  $Ca^{2+}$  handling and induction of cardiac hypertrophy or heart failure. Our goal is to determine experimentally the threshold of  $G_q$  activation for induction of such heart pathologies with a focus on duration, repetition and refractoriness of  $G_q$  signaling. For this purpose, we here describe Neuropsin (OPN5) as a new optogenetic receptor enabling precise temporal control of  $G_q$  activation.

Transgenic mouse embryonic stem cells (mESC, R1 line) were generated to express human OPN5 and differentiated into beating embryonic bodies containing cardiomyocytes. These cells have an embryonic phenotype in which  $G_q$  signaling is accelerating the pacemaking machinery. In line with this, illumination with UV light (385 nm) instantaneously accelerated the spontaneous beating rate (delay < 0.1s, time to peak  $0.5 \pm 0.1$  s, n=7). Compared to pharmacological stimulation with 100nM Endothelin1 (time to peak  $21.9 \pm 4.2$  s, n=7), the light-induced increase in beating rate was significantly faster ( $p=0.002$ , two-tailed paired t-test) and higher ( $p=0.0006$ , two-tailed paired t-test). Importantly, the specific  $G_q$  inhibitor FR900359 (1  $\mu$ M) completely blocked UV light-induced frequency increase. Light intensity-response relationships were analyzed using light pulses with different intensities (from 30 to 3000  $\mu$ W/mm<sup>2</sup>) and durations (1 ms to 30 s). Interestingly, we found that human OPN5 activation can be fine-tuned by duration of light pulses as well as light intensity and its light sensitivity is best described by the overall amount of photons applied (product of light duration and intensity). Furthermore, we analyzed which retinal isomer must be supplied for best function of human OPN5 and found similar maximal light-induced frequency increase and light sensitivity when supplementing cells with 11-cis retinal, all-trans retinal, and even without retinal ( $p=0.34$ , one-way ANOVA, n=10-12). Finally, we compared the human OPN5 with the mouse OPN5 variant that has a slightly longer c-terminus. Overall human and mouse OPN5 showed no significant differences in light sensitivity ( $p=0.63$ , two-tailed unpaired t-test, N=3 clones) and maximal frequency increase ( $p=0.57$ , two-tailed unpaired t-test, N=3 clones). However, mESC clones expressing mouse OPN5 showed a much higher variability.

In summary, human OPN5 is a well-suited optogenetic tool to investigate  $G_q$  signaling with high temporal precision in cardiomyocytes *in vitro*. In the future, we will use various stimulation protocols to investigate the different timings between physiological and pathophysiological  $G_q$  signaling.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB034

**Photopharmacological modulation of cardiac ion channels**D. Malan<sup>1</sup>, T. Fehrentz<sup>4</sup>, T. Bruegmann<sup>1</sup>, F. Huber<sup>2</sup>, K. Huell<sup>3</sup>, N. Hartrampf<sup>2</sup>, N. Klöcker<sup>4</sup>, D. Trauner<sup>3</sup> and P. Sasse<sup>1</sup><sup>1</sup>*Institute of Physiology I, University Bonn, Bonn, Germany,*  
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We have previously demonstrated that optogenetic defibrillation can terminate ventricular tachycardia using pain-free illumination (Bruegmann et al, 2016). However, optogenetic technologies require long lasting cardiac gene transfer of light-gated ion channels, which is very challenging. In contrast, photopharmacological methods use photoswitchable compounds that change their conformation upon isomerization by light.

Here, we analyzed photopharmacological modulation of cardiac ion channels and initially tested FHU779, a photoswitchable derivative of the L-type  $\text{Ca}^{2+}$  channel (LTCC) blocker diltiazem. Patch clamp experiments of adult ventricular cardiomyocytes showed a block of LTCCs when switching FHU779 (25  $\mu\text{M}$ ) to the active trans-state by 480 nm illumination compared to a release of block when illuminating with 385 nm to switch FHU779 to the inactive cis-state (20.5 $\pm$ 8.8% current switchable, n=12). The spatial precision of light was tested by plating human iPS-derived cardiomyocytes (Cor4U cardiomyocytes, Ncardia) on multi-electrode arrays. In the presence of FHU779 (10  $\mu\text{M}$ ) local illumination with 480 nm significantly (p=0.016, paired t-test, n=4) shortened field potential durations (170.4 $\pm$ 14.8 ms) compared to 385 nm light (186.3 $\pm$ 15.9 ms) but only on illuminated electrodes. This indicates a localized shortening of action potential plateaus by photoswitchable LTCC block. Because LTCC are important for sinoatrial pacemaking, the effect of FHU779 on heart rate was tested in explanted Langendorff-perfused hearts. Illumination of the right atrium with 480 nm reduced the heart rate, which was quickly reversed by 380 nm light (5.8 $\pm$ 1.8% of heart rate switchable, n=4).

Previous optogenetic defibrillation experiments suggested an importance of  $\text{Na}^+$  channel block for successful defibrillation (Brügmann et al, 2016). We therefore next tested azobenzene trimethylammonium, which was reported to block both  $\text{Na}^+$  channels, LTCC and excitability in cardiomyocytes (Frolova et al, 2016; Magome et al, 2011). After application of the iodide derivate (AzoTAI, 10  $\mu\text{M}$ ) on a monolayer of Cor4U cardiomyocytes, spontaneous beating was reduced by 520 nm illumination (trans-AzoTAI), which could be repetitively restored by 385 nm light (21.3 $\pm$ 3.7% of heart rate switchable, n=16). Patch clamp analysis of Cor4U cardiomyocytes showed a 470 nm-induced reduction of  $\text{Na}^+$  and LTCC currents and a block of spontaneous action potential generation that was reversed by application of 385 nm light (n=3-7). Preliminary experiments on Langendorff-perfused hearts indicate that AzoTAI can block AV-node conduction by 520 nm illumination which is reversed by 385 nm light.

Our results demonstrate that photopharmacological methods can be successfully applied in cardiomyocytes and the intact heart to reversibly block ion channel function and may therefore be useful for light-induced termination of cardiac arrhythmia.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB035

**Role of the late sodium current in determining electrophysiological characteristics of cardiac ventricular myocytes**D. Baranyai<sup>1</sup>, R. Veress<sup>1</sup>, B. Horvath<sup>1</sup>, N. Szentandrássy<sup>2</sup>, J. Magyar<sup>1</sup>, D. Kiss<sup>1</sup>, B. Kurtán<sup>1</sup>, C. Dienes<sup>1</sup>, T. Bányász<sup>1</sup> and P. Nánási<sup>2</sup><sup>1</sup>*Department of Physiology, University of Debrecen, Debrecen, Hungary and*  
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The late sodium current ( $I_{\text{Na,L}}$ ) plays pathophysiological role in several heart diseases. Despite this, the contribution of  $I_{\text{Na,L}}$  to the cardiac ventricular action potential (AP) has not been characterized yet under physiological circumstances. We wanted to visualize  $I_{\text{Na,L}}$  during a canine ventricular AP and to determine the effect of  $I_{\text{Na,L}}$  blockade on the AP shape and on the short term variability of the AP duration (APD).

Experiments were performed in isolated canine left ventricular myocytes produced by enzymatic digestion. Intramuscular application of 10 mg/kg ketamine hydrochloride and 1 mg/kg xylazine hydrochloride was used to achieve complete narcosis in adult healthy mongrel dogs of either sex as approved by the local ethical committee. We used the AP voltage clamp technique to visualize  $I_{\text{Na,L}}$  during the AP by 1  $\mu\text{M}$  GS-45967 (GS). Conventional microelectrode technique was used to determine the effects of  $I_{\text{Na,L}}$  blockade on AP morphology. Under the AP, the GS sensitive current showed a peak at the AP upstroke (likely because of early sodium current ( $I_{\text{Na,E}}$ ) inhibition), and then showed a sustained, gradually decreasing current due to  $I_{\text{Na,L}}$ . At 1 s pacing cycle length (PCL) GS reduced the maximal rate of depolarization ( $V_{\text{max}}$ ) by 44% (from 278.5 $\pm$ 9.5 to 155.0 $\pm$ 17.8 V/s) and AP amplitude (APA) by 15% (from 110.2 $\pm$ 1.8 to 93.8 $\pm$ 4.4 mV). These effects are likely due to the small  $I_{\text{Na,E}}$  blocking effect of GS. GS shortened the APD by 14% (from 209.4 $\pm$ 5.8 to 179.6 $\pm$ 10.9 ms) measured at 90% of repolarization and depressed the mid-plateau membrane potential by 6 mV. GS decreased short term APD variability by 18% (from 2.7 $\pm$ 0.3 to 2.2 $\pm$ 0.5 ms). At shorter PCL, effects of GS on APA and  $V_{\text{max}}$  were more pronounced, indicating a stronger  $I_{\text{Na,E}}$  blockade under these conditions. Effects of GS on APD, mid-plateau potential and short term APD variability were less prominent with shorter PCL. In our experiments the profile of  $I_{\text{Na,L}}$  was successfully recorded under physiological conditions. The blockade of  $I_{\text{Na,L}}$  has been shown to affect AP morphology and APD short term variability. Based on these



results,  $I_{NaL}$  significantly contributes to the electrophysiological characteristics of cardiac ventricular myocytes. *Support: GINOP-2.3.2.-15-2016-00040, EFOP-3.6.2-16-2017-00006 by the European Union and the European Regional Development Fund; NKFIH-K115397.*

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB036

### Simultaneous patch clamp and video imaging of CPVT patient specific iPSC derived cardiomyocytes

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Catecholaminergic polymorphic ventricular tachycardia (CPVT) is a life threatening genetically inherited cardiac disease characterized by arrhythmias under adrenergic stress. Mutations in the cardiac ryanodine receptor (RyR2) are the leading cause for CPVT. The role of calcium in CPVT has been previously studied [1], but the association between membrane potential and contractile function in arrhythmia is not well understood.

In this study, we characterized electrophysiological properties of CPVT patient-specific induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) carrying different mutations in RyR2. In addition, we evaluated effects of carvedilol and flecainide on action potential (AP) and contractile properties of hiPSC-CMs. Both drugs have been shown to directly affect RyR2.

iPSC-CMs were generated from skin biopsies of CPVT patients carrying exon 3 deletion (E3D) and L4115 mutation in RyR2 and of a healthy individual. Cardiac differentiation was carried out by the END2 method and CMs were dissociated and plated as single or miniaggregates. APs and video were recorded simultaneously from the same hiPSC-CMs. Conventional perforated patch clamp technique was synchronized with imaging system to acquire membrane voltage and contractile dynamics.

Only ventricular CMs (APD90/APD50 < 1.3) were analyzed. Differences in AP properties (Table1) were seen in CPVT and control CMs: APD90 of both E3D (n=37) and L4115F (n=57) CPVT CMs was shorter than control CMs (n=41). E3D-CPVT CMs had shortest APD90, lowest APA, dVdT and more positive MDP than control or L4115F CPVT CMs. Furthermore, more arrhythmic events, DADs, EADs and alternans were seen in CPVT CMs compared to controls. Adrenaline decreased APD10, 50 and 90 and increased beat rate in all hiPSC CMs. Carvedilol and flecainide increased APD10, 50 and 90 and decreased beat rate in all hiPSC CMs. Both drugs increased the APD90 the most in L4115F CPVT CMs. EADs were pronounced also in contractile profile unlike DADs. APD alternans was characterized by amplitude changes in contractile profile.

iPSC-CMs provide a unique platform for disease modeling and testing drugs for CPVT. Combining electrophysiological measurements we can gain deeper insight into mechanisms of arrhythmias.

Table 1: AP parameters.

| Ventricular hiPSC CMs | BPM (beats/min) | APD10 (ms)  | APD50 (ms)   | APD90 (ms)   | APA (mV)    | MDP (mV)    | dVdT (V/s)  | ratio (APD90/APD50) |
|-----------------------|-----------------|-------------|--------------|--------------|-------------|-------------|-------------|---------------------|
| CONTROL               | 59.9 ± 19.8     | 87.8 ± 30.9 | 233.3 ± 89.0 | 269.2 ± 94.3 | 114.1 ± 7.5 | -72.6 ± 5.5 | 30.5 ± 15.2 | 1.17 ± 0.05         |
| L4115F CPVT           | 71.9 ± 20.9     | 95.7 ± 35.1 | 212.0 ± 84.2 | 248.0 ± 91.9 | 111.2 ± 6.5 | -72.0 ± 6.1 | 24.8 ± 20.3 | 1.18 ± 0.05         |
| E3D CPVT              | 54.6 ± 17.6     | 87.5 ± 17.8 | 192.5 ± 43.1 | 230.4 ± 52.2 | 106.2 ± 8.7 | -66.6 ± 7.6 | 16.3 ± 4.7  | 1.20 ± 0.05         |

Values are averages and standard deviations. BPM=beats per minute, APD=action potential duration, APA=action potential amplitude, MDP=maximum diastolic potential, dVdT=upstroke velocity.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB037

### The role of nuclear envelope protein TMEM43 in cardiac development and function in mice

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TMEM43 is a recently discovered, evolutionarily conserved protein expressed in mammalian heart that is associated with the Linker of Nucleoskeleton and Cytoskeleton (LINC) complex. The LINC complex structurally integrates the nucleus and the cytoplasm, and plays a critical role in mechanotransduction across the nuclear envelope[1]. A single amino acid substitution of serine 358 to leucine (S358L) in TMEM43 is the unequivocal cause of a distinct form of arrhythmogenic cardiomyopathy (AC)[2]. However, the role of TMEM43 in heart has remained obscure. In addition, it also remains to be determined how the S358L mutation in TMEM43 leads to cardiomyopathy.

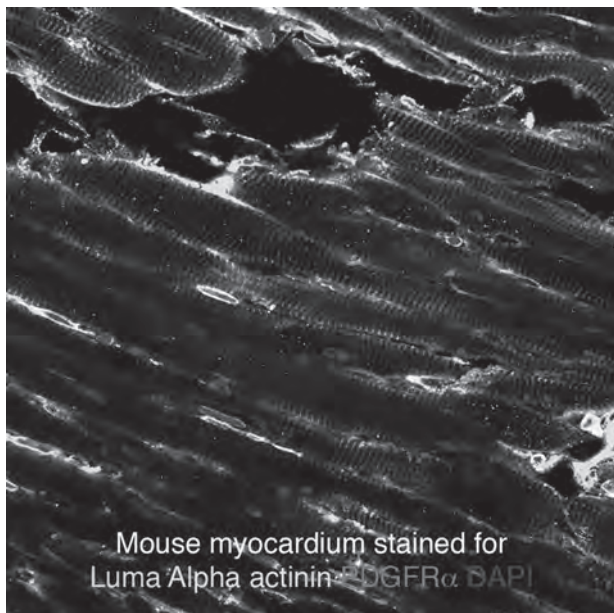
To investigate the *in vivo* role of TMEM43 in cardiac function, we generated TMEM43 knockout (KO) mice. We evaluated cardiac function of TMEM43 KO(n=14) and wildtype (WT) (n=13) mice over the course of 20months by echocardiography. Cardiac function of TMEM43 KO mice was comparable to WT littermates (32% vs. 35% Fractional Shortening (FS)), as were Left Ventricle Internal Dimensions in diastole (LVIDd both 3.9mm) and systole (LVIDs 2.6mm vs.2.5mm), Left Ventricle Posterior Wall thickness in diastole (LVPWd 0.79mm vs.0.72mm), and Inter-Ventricular Septum thickness in diastole (IVSd 0.83mm vs.0.81mm) at 88weeks.

Because LINC complex proteins have been shown to be important in cardiac hypertrophy following pressure overload by transverse aortic constriction(TAC)[3], we hypothesized TMEM43 might play a similar role. To test this, we performed TAC on TMEM43 KO mice and monitored them at 1 and 2 weeks post-TAC using serial echocardiography. However, there were no significant differences in any of these parameters between

TMEM43 KO(n=20) and WT(n=20) littermates( $P>0.05$ , 2-way ANOVA), suggesting that TMEM43 KO hearts exhibit a normal hypertrophic response to pressure overload.

From these data, we reasoned that the S358L mutation in TMEM43 might be gain-of-function. To test this, we generated and characterized TMEM43 S358L knock-in(KI) mice using CRISPR/ Cas9. Echo at 1year revealed no changes between S358L heterozygous (n=6), homozygous (n=9), and WT (n=11) littermates in cardiac function (FS, 41%, 40%, 37%, respectively); LVIDd (3.0mm, 3.1mm, 3.1mm, respectively); LVIDs (1.8mm, 1.8mm, 2.0mm, respectively); LVPWd (0.79mm, 0.79mm, 0.72mm, respectively); IVSd (0.85mm, 0.83mm, 0.79mm, respectively)( $P>0.05$ , 2-way ANOVA). Consistent with the lack of an AC-like phenotype, we observed no incidences of sudden death or premature lethality of S358L mutant mice compared to WT littermates.

Our data suggest that TMEM43 is dispensable for murine cardiac development and function and that the TMEM43 S358L mutation alone may not cause cardiomyopathy in mice. Future studies are needed to determine whether TMEM43 S358L KI mice in C57/B6 or other genetic backgrounds would eventually develop cardiomyopathy with greater age, and to determine whether TMEM43 S358L KI mice respond normally to cardiac stress, such as TAC-induced pressure overload.



Murine cardiac section stained with alpha actinin to mark myocytes (cyan), PDGFR alpha to mark fibroblasts (red), a nuclear envelope protein Luma (green) and DAPI (blue).

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB038

### Improved analysis procedures enhance the quantification of spatiotemporal excitation in isolated guinea pig hearts

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Optical mapping techniques have been used to measure cardiac ventricular electrical excitation patterns, with high spatial and temporal resolutions, in whole hearts isolated from several species including mouse, rat, rabbit and guinea pig [1-5]. The shapes of the ventricular action potential (AP) are species-specific but they generally share a common feature that consists of a very fast upstroke and a much slower repolarisation. These AP kinetic differences pose a pragmatic challenge of how to reliably quantify dynamic features of membrane potential changes across each entire AP recorded simultaneously from several thousand pixels. In support of our studies of electrical excitation in isolated guinea pig hearts, we have developed algorithms and novel analysis procedures that improve the quantification of several dynamic parameters of cardiac APs.

We have routinely recorded spatiotemporal propagation (1kHz, 128x128 pixels of  $\sim 230\mu\text{m}$ ) of left ventricular APs from isolated guinea pig hearts for up to 60 seconds. Dynamic features of individual pixel APs are measured using a piecewise digital filtering algorithm to: (i) securely separate the upstroke of the AP (ranging 8-15msec depending on biological setting) from the remainder of excitation; this enables accurate quantification of upstroke duration and speed. (ii) apply a suitable noise reduction filter on the plateau and repolarisation phase. This improves signal:noise, consistency of APs recorded between pixels, and facilitates accurate determination of AP durations (APDs) and repolarisation rates. Another notable improvement is made on the computation of the conduction velocity (CV) across the ventricle by enacting an algorithm that ensures the validity of the spread of data points surrounding each and every point of interest. These procedures facilitate the accurate quantification of entire AP spatiotemporal parameters including upstroke duration, APD and CV.

The ventricular AP form and dynamics can vary considerably between commonly-used experimental models. For example, the APDs in mouse and rat ( $\sim 40 - 50 \text{ msec}$  [1,2]) are considerably shorter than that of guinea pigs (174 msec, 186 msec [3]) or rabbits ( $\sim 155 - 176 \text{ msec}$  [4,5]). The new method of analysis of AP dynamics derived from optical mapping signals described herein offers the opportunity to provide consistency to data measurements across different experimental models

and, thereby, improve the assessment of cross-species differences in cardiac spatiotemporal excitation patterns.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB039

### Exploring caveolar protein organisation in cardiac myocytes

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Caveolae are small 50 – 100 nm invaginations found in the membrane of most cells, including cardiac myocytes. We have recently shown that around 250 proteins are resident within cardiac caveolae<sup>1</sup> which, given the size of caveolae, suggests there are heterogeneous populations of these nanodomains. Isolation of the caveolae coat complex (CCC) and super-resolution imaging of caveolae have given new insights into the organisation of caveolar proteins (caveolin 1 (cav1) and cavin 1) in HeLa and endothelial cells. However, these complexes have not been described in cardiac caveolae, which also contain the muscle-specific caveolar proteins caveolin 3 (cav3) and cavin 4. Here, using the latest techniques in quantitative western blotting (Q-WB)<sup>2</sup> and super-resolution imaging, alongside established methods to isolate the CCC<sup>3</sup>, we are beginning to identify how caveolar proteins are organised in cardiac myocytes.

Single cardiac myocytes, isolated from male Wistar rats (250-280 g), were homogenised in Laemmli sample buffer for protein analysis, attached to laminin-coated coverslips for imaging or incubated with the cross-linker dithiobis(succinimidyl propionate) (DSP) for isolation of the CCC. Q-WB was performed on a Protein Simple® WB system with the analysis of recombinant protein calibration standards alongside biological samples to permit absolute quantitation of specific protein concentrations (cav 1, 3, cavin 1, 4)<sup>2</sup>. For imaging cells were fixed in paraformaldehyde, labelled using antibodies against Cav 3 in combination with cavin 1, cavin 2 or cavin 4 and visualised by Airyscan or dSTORM. For CCC isolation, complexes were fixed in cells *in situ* by incubation with 2 mM DSP for 1 h. Following lysis, samples were run on a sucrose velocity gradient to separate protein complexes based on size<sup>3</sup>.

Cav 1, cav 3 and cavin 1 are expressed at similar levels in cardiac myocytes. However, cavin 4 expression is much lower in these cells (26% of cavin 1, n=3). Airyscan imaging shows that a greater proportion of cav 3 co-localises with cavin 1 (53.4 ± 1.4%) than either cavin 2 (23.8 ± 3.7%) or cavin 4 (20 ± 2.7%) supporting the hypothesis that cavin isoforms may contribute to the heterogeneous caveolar populations (animal n=3, mean ± SEM). At a finer resolution (~40 nm - dSTORM), we observe the cavin distribution is the likely result of the larger and more numerous clusters formed by cavin 1 than cavin 4. The current protocol used to isolate the CCC

appears to selectively isolate complexes containing only cav 1 and cavin 1; the presence of the cross-linker DSP does not affect the cav 3 complex size. Whether this is because cav 1 forms a distinct caveolar population or because DSP does not cross-link cav 3 requires further investigation.

This is the first description of the heterogeneous population of caveolae in cardiac myocytes. Understanding the protein composition of the different caveolae is essential in understanding the multiple functions that caveolae perform.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB040

### Optogenetic stimulation of G<sub>s</sub> signaling in intact hearts to analyze Ca<sup>2+</sup>-leak induced arrhythmia

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Ventricular arrhythmias, which have a high risk of resulting in sudden cardiac death, can be due to structural remodeling after myocardial infarction, ion channel imbalances as well as hereditary mutations. Catecholaminergic polymorphic ventricular tachycardia (CPVT) is an inherited cardiac arrhythmia disease caused by mutations in the ryanodine receptor or the sarcoplasmic reticulum (SR) protein calsequestrin (Casq2). In CPVT, b-adrenergic G<sub>s</sub>-stimulation promotes premature ventricular contractions (PVC) and ventricular tachycardia (VT) by enhancing SR Ca<sup>2+</sup> leak during diastole in ventricular cardiomyocytes. b-adrenergic stimulation of the sinus node, however, may act protective by accelerating the heart rate and thus counteracting the development of PVC because of shorter diastolic intervals.

In order to investigate the localized effect of β-adrenergic signaling on heart function and arrhythmia generation and to distinguish between regional specific protective and adverse effects of a high adrenergic tone, we developed a mouse model expressing Jellyfish opsin (JellyOP), a light-sensitive G<sub>s</sub>-coupled receptor from box jellyfish, in cardiomyocytes.

Illumination of isolated atrial tissue resulted in an increase of cAMP levels similar to application of isoprenaline. In Langendorff-perfused JellyOP hearts, sustained supramaximal illumination of the dorsal right atrium led to an acceleration of heart rate with a maximal increase of +44.0±9.3% (n=5), which was slightly lower than perfusion with 1 μM isoprenaline (+58.7±10.2%). However, the maximal effect on heart rate after start and wash-out after end of stimulation were much faster using JellyOP stimulation than isoprenaline application. Illumination of isolated JellyOP ventricular cardiomyocytes resulted in an instantaneous increase in the L-type Ca<sup>2+</sup>



current by  $+20.5 \pm 5.4\%$  ( $n=8$ ) and to a maximal increase in contractility by  $+218 \pm 70\%$  ( $n=10$ ).

To investigate local effects of  $G_s$ -signaling on PVC and VT in a CPVT model, we crossed the JellyOP mouse line with a CPVT mouse line with heterozygous deletion in the *Casq2* gene (JellyOP x *Casq* +/-). Upon atrial illumination we observed junctional arrhythmia in 80% of JellyOP x *Casq* +/- mice ( $n=5$ ) but never in JellyOP x *Casq* +/+ mice ( $n=4$ ). This type of arrhythmia was characterized by accelerated heart rates with regular narrow-shaped QRS complexes but without properly timed prior p-waves. This indicates that *Casq2* heterozygous knockout might sensitize pacemaking mechanisms towards adrenergic stimulation specifically in the AV node or the ventricular conduction system.

Unfortunately, we never observed the expected PVC or VT induction upon ventricular illumination of JellyOP x *Casq* +/- mice ( $n=5$ ). Thus, next experiments will be performed on homozygous *Casq2* knockout mice, which are reported to display a stronger CPVT phenotype.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCB041

### Correlating nanoscale Ryanodine Receptor organizations to spontaneous calcium sparks in live cardiomyocytes

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Understanding of cardiomyocyte subcellular structure has benefited greatly from the advent of sub-diffraction limit super resolution microscopy, particularly regarding the organization of Ryanodine Receptors (RyRs). These investigations have indicated that RyRs are present in clusters, and suggested that neighbouring clusters may cooperatively generate calcium sparks, the fundamental units of calcium release underlying the calcium transient. However, directly linking the structure of these RyR conformations to their function requires connecting super resolution imaging with calcium imaging. To this end, we presently employed cardiac myocytes isolated from mice expressing a photo-activated red fluorescent protein tagged ryanodine receptor 2 (PA-tag-RFP-RyR2). A commercial Zeiss Elyra dSTORM setup was used to image live PA-tag-RFP myocytes loaded with Fluo 4 calcium indicator. Calcium measurements were first obtained through HiLo illuminated widefield imaging, followed by PA light microscopy (PALM) imaging using the same camera setup. We observed that careful adjustment of the rate of fluorophore activation and photobleaching optimized the generation of blinking phenomena necessary for live cell PALM imaging. RyR localisation was obtained with accuracies of 30-40nm in the X, Y axis in live cell images, whereas somewhat higher resolution (5-15nm) was obtained in parallel experiments performed in fixed cells with RyR antibody labeling. RyR cluster arrangements were observed to be broadly consistent between the two imaging modalities. Spontaneous calcium sparks recorded in PA-tag-RFP-RyR2 myocytes were directly paired to underlying RyR constellations, allowing assessment of cooperative functional interactions between neighbouring RyR clusters. In ongoing work, this approach will quantitatively link calcium handling dynamics to the opening of individual

RyRs, and allow real-time insight into the stochasticity and plasticity of this structure/function relationship.

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PCB042

### 3D imaging and quantitative analysis of mouse coronary vasculature

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Mice are widely used in cardiovascular research. Quantitative characterization of the vascular structure is crucial for the analysis of the coronary network, which can be imaged by microtomodensitometry (microCT) after perfusion with a contrast medium. The aim of this study was to propose a computational processing of microCT imaging of mouse heart that ensures objective segmentation of the coronary vasculature and quantification of its 3D architecture. All procedures, performed on 6 C57BL/6J mice, were done accorded with current national and European legislations, and agreed by local ethical committee. 2 hours after injection of anticoagulant and vasodilator treatment, mice were euthanatized by pentobarbital intraperitoneal injection ( $600 \text{ mg.kg}^{-1}$ ), exsanguinated, and perfused with a contrast medium (Neoprene latex and barium sulphate) via the brachiocephalic artery trunk. After dissection, hearts were imaged with a microCT system (Skyscan, Brucker) with  $12 \times 12 \times 12 \text{ } \mu\text{m}$  resolution, followed by 3D reconstruction (NRecon, Brucker). Image processing was done using CTAn (Brucker) and Image J with BoneJ plugin. The cardiac muscle was segmented from background and ventricular cavities, and then the coronary artery from the myocardium, by 2 successive thresholds based on the statistical distribution of pixels according to their grey levels and independently from any visual estimate. In 3 specimens, the aortic valve did not resist the perfusion pressure, and the left ventricle resulted perfused. A shape-based segmentation was applied to delete the ventricle from the coronary network. After segmentation, the following parameters were calculated: the volumes of the organ ( $V_h$ ) and of the coronary arteries network ( $V_c$ ), and their ratio as an estimate of the density of the coronary artery network ( $D_c$ ), and its fractal dimension ( $D_f$ ), which measures the capacity of a self-similar structure to fill the three-dimensional space it occupies. The thickness ( $T_c$ ) and the length ( $L_c$ ) of the vascular segments, and their frequency distribution, and the total length of the coronary arteries ( $L_{cn}$ ), were calculated. Statistical analyses were done with Gradpad Prism software. Data are given as mean  $\pm$  SEM.  $V_h$  was  $134.7 \pm 9.0 \text{ mm}^3$ ,  $V_c$  was  $1.4 \pm 0.1 \text{ mm}^3$ ,  $D_c$  was  $1.05 \pm 0.06\%$ , and  $D_f$  was  $1.65 \pm 0.02$ .  $T_c$  was  $9.8 \pm 0.2 \text{ } \mu\text{m}$ . Thickness frequency distribution followed a Gaussian law, and showed that vessels of  $12 \text{ } \mu\text{m}$  diameter or more can be detected, but with underestimation of the smaller ones. Total  $L_{cn}$  was  $158.3 \pm 13.9 \text{ mm}$ , and average  $L_c$  was  $249 \pm 18 \text{ } \mu\text{m}$ . Segment length frequency distribution followed a one-order exponential decay from shortest ( $0\text{--}100 \text{ } \mu\text{m}$ ) to longest segments. We conclude that this computational processing applied to 3D microCT imaging ensures objective calculation of quantitative parameters that characterize the 3D structure

of the coronary vasculature, including the microcirculation, down to 36  $\mu\text{m}$  (3 pixel size).

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB043

### Echocardiographically-Assessed Right Ventricular Diastolic Function in Hypertrophic Cardiomyopathy

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#### Background

Hypertrophic cardiomyopathy (HCM) is considered a biventricular myopathic process (1), the hallmark of which is diastolic dysfunction. Whilst resting right ventricular (RV) systolic function is reported to be normal in HCM (2), there is a lack of published data regarding RV diastolic function in HCM.

#### Methodology

A prospective study was undertaken in a NHS tertiary centre echocardiography department to collect RV diastolic function parameters in patients with HCM and in healthy control subjects when they attended for transthoracic echocardiography (TTE).

Inclusion criteria were; confirmed diagnosis of HCM, or normal TTE in a subject with no cardiovascular diagnoses/risk factors. Exclusion criteria were; diagnosis of another condition independently associated with RV diastolic dysfunction [see reference (3)], previous cardiac surgery, previous alcohol septal ablation, congenital abnormality which may affect right heart flow/pressure, or >moderate valvular regurgitation/stenosis. Statistical analyses were performed using SPSS v24 (IBM). P values <0.05 were considered significant.

#### Results

There were significant differences in age (51[22] vs 38[18],  $p<0.0005$ ), gender (male 56% vs 76%,  $p=0.035$ ), sinus rhythm (100% vs 88%,  $p=0.012$ ), body mass index (BMI) ( $24.7\pm3.2$  vs  $28.1\pm4.1$ ,  $p<0.005$ ) & heart rate (64[15] vs 60 [12],  $p=0.012$ ) respectively between 50 control & 50 HCM patients. There were no significant differences in blood pressure.

As shown in Table 1, RV diastolic function parameters (TV E/A, RV e' velocity, RV E/e' & RV isovolumic relaxation time) are significantly impaired in HCM compared to controls, with the exception of TV E-deceleration time.

After controlling for baseline differences in age/gender/BMI/heart rate/rhythm, RV e' ( $p<0.001$ ) and the surrogate of RV filling pressure RV-E/e' ( $p=0.007$ ) remained significantly impaired in HCM whilst TV E/A ( $p=0.287$ ) and RV IVRT ( $p=0.404$ ) did not.

#### Conclusions

Some RV diastolic function parameters are impaired in HCM compared to healthy controls. Echocardiographically-derived RV filling pressures are significantly elevated in HCM, suggesting that the disease has pulmonary haemodynamic consequences.

Table 1: Independent t-test analysis of RV diastolic variables.

|                                    | Control         | HCM             | p-value |
|------------------------------------|-----------------|-----------------|---------|
| TV E/A ratio                       | 1.65 $\pm$ 0.40 | 1.40 $\pm$ 0.37 | 0.002   |
| TV E-deceleration time (sec)       | 0.230 [0.098]   | 0.260 [0.130]   | 0.139   |
| RV e                               | 11.3 [2.6]      | 8.3 [3.0]       | <0.001  |
| RV E/e                             | 4.55 [1.37]     | 5.43 [2.55]     | 0.002   |
| RV isovolumic relaxation time (ms) | 50 [40]         | 60 [60]         | 0.023   |

Presented as mean $\pm$ standard deviation or median[interquartile range]. TV=tricuspid valve. E=early passive diastolic filling. A=late active diastolic filling.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB044

### Identification of proteomes of caveolae subpopulations in cardiac muscle.

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The lipid raft concept proposes that membrane environments enriched in cholesterol and sphingolipids cluster certain proteins and form platforms to integrate cell signalling. In cardiac muscle, caveolae concentrate signalling molecules and ion transporters, and play a vital role in adrenergic regulation of excitation-contraction coupling and consequently cardiac contractility. Evidence from non-cardiac cells has shown that there are multiple caveolar subpopulations, defined by different content of cholesterol, caveolin isoforms, signal effectors (e.g. kinases) and targets (e.g. ion channels). The aim of this investigation was to identify caveolar proteins in cardiac muscle, investigate dynamic regulation of caveolar content, and define co-localised subpopulations of caveolar proteins.

We defined the cardiac caveolar proteome using quantitative proteomics to identify proteins depleted from caveolar membranes prepared from rat ventricular myocytes using a standard discontinuous sucrose gradient after treatment of these myocytes with methyl- $\beta$ -cyclodextrin (M $\beta$ CD) to deplete cholesterol and disrupt caveolae. We defined 249 proteins as high-confidence caveolar residents. Functional annotation clustering indicates cardiac caveolae are enriched in integrin signalling, guanine nucleotide binding, ion transport, and insulin signalling clusters.

To define subpopulations of cardiomyocyte caveolae we enriched caveolar membranes using a sucrose gradient.

Membranes were fractionated by size exclusion chromatography, and fractions blotted for Caveolin-1, Caveolin-3, Na pump  $\alpha 1$  subunit and Cavin-1. Preliminary results show that Caveolin-1 and Caveolin-3 are not enriched in the same fractions, suggesting a subset of cardiomyocyte caveolae is Caveolin-1-free. We find cavin-1 is associated preferentially with Caveolin-3 over Caveolin-1/3-enriched membranes. To further extend caveolae characterisation we developed a new profile of sucrose gradient which allows separation of caveolar membranes with higher resolution. We characterised these populations by peptide mass spectrometry. All cavins and caveolins are predominantly localised in more buoyant caveolar membranes (60-80%) except Cavin-4 (46%). Moreover, co-localisation fluorescence microscopy (proximity ligation assay) showed that Caveolin-3 share the same location with Cavin-1 in sarcolemma and transverse tubules while with Cavin-4 in sarcolemmal membranes only. This confirms that myocyte caveolae exist in different subpopulations. Hence physical and functional co-localisation of subpopulations of caveolar residents may contribute to the complexity of signalling through these microdomains in cardiac muscle.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCB045

#### **Deletion of Akt1 and Akt2 leads to cardiac atrophy and loss of cardiac function**

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Akt1 and Akt2 are the main isoforms of protein kinase B (Akt) expressed in the mammalian heart. Knockout mice for Akt1 and Akt2, respectively, have demonstrated that Akt1 primarily appears to regulate growth whereas Akt2 is rather involved in regulating metabolism.

In order to investigate common as well as divergent functions of AKT isoforms in the adult heart, we generated and analyzed tamoxifen inducible, cardiac myocyte specific Akt1 and Akt2 single knockout as well as Akt1/Akt2 double knockout mice. We observed a maximal loss of both isoforms 8 days after start of 5 consecutive IP injections of OH-Txf. Single KO mice were fully viable and showed no functional or structural alteration of the heart. In contrast, deletion of both isoforms induced progressive cardiac atrophy and loss of heart function leading to terminal heart failure 3 to 4 weeks after first injection of OH-Txf. Echocardiography revealed a progressive decline of pump function characterized by a drop of ejection fraction from initially 62 to 37 % on day 10 and 31 % on day 21. Wall thickness of iCM-Akt1/2 KO hearts declined from day 10 to day 21. Concomitantly, cardiac mass dropped by 30 %. This effect was mainly due to a loss of cardiomyocyte size, which progressively declined after Akt1/2 deletion.

Increased nuclear localization of the pro-autophagic transcription factor Foxo3A as shown by immunohistochemistry was identified as one possible cellular mechanism contributing to cellular atrophy in Akt1/2 KO hearts. Simultaneously, the expression of Foxo3A-regulated pro-autophagic genes like Gabarapl1, Bnip3 or Sqstm1 was increased early during

development of heart failure (d10 after start of OH-Txf) as demonstrated by quantitative real time PCR and western blot analyses. Increased proteasomal (chymotrypsin-like and caspase-like) and calpain activities were detected at a late stage of heart failure (d21) which might further promote the loss of cardiac cell mass.

Global cardiac gene expression analysis using Agilent 60 K microarrays demonstrated a concerted downregulation of transcripts for mitochondrial proteins suggesting a compromised ATP generation. Therefore, we further investigated the impact of AKT1/2 deletion on cardiac energetics. Repetitive <sup>31</sup>P-NMR spectroscopy performed in anesthetized mice revealed progressive energetic depletion of Akt1/2 KO hearts. Phosphocreatine:ATP ratios (PCr/ATP) declined to values of 1.5 which in humans is associated with serious heart failure. In parallel, free creatine levels considerably increased as shown by ceratine chemical exchange saturation transfer (CrCEST) imaging.

Conclusion: Common functions of AKT1 and AKT2 provide essential backup control mechanisms which promote cardiac energy generation and restrict FOXO-mediated degradation processes and thereby mediate the structural and functional integrity of the adult mammalian heart.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCB046

#### **The effect of infarction method on the development of ventricular arrhythmias in a porcine model of acute myocardial infarction**

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Background: Electrical instability during acute myocardial infarction (AMI) can lead to ventricular fibrillation (VF) and sudden cardiac death. Large animal models play a pivotal role in the search for mechanisms and treatment. Models use open and closed thorax methods, yet it is unknown how these affect the development of arrhythmias. In a porcine model, we compared an open thorax (OTH) and a closed thorax (CTH) method of left anterior descending artery (LAD) occlusion on the development of ventricular arrhythmias during a 1 hour infarction.

Methods: In Danish landrace pigs (female, 50±1 kg, n=42) anaesthesia was induced with tiletamin/zolazepam (5 mg/kg), xylazine (2.5 mg/kg), ketamine (2.5 mg/kg), butorphanol (0.4 mg/kg), methadone (0.4 mg/kg; all i.m.) and maintained with fentanyl (0.1 mg/kg/h) and propofol (12.5 mg/kg/h; both i.v.). The study conformed to European Directive 2010/63/EU and was approved by the Danish Animal Inspectorate. OTH (n=20): a mid-sternal thoracotomy was performed with isolation and ligation of the mid-LAD. CTH (n=22): an angiogram was performed with mid-LAD balloon occlusion. We recorded ECG and blood pressure continuously and tested cardiac output (CO) and femoral arterial blood every 15 minutes. Values are mean±S.E.M, compared by ANOVA unless otherwise indicated.



Results: In the OTh group 7/20 pigs developed VF compared to 12/22 in the CTh group (Fisher's exact,  $p>0.05$ ). The time to VF was longer in the OTh pigs (OTh:  $23\pm1$ , CTh:  $17\pm1$  min; t-test,  $p=0.012$ ). The count of premature ventricular contractions (PVCs) did not differ (OTh:  $146\pm27$ , CTh:  $159\pm22$  PVCs; t-test,  $p>0.05$ ). The characteristic phase 1a and 1b peaks of PVCs were observed in both groups, but arose later in the OTh pigs. Ventricular tachycardia was rare. Upon thoracotomy, the OTh pigs showed a drop in mean arterial pressure (MAP; OTh:  $91\pm3$  vs  $74\pm4$  mmHg,  $p<0.001$ , CTh:  $99\pm3$  vs  $96\pm4$  mmHg;  $p>0.05$ ) and CO (OTh:  $4.4\pm0.2$  vs  $3.0\pm0.2$  L/min,  $p<0.001$ , CTh:  $4.8\pm0.2$  vs  $4.8\pm0.2$  L/min,  $p>0.05$ ). Infarction caused a hemodynamic drop in both groups, but MAP (OTh:  $57\pm3$ , CTh:  $90\pm4$  mmHg,  $p<0.0001$ ) and CO (OTh:  $2.6\pm0.2$ , CTh:  $3.9\pm0.2$  L/min,  $p<0.0001$ ) remained lower in the OTh group. The drop in MAP and CO was partly attributable to lower heart rate in OTh pigs upon thoracotomy (OTh:  $73\pm5$ , CTh:  $102\pm4$  bpm,  $p<0.0001$ ) and infarction (OTh:  $79\pm5$ , CTh:  $103\pm4$  bpm,  $p<0.001$ ). Lactate was unchanged after thoracotomy (OTh:  $1.2\pm0.1$ , CTh:  $1.2\pm0.1$  mmol/L,  $p>0.05$ ) and infarction (OTh:  $1.4\pm0.2$ , CTh:  $1.2\pm0.1$  mmol/L,  $p>0.05$ ).

Conclusion: Both AMI methods produce a similar incidence of ventricular arrhythmia and VF. However, these events occur later in the OTh method. Additionally, while not causing global tissue hypoxia, thoracotomy compromises CO and MAP independent of infarction, providing a less suitable reflection of the clinic. These differences are of interest when choosing an AMI model.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB047

## Comparing the Effects of Anaesthesia on Cardiac Function in 'Awake' and 'Slept' Mice by Echocardiography

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Cardiovascular diseases (CVDs) are the Number-1 killer worldwide with over 17.5 million deaths per year<sup>1</sup>, and the use of preclinical murine models has greatly aided such understanding. Live, real-time, in-vivo ultrasound imaging is one great tool for studying as dynamic an organ as the heart and is especially successful for small animals. However, preclinical studies rely on imaging under anaesthesia due to difficulties in compliance of the animals to stay still, which has several limitations: 1) It is not a true physiological representation of the disease, potentially modifying the therapeutic-reponse, and measurements are likely to be confounded by effects of anaesthesia; 2) With numerous studies documenting the cardio-depressive effects of anaesthesia<sup>2,3</sup>, it is especially not suitable for studies of the heart; 3) Clinically, patients are imaged in the 'awake' (aka conscious) state without the use of anaesthetics, hence the difference in method does not allow horizontal-comparison of results and often leads to promising preclinical research not translated into the clinic. Here we have developed a clinically-relevant preclinical method of measuring cardiac function in 'awake' and 'slept' mice using real-time cardiac ultrasound. We aim to inform researchers on the various differences in cardiac function between animals imaged under anaesthesia and in the 'awake' state, and hope to propose a new regime for studying cardiovascular function via echocardiography in the preclinical setting.

Ten female BALB/c mice were shaved one-day prior (-1d) to echocardiography and were consecutively imaged on a daily basis (1d to 5d), first in the 'awake' state (securely-but-gently-scruffed) and then in the 'slept' state (anaesthetised, 2% isoflurane in 100% oxygen). Parasternal long- and short-axis views in B- and M-modes were obtained.

Ejection fraction (EF) was significantly higher in awake mice and showed less variability at all time-points compared to anaesthetised (96.0 $\pm$ 5.3 vs 47.9 $\pm$ 9.6%). Stroke volume (SV) initially-similar, but reduced overtime (22.8 $\pm$ 6.7 vs 27.7 $\pm$ 4.4 $\mu$ l at 1d; 9.5 $\pm$ 4.7 vs 25.1 $\pm$ 6.6 $\mu$ l at 5d). Cardiac output (CO) was comparable (12.2 $\pm$ 5.5 vs 11.6 $\pm$ 2.5ml/min).

In conclusion, we have developed a clinically-relevant preclinical method of measuring cardiac function and showed the feasibility of imaging conscious mice using real-time cardiac ultrasound. EF was higher in 'awake' state, as expected without the cardiac-depressive-effects of anaesthesia, thereby allowing greater-sensitivity in detecting changes after therapy/treatment. Variability was also reduced, possibly due to the absence of sinus rhythm-disturbance caused by anaesthesia, thus increasing the chances of observing a true effect. SV reduced overtime, possibility-reflecting training of the animals to the procedure. CO was similar between the two states. Taken all together, we propose this new method of imaging to better-align preclinical to clinical ultrasound imaging of cardiac function.

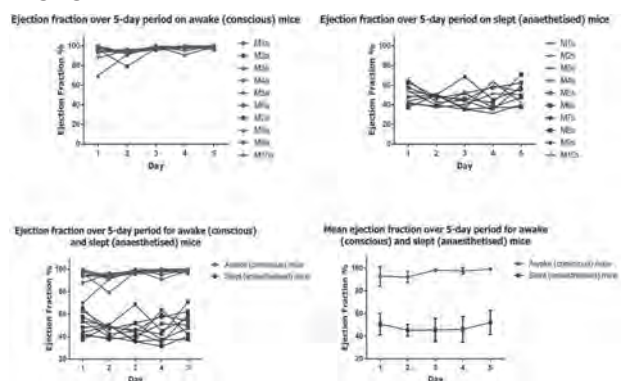


Fig. 1 Ejection fraction (EF) over the 5-day study period in A) 'awake' mice; B) 'slept' mice; C) 'awake' and 'slept' mice; and D) average EF in 'awake' and 'slept' mice. Plotted using measurements taken from M-mode images of both parasternal long- and short-axis view at diastole and systole. Ejection fraction, displayed as a percentage, is representative of the left ventricular systolic function, and takes into account the left ventricular internal dimensions at diastole and systole. The two groups are distinguished by different colours, deep purple for awake mice and deep blue for slept mice; each animal is denoted by a separate symbol; and ejection fraction readings for the same animal are interconnected with a solid line.

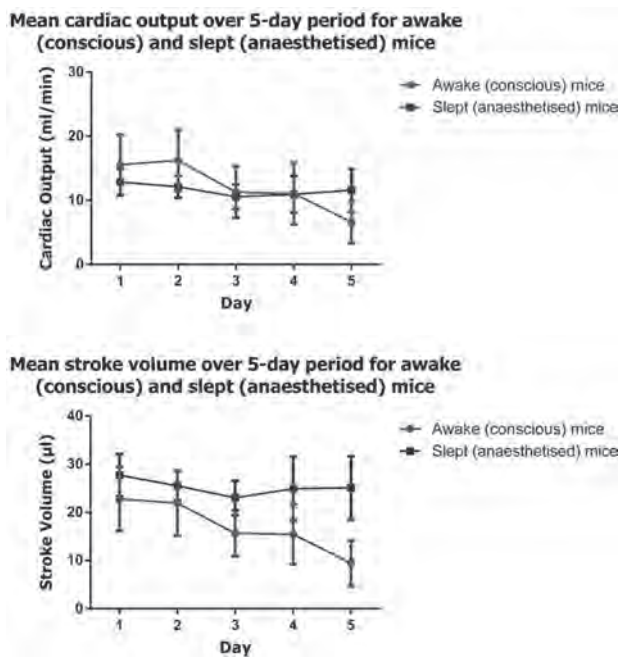


Fig. 2 A) Mean cardiac output (CO), and B) Mean stroke volume (SV) over the 5-day study period in 'awake' and 'slept' mice, plotted using calculations based on measurements of EDV and ESV and manual heart rate deduction from M-mode images of both parasternal long- and short-axis view at diastole and systole. Cardiac output, displayed in millilitre per minute, is the amount of blood the heart pumps in 1 minute. Stroke volume, displayed in microliter, is the volume of blood pumped from the left ventricle per heartbeat. Each animal is denoted by a separate symbol; and cardiac output readings for the same animal are interconnected by a solid line.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB048

## GPR30 mediated cardioprotection of estrogen against stress through rapid signaling pathway

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Presently, there are no conventional treatments for cardiac stress injury, and the existing therapies are not always effective. Estrogen(E2) plays an important cardioprotective effect when  $\beta$ -adrenergic receptors ( $\beta$ -ARs) were over stimulated by catecholamine storm. The lately discovered estrogen receptor GPR30 executes rapid effects of E2. In this study, we investigated GPR30 effect on myocardium and analyzed its mechanism in stress state. Stress model was developed by epinephrine (Epi) both *in vivo* ( $8.56 \times 10^{-8}$  mol/100g) and *in vitro* (100nM/L) in rat and human induced pluripotent stem cells-derived cardiomyocytes (hiPSC-CM) (Paur et al. 2012). G1 (48μg/100g) or G15 (76μg/100g) were used as agonist

or antagonist of GPR30(Cao et al. 2015). Values are means  $\pm$  S.E.M., compared by one-way ANOVA followed by Bonferroni post hoc tests. (1) In stress state, GPR30 activation with G1 improved heart function by decreasing LVIDs (G1+Epi:  $1.92 \pm 0.20$  vs.  $3.80 \pm 0.04$  mm Epi treated,  $P < 0.01$ ,  $n=4$ ) and increasing the EF% (G1+Epi:  $92.46 \pm 2.41$  vs.  $73.22 \pm 1.63$  % Epi treated,  $P < 0.01$ ,  $n=4$ ) measured by echocardiography, and increasing cardiomyocyte contraction amplitude (G1+Epi:  $10.50 \pm 0.86$  vs.  $7.86 \pm 1.09$  % Epi treated,  $P < 0.01$ ,  $n=10$ ). It also decreased LDH (G1+Epi:  $651.20 \pm 20.60$  vs.  $858.60 \pm 27.18$  U/L Epi treated,  $P < 0.01$ ,  $n=3$ ) and BNP (G1+Epi:  $73.41 \pm 1.96$  vs.  $90.50 \pm 2.10$  μg/L Epi treated,  $P < 0.01$ ,  $n=3$ ). (2) Both E2 and acute GPR30 activation by G1 reversed stress induced increases of  $\beta_2$ AR phosphorylation ( $P < 0.01$ ,  $n=4$ ) and cytoplasmic expression ( $P < 0.01$ ,  $n=4$ ), and the effect of E2 was canceled by G15 ( $P < 0.01$ ,  $n=4$ ). (3) Both E2 and acute GPR30 activation by G1 decreased Gi activation ( $P < 0.01$ ,  $n=3$ ) and increased cAMP level in stress state (E2+Epi:  $10.82 \pm 0.63$  vs.  $7.40 \pm 0.34$  pmol/ml Epi treated,  $P < 0.01$ ,  $n=4$ ; G1+Epi:  $10.90 \pm 0.62$  vs.  $7.40 \pm 0.34$  pmol/ml Epi treated,  $P < 0.01$ ,  $n=4$ ). (4) Both E2 and acute GPR30 activation increased L-type calcium current ( $I_{Ca-L}$ ) in stress state in cardiac myocytes of rat (E2+Epi:  $-9.11 \pm 0.95$  vs.  $-4.87 \pm 0.88$  pA/pF Epi treated,  $P < 0.05$ ,  $n=5-6$ ; G1+Epi:  $-7.57 \pm 0.44$  vs.  $-4.87 \pm 0.88$  pA/pF Epi treated,  $P < 0.05$ ,  $n=5$ ) and hiPSC-CM (E2+Epi:  $-13.63 \pm 1.28$  vs.  $-7.25 \pm 0.66$  pA/pF Epi treated,  $P < 0.01$ ,  $n=7-8$ ) which were abolished by G15 (G15+E2+Epi:  $-4.31 \pm 0.81$  vs.  $-9.11 \pm 0.95$  pA/pF E2+Epi treated,  $P < 0.01$ ,  $n=6$ ) or GPR30 knockdown (siGPR30+E2+Epi:  $-7.29 \pm 1.52$  vs.  $-13.63 \pm 1.28$  pA/pF E2+Epi treated,  $P < 0.01$ ,  $n=7-8$ ) measured by whole-cell patch clamp. These data suggest that GPR30 mediated cardioprotection of estrogen against stress through rapid signaling pathway via suppression of  $\beta_2$ AR phosphorylation, increasing the activity of G $\alpha$ s-cAMP signal pathway, strengthen  $I_{Ca-L}$  and calcium transient amplitude to improve the cardiac function. Our findings provide a light for treating patient with stress induced heart disease especially for those with estrogen deficiency.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB049

## Inotropic interventions that almost double the systolic force of a cardiac trabecula do not affect the OFF state of the thick filament in diastole

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We study the regulation of contraction of cardiac muscle by using ultra low-angle X-ray diffraction at the ID02-beamline of the European Synchrotron (ESRF, Grenoble, France) to record

the nanometer-scale structural signals from the contractile proteins along the thin and thick filaments and the micrometer scale changes in the sarcomere length (SL). By recording the X-ray diffraction signals that mark the state of the thick filament during the contraction-relaxation cycle of an electrically paced trabecula (0.5 Hz, SL 2.2  $\mu\text{m}$ , 27 °C), dissected from the right ventricle of the heart of male rats (230-280 g, aged 2-3 months) under isoflurane-induced deep anaesthesia (5% v/v), we show that (i) during the diastole most of the myosin motors lie on the surface of the thick filament packed into helical tracks in the OFF state, unavailable for actin binding and ATP hydrolysis (Woodhead *et al.* 2005; Stewart *et al.* 2010); (ii) during the systole the myosin motors leave the OFF state in relation to the loading condition, revealing a positive feedback mechanism based on thick-filament mechano-sensing (Reconditi *et al.* 2017). The same mechanism has been previously demonstrated in skeletal muscle (Linari *et al.* 2015). Thus, even if in an isometric tetanus of skeletal muscle force is under the control of the firing frequency of the motor unit, while in a heartbeat force is controlled by the afterload, the stress-sensor switching the motors ON plays the same role in adapting the energetic cost of the contraction to the force (Piazzesi *et al.* 2018).

In a heartbeat the internal  $[\text{Ca}^{2+}]$  ( $[\text{Ca}^{2+}]_i$ ) may not reach the level for full thin filament activation and the mechanical response depends on both  $[\text{Ca}^{2+}]_i$  and  $\text{Ca}^{2+}$ -sensitivity of the filament, parameters that are under the control of several regulatory systems, like the relation between SL and systolic force (a property known as Length Dependent Activation) and the degree of phosphorylation of contractile, regulatory, and cytoskeletal proteins. Here we test the integration between thin and thick filament regulation mechanisms by recording the X-ray signals that mark the state of the thick filament during two thin-filament mediated inotropic interventions able to potentiate up to twofold the twitch force developed by trabeculae at SL 2.0  $\mu\text{m}$  and with 1 mM external  $[\text{Ca}^{2+}]$ : either SL increase to 2.25  $\mu\text{m}$  or addition to the solution of the  $\beta$ -adrenergic effector isoprenaline ( $10^{-7}$  M). In diastole none of the signals related to the OFF state of the thick filament is affected by either intervention. The results further clarify the role of the thick filament mechano-sensing as the energetically well-suited downstream mechanism that rapidly adapts the number of myosin motors leaving the OFF state to the systolic force. Supported by Ente Cassa di Risparmio di Firenze. Woodhead, J.L., Zhao, F.Q., Craig, R., Egelman, E.H., Alamo, L., and Padron, R. (2005). Atomic model of a myosin filament in the relaxed state. *Nature* 436, 1195-1199.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## Computational assessment of the role of myocardial structural variability in ventricular arrhythmia dynamics

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Cardiac tissue microstructure influences propagation of cardiac electrical excitations under normal and arrhythmic conditions, but quantitative characterisation of this relationship remains a significant research challenge. Reaction-diffusion computational models of cardiac electrophysiology offer a viable approach for studying the influence of microstructure on complex excitation patterns which underlie arrhythmias (Benson *et al.* 2011). In this study, the influence of tissue microstructure (cardiomyocyte and sheetlet orientations) on organ-scale excitation patterns was investigated using 5 healthy rat ventricle reconstructions, obtained from diffusion tensor MRI (DTI) at 100  $\mu\text{m}$  isotropic resolution (Teh *et al.* 2016). The primary, secondary, and tertiary eigenvectors from DTI have been shown previously to align with the myocyte, sheetlet plane, and sheetlet normal directions, respectively. The rat action potential duration and its restitution were simulated using the Fenton-Karma 3 variable cellular activation model (Fenton *et al.* 1998). Scroll wave re-entry was initiated in the 5 anatomical models at 10 prescribed locations for 3 different microstructure scenarios (giving 150 simulations in total): (i) isotropic (no microstructure); (ii) anisotropic (myocyte but no sheetlet microstructure); and (iii) orthotropic (myocyte and sheetlet microstructure). DTI-based microstructure was shown to increase dispersion of repolarisation following pacing at the left ventricular apex. In addition, inclusion of microstructure increased mean number of scroll wave filaments, and fast Fourier transform analysis of pseudo ECG waveforms revealed that anisotropic and orthotropic microstructure favoured the transition from narrow, single-peaked spectra to a broader range of peaks, associated with increased complexity of electrical activity. Arrhythmia dynamics differed between the 5 reconstructions, where myocardial structural variability between datasets could account for whether or not ventricular tachycardia degenerated into ventricular fibrillation, highlighting the important and under-appreciated role of structural variability. This study shows that myocardial structural variability alone can account for large inter-subject variability in arrhythmia dynamics.

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PCB051

### Regional differences in $\text{Ca}^{2+}$ signaling and transverse-tubules across left atrium from adult sheep

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In ventricular myocytes, key proteins involved in excitation-contraction coupling are located predominantly at the t-tubules (TT) membrane. While TT have also been described in atrial myocytes of large species, information regarding  $\text{Ca}^{2+}$  cycling and TT network in different regions of left atrium (LA) are lacking. Knowing that LA plays a prominent role in atrial fibrillation, we have examined  $\text{Ca}^{2+}$  transients (CaTr) and TT characteristics in 4 different LA regions in an animal model that translates to human.

Procedures were in accordance with guidelines from Directive 2010/63/EU of the European Parliament. Sheep (N=20, 50±2 Kg) were pre-medicated with 20 mg/kg ketamine and 10-20 mg/kg acepromazine (both i.m.). Anesthesia was induced with sodium pentobarbital (10 mg/kg, i.v.) and maintained with 2-3% isoflurane. Heparin (200 IU/Kg, i.v.) was injected and sheep were euthanized with pentobarbital (20 mL, i.v.). Heart was excised and rinsed with cardioplegic solution. LA was cut, cannulated via the circumflex artery and perfused 20 min with 0.1 mM EGTA isolation solution (IS), followed by ~30 min perfusion with IS containing 1 mg/mL type II collagenase (Worthington), 0.1 mg/mL type XIV protease (Sigma) and 0.08 mM  $\text{Ca}^{2+}$ . Enzymes were washed out with 0.2 mM  $\text{Ca}^{2+}$  IS. Myocytes were obtained from epicardial (EPI) and endocardial (Endo) surface of left appendage, free wall (FW) and pulmonary veins sleeves (PV). CaTr was recorded (Fura-2, 0.5Hz) using an IonOptix system. Cell membrane was stained with di-8 ANNEPS and visualized under confocal microscopy. Values are mean±S.E.M. compared by ANOVA.

No difference were observed for diastolic  $\text{Ca}^{2+}$  nor CaTr amplitude (diastolic  $\text{Ca}^{2+}$ : EPI= 0.95±0.01 Ratio Unit, [RU] (n=24), Endo= 0.93±0.01 RU (n=26), FW= 0.91±0.01 RU (n=30) and PV= 0.92±0.01 RU (n=22); NS; CaTr: Epi= 0.013±0.002 RU, Endo= 0.013±0.002 RU, FW= 0.015±0.002 RU and PV= 0.016±0.002 RU; NS). Compared to left appendage, time to peak (TTP) and time to 50% decay (TTD) were longer in the 3 other regions (TTP in ms: 142±10 Endo, 211±22 FW and 198±28 PV vs 97±8 EPI;  $P<0.05$ ; TTD in ms: 509±23 Endo, 676±21 FW and 640±20 PV vs 380±22 EPI,  $P<0.05$ ). Longest TTD was observed in FW and PV cells;  $P<0.05$  compared to Endo. TT were present in all cell types albeit the percentage of cells displaying organized TT network varied (% of cells expressing TT: 75% Epi (n=81), 85% Endo (n=79), 63% FW (n=59) and 57% PV (n=76)). TT density (in  $\mu\text{m}^{-1}$ ) was significantly lower in PV and FW compared to Epi and Endo cells (0.021±0.001 Epi (n=48), 0.019±0.001 Endo (n=63), 0.014±0.001 FW (n=43), 0.012±0.001 PV (n=65),  $P<0.05$ ).

LA displays region-specific kinetics of  $\text{Ca}^{2+}$  signaling. These data are consistent with TT density observed. Overall, those results suggest regional differences in CaTr and TT in LA may play a major role in AF genesis.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB052

### Optogenetic $\text{Ca}^{2+}$ -induced $\text{Ca}^{2+}$ release from intracellular stores in cardiomyocytes

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In cardiomyocytes, the ryanodine receptor-2 (RyR2) gates  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release from the intracellular stores of the sarcoplasmic reticulum (SR). Pathological  $\text{Ca}^{2+}$  leak from the SR caused by gain-of-function mutations or altered phosphorylation of RyR2 plays an important role in the development of arrhythmias and heart failure. It remains challenging to investigate the importance of location and duration of pathological SR  $\text{Ca}^{2+}$  leak because of the diffusion-limited pharmacological RyR2 opening.

We aim to develop an optogenetic tool for precise spatial and temporal control of SR  $\text{Ca}^{2+}$  release by targeting a light-gated  $\text{Ca}^{2+}$ -permeable cation channel Channelrhodopsin-2 (ChR2) with L132C, H134R, T159C mutations (CatChUP) to the SR close to the RyR2 in order to obtain localized light-induced  $\text{Ca}^{2+}$  release that is subsequently triggering RyR2 opening. Therefore, CatChUP-EYFP was c-terminally fused to the SR membrane-spanning and RyR-interacting protein Junctin (CatChUP-EYFP-Junctin). After expression in HL-1 cells, CatChUP-EYFP-Junctin was detected in intracellular compartments but not on the plasma membrane (PM).  $\text{Ca}^{2+}$  imaging with X-Rhod-1 was performed to analyze effects of the RyR2 opener caffeine (10 mM) and blue-light (470 nm). Interestingly, blue-light illumination led to  $\text{Ca}^{2+}$  release in CatChUP-EYFP-Junctin HL-1 cells ( $\Delta F/F_0$ : 10.6±1.3% for 1.1 mW/mm<sup>2</sup>, n=6 and 18.3±2.0% for 2.4 mW/mm<sup>2</sup>, n=6), which was significantly higher ( $p<0.01$ , students t-test) than in wild-type HL-1 cells but  $\text{Ca}^{2+}$  release by caffeine was not different ( $\Delta F/F_0$ : ~45%,  $p>0.4$ , students t-test).

Next, we generated a transgenic mouse embryonic stem cell (ESC) line with stable expression of CatChUP-EYFP-Junctin. ESCs were differentiated into cardiomyocytes in spontaneously beating embryoid bodies (EBs). Using beating EBs from ChR2-EYFP ESCs with PM targeted ChR2 as the controls; spontaneous beating rate of EBs was examined by infrared video microscopy. Application of low-intensity blue-light (20 s, 4-20  $\mu\text{W}/\text{mm}^2$ ) led to an instantaneous and sustained increment of spontaneous beating rate in CatChUP-EYFP-Junctin EBs but not in ChR2-EYFP EBs, which required higher light intensities ( $>20 \mu\text{W}/\text{mm}^2$ ) to accelerate beating. Importantly, at light intensities between 30-300  $\mu\text{W}/\text{mm}^2$  rate acceleration was always higher in CatChUP-EYFP-Junctin EBs (e.g. increase by 159.2±27.8%, n=7 at 102  $\mu\text{W}/\text{mm}^2$ ) compare to ChR2-EYFP EBs (increase by 67.4±12.9%, n=4 at 102  $\mu\text{W}/\text{mm}^2$ ) ( $p=0.04$  students t-test). This indicates the light-induced  $\text{Ca}^{2+}$  release in ESC-derived CatChUP-EYFP-Junctin expressing cardiomyocytes that speeds up the  $\text{Ca}^{2+}$  clock machinery, which is the main pacemaking mechanism in these cells. Further patch-clamp and  $\text{Ca}^{2+}$  imaging experiments are required to discriminate between PM-bound ChR2 photocurrent and SR  $\text{Ca}^{2+}$  release-induced  $\text{Na}^+/\text{Ca}^{2+}$  exchanger current and to proof light-induced  $\text{Ca}^{2+}$  release by CatChUP-EYFP-Junctin.

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PCB053

### Gene therapy for cardiac conduction system dysfunction in heart failure

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**Background and objectives:** Heart failure (HF) is characterised by generalised dysfunction of the cardiac conduction system (CCS). Ion channel and structural remodelling in the CCS have been demonstrated in animal models of cardiovascular disease. As Purkinje fibres (PFs) are minute strands of tissue, little is known about their ultrastructure and remodelling in disease. Furthermore, given the role for microRNAs (miRs) in CCS molecular remodelling, we aimed to develop a tissue specific method for delivering therapeutic transgenes, such as miR sponges.

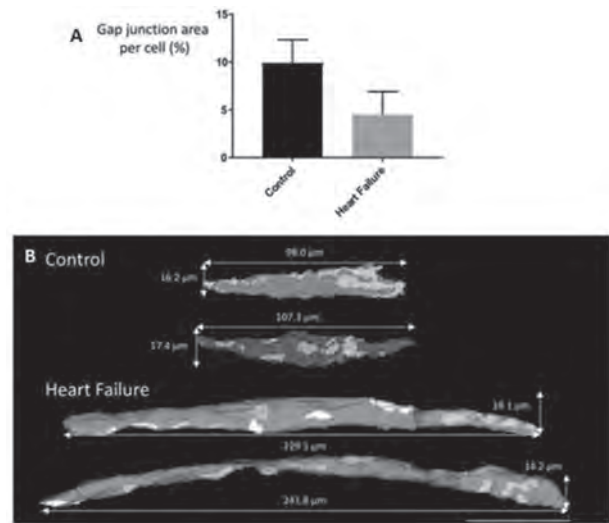
**Methods:** New Zealand rabbits were used for PF ultrastructural studies. HF was induced via pressure and volume overload as previously described<sup>(1)</sup>. Free running PFs were stained and embedded for serial block face scanning electron microscopy (SBF-SEM). Manual contrast-based segmentation techniques were used on IMOD software to determine the 3D ultrastructure of free running PFs in control and HF animals.

To target transgene expression to the CCS, adenoviral plasmids were cloned expressing a GFP reporter gene. GFP transcription was placed under control of the KCNE1 promoter, a potassium channel subunit expressed throughout the CCS, or the HCN4 promoter, a key pacemaker ion channel, to target the SAN. Adenovirus was then produced using Gateway Cloning Technology (Thermo-Fisher Scientific) in combination with the ViraPower Adenoviral Expression system (Invitrogen) and transfected into the 293A cell line for amplification. For in vivo use, all plasmids were also cloned into AAV packaging plasmids.

**Results:** Purkinje cells (PCs) formed a central core within PFs, encapsulated by an extensive collagen matrix. 3D reconstructions revealed a central core of longitudinally running cells, running parallel to the direction of the fibre. PCs were uninucleated and spindle shaped with an irregular membrane, and an average length of 108µm, diameter 15µm and volume of 7965µm<sup>3</sup> (n=9). Gap junctions were abundant and distributed along the lateral surface of cells, and there was a trend towards decreased expression in HF (Figure 1B, p=0.0526, n=3 cells analysed per group). Hypertrophy (figure 1B) and nuclear membrane breakdown were evident in HF PCs, the latter facilitating mitochondrial entry.

Testing the various promoter constructs for therapeutic gene delivery to the CCS is ongoing in vitro using isolated neonatal rat cardiomyocytes and ex vivo using SAN tissue isolated from Wistar rats. These tests will show the efficacy of transgene expression under each promoter as well as tissue specificity. All adenoviral plasmids were successfully cloned, verified by restriction digest and Sanger sequencing.

**Conclusions:** SBF-SEM revealed ultrastructure of free running PFs in situ, and uncovered novel structural changes in HF that are likely to be pro-arrhythmic. The efficacy of our proposed gene therapy approach in targeting the CCS will be further elucidated in the coming months whilst testing the final viral expression constructs.



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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB054

### 3D-reconstruction of ventricular cardiomyocyte mitochondria from the Greenland shark (*Somniosus microcephalus*) the World's Oldest Vertebrate

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The life span of the Greenland shark (*Somniosus microcephalus*) is at least 272 years and may be as long as 500 years (1). This extreme longevity is particularly interesting with respect to the heart, because ageing in humans is associated with cardiovascular dysfunction including fibrosis, atherosclerosis and heart failure. Accumulating evidence has suggested a causative link between mitochondrial dysfunction and major phenotypes associated with ageing (2). In this study we have performed quantitative analysis on mitochondrial morphology and subcellular organisation on mitochondria for the compact layer of the Greenland shark. We have used scanning electron micrographs of ultrathin serial sections to segment individual mitochondria (n=208) and performed three-dimensional computational reconstructions of these within the cardiomyocyte. We report an average mitochondrial volume of 1.33±1.21 µm<sup>3</sup> and a mitochondrial fraction/volume density of 0.251. Furthermore, we report cases of severe mitomegaly and a large number of caveolae. This is of

interest because, caveolins, proteins associated with caveolae (small cave-like invaginations of the sarcolemmal membrane), have been implicated as signalling mediators coordinating cellular protection responses during oxidative stress and ageing (3). To our knowledge, this is the first study to present a deep qualitative and quantitative analysis of an elasmobranch cardiomyocyte and its organelles. Further studies will lead to better understanding of mitochondrial morphology and subcellular associations in a model of extreme longevity. Nielsen J *et al.* (2016). *Science* 353, 702-704.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## PCB055

### Investigating $\text{Ca}^{2+}$ Mediated Arrhythmias in a Computational Model of Rabbit Atrial Myocytes

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**Background:** The system of transverse and longitudinal sarcolemmal tubules (T-system) is observed to remodel in two cardiovascular conditions of increasing incidence: atrial fibrillation (AF) and heart failure (HF). The relationship between these conditions, wherein patients with HF often develop AF, and vice versa, confounds this problem further. Dysfunctional calcium regulation, as a result of altered T-system morphology, has been suggested to underlie disruptions in excitation-contraction coupling, as well as an increase in the frequency of arrhythmic events at the cellular scale; however, these mechanisms and their importance remain to be fully described.

**Methods:** A contemporary computational model describing rabbit atrial electrophysiology (Aslanidi, *et al.* 2009) was integrated with our novel model describing stochastic spatio-temporal  $\text{Ca}^{2+}$  dynamics (Colman *et al.* 2017). In isolation to other potential forms of atrial remodelling, morphological T-system remodelling associated with HF was simulated through removing sarcolemmal ion-channel currents from individual calcium release units (CRUs), either assigned randomly or in pre-defined regions of varying extent. Rapid pacing protocols were applied to induce  $\text{Ca}^{2+}$  transient alternans, and load the Sarcoplasmic Reticulum (SR) with  $\text{Ca}^{2+}$ : the resulting statistics of spontaneous release events were analysed.

**Results:** The model reproduced rabbit atrial action potential and  $\text{Ca}^{2+}$  transient morphology associated with healthy cardiac function. Variation in T-system density and organisation, in isolation, demonstrated an inverse correlation between T-system density and the susceptibility of spontaneous  $\text{Ca}^{2+}$  release events, determined by an interaction of localised SR  $\text{Ca}^{2+}$  loading and reduced efflux, promoting successful  $\text{Ca}^{2+}$  wave propagation. Lower T-system density also promotes  $\text{Ca}^{2+}$  alternans, by alternating successful and failed propagations of  $\text{Ca}^{2+}$  into regions without T-tubules.

**Conclusion:** A novel model of rabbit atrial electrophysiology has been developed, that includes a stochastic spatio-temporal description of  $\text{Ca}^{2+}$  handling. Investigations using this model suggest that alterations in T-system morphology may play an important role in the predisposition of atrial myocytes to arrhythmic events in the presence of HF. The model presents a powerful research tool to explore how these

cell-level phenomena manifest at the tissue level and result in arrhythmias.

Aslanidi, *et al.*, *Biophys. J.* 96(3):798-817, 2009 Colman *et al.* *PLOS Comp. Biol.* 13, e1005714, 2017

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## PCB056

### Complexity analysis of equine telemetric electrocardiogram recordings

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#### Background

Arrhythmic tendency has been paradoxically associated with intensive exercise in both human and equine athletes. Our previous research has indicated that analysis of electrocardiogram (ECG) complexity might be a valuable tool for early arrhythmia detection in humans. We aimed to determine if ambulatory equine ECG recorded by in vivo telemetric electrocardiography might be amenable to semi-automated complexity analysis.

#### Methods

Non-invasive ECG recordings were collected during routine clinical work up from 51 healthy horses using telemetric ECG recorder. No ethical approval was required as no recordings were performed specifically for this study. The study subjects were participating in race training and none showed clinically significant cardiac abnormalities on prior routine examination. Data were recorded at 500 Hz sampling rate in continuous episodes, before and during a period of acceleration from walk to canter. The artefact-free segments of >60 seconds were resampled to 125 Hz rate and filtered using the digital band-pass Butterworth filter (0.13 Hz to 21 Hz). Filtered recordings were converted to binary strings using a custom beat detection algorithm. Only the strips where beat detection was 100% correct were used for subsequent complexity analysis.

Binary beat-detection strings were subjected to complexity analysis using several previously published methods: Lempel-Ziv '76 [LZ76] (1), Lempel-Ziv '78 [LZ78] (2), Titchener T-complexity [TC] (3) and Croll's BiEntropy [CE] (4).

#### Results

To estimate the minimum duration of ECG required for the reliable complexity analysis we evaluated the dependence of complexity of ECG strip on its duration. Three 120-sec lead I ECG strips displaying stable heart rate were used to generate individual sets of random sub-strips of different duration (20, 24, 28, 35, 42, 50, 60, 72, 86 and 100 sec; 60 sub-strips each). It was found that LZ78 and CE values are progressively decreasing with the increase of analysed strip length while LZ76 and TC values are asymptotically closing to the "true value". Variation coefficients for both LZ76 and TC decreased from 10-12% for 20-sec strips to 4-5% for strips longer than 72 sec.

#### Discussion

Our results suggest that Lempel-Ziv 76 or Titchener T-complexity, but not LZ78 or Croll's BiEntropy might be the feasible tools to estimate the ECG recording complexity. Further study



will be needed to improve the performance of complexity analysis and decrease the errors due to random sampling.

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Titchener MR. Deterministic computation of complexity, information and entropy. Inf Theory, 1998 Proceedings 1998 IEEE Int Symp 1998;22(1):326.

Croll G. BiEntropy-The Approximate Entropy of a Finite Binary String. ArXiv Prepr ArXiv13050954 2013:1–16.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB057

## Multi-scale Coupling between Cardiac Sub-cellular Calcium Release Events and Re-entrant Excitation

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**Motivation:** The role of sub-cellular spontaneous calcium release events (SCRE) in the development of arrhythmia at the tissue-scale has yet to be investigated in detail. SCRE may underlie the emergence of spontaneous excitation in single cells, resulting in arrhythmic triggers in tissue. However, translation of single-cell data to the tissue scale is non-trivial due to the complex substrate considerations associated with arrhythmic conduction patterns. Computational modelling provides a viable approach to dissect these multi-scale mechanisms, yet there remains a significant challenge in accurately and efficiently modelling this probabilistic behaviour in large-scale tissue models. The aim of this study was to develop an approach to overcome this challenge and investigate the potential organ-scale implications of cellular SCRE.

**Methods:** A computational model of stochastic, spatio-temporal calcium handling [1] was used to investigate the dynamics of SCRE under multiple conditions (pacing rate, beta-stimulation, disease remodelling) in single cells. Analysis of these data allowed the derivation of spontaneous release functions, which capture the variability and properties of SCRE matched to the full cell model [2]. These functions were then integrated with tissue models, comprising idealised 2D sheets as well as full reconstructions of ventricular and atrial anatomy. Mechanisms of synchronisation of independent cellular events into spontaneous focal excitation were investigated under calcium overload conditions; sustained re-entry was implemented to study the potential long-term interactions between re-entry and focal excitation.

**Results:** The developed approach allowed dynamic reproduction of SCRE in efficient single cell models and resulting large-scale tissue models, validated against the behaviour observed in the detailed cell models. In homogeneous tissue, the emergence of a spontaneous beat from a single source was observed and the positive role of electrotonic coupling was demonstrated. Sustained re-entrant excitation promoted calcium overload, and led to the emergence of focal excitations both after termination of re-entry and also during re-entrant excitation. These results demonstrated a purely functional mechanism of re-entry and focal activity localisation, related to the unexcited spiral wave core.

**Conclusions:** A novel approach has been developed to dynamically and accurately model SCRE at the tissue scale, in-line with behaviour observed in detailed single-cell models. Mechanisms of the interaction of cellular SCRE with organ-scale arrhythmia were demonstrated, providing novel insight into the coupling between focal excitation and re-entry.

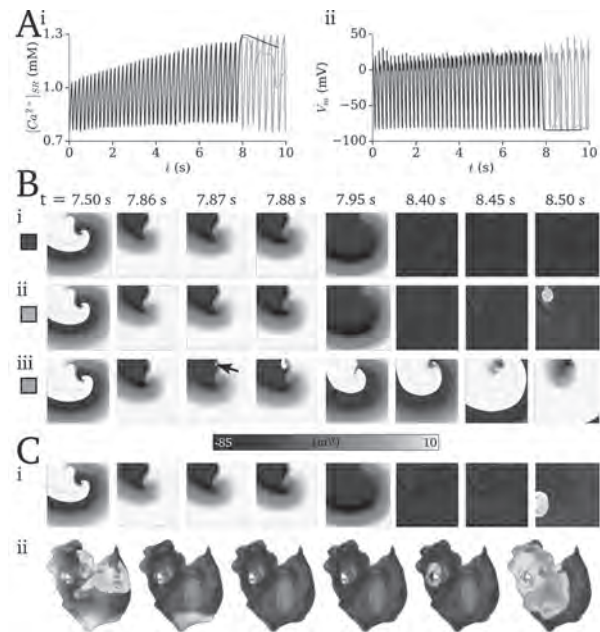


Figure: Coupling between re-entry and spontaneous calcium release events. A – SR-Ca<sup>2+</sup> concentration (i) and V<sub>m</sub> (ii) associated with sustained re-entry followed by self-termination (at around 8 s), simulations without SCRE (purple) and with the General Dynamic SRF model with two different thresholds (orange, 1.125 mM; blue, 1.0 mM). B – Temporal snapshots of voltage in the 2D sheet associated with the traces shown in A, showing self-termination (i) and the emergence of delayed (ii, corresponding to the orange traces in A) and rapid (iii, corresponding to the blue trace in A) focal excitations. C – Examples of non-localised focal excitations emerging in the 2D sheet (i) and 3D whole atria models (ii).

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[2] Colman MA, Parra-Rojas C, Perez-Alday EA. From microscopic calcium sparks to the ECG: Model reduction approaches for multi-scale cardiac simulation. In: 2015 Computing in Cardiology Conference (CinC). 2015. p. 325–8.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB058

## Quantification of collagen deposition in the ventricle of the Greenland shark (*Somniosus microcephalus*) the World's Oldest Vertebrate.

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The exciting discovery of the Greenland sharks (*Somniosus microcephalus*) life span, which is at least 272 years and may extend to 500 years, is of particular interest with regards to the cardiovascular system and age associated diseases such

as fibrosis [1]. Age-related fibrosis is currently accepted as an unavoidable process characterised by harmful degenerative changes that result in collagen deposition within the extracellular matrix [2]. In this study we investigated the epicardium, compacta, transition zone and spongiosum of Picrosirius red stained tissue sections of seven Greenland shark ventricles that ranged from 150 to 250 years old. We used data from brightfield and cross-polarised light microscopy to quantitatively analyse these stained sections using Image J. We report significant collagen deposition in all layers of the ventricle with the epicardium especially, demonstrating extensive ( $45.97 \pm 2.96\%$ ) fibrosis across all specimens. Variations in collagen deposition showed a positive correlation with the age of the sharks as expected. The coherency of the collagen fibres in all four layers of the ventricle across all specimens varied significantly, with the spongiosum being the least ordered and the epicardium being the most. The extent of fibrosis in the Greenland shark may impact its ability to fill with blood and volume-regulate cardiac output [3]. This is the first quantitative analysis of fibrosis in the heart of the world's oldest vertebrate and it allows rare insight into collagen deposition at varying extremes of age.

[1] J. Nielsen, R.B. Hedeolm, J. Heinemeier, P.G. Bushnell, J.S. Christiansen, J. Olsen, C.B. Ramsey, R.W. Brill, M. Simon, K.F. Steffensen, J.F. Steffensen. (2016) 'Eye lens radiocarbon reveals centuries of longevity in the Greenland shark (*Somniosus microcephalus*)' *Science* 353(6300) pp. 702-4.

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[3] Moreo, A., Ambrosio, G., De Chiara, B., Pu, M., Tran, T., Mauri, F. and Raman, S. V (2009) 'Influence of myocardial fibrosis on left ventricular diastolic function: noninvasive assessment by cardiac magnetic resonance and echo.' *Circulation. Cardiovascular imaging*. NIH Public Access, 2(6) pp. 437-43.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB059

### Effects of external calcium concentration on diastolic, systolic and time-averaged calcium concentration in mouse ventricular myocytes

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The heart spends about half of its time in diastole as relaxation is required for normal cardiac function. Impairment of relaxation can lead to diastolic heart failure. Although much is known about the regulation of systolic Ca concentration ( $[Ca^{2+}]_o$ ), less is known about the regulation of diastolic. In a recent study on rat ventricular myocytes, we found that the time-averaged level of  $[Ca^{2+}]_i$  was determined by Ca influx into the cell, thereby controlling diastolic  $[Ca^{2+}]_i$  [Sankaranarayanan et al.]. In this study, therefore, we aimed to investigate the effects of a simple inotropic manoeuvre, elevation of external Ca concentration, on both diastolic and systolic calcium concentration.

Cardiac ventricular myocytes were isolated from mice and loaded with the AM form of the fluorescent calcium indicator Fluo-3. Calcium transients and membrane currents were recorded with the perforated patch clamp technique. Different stimulation frequencies (0.5, 3 Hz) were achieved by stimulating the cells through the patch electrode with square depolarizing pulses. External Ca concentration was varied over the range 0.5 to 5 mM. Data are reported as the mean  $\pm$  SEM for "n" cells.

At normal (1 mM) or low (0.5 mM) external Ca, increasing frequency from 0.5 to 3 Hz decreased the amplitude of the Ca transient while increasing diastolic  $[Ca^{2+}]_i$ . (For example, at 0.5 mM Ca, increasing frequency decreased amplitude from  $322 \pm 60$  nM to  $133 \pm 22$  nM;  $n=16$ ,  $P<0.001$  and increased diastolic from  $76 \pm 12$  nM to  $84 \pm 10$  nM;  $n=15$ ,  $P<0.005$ ). In contrast, at 5 mM Ca, increasing frequency had no effect on the amplitude ( $567 \pm 88$  nM vs  $571 \pm 75$  nM;  $n=10$ , NS) but still increased diastolic  $[Ca^{2+}]_i$  (from  $203 \pm 46$  nM to  $407 \pm 95$  nM;  $n=9$ ,  $P<0.05$ ). At all Ca concentrations, however, increasing frequency increased the time-averaged  $[Ca^{2+}]_i$  as has been found in rat myocytes. These changes of time-averaged  $[Ca^{2+}]_i$  were compared with the measured Ca influx per unit time, estimated from the L-type Ca current. Increasing frequency increased Ca influx per unit time. The fractional change of measured influx was, however, greater than that of average  $[Ca^{2+}]_i$  suggesting a contribution of a significant, frequency-independent Ca influx mechanism in addition to the L-type Ca current [Sankaranarayanan et al.].

These results demonstrate that changes of time-averaged  $[Ca^{2+}]_i$  result from those of Ca influx. Future work needs to identify this additional Ca influx pathway and how it is affected by physiological and pathological changes.

Sankaranarayanan R, Kistamas K, Greensmith DJ, Venetucci LA, Eisner DA (2017) Systolic  $[Ca^{2+}]_i$  regulates diastolic levels in rat ventricular myocytes. *J Physiol*. 595, 5545-5555.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB060

### A novel method of studying intercellular and intracellular $Ca^{2+}$ signalling in human cardiomyocytes from the right atrial appendage

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The current investigation aimed to develop a novel technique that allows the study of intracellular and intercellular  $Ca^{2+}$  signalling in strips of atrial cardiomyocytes. There is evidence of cell heterogeneity within the atrial appendage, which may contribute to the failure in the treatment of antiarrhythmias. Right atrial appendage biopsies from patients undergoing valve replacement surgery were placed in ice-cold  $Ca^{2+}$ -free HEPES-buffered solution and transferred from the surgery to the laboratory on ice. Once in the laboratory, samples were placed in  $Ca^{2+}$ -containing HEPES-buffered solution and 3 mm long strips of tissue were dissected from the appendage and loaded with the  $Ca^{2+}$ -sensitive indicator Cal-520 AM ( $10\mu M$ ) or Calbryte-520 AM ( $10\mu M$ ). Real-time confocal microscopy was then utilized to observe fluctuations in  $Ca^{2+}$  activity. The major advantage of this novel method is the minimal disturbance of cell-to-cell contacts within atrial bundles to allow the study of intercellular communication between atrial cardiomyocytes.

We observed a different pattern of spontaneous  $\text{Ca}^{2+}$  activity in atrial cardiomyocytes. Spontaneous activity varied from synchronized  $\text{Ca}^{2+}$  spikes to asynchronous  $\text{Ca}^{2+}$  waves and sparks. Another striking observation was the degree of atrial myocyte heterogeneity within muscle bundles.

In conclusion, our preliminary investigation demonstrates how live confocal imaging of tissue strips isolated from the right atrial appendage is a useful technique for the study of intracellular and intercellular  $\text{Ca}^{2+}$  signalling in the highly heterogeneous population of human atrial cardiomyocytes. A detailed study of the distinct atrial myocytes and their potential link to patient history may improve our understanding of atrial fibrillation and other arrhythmias, and inform how such diseases are treated in the future.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## PCB061

### Increased systemic oxidative stress index and DNA damage in patients with age-related macular degeneration

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Molecular pathogenesis of AMD remains unclear and intricate. Although a few number of studies investigated components of oxidant/antioxidant pathways and DNA damage/repair systems separately in AMD, there is no comprehensive research study available on this issue to best of our knowledge. The aim of the current study was to investigate the evidence of systemic oxidative stress and DNA damage in patients with exudative age-related macular degeneration (AMD). This prospective controlled study comprised 22 patients with exudative AMD (67.3±2.2 years of age) and 24 age- and sex-matched (62.1±2.2 years of age) healthy controls (p>0.05). Serum total oxidant status (TOS) and total antioxidant status (TAS) were measured using a commercial kit and oxidative stress index (OSI) was calculated. DNA damage was assessed using comet assay following lymphocyte isolation from venous whole blood. Parameters including tail length and tail moment were evaluated for quantitative analysis of DNA damage. Serum 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels were also measured to assess oxidative DNA damage. The AMD group had lower TAS and higher OSI values than the control group, while TOS values did not differ between the two groups (p= 0.0001, p= 0.025 and p= 0.930, respectively). Furthermore, tail length (p= 0.0001) and tail moment (p= 0.003) were significantly higher in the AMD group compared to the control group. However, serum 8-OHdG (p= 0.892) value was similar between the AMD and control groups. Systemic oxidative stress index and cumulative DNA damage appear to be increased in patients with exudative AMD.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## PCB062

### FISIOSINAL, A NEW TOOL FOR HRV ANALYSIS IN SPACE LIFE SCIENCES

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Throughout these 17 years of research and experience on board of the International Space Station, it is now clear that prolonged exposure to the extreme conditions of space, induces severe changes to human health and physiology, which can compromise astronauts performance, safety and ultimately mission success. Indeed, microgravity, and surely, deep Space exploration, affects all physiological systems, in particular, the cardiovascular system and the autonomic control mechanisms, with extreme variations in heart rate variability (HRV) and blood pressure. Most data obtained so far, indicate a decrease in heart rate (HR) during space flight, mainly explained by a decrease in the low frequency component (LF) of HRV and an increase in the high frequency band (HF), indicating a predominant vagal modulation of the sinus node during short-term space flights. However, this sympathovagal relation between the low frequency and the high frequency components (LF/HF) of heart rate variability, has yielded a few contradicting results. In order to validate our laboratory and in-house HRV analysis software, Fisiosinal, as a potential new tool for space life sciences and physiology studies, 14 normal human subjects volunteered to participate in a protocol of simulated weightlessness, consisting of lying down on a TILT table with -6 ° (TILT down), for two hours. During the study, volunteers were subjected to an autonomic maneuver, in order to stimulate the sympathetic nervous system (Mental Stress Maneuver). All cardiac components were collected, and after wavelets analysis, our preliminary results show a significant decrease in HR, with a p value of 0,0128. Even though, not quite significant, the LF/HF ratio shows a clear tendency to decrease, associated with a comprehensible increase in parasympathetic nervous system activation. During the mental stress sympathetic maneuvers, HR was increased, which correlated with an enhanced sympathetic modulation (higher values of LF), alongside a general decrease in the high frequency band. Despite the fact that, some of these findings are not directly related to what has been previously stated, i.e., a decrease in LF/HF during space flight mainly due to a strong parasympathetic activation, the results are indeed related to the effects of simulated weightlessness. The increased values of LF are mostly consistent with the autonomic maneuver performed, which stimulated sympathetic nervous system activation, thus overthrowing the effects of TILT down. Overall, our preliminary results are in accordance with the current literature, therefore validating our facilities and more importantly our in-house software, Fisiosinal, as suitable tools for studies in the field of space life sciences, and the effects of microgravity (simulated) upon human physiology.

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Vandeput S et al. (2013). *Med Sci Monit* 4(19): 9-17



Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB063

### Abnormal L-type calcium channel properties in ventricular cardiomyocytes derived from adult dystrophic mdx mice

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Duchenne muscular dystrophy (DMD), induced by mutations in the gene encoding for the intracellular protein dystrophin, is a severe inherited disease characterized by progressive muscle weakness and degeneration. Besides the well-studied skeletal muscle degenerative processes, DMD is also associated with cardiac complications. These include dilated cardiomyopathy development and cardiac arrhythmias. The present understanding of the pathomechanisms in the DMD heart is very limited, but evidence has been accumulating that dysfunctional ion channels in dystrophic cardiomyocytes play a significant role. The aim of this study was to characterize potential abnormalities in L-type calcium channel function, expression, and regulation in ventricular cardiomyocytes derived from dystrophic *mdx* mice. The *mdx* mouse is dystrophin-deficient, and the most commonly used mouse model for human DMD. For the experiments, ventricular cardiomyocytes were isolated from the hearts of adult normal (wild type) and dystrophic *mdx* mice, and the properties of their calcium channels were compared by using the whole cell patch-clamp technique. In addition, RT-PCR, western blot and immunocytochemical studies were performed. We found that currents through L-type calcium channels were significantly increased, and channel inactivation was reduced in dystrophic ventricular cardiomyocytes. These gain-of-function calcium current abnormalities will increase the Ca influx into the dystrophic myocyte during an action potential. Calcium channel subunit expression and localization in dystrophic cardiomyocytes was normal. This suggested that altered channel regulation was responsible for the observed calcium current abnormalities in dystrophic cells. Accordingly, inhibition of neuronal nitric oxide synthase (nNOS) increased the calcium currents only in wild type but not in dystrophic cardiomyocytes. This points to impaired nNOS activity in dystrophic cardiomyocytes as potential source for functional L-type calcium channel abnormalities. We conclude that gain-of-function L-type calcium channel abnormalities enhance the risk of calcium-dependent arrhythmias and cellular calcium overload in the dystrophic heart.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB064

### Mitochondrial impairment downregulates inward-rectifying K<sup>+</sup> channel (KIR2.1) activity in L6 myoblasts via oxidative phosphorylation dysfunction.

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During myogenesis, myoblast fusion depends on mitochondria and ion-channel-regulated intracellular Ca<sup>2+</sup> signaling. However, the relationship between mitochondrial function and ion channels that affect calcium signaling in myoblasts is unknown. Here, we compared ionic currents associated with intracellular Ca<sup>2+</sup> influx in normal and mitochondria-depleted ( $\rho 0$ ) L6 GLUT4myc myoblasts. In  $\rho 0$  myoblasts, we observed a decrease in the inward-rectifying K<sup>+</sup> current (KIR2.1), which hyperpolarizes the membrane to generate the electromotive force for Ca<sup>2+</sup> influx. The downregulation of KIR2.1 was induced by not only a decrease in KIR2.1 expression but also mitochondrial dysfunction of electron transport-coupled ATP synthesis in  $\rho 0$  myoblasts that might be affect intracellular Ca<sup>2+</sup> signaling in  $\rho 0$  myoblasts. Our work elucidates the functional effect of mitochondria on myogenesis-related ion channels during myoblast differentiation.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB065

### Combining electrophysiology and contractility recordings for more complete assessment of hiPSC-CMs

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Human induced pluripotent stem cells (hiPSCs) are becoming increasingly important for cardiac safety testing due to their recapitulation of native behaviour, relative abundance and ease-of-use. We combine automated patch clamp (APC), impedance and extracellular field potential (EFP) measurements to study hiPSC-derived cardiomyocytes (hiPSC-CMs) from different sources. In line with the comprehensive in vitro proarrhythmia assay (CiPA) initiative, cardiac ion channels expressed in heterologous expression systems have been recorded on APC devices at room temperature and at physiological temperature and the effects of different pro-arrhythmic compounds on ion channels was investigated. In addition hiPSC-CMs were used on APC platforms recording from 1, 8 or 384 wells simultaneously. Voltage-gated Na<sup>+</sup> (Na<sub>v</sub>), Ca<sup>2+</sup> (Ca<sub>v</sub>) and K<sup>+</sup> (K<sub>v</sub>) currents were recorded. The Na<sub>v</sub> channel was blocked by tetracaine with an IC<sub>50</sub> of 2.9 ± 1.4 µM (n = 5) and by TTX with an IC<sub>50</sub> of 1.1 ± 0.5 µM (n = 4), values which are consistent with the cardiac Na<sub>v</sub>

channel,  $\text{Na}_v1.5$ . Nifedipine blocked the  $\text{Ca}_v$  currents recorded from hiPSC-CMs with an  $\text{IC}_{50}$  of 84 nM, consistent with the cardiac  $\text{Ca}_v1.2$  channel. In addition to voltage clamp measurements, action potentials from hiPSC-CMs were also recorded in the current clamp mode and the effect of nifedipine on action potential amplitude and duration will be shown. Using the dynamic clamp technique coupled with an APC device, electrically modelled  $\text{IK}_1$  was injected into the cell under current clamp conditions and this resulted in a more hyperpolarized and stable resting membrane potential. The amount of  $\text{IK}_1$  injected into the cell affected action potential (AP) duration. Pharmacology using a calcium channel activator, BayK 8644, and blocker, nifedipine, was also performed. BayK 8644 at 1  $\mu\text{M}$  significantly prolonged AP duration and nifedipine (30  $\mu\text{M}$ ) shortened AP duration.

Within the myocyte phase II study of the CiPA initiative, a device combining impedance-based contractility and extracellular field potential (EFP) recordings was used to investigate the effects of different compounds deemed low, intermediate and high risk by the FDA. Different hiPSC-CMs were used in a large cross-site evaluation which found that high risk compounds such as dofetilide prolong field potential duration and arrhythmic events were detected in both impedance and EFP recordings. Intermediate risk compounds induced arrhythmia in almost all cases at the highest dose and either no significant effect or a decrease in  $\text{FPD}_{\text{max}}$  was recorded for low risk compounds. In addition, cells can be optically stimulated and impedance and EFP signals simultaneously recorded from the same cellular monolayer. In this way, contractility and EFP signals can be exactly overlaid giving in-depth mechanistic information about contractile behaviour and electrophysiology of iPSC-CMs.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCB066

### **L-type calcium channel inactivation processes during action potential in newborn rat cardiomyocytes stimulated with isoproterenol**

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In cardiomyocytes, the L-type calcium channel inactivation processes (voltage- and calcium-dependent; VDI and CDI respectively) has been associated with the determination of the action potential duration. Interestingly, in rat cardiomyocytes although the  $\beta$ -adrenergic stimulation has been shown to increase L-type calcium current when studied under square pulses, the action potential duration is reduced. In this work, we explored this apparent contradiction combining self-action potential clamp (sAP-clamp) with the molecular inhibition of VDI or CDI (by overexpressing  $\text{Ca}_v\beta_{2a}$  or calmodulin mutants respectively) to directly record the L-type calcium current during the action potential in newborn rat cardiomyocytes. Our results show that, in control cardiomyocytes, L-type current coincides with the action potential kinetic, and while the inhibition of either inactivation processes prolongs the action potential duration, the L-type current extends its duration accordingly, suggesting that both processes are important in controlling L-type current during an action

potential. In contrast, after  $\beta$ -adrenergic stimulation, L-type calcium current finishes before than the action potential, same effect observed in cells where the L-type channel has a reduced VDI, but not in cells without CDI, where the L-type current maintains the same kinetic than the action potential, demonstrating experimentally that the CDI is the inactivation process that governs the L-type current kinetic during  $\beta$ -adrenergic stimulation.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCB067

### **Collective and individual dynamics of endothelial cells (HUVEC) in confluent cultures**

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Endothelial cells in confluent monolayers show various signatures of temporal intermittent collective dynamics, e.g. the oriented movement of clusters or the formation of spirals. These effects are the result of a complex interplay of individual active cell dynamics and cell-to-cell interactions. It is the aim of this study to disentangle and quantify these two fundamental aspects. Therefore, we seeded human umbilical vein endothelial cells (HUVEC) on a fibronectin-collagen IV gelatin coated surface, stained the cell nuclei with low concentrations of Hoechst 33342 (1:20000) and observed the dynamics over 24 h with a sampling interval of 10 min. A self-developed image processing software allowed the automated tracking of individual cells resulting in several 10000 cells per time point. An increasing cell number over time showed that cells were viable. In parallel, the mean squared velocity of individual cells as a measure of active motility slowed down over time by about 50 % indicating a non-stationary behavior. In addition, we characterized the dynamics of individual cells from their paths and the corresponding mean squared displacement. We found that endothelial cells showed a normal diffusive behavior with temporal correlations during the first 8 h, but transformed to sub-diffusive dynamics within the following two 8-h-intervals indicating a more persisting cell localization. In parallel, we observed a temporal change of the spatial velocity autocorrelation function of cells indicating changes of interactions between cells. In summary, we have characterized an interesting dynamical behavior of interacting endothelial cells showing anomalous dynamics and complicated time-dependent interaction correlations. In future, this approach will allow to assess quantitatively the influence of modifications of cell contacts and the cell migration machinery onto the individual and collective dynamics of confluent cells.

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PCB068

### Fetal hemodynamic response to acute maternal hyperoxygenation in the setting of intrauterine growth restriction

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**Introduction:** Placental insufficiency results in chronic fetal hypoxemia that leads to intrauterine growth restriction (IUGR) because oxygen delivery to the fetus is an independent determinant of fetal growth (1). Furthermore, IUGR offspring are at an increased risk of adverse perinatal and long-term outcomes, including neurodevelopmental and cardiovascular complications (2). One approach to preventing these negative effects may be to increase fetal oxygenation via maternal hyperoxygenation (MH), which improves outcomes in severely IUGR pregnancies (3). We present the hemodynamic response to acute MH in normoxemic and chronically hypoxemic IUGR fetuses.

**Methods:** At 109-111 days (d) gestation (term=150d), pregnant Merino ewes (n=6, control; n=4, IUGR) underwent surgery, with general anesthesia induced by intravenous injection of diazepam (0.3 mg/kg) and ketamine (7 mg/kg) and maintained with inhalation of isoflurane (1-2%) in oxygen with post-operative antibiotics and analgesia, to implant vascular catheters in the maternal jugular vein, fetal femoral artery and vein as well as the amniotic cavity. At 126±1d, ewes underwent cardiovascular MRI assessment using a 3T Siemens Skyra system in both a normoxic and hyperoxic (100% O<sub>2</sub>) state under general anesthesia induced and maintained as above (isoflurane, 2.5-2.75%). MRI assessment was performed using phase contrast (PC) measurements of blood flow in major fetal and placental vessels including uterine arteries (UTAs), umbilical vein (UV), ductus venosus, ascending aorta (AAO), main pulmonary artery, branch pulmonary arteries (PAs), ductus arteriosus, common carotid arteries (CCAs), superior vena cava and descending aorta. Foramen ovale (FO) shunting was calculated by subtracting flow in PAs from AAO. All blood flow measures were indexed to fetal weight calculated from the 3D MRI acquisition of fetal volume (4).

**Results:** MH significantly increased fetal arterial PaO<sub>2</sub> (Control: 19.1±1.6 vs 22.7±1.4 mmHg; IUGR: 16.1±1.7 vs 21.4±2.6). IUGR fetuses had significantly reduced uterine artery blood flow in comparison to controls which did not change in response to MH. Acute MH significantly increased blood flow in the fetal PAs and decreased blood flow through the FO whilst other major vessels were unaltered. The data was analyzed using a two-way ANOVA (statistical significance, P<0.05).

**Conclusions:** We used PC-MRI to perform a comprehensive assessment of the fetal circulation and show that acute MH increases PA and decreases FO blood flow in both controls and IUGR fetuses. Unaltered flow in the UV and CCAs with increased arterial fetal oxygen content suggests increased oxygen delivery to the fetus and the brain. Despite reduced UTAs blood flow, a chronic MH treatment can be of great benefit to an IUGR fetus to improve oxygen delivery, particularly to important organs like the brain, heart, lungs and liver

that may help the timely development and maturation of its vital organs and improve its growth trajectory.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB069

### The Role of Receptors for Advanced Glycation End-products (RAGE) in the development of secondhand smoke (SHS)-induced intrauterine growth restriction (IUGR).

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Intrauterine growth restriction (IUGR) is a disease affecting 10% of all pregnancies. IUGR is associated with prominent maternal, fetal, or placental abnormalities, but studies investigating the effects of secondhand smoke (SHS) exposure and IUGR induction are limited. Receptors for Advanced Glycation End-products (RAGE) are pro-inflammatory transmembrane receptors induced in the placenta during SHS exposure. We tested the hypothesis that RAGE activation propagates smoke-induced IUGR and is functionally relevant in health complications associated with IUGR offspring. Pregnant C57/Bl6 mice (n=8) were exposed to SHS or SHS and semi-synthetic glycosaminoglycan ethers (SAGEs) known to inhibit RAGE signaling. RAGE<sup>-/-</sup> mice were also exposed to SHS. At 4 weeks of age, offspring were weighed, and blood pressure and heart rate were determined. Whole body, heart and liver weights were also determined. Lastly, organ-specific mitochondrial function tests were performed. SHS treated mice demonstrated a significant reduction in fetal and placental weight compared to controls. Mice co-treated with SHS and SAGEs were protected from SHS-induced fetal weight decreases. SHS treatment of C57Bl6 mice activated placental ERK, JNK, and p38 and the expression of inflammatory mediators including TNF- $\alpha$  and IL-1 $\beta$ . SHS-mediated activation of these molecules was reduced to basal levels when RAGE signaling was targeted by SAGE. At 4 weeks of age, antenatal exposure of SHS still caused: 1) a significant reduction in total body weight; 2) significantly elevated systolic and diastolic blood pressure; 3) no differences in the animal's heart rate; 4) significantly decreased heart and kidney weights; and 5) significantly decreased oxygen consumption related to cellular respiration in SHS-exposed hearts and lungs when compared to organs from room air exposed controls. We conclude that inhibition of RAGE protects against fetal weight loss during SHS-induced IUGR. Our results further suggest that there is a direct correlation between RAGE activation and the development of IUGR during SHS. Furthermore, we conclude that antenatal exposure to SHS impacts the placenta and induces detrimental effects on growth rates and cardiopulmonary energetics through the 4<sup>th</sup> week of age, or the equivalent of early adolescence. These studies provide insight into tobacco-mediated IUGR development and clarify avenues that may be helpful in the alleviation of placental complications. Also,



these studies may be beneficial in understanding the long-term effects of antenatal SHS exposure.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## PCB070

### Mechanical stretch induces phosphorylation of keratin intermediate filaments 8 and 18

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Keratin 8 (K8) and 18 (K18) form the intermediate filament (IF) network in cells of simple epithelia. The most widely proposed function of keratin filaments (KFs) is the mechanical stabilization of cells but several studies suggest that KFs are also involved in signaling and other functions. Phosphorylation is the most important post-translational modification of K and it was shown that K8 and K18 are phosphorylated at different serine residues in their head and tail domains. Various kinds of cell stress can induce K phosphorylation. K phosphorylation fulfills different functions in the cell. It changes the distribution of keratins within the cell, alters its polymerization behavior and is associated with keratin granule formation. Previous work of our group showed that K8 serine 431 (ser431) phosphorylation can reduce the tensile force on the desmosome upon stretch and that dephosphorylation of K18 serine 52 (ser52) alters the mechanical properties of KF. Since mechanical stress – in particular cell stretch – can pose a serious threat to epithelia by disrupting cell-cell contacts and destroying the integrity of an epithelial cell layer, we hypothesize that K phosphorylation acts as a mechanoprotective mechanism. Besides confirming this novel role of K phosphorylation, we also investigate how mechanical stimulation induces K phosphorylation, which includes the identification of the involved signaling pathways as well as the primary mechanosensor. In our experiments, we exposed A549 lung carcinoma cells to biaxial stretch for different time durations. A549 cells were grown on elastic PDMS membranes, which were stretched to a maximum of 50 % longitudinal distension. Subsequently, the phosphorylation levels of two potential phosphorylation sites in each K8 and K18 were determined by In-Cell ELISAs and Western blots. We observed an increase in phosphorylation on keratin 8 serine 73 (ser73) and serine 431 and keratin 18 serine 33 (ser33) and serine 52 upon stretch. The increase in phosphorylation was dependent on stretch duration. To elucidate the signaling pathway(s) responsible for phosphorylation of the keratin residues, A549 cells were incubated with various kinase inhibitors prior and during the stretch. Thereby we identified ERK1/2 kinases to be in part responsible for the stretch induced phosphorylation of K8ser73 and K8ser431 and K18ser52, as their inhibition with the potent and selective ERK1/2 inhibitor FR180204 reduced phosphorylation levels significantly. These results confirm that stretch as a form of mechanical cell stress leads to increased phosphorylation of KIF.

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## PCB071

### Effect of the endocannabinoid anandamide on airway tone in healthy and asthmatic mice

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The endocannabinoid system has emerged to be an important modulator of different organ functions. Anandamide (AEA), the currently best-characterized endocannabinoid, can act either via CB1 or CB2 receptors or via its degradation to eicosanoids by the enzyme fatty acid amide hydrolase (FAAH). In a recent study we could show that AEA is a key mediator of hypoxic vasoconstriction in the lung. Therefore, we wondered if endocannabinoids can also regulate airway function.

We have investigated the effect of AEA on airway tone of mouse (C57BL/6 WT, FAAH<sup>-/-</sup>, Cnr1/2<sup>-/-</sup>) ex vivo. Large airways were examined by isometric force measurements in a wire-myograph and small intrapulmonary airways were analyzed in precision cut lung slices. We have also tested AEA in lung slices of ovalbumin (OVA)-induced asthmatic mice. Therefore, Balb/c mice were injected i.p. with 20 µg OVA in 2 mg Alum on days 0 and 14, then they were challenged by inhalation of 1% OVA 3 times a week and analysis was performed on d24 for acute asthma and on d37 for chronic asthma. Successful asthma induction was confirmed by a prominent cell invasion around the airways as determined in H&E stainings of lung sections. Moreover, qRT-PCR was performed to investigate the expression levels of FAAH in the airways of the upper and lower respiratory tract.

In isometric force measurements AEA induced a strong airway relaxation in tracheal rings after serotonin (5-HT) pre-constriction (95.4±3.3%, n=8), while the effect was weaker after methacholine (MCh) pre-constriction (30.5±6.1%, n=12). In the small intrapulmonary airways of precision cut lung slices AEA induced a similar relaxation after 5-HT (51.0±3.9%, n=25) and MCh (35.3±4.6%; n=22) pre-constriction. This bronchorelaxing effect was mediated by FAAH-dependent metabolites as it was strongly reduced in FAAH<sup>-/-</sup> animals (18.2±3.0% n=8 (myograph after 5-HT)) but remained unaffected in Cnr1/2<sup>-/-</sup> mice. In lung slices of asthmatic animals AEA-dependent bronchorelaxation was unchanged compared to healthy mice (acute OVA: 62.6±5.4%, n=18 (5-HT); chronic OVA: 49.0±5.9, n=19 (5-HT)). QRT-PCR experiments demonstrated that expression levels of FAAH were elevated in the lower respiratory tract (4.0±0.4 (2<sup>-ΔCt</sup> × 10<sup>6</sup>), n=3) compared to the trachea (2.2±0.4, n=3) or whole lung tissue (1.1±0.3, n=3). This may in part explain the limited relaxation by AEA in the upper respiratory tract after MCh pre-constriction.

These results indicate that AEA is a strong airway relaxant in the upper and lower respiratory tract. The effect is mediated via metabolites of the FAAH enzyme and it is also present in asthmatic airways ex vivo. Thus, targeting the endocannabinoid system may be a promising approach for future therapies for obstructive airway disease.

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PCB072

# **Anti-oxidant and Anti-inflammatory effects of Melatonin on TBARS, GSH-Px, SOD, and YKL-40 in Endotoxemic Lung Tissue of Wistar albino Rats**

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**Motivation/problem statement:** In the literature, there are studies that melatonin has protective effects on oxidative stress and excessive cytokines release (1). However, there are a few study about relation with lung tissue damage using high and multi doses of melatonin during endotoxemia (2). Lipopolysaccharide (LPS) as bacterial endotoxin, is used agent for forming experimentally endotoxemia (3). YKL-40 is known carbohydrate binding protein, a member of chitinase family, that have been shown degrade and remodelling of extracellular matrix in endothelial cells. YKL-40 identified by proteomic analysis is considered as a biomarker of sepsis. (4). Oxidative stress is occur the imbalance between antioxidants defence mechanisms and increased free radical species in cells (5). In the organism, Superoxide dismutase (SOD), Glutathione peroxidase (GSH-Px), and Glutathione reductase (GR), are an important antioxidant enzymes, scavenges reactive radicals that occurred in oxidative stress conditions in chronic diseases such as sepsis, inflammatory diseases (6). We aimed to investigate the effects of melatonin on inflammatory and oxidative stress parameters and tissue histology of Wistar albino male rats endotoxemia induced by LPS.

**Methods/procedure/approach:** We separated male Wistar albino rats (210-230g) to 4 groups as; control, LPS (10mg/kg i.p.), Melatonin (3 \* 10 mg / kg i.p.), Melatonin + LPS. Six hours after the first injection, all animals were sacrificed taking blood from the heart under anesthesia for histological and biochemical parameters. Lung tissues were removed at -80 °C for ELISA and TBARS analyzes, and in 10% formaldehyde for histological examinations.

**Results:** TBARS values were significantly ( $p < 0.05$ ) higher both in the melatonin + LPS and LPS groups than that of control group ( $p < 0.01$ ). Glucose, Alanine Amino Transferase and Aspartate Aminotransferase enzymes values were significantly higher in the LPS group than those of control group ( $p < 0.01$ ). It was observed compared other groups, GSH-Px ( $p < 0.01$ ) and YKL-40 ( $p < 0.05$ ) were decreased, and both of levels of GR and SOD ( $p < 0.05$ ) were increased in the LPS group. In the histological investigation, control and Melatonin group's lung tissues were observed to be in normal structure. In the LPS group, it has been found that alveoli in the lung tissue are destroyed in several areas, in which the septum is thickened and contains inflammatory cells infiltration. In the LPS+Melatonin group, the indications in the LPS group were delayed, but the findings were still continuing in a few areas.

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PCB073

# **Apocynin reduces apnoea index in a mouse model of chronic intermittent hypoxia in a non-NADPH oxidase 2 dependent manner**

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Chronic intermittent hypoxia (CIH) is a hallmark feature of obstructive sleep apnoea syndrome (OSAS) as a consequence of repetitive upper airway occlusions in patients during sleep. These recurring hypoxia-reoxygenation cycles result in the overproduction of reactive oxygen species (ROS). Excessive ROS are associated with aberrant plasticity at multiple levels of the respiratory system including impaired respiratory control, which manifests as destabilised breathing during sleep. NADPH oxidase (NOX) enzymes are implicated in CIH-induced respiratory plasticity. We hypothesize that NOX-derived ROS underlie CIH-induced respiratory maladaptation, which perturbs respiratory homeostasis and exacerbates the pathology of OSAS.

A mouse model of CIH was generated by the cycling of gas from normoxia (21% O<sub>2</sub>) for 210 seconds to hypoxia (5% O<sub>2</sub> at the nadir) over 90 seconds for 8hr/day for 2 weeks. 10-11 week old male (C57BL/6J) mice were assigned to one of 3 groups: normoxic controls (sham; n=12), CIH exposed (n=12) and CIH+apocynin (NADPH oxidase 2 inhibitor, 2mM; n=12) given in the drinking water throughout the CIH exposure. In separate studies, 10-11 week old NOX2 null (B6.129S-Cybb<sup>tm1Din</sup>/J) male mice were assigned to sham (n=12) or CIH (n=12) exposure. On day 15, whole body plethysmography was used to measure breathing parameters in unrestrained, unaesthetized mice on a breath-by-breath basis in room air. Flow signals recorded from the plethysmograph were analysed offline for the determination of apnoea count. An apnoea was defined as  $\geq 2$  missed breaths. All values are expressed as mean  $\pm$  SD and data were statistically compared by unpaired Student *t*-test. Mice exposed to 2 weeks of CIH showed almost double the number of apnoeas per hour compared with sham exposed mice (11.2  $\pm$  10.3 vs. 19.0  $\pm$  12.5;  $p=0.11$ ), although this increase was not statistically significant. Apocynin intervention significantly reduced the frequency of apnoeas compared with the CIH group (19.0  $\pm$  12.5 vs. 9.5  $\pm$  5.9;  $p=0.03$ ). Apnoea index was increased in NOX2 null mice exposed to CIH

compared with NOX2 null sham mice ( $12.0 \pm 12.5$  vs.  $19.5 \pm 13.8$ ;  $p=0.18$ ) reminiscent of that observed in wild-type mice. CIH-induced increase in the propensity for apnoea may be of biological relevance as it may underpin progression in the severity of OSAS pathology (i.e. mild to moderate OSA). The reduction in apnoea frequency following treatment with apocynin implicates ROS in the manifestation of CIH-induced respiratory disturbances. However, these ROS do not appear to be NOX2-derived as apnoea index in NOX2 null mice largely mirrors that of wild type mice. Our results reveal the proclivity for CIH to perpetuate respiratory maladaptation perhaps relevant to 'complex' apnoea in OSAS patients, and point to antioxidant intervention as a potential therapeutic strategy in OSAS.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB074

### Body mass and Body shape Versus Two Indices of Pulmonary Function

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**Introduction:** Obesity is a cumbersome problem all over the globe. Conventionally, Body Mass Index(BMI) is used as a parameter to grade Obesity. As BMI fails to distinguish between muscle mass and fat; and fails to differentiate between central and peripheral obesity, there is a need for better parameters to be correlated with Pulmonary Function. Several studies had emphasized Waist Circumference (WC) and Waist – Hip ratio (WHR) as alternatives to BMI [1]. But, these serve as insufficient indicators as they measure only body size. A Body Shape Index (ABSI) [2] and Surface Based Body Shape Index (SBSI) [3] are two new parameters that consider both body shape and body size into account and are better measures of mortality than BMI. They are calculated using the formulae,  $ABSI = WC(m) / [BMI^{2/3} \times height^{1/2}(m)]$  and  $SBSI = (H^{7/4})(WC^{5/6}) / [(BSA)(VTC)]$ . The study was aimed to appraise the work done associated with BMI and Pulmonary Function and to correlate Body Shape with Pulmonary Function.

**Methods:** The study was done in 100 medical and paramedical students. Spirometric measurements namely,  $FEV_1/FVC$  and MVV were measured using a computerized spirometer. Anthropometric Measurements such as Height, Weight, Waist Circumference, Vertical Trunk Circumference[4] were made for calculating BMI, ABSI and SBSI and were correlated with  $FEV_1/FVC$  and MVV.

**Results:** (Table1): ABSI and SBSI are found to be significantly and negatively correlated with MVV. Correlation of BMI with  $FEV_1/FVC$  showed significance. This finding correlated with previously carried out studies[5]. ABSI was positively correlated with  $FEV_1/FVC$ . The significance is so low that it can be considered that the association between ABSI and  $FEV_1/FVC$  was not picked up. There was a positive correlation between SBSI and  $FEV_1/FVC$ .

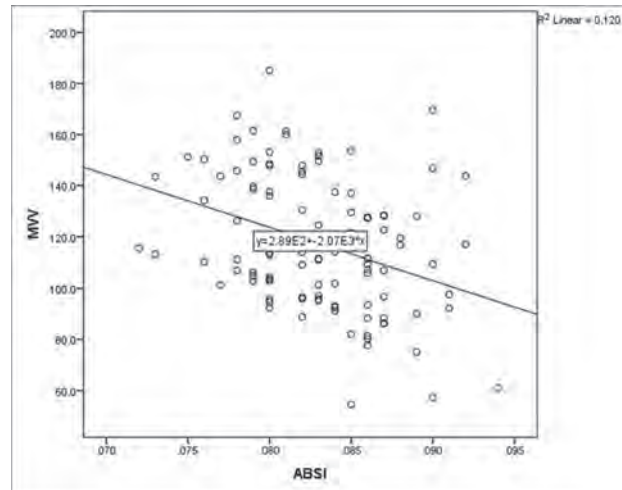
**Conclusions:** Our study is unique as it is the first study, which made efforts to correlate ABSI with  $FEV_1/FVC$  and MVV in this part of the world. Increase in ABSI and SBSI were found to decrease MVV. Both ABSI and SBSI have outperformed BMI in correlation with MVV. Our study has paved way for future long term researches on body shape and pulmonary function, which has not been reported in this part of the world, so that it could facilitate screening of respiratory compromise. Also, increased ABSI and SBSI can serve as better indicators of

obesity related respiratory problems. Change in ABSI and SBSI of a person over a period of time can be used as a prognostic indicator. Respiratory tolerance to exercise can be assessed using ABSI and SBSI since MVV is a marker of Respiratory muscle function.

Table 1 Results

|             | BMI | ABSI    | SBSI    | $FEV_1/FVC$ | MVV     |
|-------------|-----|---------|---------|-------------|---------|
| BMI         | 1   | -.276** | -.388** | -.324**     | -.055   |
| ABSI        |     | 1       | .827**  | .024        | -.347** |
| SBSI        |     |         | 1       | .068        | -.386** |
| $FEV_1/FVC$ |     |         |         | 1           | .318**  |

\*\* . Correlation is significant at the 0.01 level (2-tailed).



ABSI Vs MVV

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB075

### Determinants of apnoea –hypopnoea-index (AHI) levels in newly diagnosed obstructive sleep apnoea patients

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Obstructive sleep apnoea (OSA) is a sleep-related breathing disorder with repeated partial or complete closure of upper airway during sleep, despite ongoing efforts to breathe. OSA



is associated with a high prevalence for cardiovascular disease, hypertension, and stroke. Patients face cyclical deoxygenation/re-oxygenation resulting in episodic hypercapnia and hypoxia. Events are graded by the apnoea-hypopnea index (AHI; the sum of apnoea and hypopnoea events per hour during sleep). The severity of OSA has been linked to various factors, including: body characteristics, upper respiratory tract muscle activity, chemosensitivity, as well as to cytokines. The contribution of chemosensitivity to the severity of OSA and its subsequent association to body characteristics is not clear. Consequently, we investigated newly diagnosed, untreated OSA patients (n=48) and assessed ventilatory responses to breathing normoxic carbon dioxide (6%) gas mixture, oxygen (13%), and combined carbon dioxide (6%) with oxygen (13%) mixture (Earing et al. 2016) to assess chemosensitivity. Moreover, body characteristics, lung function, and blood parameters, including adiponectin, CRP, leptin, and endocannabinoids (AEA and 2AG) were measured. In addition, a group of healthy people (n=49) were assessed on all parameters, except blood factors, for comparison with OSA patients. In OSA patients, AHI was significantly correlated with ventilatory response to carbon dioxide (6%),  $r=-0.511$ ,  $p=0.001$ ; there was no significant association with response to hypoxic gas breathing. Additionally, there were significant correlations of AHI with body characteristics, in particular with neck circumference ( $r=0.509$ ,  $p=0.001$ ). Blood parameters showed no significant correlations with AHI. Healthy participants, revealed significant differences in all measured parameters. To investigate whether body characteristics were likely to be a contributing factor to reduced chemosensitivity to  $\text{CO}_2$ , we performed multiple regression analysis for prediction of  $\text{CO}_2$  sensitivity variance in the whole participant group. From this analysis, neck circumference was the best predictor, explaining about 30% ( $r^2=0.286$ ,  $p<0.0001$ ,  $n=87$ ) of  $\text{CO}_2$  response variance. Conclusion: AHI is strongly associated with  $\text{CO}_2$  sensitivity and body characteristics in OSA patients; however, only a smaller proportion of the altered  $\text{CO}_2$  sensitivity can be attributed to selected body characteristics. It is likely that factors other than those related to body characteristics influence the observed alteration in chemosensitivity in OSA.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB076

### Evaluation of the methods used to measure sniff nasal inspiratory pressure in healthy human subjects

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Maximal inspiratory pressure (MIP) is currently used to assess respiratory muscle strength (RMS), however it requires significant effort. Falsely low values are common, especially in patients with neuromuscular disorders [1]. In contrast, sniff nasal inspiratory pressure (SNIP) is a natural manoeuvre, so subjects find it easier. Therefore SNIP has been recommended to scrutinise inspiratory muscle weakness after a low MIP [2]. However, limited guidelines and varied approaches regarding the optimal method of measuring SNIP exist [3, 4]. The aim of this study was to investigate the effects of inter-measurement time, left/right nostril and contralateral occlusion on SNIP measurements in healthy subjects.

We performed a pilot study comparing inter-measurement time differences in SNIP measured in 10 healthy subjects, depending on whether repeats were performed 30, 60 or 90 s apart. Furthermore, our study tested four methods of measuring SNIP in a randomised order in 52 healthy subjects, aiming to compare results obtained from right nostril left occluded, right nostril left non-occluded, left nostril right occluded and left nostril right non-occluded. We also attempted to determine the optimal number of repeats that would eliminate any learning effect, but not pose risk of fatigue. The study was given ethical approval by the Research Ethics committee, University of Plymouth. All participating subjects gave informed and signed consent. SNIP was measured from functional residual capacity via a probe (MicroRPM, Micromedical) in one nostril, and subjects were instructed to perform a short, sharp sniff. A repeated-measures ANOVA tested differences between the mean maximal SNIP for each method and position. Unpaired t-testing compared differences between occluded and non-occluded and also between left and right nostrils.  $p<0.05$  was considered significant for t-tests, while a  $p<0.0125$  was considered significant for ANOVA.

There were no significant inter-measurement time differences in SNIP depending on the interval between repeats ( $p=0.98$ ). Occluded SNIP was significantly higher than non-occluded (73.4 vs. 56.1  $\text{cmH}_2\text{O}$ ,  $p<0.00001$ ), while left or right SNIP did not significantly differ (63.6 vs. 66.0  $\text{cmH}_2\text{O}$ ,  $p=0.60$ ). There was no learning effect continuing after 20 repeats ( $p=0.64$ ), and SNIP did not decline during 80 repeats.

Overall, occluded SNIP is a more reliable RMS indicator than non-occluded SNIP, as there is reduced risk of underestimation. Allowing subjects to choose which nostril to insert the probe is appropriate, as this did not significantly affect SNIP, but may increase ease of performance. Ultimately, if our findings are replicated in similar trials performed on patients with respiratory muscle weakness, they could be used to develop guidelines on how to perform a reliable SNIP, increasing the use of this test in clinical settings.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB077

### Role of TMEM16A in mucus secretion in airways inflammatory diseases

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Cystic Fibrosis (CF) is an inherited disease affecting mucin-producing organs. In fact in CF mucus is particularly viscous and adhesive due to a compromised ions secretion caused by defective channels called CFTRs (cystic fibrosis transmembrane conductance regulators). Mucus hyperproduction causes airway obstruction, reduced mucociliary clearance and chronic inflammation. CFTR is not the only channel responsible for electrolytic balance in secretory epithelia: TMEM16A is a  $\text{Ca}^{2+}$ -activated chloride channel strongly up regulated in CF and asthma, which parallels goblet cell metaplasia. We report that airway epithelial knockout of TMEM16A leads to accumulation of mucus in secretory (Clara) cells and we

demonstrate that this channel is indispensable for their basal and ATP-driven mucus release. While acute ATP-induced mucus secretion by airway club cells is strongly compromised in the absence of TMEM16A, the cholinergic mucus secretion due to compound exocytosis is independent of TMEM16A. The present data demonstrate for the first time that the calcium-activated chloride ion channel TMEM16A controls constitutive exocytosis in a ATP-dependent manner. The results provide the basis for an alternative therapeutical target for Cystic Fibrosis, as well as for other inflammatory lung diseases such as asthma and COPD.

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The supply of mice with a floxed TMEM16A gene by Prof. Jason R. Rock (Boston University School of Medicine) and Foxj1-Cre mice by Prof. Michael J. Holtzman and Dr. Y. Zhang (Washington University School of Medicine) is gratefully acknowledged.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCB078

##### Is there correlation between GAP and BODE indexes in Interstitial Lung Disease?

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##### Background

Compared to GAP index widely used in prognosis assessment of Interstitial Lung Disease (ILD), BODE index is rarely used despite its strong and independent prognosis value in chronic respiratory diseases.

##### Aim

The aim of this study was to investigate possible correlations between GAP (Gender, Age, Physiology) and BODE (Body Mass Index, Airflow Obstruction, Dyspnea, Exercise Capacity) indexes.

##### Methods

It is a longitudinal cohort study including 72 patients with ILD. All patients performed forced spirometry, Diffusing capacity for Carbone Monoxide Test (DLCO) and Six-Minute walk Test (6MWT). All patients were staged according to the GAP and BODE indexes. Khi-square test was used to compare two percentages on paired samples.

##### Results

The average values of age and BMI were respectively  $56 \pm 12$  years and  $30,1 \pm 5,5\%$ . The sex ratio was 0,63 (28M/44F). Pulmonary Function Tests revealed a Forced Expiratory Volume in one Second (FEV1) at  $76 \pm 19\%$ , Forced Vital Capacity (FVC) at  $75 \pm 19\%$ , Total Lung Capacity (TLC) at  $84 \pm 23\%$  and DLCO at  $64 \pm 24\%$ . In 6MWT, distance was  $459 \pm 76$  m and 20,8% of the study group experienced significant fall of hemoglobin saturation during or at the end of the test.

The mean of GAP score was  $2,5 \pm 1,9$  and 64% of our study group was ranked in stage 1, 30% in stage 2 and 5% in stage 3. The predicted mortality at three years is 16,1% according to the GAP score.

The mean of BODE index was  $1,9 \pm 1$  and predicted mortality at four years is 15% according to the BODE index.

Significant correlations ( $p < 0.05$ ), were found between GAP and BODE indexes.

##### Conclusion

This preliminary study revealed, for the first time, significant correlations between GAP and BODE indexes and confirmed reliability of prognosis value of BODE index in ILD.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCB079

##### Chronic Intermittent Hypoxia Enhances Respiratory Muscle Weakness in Dystrophin-deficient *mdx* Mice

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Sleep-disordered breathing (SDB) is a prominent feature of neuromuscular disease. Boys with Duchenne muscular dystrophy (DMD) experience nocturnal desaturation during sleep as a result of obstructive and central apnoea. We have previously described maladaptive changes in respiratory muscle structure and function in rodent models of chronic intermittent hypoxia (CIH), a hallmark feature of SDB driving many pathologies. We hypothesized that CIH exacerbates respiratory muscle dysfunction in muscular dystrophy. We sought to explore the effects of CIH on diaphragm muscle in wild-type (WT) and dystrophin-deficient *mdx* mice.

Six-week-old male WT (n=9) and *mdx* (n=9) mice were exposed to CIH, consisting of alternating bouts of hypoxia (90s; 5-6.5% O<sub>2</sub> at the nadir) and normoxia (210s; 21% O<sub>2</sub>) for 12 cycles per hour, 8 hours per day for 14 days. WT (n=9) and *mdx* (n=9) sham animals were exposed to normoxia in parallel. Diaphragm muscle was excised and functional capacity was examined *ex vivo*. Diaphragm muscle structure was examined along with the expression of inflammatory-, redox-, autophagy- and mitophagy-related genes. Data were statistically compared by two-way ANOVA with Bonferroni *post hoc* test.  $P < 0.05$  was considered statistically significant.

Substantial muscle weakness was observed in dystrophic diaphragm compared with WT diaphragm. Exposure to CIH elicited significant muscle weakness in the diaphragm of WT mice. In *mdx* mice, exposure to CIH resulted in further weakness in the compromised diaphragm. *Mdx* diaphragm had significantly increased area of mononuclear cell infiltration, increased muscle fibre central nucleation, increased

number of muscle fibres expressing embryonic myosin and elevated collagen content compared with WT. CIH significantly decreased mononuclear cell infiltration and central nucleation in *mdx* diaphragm. There was elevated expression of NF- $\kappa$ B, TNF- $\alpha$ , Nrf2, NOX-2, atrogin-1 and PARK2 mRNA in *mdx* diaphragm compared with WT. CIH increased the expression of BNIP3 and PINK1 in *mdx* diaphragm.

Our findings demonstrate that exposure to CIH exacerbates diaphragm muscle weakness in the *mdx* model of muscular dystrophy. Since SDB is a feature of DMD, we reason that SDB may accelerate respiratory insufficiency in DMD. An understanding of the pathogenic factors driving CIH-induced muscle weakness in *mdx* may help inform therapeutic strategies that may have relevance to DMD boys.

Supported by the Department of Physiology, UCC. Work carried out in TCD was supported by funding from The Physiological Society.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB080

### RAGE and SAGE: Therapeutic Modalities for smoke-induced COPD

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Chronic obstructive pulmonary disease (COPD) is soon to be the third leading cause of death worldwide and it is characterized by debilitating inflammation and deleterious tissue loss in the respiratory compartment. Voluntary and involuntary exposure to tobacco smoke is the major cause of COPD. Past studies identified the receptor for advanced glycation end-products (RAGE) as a smoke-induced pattern recognition receptor with potent pro inflammatory characteristics. Our research demonstrated that RAGE is increased in the lung following first and secondhand smoke (SHS) exposure and that transgenic mice that conditionally up-regulate RAGE manifest characteristics of a smoker's lung in the absence of smoke. SAGEs are semi-synthetic glycosaminoglycan ethers that are potent modulators of inflammation in numerous animal models of human disease, and are in preclinical development for periodontitis, oral mucositis, and bladder inflammation. We have shown that SAGEs significantly inhibit interactions between RAGE and its many ligands necessary for signaling. The current research evaluated the *in vivo* effects of short-term SHS exposure in RAGE null, conditional RAGE over-expressing, and control mice compared to identical animal groups exposed to room air only. Mice were subjected to daily SHS via a nose only inhalation system (Sireq Scientific, Montreal, Canada) as approved by institutional review boards. In short, mice were exposed to a 10 second computer-controlled puff of smoke generated every minute followed by 50 seconds of fresh air. Mice were exposed to SHS from two cigarettes over 10 minutes, allowed to breathe room air for 10 minutes, then exposed to smoke from one cigarette for an additional 10 minutes. This procedure was repeated five days a week for four weeks and compared to mice restrained and exposed to room air over the same period of time ( $n=8$  per group). Select groups of mice were also co-treated with SAGEs via weekly ip injections. Molecular characterization of primary and SHS revealed significant pulmonary inflammation mediated at least in part by RAGE. Inflammatory cell behaviors were assessed

by determining the activation of Ras, intracellular signaling kinases, and cytokine synthesis and secretion. Bronchoalveolar lavage fluid (BALF) was procured from our mouse models and assessed for inflammatory cells and secreted molecules. Lastly, pulmonary physiology tests revealed RAGE-mediated impairment. As a general theme, inflammation induced by tobacco smoke exposure was influenced by the availability of RAGE and significant amelioration of tobacco smoke-induced inflammation was observed in mice co-treated with SAGEs. These data reveal captivating information suggesting a role for RAGE signaling in lungs exposed to tobacco smoke and implicates plausible therapeutic modalities.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB081

### On Application of Cardiac Preparations of Fish In Physiological Education and Research

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#### INTRODUCTION

In medical education&research are till today usual cardiovascular preparations of frog, rat, rabbit, etc. An essential part of experiments with isolated angio-cardiac preparations could be replaced by fish organs. About pharmaco-physiological reactions of fish heart of *salmo gairdner* is reported [1,3]. Now is given information about ionic-dependent thermo-induced reactions: These demonstrate some common action mechanisms with mechanosensitive ionic channels of vesical (detrusor, trigone) myocytes [2].

#### METHOD

Isolated heart and vascular strips from rainbow-trout (about 300g) from fish-shop are investigated about elementary physiological properties incl. motor activity recording) & influence of physiological factors in Krebs-Henseleit-solution [1].

#### RESULTS

Atrium&ventriculus carotis generate spontaneous contractions: [Ca<sup>++</sup>]-increase has positive, [K<sup>+</sup>] negative chrono- and inotropic effects. Negative acetylcholine and positive adrenaline effects (10nM-1 $\mu$ M) are antagonized by atropine&propranolol resp. (1 $\mu$ M). Ouabain (strophanthine, 0.1-1 $\mu$ M) induce strong augmentation of cardiac contractions. Thermo-induced effects are strong dependent on ionic concentration. Change of some pharmaco-physiological factors (hormones, drugs) induce different rhythmic burst-like motor pattern at 14-24°C, contrary to regular&uniform motor activity at 6°C.

#### CONCLUSION

Fish cardiac preparations could reduce enormous application of animal organs in pharmacological industry and education&research at universities, i.e. this could lead to large animal protection.

#### Table

Thermo-induced reactions in trout atrium in dependence of CaCl<sub>2</sub> and KCl



At 24°C Atrium contraction frequency  $67 \pm 23/\text{min} = 100\%$ , amplitudes  $35 \pm 51 \text{ mm} = 100\%$ , i.e. 4.7% of the prep.length of  $0.6 \pm 1.3 \text{ mm}$

Isotonic recording, mean  $\pm$  standard deviation,  $p < 0.05 < 0.01$

A.  $\text{CaCl}_2$  2.1mmol/l=1x

contr. ampl. in % of initial value ( $n=18$ )

24°C, 0.25x:  $78 \pm 60$ , 1x:  $213 \pm 118$ , 2x:  $271 \pm 170$

6°C, 0.25x:  $24 \pm 16$ , 1x:  $89 \pm 75$ , 2x:  $156 \pm 152$

contr. freq./min in % of initial value

24°C, 0.25x:  $106 \pm 62$ , 1x:  $87 \pm 47$ , 2x:  $52 \pm 35$

6°C, 0.25x:  $28 \pm 15$ , 1x:  $35 \pm 16$ , 2x:  $31 \pm 14$

B.  $\text{KCl}$  4.6mmol/l=1x

contr. ampl. in % of initial value ( $n=18$ )

24°C, 0.25x:  $149 \pm 70$ , 1x:  $113 \pm 65$ , 2x:  $43 \pm 24$

6°C, 0.25x:  $118 \pm 120$ , 1x:  $69 \pm 61$ , 2x:  $3 \pm 6$

contr. freq./min in % of initial value

24°C, 0.25x:  $106 \pm 60$ , 1x:  $97 \pm 93$ , 2x:  $71 \pm 73$

6°C, 0.25x:  $34 \pm 14$ , 1x:  $24 \pm 13$ , 2x:  $2 \pm 4$

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DEDICATION for moral-scientific support 2018-1980/Nobel Laureates:

Australia: Sir J.Eccles, Austria: K.Lorenz, Belgium: I.Prigogine, Canada: G.Herzberg, J.Polanyi, France: J.Dausset, J.-M. Lehn, Germany: M.Eigen, K.v.Klitzing, H.Michel, E.Neher, GB: Sir A.Hewish, B.Josephson, Lord A.Todd, India/USA: S.Chandrasekhar, H.B.Khorana, Japan: K.Fukui, Sweden: S. Bergström, B.Samuelsen, USA: P.Anderson, J.Deisenhofer, D.Hubel, L.Pauling, E.Wiesel

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB082

### Perceptions of medical students about an elective on shock physiopathology

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Introduction: Student-centered teaching methods have been considered important in the preclinical phase of the first three years of medical education. For third-year students, an elective course module named "Advanced Approach to Shock Physiopathology and Therapy" has been carried out in Trakya University Medical Faculty during four consecutive years between 2014 and 2017 in cooperation with physiology, emergency medicine, cardiology, infectious diseases, neurology departments and intensive care unit. This course, with a total of 170 hours workload, consisted of a pretest, a total of 10 hours theoretical lectures followed by a problem based learning meeting, observational studies in the clinical setting (two 24-hour emergency service observations including night shift works, 12-hour cardiology clinic, 2-hour intensive care unit observations), preparation of personal impression reports about the clinic observations, attendance meetings to discuss clinic observations, presentations of articles and case reports on shock physiopathology, presentation of an individually prepared project and a post-test. The aim of

this study was to define students' views on different teaching methods applied in the elective on shock physiopathology.

Materials and methods: The study was carried out using case analysis design of qualitative study designs. The data were collected using three different qualitative collection techniques (triangulation: individual and focus group interview, document review). Individual interviews and focus group interviews were conducted with volunteer participants. Nine questions were prepared by researchers. Face-to-face interviews and focus interviews were conducted with audio and video recordings. Qualitative data set; document analysis, descriptive analysis and content analysis techniques.

Results: 25 students (52% female, 48% male) who volunteered for individual and focus group interviews were initially satisfied with elective module (68%), the module was found different (40%) student centered (36%) and permanent (44%). Participants stated that briefly expressing the basic and clinical features of subjects by teachers from different departments supported the permanency. Students indicated that active learning methods in this module encouraged them to research (64%) and gained academic research skills (28%).

Conclusion: When evaluated from the perspective of the medical students, the elective module on shock physiopathology that contains less theoretical lectures and active learning methods provides satisfactory and permanent learning. Students described lectures as effective in terms of providing permanency. Active learning methods are described effective to gain academic research skills.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB083

### Use of student-created video resources to enhance practical training in Objective Structured Practical Examinations (OSPE's).

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We have pioneered the use of Objective Structured Practical Examinations (OSPE's) in science teaching and previously piloted successful delivery of theoretical, practical and problem-solving skills at multiple stations to formally examine a wide range of communication and science laboratory practical skills. As the range of disciplines being examined has expanded, the number of students participating have also increased, leading to challenges delivering the required practical skills to all students within the time and space available. Previously, students learned and practiced in advance of the examination in a large, busy all-day practical class. However, teaching delivery had the potential to be variable and some students required more time to practice. To address this, two students created a series of video teaching resources in collaboration with academic and technical staff. These training videos were created using free recording/editing software and recorded using mobile phones. Videos were no longer in duration than 5 minutes and were designed to help students understand how best to complete the tasks at each assessment station. Videos were released to students through the VLE and they were instructed to prepare prior to the practical class. Smaller groups of students were allocated to 2 hour practical classes where focussed practice time and further instruction was available.

No significant difference in mean score achieved by physiology students (2017-18 cohort,  $20.11 \pm 2.00$ ,  $n = 43$ ) was identified when comparing with data recorded last year from students who did not have access to the video resources (2016-17 cohort,  $19.54 \pm 1.26$ ,  $n = 52$ ) (Data represents mean score  $\pm$  stdev). This suggests that student performance did not suffer by reducing the practice time before the assessment. The same result was observed in a separate anatomy cohort of students when comparing performance between this and last year. Feedback recorded in the university central course evaluation forms from both physiology and anatomy students cited the OSPE assessment and the new videos as being one of the best things about their respective courses. Staff reported that use of the video resources meant that students appeared to be better prepared and less nervous, and were more focused when attending their practice sessions. Data from the VLE showed a surge in video watch time for the majority of students in the day before their assessment. These results suggest that larger, diverse practical classes can be trained in a consistent and effective manner by using student-created video resources. Future work will involve the development of further videos for a wider range of practical skills, as well as analysing whether any correlation exists between video watch time and score achieved in the OSPE assessment.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB084

### The effectiveness of drama-based activities to teach the cardiac cycle to undergraduate students.

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Active learning modes are thought to promote higher levels of student engagement, understanding and thus, improve test scores and satisfaction. Nevertheless, few studies have used drama as an active learning tool to learn complex physiological concepts despite an understanding that participating in the arts not only engages all learning styles but can improve learning in a variety of different topics (Braund, 2015).

As such, we evaluated the use of drama-based activities in comparison to traditional didactic lecture delivery using powerpoints, to teach the cardiac cycle to first year students. Our aim was to ascertain whether this active learning mode could improve their test scores and enhance knowledge retention of the topic.

Ethical approval for the experimental protocol and the questionnaires was permitted by the School of Medicine Research Ethics Committee, University of Nottingham. Participants had no prior knowledge of the subject matter and comprised of a convenience sample of 34 undergraduate students. There was no personal reward or monetary incentive for participation. Of the initial pool, 28 students (82.4%, age  $19.6 \pm 2.01$  years (mean  $\pm$  SD) consented, participated and completed the study. To protect anonymity, the students were randomly assigned a number which was subsequently used to divide them into a Control group ( $n = 14$ ) to receive the topic via lecture, and a Test Group ( $n = 14$ ) to engage in drama-based activities. The assigned numbers also allowed tracking of individual test scores in online quizzes which were completed immediately after the delivered sessions and three weeks later with the

aim to test understanding of the topic and knowledge retention respectively. Completion of brief online questionnaires requiring responses on the Likert scale (Strongly Agree – 5 to Strongly Disagree = 0) was also requested to gain insight in perceived confidence in knowledge gained and satisfaction of the teaching method used. Unpaired t-tests were performed on the results obtained with  $P < 0.05$  deemed significant.

No significant difference was found between the Control and Test group grades from either the post-session quizzes ( $P = 0.43$ ) or those completed three weeks after the delivered content ( $P = 0.15$ ) suggesting the drama activities did not improve immediate knowledge gain or retention of this topic matter. However, students participating in the drama session reported significantly higher levels of confidence ( $P < 0.05$ ) and satisfaction ( $P < 0.0001$ ) with the mode of delivery for learning compared to students receiving the lecture. These findings suggest that drama activities were just as effective in a traditional lecture for students to learn about the cardiac cycle but beneficially improved their learning experience of this scientific topic.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB085

### On Philosophical Physiology in Context of Anthropology and Philosophy

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INTRODUCTION: Central position of physiology (Nobel discipline) in biology, medicine, psychology, philosophy needs creation of *philosophical physiology* (independently from medical philosophy) incl. not only ethics, but also scientific-theoretical&aesthetic fundamentals. About relation of physiology to anthropology&philosophy is given information to phil. (1-3)&phys. congresses (4-5).

CONCEPTION for Discussion:

A. EPISTEMOLOGY. An *integrative physiology* needs enlarged *fundamentals* in normal&pathological physiology, psycho-neurology (e.g. p.-n.-immunomodulation). Physiological approaches to Chinese-Indian&other traditional medicine in education&research has to be discussed. Reconsideration of *physiol. notions* acc. to *axiology-logic-semantic* is recommendable.

B. MORAL PHILOSOPHY. Independently from various modern ethical *theories* (deontology, utilitarianism, etc.) has to be considered *Kant's human obligations to himself*-physiologists, other *humans*-patients, *sub-human*, e.g. reduction of animal experiments, *suprahuman beings*: moral limits about&applications of physiological practices for investigations of probands&patients in context of theology (Brahmanism-Buddhism/Christianism-Mosaism/Confucianism-Taoism/Mohammedanism).

C. AESTHETICS. In relation to A-B interdisciplinary consideration is necessary to destine volume of *paradigm-changes* in physiology by clinical-physiological investigations leading to pathophysiological & psychopathological effects (*primum non nocere*).

D. PSYCHOSOMATICS. Central philosophical question "What is the Human?"/KANT is since PLATON not answered: He distinguishes *physiological* and *pragmatic anthropology*. Human reflects by "pure apperception ... from physical determinations independently personality (*homo noumenon*) ..., different to ... (*homo phenomenon*)".

Emperor AKIHITO honoured World Congr.Psychosom.Med. (ICPM-2005-Kobe) by strategical ideas: "total symptoms of mind&body, seeking ways of holistic care ... it is extremely important for patients ... my hope contributes ... the progress of medical science and people's happiness in the entire world".

CONCLUSION: Establishment of *regular common congress-sessions* of physiological (IUPS/FEPS etc.) with e.g. philosophical (FISP-ISB-EACME)/psychological (IUPsyS)/medical societies (ISIM-ICC-FIGO-SIU) could open new scientific&political dimension in bio-medical sciences, leading to humanization, higher efficacy & internationalization of science-medicine-ecology in context of UNO-Agenda21 for better health-education, etc. on global level.

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Michailov, Neu, Welscher et al.: IUPS-2017-Rio de Janeiro, Abs-Book ID1001, Proc.XXIV

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB086

### **Chlorogenic acid (CGA) ameliorates Ischemia-Reperfusion-induced Renal Damage through scavenging reactive oxygen species**

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Reduction of oxygen distribution produces reactive oxygen radicals (ROS) such as hydrogen peroxide, superoxide anion and causes cellular dysfunction and necrosis. The release of massive ROS causes activation of stress-induced transcription factors and production of proinflammatory and anti-inflammatory molecules. Numerous molecules have been investigated to prevent oxidative damage. p-Coumaric acid (p-CA), also known as 4-hydroxycinnamic acid, is one of the potential molecules which has high free radical scavenging, anti-inflammatory and antimicrobial activities. To evaluate the role of p-CA in renal ischemia/reperfusion-induced remote lung injury, thirty-two Wistar Albino adult female rats weighing 200-250 grams (12-16 weeks) were used. These rats were randomly divided into 4 equal groups (n=8). The groups were identified as: sham (S), ischemia-reperfusion (IR), IR+ 50 mg/kg p-CA (p-CA 50), IR+100mg/kg p-CA (p-CA 100). Treatment groups were subjected to surgery in the IR group. Rats in the

Sham group underwent right dorsal side nephrectomy. Other groups were clamped in the left renal artery after right dorsal side nephrectomy. p-CA was administered intraperitoneally at single dose immediately before reperfusion. Then the clamp was removed and the reperfusion period started for 3 hours. After the applications were completed, total antioxidant level (TAS), total oxidant level (TOS), superoxide dismutase (SOD), myeloperoxidase (MPO) and malondialdehyde (MDA) levels were measured spectrophotometrically. Oxidative stress index (OSI) was measured. Values are means  $\pm$  SD., compared by ANOVA. In IR, TOS, MDA, MPO and OSI increased in significant level, while TAS and SOD decreased ( $p<0.001$ ). An increase in TAS and SOD levels and a decrease in TOS, MDA and MPO values was observed in CGA applied groups ( $p<0.001$ ). Therefore, our findings suggested that the active ingredient p-CA may exert its antioxidant effects in remote lung injury induced by renal ischemia-reperfusion in rats.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB087

### **The role of the glycocalyx in cell-cell interactions in human melanoma**

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Melanoma is responsible for most skin cancer related deaths and metastasis is a major factor in patient prognosis. Migration importantly contributes to the metastatic cascade. It is affected by the extracellular pH ( $pH_e$ ) of the tumour stroma and the pericellular  $pH_e$  of individual cells, which is maintained by the glycocalyx. The aim of this project is to study whether the impact of  $pH_e$  on migration is modulated by the glycocalyx. The glycocalyx of human melanoma cells (MV3) was targeted with heparinase and neuraminidase, enzymes which degrade different N-glycosidic bonds.

The effects of the enzymes on the glycocalyx were quantified by fluorescence microscopy with TRITC-labeled wheat germ agglutinin (WGA). Height and stiffness of the glycocalyx were determined with atomic force microscopy (AFM). The functional influence of the treatment at different  $pH_e$  values was assessed in migration wound assays with time-lapse video microscopy.

Values are presented as means  $\pm$  S.E.M., compared by ANOVA, N=3-4, n=30-40 for all experiments.

After 4 hours of heparinase or neuraminidase treatment WGA staining is reduced by  $18 \pm 0.5\%$  or  $31 \pm 0.5\%$ , respectively (both  $p<0.05$ ). The stiffness of the glycocalyx slightly decreases upon neuraminidase treatment (from  $0.22 \pm 0.01\text{nm/pN}$  to  $0.19 \pm 0.01\text{nm/pN}$ ,  $p<0.05$ ). The combination of both enzymes elicits a stronger effect ( $0.15 \pm 0.01\text{nm/pN}$ ,  $p<0.05$ ). The height of the glycocalyx is also reduced ( $p<0.05$ ) by both enzymes individually and in combination (control:  $210 \pm 3\text{nm}$ , heparinase:  $186 \pm 4\text{nm}$ , neuraminidase:  $170 \pm 5\text{nm}$ , both enzymes:  $162 \pm 2\text{nm}$ ). Collectively, fluorescence microscopy and AFM suggest a pruning of the glycocalyx.

Migration was observed at pH 6.4 and 7.4 for 10 hours in wound assays following 4 hours of enzymatic pretreatment. The velocities of MV3 cells at 6.4 and 7.4 are not different from each other ( $\sim 0.45\mu\text{m/min}$ ). Under control conditions, MV3 cells have the highest directionality ( $0.53 \pm 0.04$ ) at pH 6.4. Directionality is lower following enzyme treatment ( $0.29 \pm 0.04$ ) and at pH 7.4 ( $0.30 \pm 0.03$ , both  $p<0.05$ ). We further analysed



how far two cells migrate together before losing their cell-cell-contact. While vastly different at the two pH levels (pH 6.4:  $94 \pm 4\mu\text{m}$ , pH 7.4:  $54 \pm 4\mu\text{m}$ ,  $p < 0.05$ ) heparinase aligned the distance at both pH values ( $75 \pm 6\mu\text{m}$  and  $76 \pm 5\mu\text{m}$ ). Adding 20mM HEPES to the bulk solution as an additional buffer lowers the velocity of the cells ( $0.23 \pm 0.01\mu\text{m}/\text{min}$ ,  $p < 0.05$ ) while the distance to separation remains unchanged even after heparinase treatment.

The data suggest a modulation of the pH-dependent effects on migration by the glycocalyx. The buffering capacity of the glycocalyx and the concentration of protons in the bulk solution both change various parameters in single cell and collective cell migration. We propose that this is due to alterations of the pericellular pH homeostasis.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB088

### Cholinergic receptor agonist, carbachol, induces $M_1$ and $M_4$ muscarinic receptors expression in megakaryocytic differentiated K562 cells

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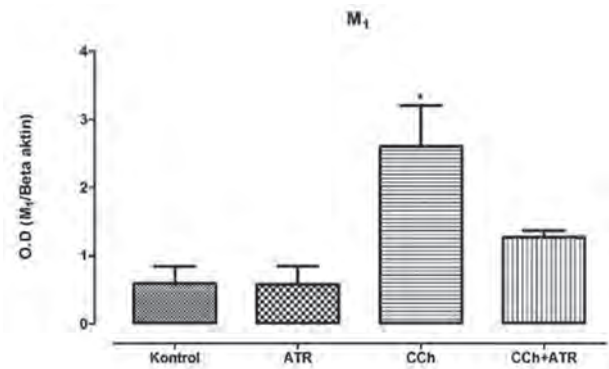
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Experimental studies show that muscarinic acetylcholine receptors are potential pharmacological targets for the treatment of cancer (1). Muscarinic receptors have been detected in different cells and tissue (2). Phorbol 12-myristate 13-acetate (PMA) causes megakaryocytic differentiation of K562 leukemia cells (3). Previously we showed that cholinergic agonist, carbachol (CCh) increased proliferation of megakaryocytic differentiated K562 cells proliferation in 24 and 48 hours (4). Preclinical studies suggested that cholinergic agents can be useful in cancer treatment prostate cancer (5). In order to detect the effects of agonist stimulation of  $M_1$ ,  $M_2$ ,  $M_3$ ,  $M_4$  and  $M_5$  subtypes in megakaryocytic differentiated K562 cells whole lysates prepared from K562 cells were analyzed with Western blot technique. Megakaryocytic differentiated K562 cells are homogenized, centrifuged and preserved at  $-80^\circ\text{C}$ . The protein content of the homogenate are analyzed via Lowry method. Statistically significant differences were determined by using the One-WayAnova and Bonferroni post test.

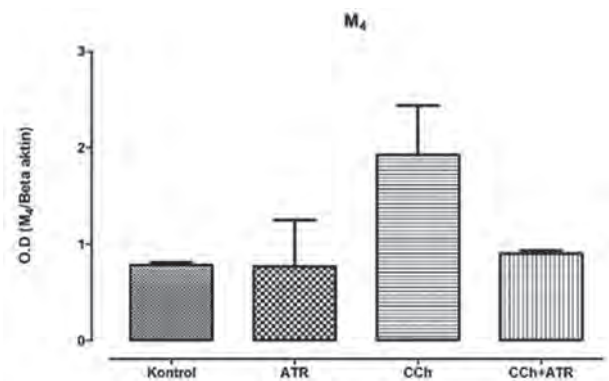
Here, we show that all muscarinic acetylcholine receptors ( $M_1$ - $M_5$ ) are expressed in megakaryocytic differentiated K562 cells. Treatment of megakaryocytic differentiated K562 cells to the cholinergic agonist, CCh, caused an increase in protein expression levels of protein expression pattern  $M_1$  and  $M_4$  mAChRs in megakaryocytic differentiated K562 cells, which was inhibited by atropine (Fig 1 a,b).

Exposure of megakaryocytic differentiated K562 cells to the antimuscarinic agent, atropine, caused increase  $M_2$ ,  $M_3$  expression level in 24 h.

Conclusions: Five muscarinic receptors were detected on megakaryocytic differentiated K562 cells. Exposure of carbachol increased  $M_1$ - $M_4$  muscarinic receptor expression. Muscarinic receptors may have a role in megakaryocytic differentiated K562 cells.



The effect of CCh, atropine on the expression of  $M_1$  (a);  $M_4$  (b) muscarinic receptors in megakaryocytic differentiated K562 cells, respectively. \* ( $P < 0.05$ ).



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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB089

### CRISPR/Cas-mediated targeting and visualization of long non-coding RNAs in human endothelial cells

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The CRISPR-Cas system is a constantly expanding toolbox for editing the genomic landscape of cells. So far, applications of CRISPR-Cas9 are limited to DNA. Targeting of RNA was mostly neglected although RNA plays a crucial role in every cellular process. The recently published CRISPR/Cas13a

from *Leptotrichia wadei* (LwaCas13a) exclusively targets and cleaves RNA with the help of a matching guide RNA. Here we used the newly developed catalytically inactive, msfGFP-labeled *LwadeadCas13a* to track down and visualize lncRNAs such as LISPR1 (long intergenic noncoding RNA antisense to S1PR1), NEAT1 and HIF1 $\alpha$ -AS1. We were able to show that LISPR1 is located in the nucleus as well as the cytoplasm which confirms previous Western Blot analysis. In contrast NEAT1 and HIF1 $\alpha$ -AS1 mainly seem to be accumulated in nuclear speckles. We addressed and optimized the transfection efficiency in HEK cells and human umbilical vein endothelial cells by fluorescence assisted cell sorting (FACS). With immunoprecipitation of *LwadeadCas13a*-msfGFP we were able to specifically enrich lncRNAs such as LISPR1. Taken together, CRISPR/Cas13 is a new and powerful tool to target RNA. It will help to elucidate mechanisms of regulation of gene expression by RNA.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB090

### Regulation of acetylcholinesterase activity, CXCL8 production and proliferation by pilocarpine in hHuman K562 eErythroleukemia cells

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Muscarinic receptors are found neuronal and nonneuronal cells (1,2). Different studies suggest that muscarinic receptors mediate some cellular events in hematopoietic cells (3). Muscarinic agonist, pilocarpine binds to M<sub>1</sub>/M<sub>3</sub> muscarinic receptors (4). We previously showed that carbachol (CCh), treatment leads to changes in muscarinic M<sub>2</sub>, M<sub>3</sub> and M<sub>4</sub> receptor transcripts in hHuman K562 eErythroleukemia cell line and muscarinic activation leads to eErythroleukemia cell proliferation dependent on the presence of fetal bovine serum (5). TNF- $\alpha$ , cytokine, induce apoptosis different tumor cells. But, different researcher also reported that TNF- $\alpha$  also stimulate cell proliferation in leukemia cells.

The present study to investigate effects of the muscarinic agonist, pilocarpine, TNF- $\alpha$  on CXCL8 release and AChE activity to determine their function in human erythroleukemia K562 cell proliferation. Human erythroleukemia K562 cells were incubated with the M<sub>1</sub>/M<sub>3</sub>R agonist pilocarpine, the M<sub>3</sub>R antagonist DAMP or TNF- $\alpha$  to investigate the role of M<sub>3</sub> receptor in human erythro leukemia K562 cells. AChE activity was determined by QuantiChrom™ Acetylcholine Assay kit. CXCL8 release was detected by using the colorimetric ELISA kit. Cell proliferation was analyzed by measuring BrdU incorporation during DNA synthesis in proliferating cells. Western blot analyses for M<sub>3</sub> muscarinic receptor protein was performed in the homogenates of human erythroleukemia cells treated with pilocarpine. All data show means  $\pm$  S.E.M. of at least six independent experiments. Statistically significant differences were determined by using the one-way analysis of variance followed by Bonferonni post tests.

Our experiments showed that pilocarpine caused a decrease in DNA synthesis in K562 cells supplemented with 0% or 1% fetal bovine serum after starvation. There was a significant inhibitory effect in pilocarpine group whereas 4DAMP treatment

not reversed this effect in K562 cell proliferation (P<0.0001) (Table 1). Pilocarpine (1 and 10  $\mu$ M) induced AChE activity in human erythroleukemia K562 cell (P<0.0001) (Table 2). Pilocarpine (10 and 100 $\mu$ M) alone inhibited CXCL8 release however, pilocarpine 1 and 10  $\mu$ M and TNF- $\alpha$  together were caused 6 fold increased CXCL8 release in human erythroleukemia K562 cell (\*\*P<0.0004). In conclusion, the findings of the current study clearly showed beneficial effects of pilocarpine on inhibition of human erythroleukemia K562 cell proliferation.

|                | % FBS   | % FBS  | % FBS    | % FBS    | % FBS   | % FBS   | % FBS    | % FBS    | % FBS    |
|----------------|---------|--------|----------|----------|---------|---------|----------|----------|----------|
|                | 0.317   | 0.408  | 0.140    | 0.125    | 0.169   | 0.273   | 0.145    | 0.163    | 0.169    |
|                | 0.270   | 0.878  | 0.152    | 0.140    | 0.344   | 0.379   | 0.149    | 0.177    | 0.193    |
|                | 0.289   | 1.001* | 0.140    | 0.128    | 0.275   | 0.299   | 0.153    | 0.172    | 0.164    |
|                | 0.449   | 1.152* | 0.136    | 0.166    | 0.465   | 0.295   | 0.164    | 0.144    | 0.145    |
|                | 0.328   | 0.939  | 0.191    | 0.169    | 0.315   | 0.204   | 0.171    | 0.136    | 0.138    |
|                | 0.385   | 0.929  | 0.165    | 0.162    | 0.284   | 0.200   | 0.196    | 0.141    | 0.129    |
| Mean           | 0.3097  | 0.8063 | 0.1597   | 0.1483   | 0.3120  | 0.2750  | 0.1630   | 0.1586   | 0.1647   |
| Std. Deviation | 0.06645 | 0.2137 | 0.02349  | 0.01977  | 0.09185 | 0.06703 | 0.01884  | 0.02072  | 0.02062  |
| Std. Error     | 0.02713 | 0.1068 | 0.009091 | 0.008069 | 0.03729 | 0.02736 | 0.007690 | 0.008429 | 0.008117 |

### Proliferation Assay Results

|                | % FBS      | P10%TNF $\alpha$ | P10%TNF $\alpha$ | P10%TNF $\alpha$ |
|----------------|------------|------------------|------------------|------------------|
|                | 0.01273101 | 0.03367557       | 0.0338809        | 0.01273101       |
|                | 0.01211499 | 0.03531827       | 0.03572895       | 0.01252667       |
|                | 0.01211499 | 0.03367557       | 0.03367557       | 0.01232033       |
| Mean           | 0.01232    | 0.04271          | 0.03580          | 0.01459          |
| Std. Deviation | 0.0003557  | 0.003491         | 0.002964         | 0.003080         |
| Std. Error     | 0.0002053  | 0.002015         | 0.001711         | 0.001778         |

### Acetylcholine Esterase Activity of Different Doses of Pilocarpine

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB091

### Antioxidant Effects of Apocynin on Ovarian Ischemia-Reperfusion Induced Remote Lung Injury in Rats

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Interruption of blood flow stimulates the release of molecules that cause accumulation of blood cells, including

polymorphonuclear leukocytes (PMN) in the lungs. Cell activation and releasing molecules can lead to acute respiratory failure. Due to the endogenous antioxidant system is inadequate because of massive reactive oxygen radicals, the exogenous molecules may be helpful in reducing oxidative damage. Inhibition of this pathway by Apocynin (Apo) has been shown to reduce oxygen radicals. We aimed to investigate the remote tissue effects of Apo in ovarian I/R injury. Wistar Albino female rats (n=40) were divided into five groups: Sham operation (Group I), ischemia/reperfusion (Group II), ischemia/reperfusion plus dimethyl sulfoxide (Group III), ischemia/reperfusion plus apocynin 20 mg/kg (Group IV) and ischemia/reperfusion plus apocynin 50 mg/kg (Group V). Bilateral adnexal 3-h period of ischemia was performed, followed by 3-h of reperfusion in study groups. Lung tissue MDA, TAS, TOS, SOD and MPO were measured using spectrophotometric methods. TOS, OSI, MDA and MPO levels were significantly higher, whereas TAS, SOD levels were lower in Group II than in Group I. Similar statistical results were determined in Group I and Group III comparisons. In treatment groups, when compared to Group II, TOS, OSI, SOD and MPO values were significantly changed. Our results suggested that, apocynin, an NADPH inhibitor, reduced I/R-induced inflammation and regulated the oxidative balance.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCB092

#### **The Effects of Apocynin on Inflammatory Cytokines And Apoptosis in Ischemia-Reperfusion Induced Remote Lung Injury**

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Under physiological conditions, in the organism, there is a protective balance between antioxidants and oxidants against harmful effects of free radicals. In ischemic reperfusion damage via the metabolic pathway named "respiratory burst" catalyzed by NOX during phagocytosis, ROS production may increase excessively, and it cannot be balanced against the antioxidant defense system, which leads to the tissue damage. Apocynin is one of the best-known inhibitors of the NOX family. We evaluated the effects of apocynin on inflammatory cytokines, cell death and apoptosis in ischemia-reperfusion (IR) induced remote lung injury. Wistar Albino female rats (n=40) were divided into five groups: Sham operation (Group I), IR (Group II), IR plus dimethyl sulfoxide (Group III), IR plus apocynin 20 mg/kg (Group IV) and IR plus apocynin 50 mg/kg (Group V). Bilateral adnexal 3-h period of ischemia was performed, followed by 3-h of reperfusion in study groups. TNF- $\alpha$ , IL-1 $\beta$ , NF- $\kappa$ B, Caspase-3 and LC3B were measured using immunohistopathological methods. Vascular congestion and hemorrhage were higher in Group II and group III. Apocynin treatment decreased the congestion and hemorrhage. Immunohistochemical staining of NF- $\kappa$ B, IL-1 $\beta$ , TNF- $\alpha$ , Caspase-3, and LC3B immunopositivities were observed at the highest values in group II and group III, whereas in the treatment groups, immunopositivities gradually decreased in inverse proportion to the dose of the apocynin. Ischemia reperfusion injury, which increases tissue apoptosis, cell death and

inflammatory cytokine content, plays an important role in the pathogenesis of remote organ damage. Apocynin administration plays a promising protective role in remote lung injury.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCB093

#### **Ligand/receptor-specific human colonic crypt calcium signatures are conferred in part by membrane receptor location and differential coupling to intracellular calcium stores.**

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A human colonic crypt is the unitary structure of the large intestinal epithelium. The crypt cell population (approx. 1000 cells per crypt) is topologically organised into an exquisite hierarchy: immature stem cells remain located within the stem cell niche at the crypt-base, while their progeny mature into functional epithelial cell types as they migrate along the crypt-axis. On reaching the surface epithelium, they are shed into the gut lumen. A number of morphogenetic gradients that were first shown to exhibit a spatial correlation with the differentiation hierarchy represented by the intestinal crypt(-villus) axis have subsequently been demonstrated to play major roles in regulating intestinal tissue renewal in health and disease. The aim of this study was to gain novel insights into the coordination of human colonic crypt physiology by correlating the spatio-temporal characteristics of receptor-mediated calcium signals with the physiological function of polarised epithelial cells along the crypt-axis. Methods: Human colonic crypts were isolated from colorectal tissue samples obtained at surgical resection (NRES approval) and placed in a 3D culture system. The spatio-temporal characteristics of intracellular calcium was monitored by Fura2/Fluo-4 imaging and the mechanism of receptor-mediated calcium mobilisation was characterised by a pharmacological approach. Calcium signalling toolkit components were visualised by fluorescence immunolabelling. Results: Muscarinic receptor activation initiated a colonic crypt calcium signal at the apical pole of intestinal stem cells (ISCs) located at the crypt base. ISCs were enriched with M3AChRs on the basal membrane and the apical site of calcium signal initiation correlated with a high concentration of endolysosomes endowed with two pore calcium channels (TPCs) and CD38 (known to synthesise NAADP). Cholinergic calcium signals proceeded to propagate across the polarised epithelial cell to the basal pole and up the crypt-axis by following an apical cell-to-cell path. Conversely, UTP (an agonist of P2Y receptor subtypes), initiated calcium signals in the upper crypt region, which propagated downward towards the crypt base. Unlike for cholinergic calcium signals, the cellular subtype responsible for purinergic calcium signal initiation was not identified, nor did there appear to be a concentrated hot spot of a specific type of intracellular calcium channel. However, whereas the muscarinic receptor coupled calcium signal was abolished by TPC inhibitors (NED-19 and diltiazem, both 250  $\mu$ M), the purinergic receptor-coupled calcium signal was blocked by the InsP3R inhibitor (2-APB, 100  $\mu$ M), but not by TPC inhibitors. Conclusions: Ligand/receptor-specific human colonic crypt calcium signatures are conferred in part by membrane receptor location



and differential coupling to intracellular calcium stores, and provide novel insights into 'bottom-up' and 'top-down' co-ordination of colonic crypt physiology.

Humane Research Trust, Big C Appeal

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB094

### Investigation of Cytotoxic Properties of Hetero-Ring Substituted Organophosphazene Compounds

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Phosphazene compounds in the group of phosphorus-nitrile compounds are also called phosphonitrile and hydroazophosphorine. Phosphazene compounds have a cyclic or linear structure in which two organic or inorganic side groups (R) are attached to each phosphorus atom, consisting of repeated -P = N-units in the structures. These compounds constitute a class of flavonoids and are abundant in edible plants. This study was designed to determine the possible cytotoxic effects of new hetero-ring substituted organophosphazene compounds on viability of two different types of human cancer cells.

New hetero-ring full substituted organophosphazene analogues (FSC 1,2) have been synthesized from the reaction of 3-(2,3-dihydrobenzofuran-5-yl)-1-(4-hidroksifenil)prop-2-en-1-one (HAF-1), and 1-(4-Hidroksifenil)-3-(6-metoksipiridin-3-yl)prop-2-en-1-one (HAF-2) with hexachlorocyclotriphosphazene (HCCP), respectively (Figure 1). The structure characterizations of all cyclotriphosphazenes were confirmed by FT-IR and NMR spectroscopy methods (<sup>31</sup>P, <sup>1</sup>H and <sup>13</sup>C-APT). The human epithelial ovarian cancer cell line (A2780) and the human prostate cancer cell line (PC-3) were treated with different concentrations of the compounds (1, 5, 25, 50 and 100 µM) [1,3]. In vitro cytotoxic properties of FSC 1,2 were determined using the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay method. The LogIC<sub>50</sub> values of FSC 1,2 were determined by using a Graphpad prism 6 programs. The high concentrations of the tested compounds caused significantly reduces in the cell viability both A2780 and PC3 cell lines (p<0.05). Our results indicated that hetero-ring substituted organophosphazene compounds can be candidate molecule for cancer treatment. However, there is need for comprehensive studies to reveal the effect of the substance.

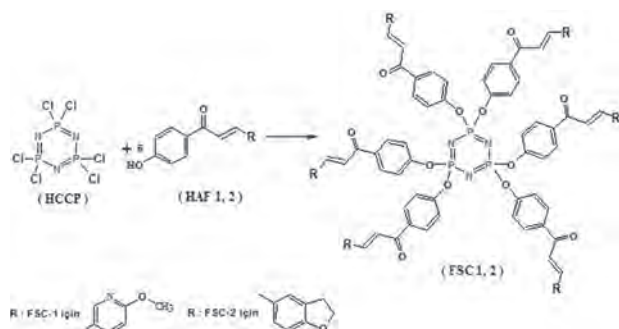


Figure 1. General structure of the compounds.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB095

### Measuring alveolar epithelial fluid reabsorption with confocal microscopy

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Alveolar reabsorption controls the thickness of the layer of alveolar lining fluid (ALF), which is of importance for alveolar gas exchange. In cultured cells, reabsorption is not measured directly but estimated from electrophysiological and biochemical measurements of activity and expression of ion transporters. The aim of this study was to establish a method to determine the rate of alveolar fluid clearance and the steady-state thickness of ALF by confocal microscopy.

Rat primary alveolar epithelial cells were grown to confluence on clear transwell filters in a tissue culture incubator in normoxia and hypoxia (1.5% O<sub>2</sub>) with or without dexamethasone (1 µM). For measurement of ALF two wells of a 6-well plate with glass bottom were filled with water and pre-warmed. The plate was closed with a special lid having an opening cut out that was tightly covered with a PCR-foil. After removal of apical culture medium filters were placed on spacers into the glass bottom plate, 150 µl of culture medium were added to the basolateral compartment, and filters were equilibrated for 20 min. Culture medium (20 µl) with and without amiloride (10 µM) containing 2 mg/ml of tetramethylrhodamine-labeled dextran (10 kD) were applied to the apical surface by puncturing the PCR-foil with a micro-syringe, and the plate was immediately transferred to a confocal microscope (Leica SP8) equipped with a climatized chamber. X/Z-scans were immediately started (t<sub>0</sub>; time after adding dextran ~ 40 sec) and were repeated after 1, 2, 3.5, 5, 8, 10, and 13 min; images of 5 positions per filter were stored for automated off-line measurement of ALF-height at each time-point using MATLAB software. The rate of reabsorption was calculated from the decrease in ALF using the first three time-points. Steady-state height of ALF was determined from the 10 and 13 min time-points.

Results show that there was no difference in ALF(t<sub>0</sub>) between control and dexamethasone treated cells in normoxia. Amiloride did not affect ALF(t<sub>0</sub>) in normoxic control cells. However, amiloride increased ALF(t<sub>0</sub>) significantly in hypoxic cells and in normoxic and hypoxic cells after treatment with dexamethasone. In contrast, there was no statistically significant difference in the calculated rate of reabsorption between the conditions. There was also no difference between conditions in the steady state height of ALF estimated from 10 and 13 min time-points.

Results indicate that measurements of the calculated neither rate of reabsorption nor steady-state ALF-height reflect the differences in Na-transport rates measured electro-physiologically, which show inhibition by hypoxia and amiloride and stimulation of transport with dexamethasone in normoxia and hypoxia (Güney et al; PM:17873005). At best, values of ALF(t<sub>0</sub>)

reflect differences in Na-transport, indicating that reabsorption rates are too fast to be detected by sequential confocal X/Y-scans.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB096

### The effect of Chitosan Nanosilver Dressing Versus Mesenchymal Stem Cells on Wound Healing

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Wounds constitute a major worldwide public health problem. Infection is the most common complication of wounds. Surgical wound infection is still a major post operative problem. New modalities for treating wound infections such as nanotechnology and stem cell therapy have been tried. However, the available studies didn't compare the effect of mesenchymal stem cells against Chitosan nanosilver on wound-healing and didn't use histological techniques in assessing their effects. This study compares the effect of chitosan nanosilver dressing with the intradermal injection of bone marrow mesenchymal stem cells on wound healing. Forty adult female albino rats were equally randomized into 4 groups: group I served the control, in group II, two surgical wounds of 4 mm diameter were induced on the back of each animal. In group III chitosan nanosilver dressing was stitched over the wounds and group IV received intradermal injection of  $1 \times 10^6$  mesenchymal stem cells derived from bone marrow after wound induction. All animals were sacrificed after seven days by decapitation. Two skin specimens with 3 mm of the surrounding skin were obtained from the two wounds of each animal and processed for histological, (Haematoxylin and Eosin stain, Masson's trichrome stain, periodic acid-Schiff reaction) immunohistochemical (Proliferating cell nuclear antigen-PCNA) and immunofluorescent (mesenchymal stem cells labeling) techniques. Wound group showed complete shedding of epidermis, decreased mean optical density of collagen fibers, negative PAS reaction and negative PCNA immunostaining. Chitosan nanosilver dressing group showed significant increase in the mean epidermal thickness ( $147.2 \pm 21.2$ ), collagen optical density ( $0.258 \pm 0.08$ ), PAS optical density ( $0.195 \pm 0.024$ ) and the positive PCNA immunoreactivity ( $0.673 \pm 0.111$ ) compared to the mesenchymal stem cells group parameters (Mean epidermal thickness:  $135 \pm 24.3$ , collagen optical density:  $0.146 \pm 0.03$ , PAS optical density:  $0.183 \pm 0.022$  & PCNA immunoreactivity:  $0.572 \pm 0.104$ ). In conclusion, both chitosan nanosilver dressing and bone marrow mesenchymal stem cells promoted wound healing, but the non-invasive chitosan nanosilver dressing had better and faster wound healing effect.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB097

### Neutraceutical targeting of the bile acid receptor, farnesoid X receptor, for intestinal disease.

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**Introduction:** Dysregulated bile acid metabolism has been linked to the pathogenesis of intestinal disorders, including inflammatory bowel disease (IBD) and cancer. The nuclear bile acid receptor, farnesoid X receptor (FXR), represents a promising target for treatment of these diseases. Previous studies suggest that a class of plant-derived phytochemicals are modulators of FXR activity. The aim of this study was to investigate the effects of a lead natural compound, denoted KFS1, on FXR activation in colonic epithelial cells.

**Methods:** Polarised monolayers of T<sub>84</sub> colonic epithelial cells were treated bilaterally with the FXR agonist, GW4064 (5µM) and KFS1 (1-100µM) for 24 hr. Expression of FXR and FGF-19 were measured by RT-qPCR. Transepithelial electrical resistance (TEER) was measured using the EVOM<sup>2</sup> Voltohmmeter. **Results:** GW4064 (5µM) induced an  $1100 \pm 457$  fold increase in FGF-19 mRNA expression in T<sub>84</sub> cells ( $n=22, p<0.05$ ). At 50µM and 100µM, KFS1 increased levels of FGF-19 expression by  $218.28 \pm 196.81$  and  $93.66 \pm 75.16$  fold, respectively ( $n=3$ ). KFS1 also reduced TEER by  $60.24\% \pm 5.02\%$  and  $64.35\% \pm 6.18\%$ , respectively ( $n=7$ ), suggesting it exerts toxic actions at these concentrations. Interestingly, at lower concentrations that did not alter TEER, KFS1, but not GW4064, increased FXR mRNA expression. KFS1 (5µM and 10µM) increased FXR expression by  $4.16 \pm 0.37$  fold and  $3.28 \pm 0.35$  fold, respectively ( $p<0.05; n=4$ ). Moreover, treatment with KFS1 (1, 5, or 10µM) for 1hr prior to treatment with GW4064 potentiated GW4064-induced FGF-19 expression by  $2.5 \pm 0.76$  fold;  $2.86 \pm 0.21$  fold;  $p<0.01$ , and  $4.64 \pm 0.5$  fold;  $p<0.01$ , respectively ( $n=6$ ).

**Conclusion:** Our data suggest that KFS1 modulates FXR signalling in colonic epithelial cells. At high concentrations, KFS1 may act as an FXR agonist, which appears to induce cellular toxicity. At lower concentrations, KFS1 increases FXR expression, which may prime cells for agonist-induced FXR activation. These findings suggest that foods, or food supplements, rich in these plant-derived phytochemicals have potential for development as FXR-targeted neutraceuticals.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB098

### Contribution of tricellulin to paracellular water permeability in epithelia of different tightness

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**Introduction:** The existence of a paracellular pathway for water through the tight junctions (TJ) was discussed controversially for many years until a molecular component of the

paracellular water transport, claudin-2, was identified. The contribution of the tricellular TJ (the site where three cell meet) and its major component tricellulin was not elucidated so far.

**Methods:** Knockdown (KD) of tricellulin was performed by transfection with shRNA targeting this protein in HT-29/B6 (human colon) or in MDCK C7 (dog kidney) cells. The expression of tricellulin, claudins, and aquaporins was investigated in KD and control cells by Western blot. The transepithelial water flux was measured using a modified Ussing chamber setup. Water flux was induced by an osmotic gradient of 100 mM mannitol or 100 mM 4 kDa-dextran.

**Results:** Tricellulin KD did not alter the expression of claudins or AQP. In HT-29/B6 cells, tricellulin KD caused a decrease of transepithelial resistance (TER) from  $1858 \pm 88 \Omega \times \text{cm}^2$  to  $969 \pm 27 \Omega \times \text{cm}^2$ . However, water flux did not significantly change in tricellulin KD at osmotic gradients induced either by mannitol or by 4 kDa-dextran. Since in HT-29/B6 cells claudin-2 is genuinely expressed, a large component of water transport may have travelled through the bicellular TJ and a possible contribution of the tricellular TJ may not have reached significant levels. In MDCK C7 cells the tricellulin KD resulted in a decrease of TER from  $12.7 \pm 0.3 \text{ k}\Omega \times \text{cm}^2$  to  $9.2 \pm 0.3 \text{ k}\Omega \times \text{cm}^2$ , but in contrast to HT-29/B6 cells, here we observed an increased water flux compared to controls under the mannitol-induced osmotic gradient (controls:  $5.17 \pm 0.51 \mu\text{l/h/cm}^2$ , KD:  $7.10 \pm 0.41 \mu\text{l/h/cm}^2$ ,  $p < 0.05$ ). The same effect was observed under a gradient induced by 4 kDa-dextran. That in these cells an effect of tricellulin KD on water transport became apparent can be explained by the fact that MDCK C7 cells lack any claudin-2 expression and therefore the large background otherwise mediated by the bicellular TJ was absent, uncovering a significant contribution of the tricellular TJ. Current size assumptions of the tricellular TJ comprise a diameter of  $\sim 100 \text{ \AA}$  that would be suitable for movement of water molecules ( $\sim 3 \text{ \AA}$ ), however through a tube of  $\sim 1 \mu\text{m}$  length.

**Conclusion:** In HT-29/B6 cells a tricellulin KD does not significantly alter transepithelial water transport due to the presence of the large contribution of other water-conducting proteins as claudin-2. In contrast, tricellulin KD in MDCK C7 cells which lack claudin-2 results in an increase in transepithelial water transport. Thus, the contribution of tricellulin and the tricellular TJ to transepithelial water transport depends on the tightness of the epithelia. In the absence of claudin-2, the tricellular TJ, controlled by tricellulin expression, contributes to paracellular water transport.

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genetic deletion of NHE3, which results in diarrhea in mice, deletion of NHE2 or NHE8 does not cause a diarrheal phenotype. For further elucidation of the function and localization of enterocyte NHE2 and NHE8, we utilized fully differentiated Caco-2Bbe cells, whose genetic expression profile closely resembles mature enterocytes. Using specific inhibitors and shRNA knockdown of NHE2, the activity of the different NHEs was analyzed by fluorometric  $\text{pH}_i$ -metry in a perfusion chamber with separate apical and basolateral perfusion and their expression was quantified by qPCR. The specificity and inhibitory potential of inhibitors for NHE1,2,3 and NHE8 were determined by overexpression in the NHE-deficient PS120 fibroblast cell line and in NHE1,2 and 3 knockout mouse intestine. Differentiated Caco-2Bbe cells express endogenous NHE1, NHE2, NHE3 and NHE8, with NHE2 having the highest and NHE1 the lowest mRNA expression levels. The acid-activated basolateral  $\text{Na}^+/\text{H}^+$  exchange rates ( $0.127 \pm 0.018 \Delta\text{pH}/\text{min}$ ) were 3-fold higher compared to the apical rates ( $0.038 \pm 0.0062 \Delta\text{pH}/\text{min}$ ).  $96.97 \pm 2.4 \%$  of the total acid-activated basolateral  $\text{Na}^+/\text{H}^+$  exchange showed a NHE1-typical inhibitor profile. On the apical membrane,  $50.9 \pm 3.1 \%$  of the acid-activated  $\text{Na}^+/\text{H}^+$  exchange rates displayed a NHE2-typical inhibitor profile,  $30.8 \pm 5.6 \%$  an NHE3-typical inhibitor profile and  $25.6 \pm 11.75 \%$  an NHE8-typical inhibitor profile. shRNA downregulation of NHE2 expression in Caco-2Bbe cells ( $\sim 30 \%$  remaining NHE2 expression) resulted in increased expression of NHE8 compared to mock transfected cells, whereas NHE1 and NHE3 expression levels did not change significantly. NHE2 silencing did not significantly influence the total apical acid-activated  $\text{Na}^+/\text{H}^+$  exchange activity. However, the percentage of apical  $\text{Na}^+/\text{H}^+$  activity with a NHE2-typical inhibitor profile was reduced, while that with a NHE8 typical inhibitor profile has increased to  $37 \pm 8.5 \%$ . Apical NHE3 activity and basolateral NHE1 activity remained constant. Caco-2Bbe cells highly express NHE2 mRNA, and NHE2 activity was functionally identified in the apical membrane, but its transport rate during  $\text{pH}_i$ -recovery on the apical membrane was modest compared to the basolateral NHE1 activity, proposing a high NHE2 turnover rate, an unknown NHE2 activation mode, or both. NHE8 activity is also found in the apical membrane and partly compensates for the loss of NHE2, but the majority of NHE8 appears localized in organelle localization and does not participate in cytoplasmic  $\text{pH}_i$ -regulation.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCB099

#### Functional activity of plasma membrane NHE isoforms expressed in the self-differentiating intestinal epithelial cell line Caco-2Bbe

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The absorption of  $\text{NaCl}$ ,  $\text{HCO}_3^-$  and fluid in the gastrointestinal tract is strongly dependent on apical  $\text{Na}^+/\text{H}^+$  exchange.  $\text{Na}^+/\text{H}^+$  exchanger NHE2, NHE3, and NHE8 are presumably expressed in the enterocyte apical membrane. Unlike the



## PCB100

### A Bacterial Signal Peptide Increases Mucociliary Clearance in Explanted Mouse Trachea by Stimulating Solitary Chemosensory Cells and Paracrine Cholinergic Signaling

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**Objective:** Mucociliary clearance (MC) is a major innate defense mechanism, driven by the coordinated beating of ciliated epithelial cells. Bacterial products such as quorum sensing molecules (QSM) alter MC (1,2). A subpopulation of airway epithelial cells, called solitary chemosensory cells (SCC), expresses taste receptors (TasR) and contributes to the recognition of these bacterial products (3,4). SCC recognize QSM via the classical taste transduction pathway involving PLC $\beta_2$  (phospholipase C  $\beta_2$ ), a rise in intracellular calcium and TRPM5 (transient receptor potential cation channel subfamily M member 5), leading to release of acetylcholine (ACh), an known activator of MC. We here asked whether formylated bacterial signal peptides modulate MC, and assessed ciliary beat frequency (CBF) and particle transport speed (PTS) on the mucosal surface of explanted murine tracheas and release of ACh.

**Methods:** The transcriptome of single tracheal ciliated and SCC was analyzed by deep sequencing. PTS was studied in wildtype C57Bl6 (WT) mice, and mice lacking components of the taste transduction cascade (TRPM5; ITPR3 (inositol 1,4,5-triphosphate receptor 3); PLC $\beta_2$ ; and Tas2r143/Tas2r135/Tas2r126 triple KO-mice (3 of 35 bitter taste receptors)) or lacking SCC (TRPM5-DTA). ACh was measured by HPLC.

**Results:** The N-formylated bacterial signal peptide FL185 (10  $\mu$ M), produced by various pathogens, e.g. *E. coli* and *Salmonella typhimurium*, increased PTS from 44 $\pm$ 2 to 75 $\pm$ 3  $\mu$ m/s (mean $\pm$ SEM; p<0.0001; n=23; paired t-test). Deep sequencing showed FPR expression in both ciliated and SCC, and presence of TRPM5, PLC $\beta_2$ , ITPR3 and several chemoreceptors including bitter receptors in SCC. FPR1/2 inhibitors cyclosporine H (1  $\mu$ M) and t-BOC2 (10  $\mu$ M) did not reduce the effect 74 $\pm$ 8 to 62 $\pm$ 3 % increase in PTS (p=0.5454; n=6; unpaired t-test). The effect was reduced in TRPM5- (74 $\pm$ 8 to 3 $\pm$ 2 %; p=0.0011; n=6), PLC $\beta_2$ - (71 $\pm$ 17 to 0.45 $\pm$ 3 %; p=0.0018; n=7), ITPR3- (34 $\pm$ 1 to 2 $\pm$ 1 %; p=0.0009; n=5), Tas2r143/Tas2r135/Tas2r126-deficient mice (37 $\pm$ 5 to 22 $\pm$ 5 %; p=0.0484; n=12) and TRPM5-DTA mice (92 $\pm$ 2 to 9 $\pm$ 1 %; p=0.0012; n=5). Atropine (1  $\mu$ M; 74 $\pm$ 8 to 18 $\pm$ 7 %; p=0.0025; n=7) and 4-DAMP (1  $\mu$ M; 66 $\pm$ 16 to 11 $\pm$ 4 %; p=0.0037; n=6), muscarinic receptor

antagonists, diminished the effect of FL185. ACh was increased in supernatants of tracheas after stimulation with FL185 (70 $\pm$ 17 to 158 $\pm$ 36 nM; p=0.0203; n=6; paired t-test). This was not the case in TRPM5-KO mice (112 $\pm$ 70 to 60 $\pm$ 20 nM); p=0.5000; n=3; Wilcoxon matched-pairs signed rank test).

**Conclusion:** A bacterial signal peptide stimulates MC independently of FPR. Instead, this effect involves typical taste transduction elements, solitary chemosensory cells, and subsequent cholinergic signaling to ciliated cells. Thus, detection of a defined set of bacterial signal peptides by SCC provides a novel defense mechanism against bacteria.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## PCB101

### Investigation of diabetes-induced changes in lung microbiome using next-generation sequencing.

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High blood glucose (hyperglycaemia) has been linked with increased glucose concentration in the airway surface liquid (ASL). This has been shown to be associated with increased risk of developing pulmonary infection in patients in the intensive care unit and in patients who have diabetes and chronic lung disease<sup>1</sup>.

Contrary to the popular belief that the lungs are sterile, it was recently discovered that the lung is inhabited by resident bacteria and that it possesses its own unique microbiome. Several chronic lung conditions, such as cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD), have been shown to induce changes to the resident lung microbiota.

We are investigating the effect of high glucose concentration in the ASL on the resident lung microbiome. Preliminary studies were performed in db/db and GK+/- diabetic mice which represent models of Type 2 and Type 1 diabetes. As the environment is a key factor in determining the microbiota in other tissues, diabetic and non-diabetic littermates were housed under similar conditions. Animals were subjected to Schedule 1 terminal anaesthesia (i.p. injection of 0.2 mL pentobarbital (60 mg/ml) diluted 1:1 with saline) and samples were collected using bronchoalveolar lavage (BAL) technique. Cannulas were inserted via the trachea and lungs were washed using 1ml of sterile PBS. Negative control samples were created by repeating all the steps without BAL in order to account for environmental contaminants.

The 16S ribosomal RNA amplification approach has become the standard for identifying bacterial species in the microbiota. 16S is present in all bacteria and allows precise species identification from a small amount of starting material, making it a strong choice for analysis of the samples with low bacterial yield, such as samples of lung microbiome. The lung microbiome of diabetic and non-diabetic mice was analysed using next-generation sequencing technique Illumina MiSeq and NanoPore. Analysis was performed using bacterial 16S rRNA gene (1500bp). Primers amplifying V3-V4 regions of 16S gene were used for the MiSeq analysis. Pure bacterial DNA was amplified using Touchdown PCR approach with annealing temperatures from 64°C to 54°C with a 1°C decrease per step. Cycle was repeated 38 times for sufficient amplification. Preliminary studies suggest a shift in diversity in the resident lung microbiome with diabetes. Abundance of several types of *Actinobacteria* was notably increased in the diabetic mice in comparison to the wild-type mice. Both *Staphylococcaceae* and *Streptococcaceae* abundance was also increased in the lungs of diabetic mice. This data supports previous findings that diabetes mellitus is a predisposing factor for nasal colonisation of *S. aureus*<sup>2</sup>.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

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PCB102

### Investigating the effect of hyperglycaemia on the airway surface liquid proteome.

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Diabetes is a disease in which the homeostatic pathways for the safe storage of glucose fail. The resultant rise in blood glucose (hyperglycaemia) is known to affect the expression and function of proteins. The complement of proteins produced by a cell type is its proteome. In diabetes hyperglycaemia is known to alter the synthesis and induce aberrant post-translation modifications to the proteomes of varied cell and tissue types.

Hyperglycaemia increases glucose flux across the lung epithelium raising the glucose levels of the liquid interface separating the cells from the environment, airway surface liquid (ASL). ASL glucose is usually maintained at ~0.4mM. However, in diabetes ASL glucose can rise as high as ~3mM when comorbid with respiratory diseases such as Cystic Fibrosis or Chronic obstructive pulmonary disease<sup>1</sup>. Raised glucose in the ASL has been associated with an increased susceptibility to lung infections<sup>1</sup>.

The effect of hyperglycaemia on the ASL proteome remains comparatively unexplored. We hypothesised that acute and chronic hyperglycaemia would modify the ASL proteome and have used an *in vitro* model to test this.

Calu3 and H441 cell were grown at airway-liquid interface and exposed to hyperglycaemic media once the cells

reach a transepithelial resistance ( $R_t$ ) of 600 and 350Ω cm<sup>2</sup> respectively. Cells were then washed with Ca<sup>2+</sup> and Mg<sup>2+</sup> enriched Dulbecco's phosphate buffered saline (DPBS) and placed in hyperglycaemic (25mM glucose) or normoglycaemic conditions (Calu3: 5.5mM, H441:11mM glucose) osmotically balanced with mannitol.  $R_t$  was measured and samples of the media and ASL were taken at 0, 24, 48 and up to 120 hours. Glucose concentration was measured with Amplex red glucose assay (Life Technologies). Samples for protein analysis were quantified using a BCA assay (Pierce) and either flash frozen for future analysis or subjected to SDS PAGE on pre-cast Bis-Tris (4-12%) gels and 16% Tricine gel (Novex). The separated proteins were then examined using silver staining (Pierce). The stained gels were imaged and the pixel density of each lane analysed and molecular weights extrapolated from LI-COR molecular weight marker (928-40000) via the GelAnalyzer 2010a program.

Glucose analysis indicated that hyperglycaemic conditions were maintained in the cell cultures.  $R_t$  declined between 120hrs and 148hrs. This data helped form a time frame for future experiments. Data from the BCA assays showed ASL in acute (24-48 hours) and chronic hyperglycaemia (120 hours) have higher protein concentrations than normoglycaemia. Several protein bands were observed to change their prominence or appear in hyperglycaemia compared with normoglycaemia.

This early data will now inform next generation shot-gun proteomic sequencing using a nano-electrospray ionisation-LC-MS/MS. ASL from primary human airway cells will also be introduced for analysis.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

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PCB103

### Impedance spectroscopy indicates multiple barriers in stratified epithelia

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For the development of efficient and sustainable treatments for skin diseases, it is essential to understand the full mechanism of skin barrier formation. Although the Stratum Corneum (SC) has been identified as an important component of the skin barrier [1], it is still a matter of debate if and to what extent the Tight Junction (TJ) is playing a role in skin barrier function [BJ1]. There is evidence, that the disruption of the TJ protein Claudin (Cldn) 1 affects skin barrier formation [2], and that Cldn1 and Cldn4 are altered in several skin diseases [3,4]. Our goal was to investigate the effects of Cldn1 and 4 disruption on barrier function and the general role of the TJ in skin. As a common method for characterizing the barrier function of epithelia, Ussing chamber based impedance spectroscopy in the kHz domain [5] was performed. Isolated cells from human foreskin were cultivated as 3D reconstructed human epidermis (RHE) in cell culture inserts to mimic skin tissue. The impact of Cldn1 and 4 was studied by comparison of RHE with

Cldn1 and 4 knockdown (KD) cells, respectively. 5% Triton X-100 in PBS was applied to the apical side of the cell layers to induce SC disassembly and cell permeabilization. Immunofluorescence staining was carried out to correlate morphological changes with barrier function.

Nyquist plots of the impedance spectra show the presence of more than one time constant. Thus, the current is forced to pass further capacitors represented by the underlying cell layers due to a high resistor in the paracellular pathway. This indicates the barrier to be formed by more than the SC. Differences in the epithelial resistance and the distribution of the phase shift over the frequency prove that the absence of Cldn1 and 4 affects barrier formation in different ways (Fig. 1). Treatment with Triton X-100 reveals the presence of multiple time constants, each of which represents a specific compartment within the tissue (Fig. 2).

In summary, these findings suggest that the TJ not only influences the formation of the SC but also acts as a barrier in the underlying layers of skin tissue.

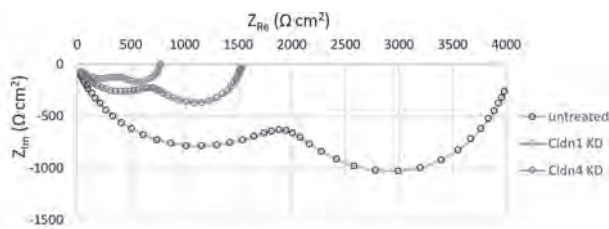


Figure 1: Impedance spectra of skin tissue. All spectra exhibit at least two time constants.

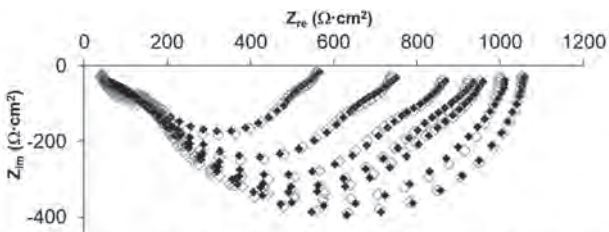


Figure 2: Impedance spectra of Triton X-100 treatment for different time points. Black: original data. Red: Fitted curve with three time constants.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB104

#### UT-B Osmoregulation in RT4 Urothelial Cell Line

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UT-B urea transporters are essential for normal bladder function. They protect bladder cells from the damaging effects of high urea concentrations (1). RT4 cells, derived from a human bladder papilloma, are a well-established cell model system for the human urothelium (2). UT-B1 urea transporters are highly expressed in human bladder tissue (3.). Aquaporin 3 (AQP3) transmembrane transporters are highly expressed in

bladder cells and are subject to osmoregulation in the presence of NaCl (4).

This study employed RT4 cells to test the hypothesis that UT-B urea transporters are subject to osmoregulation when exposed to increased NaCl concentrations.

RT4 cells were cultured in media with a range of osmolalities for 48 hours, using either mannitol or sodium chloride. End-point polymerase chain reaction (PCR) and Western blotting experiments were carried out. Densitometry measurements were analysed.

Western blotting results for mannitol treatments show visibly significant increases in AQP3 protein abundance ( $P < 0.05$ ,  $n = 9$ , ANOVA). UT-B, GAPDH and NaKATPase gave no statistically significant change with increasing mannitol concentration. NaCl-treated cells had statistically significant changes in protein expression for both UT-B ( $P < 0.01$ ,  $n = 7$ , ANOVA) and AQP3 ( $P < 0.01$ ,  $n = 7$ , ANOVA), while GAPDH and NaKATPase protein expression remained unchanged.

NaCl-induced, but not mannitol-induced, changes in external osmolality appear to regulate UT-B protein abundance in the RT4 cell line.

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This work was supported by the Jack Pickard Research Foundation and University College Dublin.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB105

#### NADPH Oxidase Inhibitor Apocynin Ameliorates Renal Ischemia-Reperfusion Injury by Regulation of Inflammation, Autophagy and Apoptosis

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The present study aimed to investigate the antioxidant, anti-inflammatory and antiapoptotic effects of NADPH Oxidase Inhibitor apocynin on the rat ovary against ischemia-reperfusion injury using histopathological methods. Forty Wistar-albino female rats were subjected to Sham operation (S), ischemia/reperfusion (IR), ischemia/reperfusion plus dimethyl sulfoxide (IR-D), IR plus apocynin 20 mg/kg (APO20), and IR plus apocynin 50 mg/kg (APO50). In all IR groups, a bilateral adnexal 3-h period of ischemia was performed, followed by 3-h of reperfusion. A single dose of apocynin was given intraperitoneally 15 min before ischemia and just before reperfusion in APO20 and APO50 groups. TNF- $\alpha$ , IL-1 $\beta$ , NF- $\kappa$ B, Caspase-3 and LC3B were measured using histopathological methods.



Immunohistochemical staining of NF- $\kappa$ B, IL-1 $\beta$ , TNF- $\alpha$ , Caspase-3, and LC3B immunopositivities were observed in the IR groups at the highest values, whereas in the treatment groups, immunopositivities gradually decreased in inverse proportion to the dose of the apocynin. In the ovary tissue hemorrhage and congestion were found to be intense in the IR groups, whereas in the treatment groups, hemorrhage and congestion were alleviated in inverse proportion to apocynin dose. In conclusion, apocynin decreases IR-induced inflammation, apoptosis and autophagic cell death in a dose-dependent manner. Thus, apocynin may be a new alternative for the treatment of diseases with increased cell death, where oxidant stress is intense.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCB106

### **Guardian goblet cells protect the human large intestinal stem cell niche via muscarinic receptor-coupled, ROS/calcium-dependent mucus secretion.**

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Mucus barrier formation is compromised in human intestinal disease. In Muc2 knock-out mice, direct contact of luminal bacteria with the gut epithelium causes inflammation and colon cancer. Recent studies of differentiated murine goblet cells (1,2) point to a pivotal role for ROS and calcium signalling in excitation-mucus secretion coupling. The aim of the current study was to investigate a potential role for ROS and calcium signalling in cholinergic-induced mucus secretion by goblet progenitor cells (GPCs) located in the human colonic stem cell niche. Methods: Human colonic crypt cultures were generated from colorectal tissue samples obtained at surgical resection (NRES approval). Intracellular calcium was monitored by Fura2 imaging. Transcript expression was assessed by RT-PCR. Protein localisation was visualised by fluorescence immunolabelling and changes in protein expression/localisation were quantified by image analysis of immunofluorescence intensity. Results: The muscarinic receptor agonist oxotremorine (1  $\mu$ M) stimulated calcium signals throughout the intestinal stem cell niche of human colonic crypts. The human colonic epithelium (N>5 subjects) expressed transcripts for: MACHR subtypes 1, 3 & 5; InsP3R subtypes 1-3; RYR subtypes 1-3; TPC1 and TPC2. Immunolabelling confirmed the expression of their protein counterparts in Muc2<sup>+</sup> GPCs located adjacent to LGR5<sup>+</sup> stem cells. The peak amplitude of the muscarinic calcium signal was inhibited by more than 80% (P<0.01, n>30 crypts, N>20 subjects) by TPC1/2 antagonists NED19 (100  $\mu$ M) and diltiazem (100  $\mu$ M). Expression of the ROS generator, NADPH oxidase 1 (NOX1) by GPCs was demonstrated by immunofluorescence labelling. A range of NOX1 inhibitors (DPI, 5  $\mu$ M; apocynin, 100  $\mu$ M; VAS2870, 56  $\mu$ M), or the antioxidant n-acetylcysteine (5 mM), suppressed the peak amplitude of the muscarinic calcium signal generation by at least 50% (P<0.01, n>10 crypts, N>3 subjects for all treatments). The Muc2-content (i.e. immunofluorescence intensity) of GPCs was reduced by approximately 50% following stimulation with muscarinic receptor agonists (N=9 subjects, n>40 crypts and n>500 GPCs); this was accompanied by the appearance of Muc-2 protein in the crypt lumen.

Inhibition of muscarinic calcium signals by pre-incubation with BAPTA-AM (66 mM), NED-19, or DPI not only inhibited Muc-2 secretion, but promoted an accumulation of intracellular Muc2 content. Finally, cholinergic input promoted an increase in Muc2<sup>+</sup> progenitor cell nuclei exhibiting mitotic figures. Conclusions: Cholinergic input to the human large intestinal stem cell niche maintains the population of goblet progenitor cells and promotes NOX/ROS-dependent calcium mobilisation via intracellular TPC channels. Increased cytosolic calcium stimulates Muc2 secretion into the crypt lumen which guards against invading luminal bacteria.

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Humane Research Trust, Big C Appeal

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCB107

### **Effect of vape carriers propylene glycol and vegetable glycerine on glucose uptake in airway epithelial cells**

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Electronic nicotine delivery systems (ENDS), more commonly known as e-cigarettes, are used as a tobacco free nicotine delivery system. ENDS utilise a liquid solution which is drawn over a heating element to produce a vapour which is then inhaled giving rise to the term 'vaping'. The solution normally contains propylene glycol and vegetable glycerine to generate the vapour together with nicotine and flavourings. Evidence has indicated these carrier chemicals may reduce growth and survival of epithelial cells, including those of the airway<sup>[1][2]</sup>.

As glucose uptake is a key metabolic requirement for cell survival and is critical for the normal function of the airway epithelium, we hypothesised that propylene glycol (PG) and propylene glycol mixed with vegetable glycerine (PG:VG) inhibited glucose uptake human airway epithelial cells.

H441 or primary bronchial epithelial cells (HBEC) were grown on plastic or cultured on permeable supports at air-liquid interface. PG (0-3%) or PG:VG (55:45, 0-3%) were applied to the medium, directly to the apical (luminal) surface, or the cells were exposed to vapour for 10 minutes. Glucose uptake across the cell membrane was measured using the Uptake Glo luminescent assay (Promega, UK). Cytotoxicity was measured using an LDH cytotoxicity assay (Peirce, UK). Mannitol (3%) was used as an osmotic control in some experiments. Fluorescence recovery after photobleaching (FRAP) was carried out on human embryonic kidney cells transfected with GLUT1-GFP. PG elicited a dose dependent inhibition of glucose uptake in proliferating H441 and HBEC. 3% PG inhibited glucose uptake to a similar level as the glucose transport inhibitor cytochalasin B (35  $\pm$  5% and 32  $\pm$  7% of control p < 0.001, n = 9 respectively). PG did not elicit any changes in LDH release. Treatment of air-liquid interface cultures with PG:VG (3%, 15ml apical) inhibited basolateral but not apical glucose uptake (p<0.01,

n=4) in H441 cells and the effect was similar in HBEC when compared to a PBS control. Inhibition was less than that induced by the GLUT transport inhibitor phloretin. No effect of PG:VG on uptake was determined when compared to 3% mannitol. FRAP analysis of GFP linked glucose transporter GLUT1 indicated that both PG:VG and mannitol reduced recovery of fluorescence compared to control.

These data indicate that exposure to PG:VG reduces glucose uptake in airway cells via mechanisms that could include osmotic effects and alteration in the turnover of glucose transporters at the membrane.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB108

### Role of neutral amino acid transporter LAT1 in acute pancreatitis regeneration

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The exocrine pancreas has a remarkable digestive enzymes synthesis capacity and therefore efficiently imports amino acids. We have identified and localized the amino acid transporters (AATs) LAT1/slc7a5, LAT2/slc7a8, SNAT3/slc38a3 and SNAT5/slc38a5 to the basolateral membrane of acinar cells suggesting they play a role in amino acids accumulation in these cells (Rooman, 2013). During acute pancreatitis (AP), enzyme synthesis is decreased and amino acids may support regeneration and proliferation. LAT1 has been shown to be overexpressed in proliferating cells, so we aimed to analyze the role of this transporter during regeneration after AP induction.

AP was induced in male and female wildtype (n=28) and tamoxifen inducible LAT1-knockout (Slc7a5<sup>fl/fl</sup> Rosa26-CreER<sup>T2</sup>) mice (n=48). After caerulein (CAE, 50 µg/Kg or saline 12 times hourly / IP) injection, animals were followed up to 7 days. For LAT1-cre mice, CAE was injected 1 week after tamoxifen gavage (160 mg/Kg for 3 days). The disease status and pancreatic regeneration was evaluated by body weight, serum amylase and histological analysis. The mRNA expression of AATs, proliferation and differentiation markers was analyzed by qPCR. Immunofluorescence and Western blot were used to analyze protein expression pattern and amount.

Mice receiving CAE presented 20-fold elevated serum amylase compared to control, evidencing the onset of AP. The body weight loss of wildtype animals (5%) was recovered 2 days after CAE injection. The morphological analysis showed maximum proliferation 3-4 days after CAE (213±30 vs. 6±2 Ki67 positive cells/10 fields). At the end of the observational period of 7 days, tissue was morphologically normal. The expression of LAT1 was unchanged at mRNA level from 1-7 days after CAE. Protein decreased from day 1 to 3, although it was not ablated. LAT1 sustained expression during regeneration

suggests it may play a role not only in protein synthesis but in cell survival, homeostasis and proliferation.

To confirm LAT1 role on acinar cells regeneration, we induced AP in LAT1-cre<sup>+</sup> and cre<sup>-</sup> mice. Increased serum amylase (49233±1557 vs. 2777±95 U/l) confirmed AP. LAT1-cre<sup>+</sup> mice had a higher weight loss (10%) and did not recover even 7 days after CAE injection. Additionally they had more acinar-to-ductal metaplasia compared to cre<sup>-</sup>, suggesting a delay in regeneration. The expression of differentiated acinar cell markers MIST and amylase was still reduced 3 and 7 days after CAE, suggesting the LAT1-cre<sup>+</sup> cells were less functional.

LAT1 is being associated in different metabolic pathways controlling cell size, survival and energy balance in cancer cells. Our preliminary results suggest LAT1 plays also a key role in physiological proliferative phase involved in AP regeneration. We are currently analyzing acinar cells fate (Ki67, p-histone 3, cleaved-Caspase 3), volume as well as up- and downstream regulators for LAT1 transporter.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB109

### Bile Acids Regulate Expression of Aryl Hydrocarbon Receptor in Colonic Epithelial Cells.

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The aryl hydrocarbon receptor (AHR) is expressed in the colonic mucosa, where it acts as a receptor for numerous substances, including bilirubin. Activation of AHR by endogenous ligands has been reported to suppress mucosal immune cell activation and to prevent inflammation. However, factors that regulate the expression of AHR in the colonic epithelium are still poorly understood. Here, we set out to conduct a preliminary investigation of the potential role for bile acids in regulation of colonic epithelial AHR expression.

T84 colonic epithelial cells were grown as polarised monolayers on transparent permeable supports until they reached a transepithelial electrical resistance of 1000 Ωcm<sup>2</sup>. Cells were treated for various periods of time (0 – 48 hrs) with the most common human colonic bile acid, deoxycholic acid (DCA, 150 µM), or with GW4064 (5 µM), a specific agonist of the nuclear bile acid receptor, FXR. Levels of the gene transcript, AHR were quantified by RT-qPCR and the data were expressed as the mean ± SEM fold change (2-ΔΔCT) in expression.

Treatment of T84 cells with DCA, resulted in an increase over time (0-48 hrs) in the expression of AHR. Maximal responses to DCA occurred after 48 hrs at which time expression of AHR mRNA was 5.48 ± 0.05 fold of that in control cells (n=4). To determine if the actions of DCA might be mediated by activation of FXR, cells were treated with the selective agonist, GW4064. However, in contrast to DCA, GW4064 downregulated AHR expression by 0.53 ± 0.01 fold after 48 hrs of treatment (n=3). These data suggest that luminal bile acids have the capacity to modulate AHR expression in the colonic epithelium. Such actions may be important in the setting of colonic inflammation and future studies will aim to elucidate

the physiological significance and molecular mechanisms involved.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB110

### ***Bordetella pseudohinzii* targets cilia and impairs tracheal cilia-driven transport in naturally-acquired infection in mice**

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**Objective:** Characterization of the pathogenic potential of the newly described *Bordetella* strain, *Bordetella pseudohinzii*, which colonizes the respiratory tract of laboratory mice.

**Methods:** Bacterial genome was analyzed using Illumina NextSeq 500 next generation sequencing system. Tracheal particle transport speed (PTS) and ciliary beat frequency (CBF) were analyzed in explanted tracheas with a high speed camera and by tracking dynabeads. Histopathological changes of the airways were analyzed by H&E staining. Cultured tracheal rings were infected with isolated *Bordetella pseudohinzii* for 4 or 24 h and attachment of bacteria was visualized by electron microscopy (SEM and TEM); CBF and PTS were recorded after 4 h of infection.

**Results:** Four bacterial isolates from lung and trachea of SPF-kept mice were identified as *Bordetella pseudohinzii* by genomic sequencing, presenting as rod-shaped coccobacilli with peritrichous flagellae in electron microscopy. Naturally-acquired infection with *B. pseudohinzii* lead to decreased number of tracheal ciliated cells (42.42 %, n=8, uninfected to 30.61.7 % infected, n=5; meanSEM), reduced CBF (18.21 0.60 to 12.121.48 Hz, n=7) and PTS (baseline: 60.72.76, n=19, to 24.512.79 µm/s, n=11). *B. pseudohinzii*-positive animals showed no clinical signs of infection, but neutrophils in BAL were highly increased (0.240.04 to 14.394.6 %; n=6). Histopathological analysis revealed tracheitis, interstitial pneumonia, and formation of tertiary lymphoid follicles (bronchus-associated lymphoid tissue = BALT) along the main and secondary bronchi. In in vitro-infected tracheal rings, *B. pseudohinzii* attached to ciliated cells, formed biofilms and damaged the epithelium. PTS and CBF were significantly reduced after incubation for 4 h with 1.6x10<sup>5</sup> CFU.

**Conclusion:** Colonization of mice with *B. pseudohinzii* leads to BAL neutrophilia, inflammation of the respiratory tract and impaired mucociliary clearance due to reduction and damage of ciliated cells. *B. pseudohinzii* may represent a novel mouse specific model organism to study closely related human lung pathogens like *Bordetella hinzii* and pertussis.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB111

### **Platelets and von-Willebrand Factor – A two-component adhesive for hematogenous metastasis**

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Metastasis is still a huge problem in clinical treatment of several types of cancer. Platelets play a pivotal role during hematogenous metastasis by protecting cancer cells from immune surveillance by forming a protective coat.

We discovered a different way how platelets are interacting with cancer cells. Own observations showed that A549 lung cancer cells phagocytize platelets and recycle platelet proteins to their plasma membrane, including GP IX (CD42a), a constituent of the von-Willebrand-Factor-receptor-complex. Platelets also contain von-Willebrand-Factor (vWF) and first data showed that vWF might be utilized as well by cancer cells.

VWF is stored in platelet alpha granules and released upon platelet activation. It is a huge multimeric protein with several negative, positive, hydrophilic and lipophilic domains which can bind to many different targets. We assume that cancer cells use platelets' vWF as a glue to adhere to the vessel wall: VWF is released upon contact to cancer cells, binds to the cancer cell by phagocytic recycled von-Willebrand-Factor-receptor-complex and serves as bridging element to the vessel wall.

#### **Methods**

AFM-based single-cell force spectroscopy (SCFS) was used to quantify the adhesion between immobilized vWF and A549 lung cancer cells. A549 cells were incubated with PKH-stained platelets for 60 minutes. Platelet uptake was verified *in situ* by fluorescence microscopy. A549 cells without platelet contact served as control.

Single A549 cells were glued to a tiplless cantilever and force-distance cycles were performed on vWF-coated surface. Results

The adhesion of A549 cells to vWF reaches a median of 416,89 pN (Q1: 357,53 pN; Q3: 515,11 pN) and a detachment work of 1,29 fJ (Q1: 1,19 fJ; Q3: 2,67 fJ) whereas A549 cells incubated with platelets prior to adhesion measurements show a much larger median adhesion 633,02 pN (Q1: 361,78 pN; Q3: 4131,75 pN) and a detachment work of 3,69 fJ (Q1: 0,9 fJ; Q3: 34,01 fJ). In some experiments including platelet incubation the adhesion was so strong that A549 cells were ripped of the cantilever. It also seems to be the case that A549 cells after platelet incubation immediately stick to the surface after adding them to the experimental dish.

#### **Conclusion**

Phagocytosis of platelets increases the adhesion of A549 lung cancer cells to vWF dramatically. We suppose that platelet uptake is accompanied with vWF release and followed by phagocytic recycling of GP IX which enables the cancer cell to adhere to vWF. It is conceivable that cancer cells use this strategy for sustained tethering to the vessel wall as the first step of metastasis.

Adhesion might be reduced and therefore hematogenous metastasis impeded by interfering with phagocytosis of platelets, the phagocytic recycling pathways of cancer cells or the von-Willebrand-Factor-receptor-complex.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.



PCB112

### HER2 and p95HER2 differentially regulate miRNA expression, downregulate MYB proteins, and increase invasiveness through miR-221/222 and miR-503 in MCF-7 breast cancer cells

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The HER2 oncogene and its constitutively active, truncated form p95HER2 play central roles in breast cancer<sup>1</sup>. The phenotypes they elicit are not identical, yet the underlying differences are incompletely understood. The aim of this study was to characterize the impact of HER2 and p95HER2 on the miRNA signature of human breast cancer cells and the resulting downstream signaling events shaping cancer invasiveness.

High-throughput miRNA profiling was carried out 15, 36 and 60 h after inducible HER2 or p95HER2 expression in MCF-7 luminal breast cancer cells, and central hits were validated by RT-qPCR. miRNAs which were strongly upregulated by p95HER2, yet not by HER2, included miR-221, miR-222, and miR-503. The c-Myb family transcription factors MYB and MYBL1, but not a third family member, MYBL2, were downregulated by p95HER2 as well as by miR-503- or miR-221/-222 mimics. A single point mutation in the MYBL1 3' untranslated region abolished the upregulation by miR221/-222. p95HER2 expression, or knockdown (KD) of either MYB protein, elicited upregulation of tissue inhibitor of matrix metalloprotease-2 (TIMP2), an important regulator of cancer invasiveness. Collectively, this suggests that p95HER2 expression elevates miR-221, -222, and -503 levels, in turn downregulating MYB and MYBL1 protein expression, which in turn favors upregulation of TIMP2. Similar results were obtained in T47D and SKBr-3 human breast cancer cells.

To investigate the effect of this pathway on migration and invasion, vector- and p95HER2-overexpressing cells were subjected to Boyden chamber migration and invasion assays. miR-221/-222 and miR-503 mimics elicited markedly increased migration in both cell types. Invasion was also increased by miR-222 (1.5 fold higher), miR-221/-222 (2.5 fold higher) and miR-503 (4 fold higher) in p95HER2 cells (all normalized to vector neg. miR 40 nM), whereas in vector cells, these miRNAs had little or no effect on invasion (n=3). TIMP2 KD decreased invasion of both vector- and p95HER2 overexpressing cells (n=3).

Collectively, this work reveals important differences between HER2- and p95HER2- mediated miRNA changes in breast cancer cells, and points to the existence of a signaling pathway from p95HER2 to miR-221/-222 and miR-503 via MYB proteins to TIMP2, which appears to contribute to breast cancer invasiveness.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCB113

### Cystic fibrosis mutations at position S549 modulate CFTR Cl<sup>-</sup> channel processing, gating and responsiveness to mutation-specific therapies

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Cystic fibrosis (CF) is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), a Cl<sup>-</sup> ion channel evolved from an ATP binding cassette (ABC) transporter, widely expressed in epithelial tissues. It is activated by protein kinase A (PKA) and gated by the binding and hydrolysis of ATP at two nucleotide-binding domains (NBDs). ATP binding opens the channel by initiating NBD dimerization, while ATP hydrolysis partially separates the NBD dimer to close the channel. Position S549 is highly conserved in ABC transporters and is believed to play a role in facilitating ATP binding and hydrolysis. Three CF mutations at this position have been reported in patients, S549N, S549R and S549I. To better understand the molecular dysfunction caused by these mutations, we studied wild-type and mutant CFTR protein processing, channel gating and responsiveness to CFTR modulators. We also investigated two additional mutations, S549C- and S549F-CFTR, to probe the role of S549 in channel gating. For our studies, we utilised recombinant Fischer Rat Thyroid (FRT) and CHO cells and Western-blotting, Ussing chambers and patch-clamp recording. The amount of fully-glycosylated S549N-CFTR expressed in FRT cells was similar to wild-type CFTR when cellular mRNA levels were held constant. By contrast, the expression of fully-glycosylated S549R- and S549I-CFTR were markedly diminished. Lumacaftor (3 µM) increased the amount of fully-glycosylated S549N- and S549R-CFTR by approximately 2-fold and enhanced the magnitude of CFTR-mediated Cl<sup>-</sup> current. By contrast, lumacaftor (3 µM) failed to rescue S549I-CFTR protein and had little effect on the magnitude of CFTR-mediated Cl<sup>-</sup> currents. Ivacaftor (5 µM) doubled the activity of S549N- and S549R-CFTR, but did not potentiate the activity of S549I-CFTR. To further understand the molecular defects introduced by these mutations, we studied single channels using excised inside-out membrane patches. To enhance the plasma membrane expression of mutant Cl<sup>-</sup> channels, we incubated transfected CHO cells at 27 °C for > 48 hours. The single channel activities of S549N- and S549R-CFTR were disrupted severely when compared to wild-type CFTR with bursts of channel openings separated by prolonged channel closures. By contrast, S549I-CFTR modulated, but did not disrupt, CFTR channel gating. Moreover, the apparent ATP affinities of S549N- and S549R-, but not S549I-CFTR, were profoundly decreased compared to wild-type CFTR. To probe further the role of S549, we examined the gating properties of S549C- and S549F-CFTR. S549C-CFTR differs from wild-type CFTR by only one atom, whereas S549F-CFTR introduces a bulky hydrophobic amino acid. Like S549I-CFTR, both S549C- and S549F-CFTR modulated, but did not disrupt CFTR channel gating. Altogether, the above investigations suggest that S549 plays a key role in CFTR folding, gating and responsiveness to CFTR modulators.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB114

### Intestinal Micro biota Translocation after hemorrhagic shock in a rat model: Role of Gut-Liver Axis

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**Introduction:** Hemorrhagic shock is a frequent complication in trauma patients, after gastrointestinal bleeding and major surgery. The intestine is one of the first organs affected by hemorrhagic shock. An early event in intestinal damage is gut wall integrity loss, including the opening or breakdown of tight junctions. In this study, the sequence of events in the gut after hemorrhagic shock which causing tight junction loss and bacterial translocation were studied. **Aim of the study:** The aim of this study was to investigate the effect of the hemorrhagic shock on intestinal epithelial barrier function in a rat model. **Material and Methods:** This study was carried out on 40 male Wistar albino rats 4-6 months old with body weight (250-300g). Rats were divided randomly into four main groups: Group 1 (Control group): It consisted of 10 normal healthy rats served as control and sacrificed without intervention. Group 2 (30 minutes group): Consisted of 10 rats which were subjected to a non-lethal hemorrhagic shock. Rats were sacrificed (30 minutes) after the onset of hemorrhagic shock. Group 3 (60 minutes group): Consisted of 10 rats which were subjected to a non-lethal hemorrhagic shock. Rats were sacrificed (60 minutes) after the onset of hemorrhagic shock. Group 4 (90 minutes group): Consisted of 10 rats which were subjected to a non-lethal hemorrhagic shock and sacrificed (90 minutes) after the hemorrhagic shock. At the end of the study: Blood samples were collected for measurement of serum tumor necrosis factor alpha (TNF- $\alpha$ ) and serum lipopolysaccharide (LPS). Distal segments of the ileum were excised immediately for histopathological study and measuring Zonula Occludens (ZO-1) gene expression. **Results:** TNF- $\alpha$  serum levels were significantly increased in all groups of hemorrhagic shock in comparison to their levels in the control group. Serum LPS levels measured in hemorrhagic groups showed significant increase in 60 and 90 min. groups when compared to the control group, while 30 min. group showed no significant change when compared to the control group. Histopathological study of the ileum segment revealed extension of the subepithelial space, focal mucosal infarction, a massive lifting down sides of the villi, some villus tips are denuded and epithelial cells are severely injured. PCR study of ZO-1 showed significant increase in ZO-1 gene expression in all groups of hemorrhagic shock as compared with the control group. PCR study of bacterial receptors in the liver (TLR4) showed significant increase in TLR4 gene expression in all groups of hemorrhagic shock as compared with the control group, which indicated bacterial translocation. **Conclusion:** This study gives more insight into the sequence of events in the gut after hemorrhagic shock. Hemorrhagic shock leads to intestinal tight junction integrity loss. This is followed by bacterial translocation and systemic inflammation. **Keywords:** hemorrhagic shock, zonula occluden-1, Bacterial translocation.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB115

### Non-neuronal secretion of acetylcholine by human colonic crypts triggers calcium signals in LGR5-positive intestinal stem cells and promotes crypt progenitor cell proliferation

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The human colonic epithelium is highly innervated by cholinergic neurons and acetylcholine (ACh) has been implicated in regulating intestinal fluid/mucus secretion, stem cell-driven tissue renewal, epithelial integrity and regeneration, and colon carcinogenesis. Recent studies have suggested that murine Tuft cells express choline acetyltransferase (ChAT) and provide a source of non-neuronal of ACh. The aim of this study was to determine whether the human colonic epithelium can synthesise ACh and regulate intestinal stem/progenitor cell biology. **Methods:** 3D cultures of human colonic crypts were generated from colorectal tissue samples obtained at surgical resection (NRES approval). Intracellular calcium was monitored by Fura2/Fluo4 fluorescence imaging. Transcript expression was assessed by RT-PCR and protein localisation was visualised by immunofluorescence labelling. The concentration of ACh secreted into the media of cultured human colonic crypts was quantified by HILIC-coupled tandem mass spectrometry (HILIC-MS/MS). Cultured human colonic crypt cell proliferation was assessed by nuclear EdU incorporation. **Results:** Bath application of ACh, carbachol or muscarinic agonists methacholine and oxotremorine triggered a calcium signal at the apical pole of slender cells located at the very base of human colonic crypts. Live cell immunofluorescence labelling with antibodies specific for LGR5 and PTK7, confirmed that the cholinergic calcium signal originated in intestinal stem cells. Co-immunofluorescence labelling of markers for ER (e.g. KDEL), endolysosomes (e.g. LAMP1 and lysotracker), InsP3Rs, RyRs, and two-pore channels (TPCs) suggested that calcium was mobilised from endolysosomes via TPCs located at the apical pole of intestinal stem cells. This was confirmed by pharmacological inhibition of calcium signal generation by TPC inhibitors NED-19 (100  $\mu$ M) and diltiazem (100  $\mu$ M), with only partial inhibition by ryanodine (50  $\mu$ M), 8-bromo cADP ribose (30  $\mu$ M), xestospongine C (2  $\mu$ M) and 2-APB (50  $\mu$ M). NED-19 autofluorescence also co-localised with the cholinergic calcium signal trigger site. RT-PCR demonstrated the expression of CHAT mRNA and the protein co-immunolocalised with markers of intestinal Tuft cells, which predominated within the stem cell niche. Media conditioned for 12-24 hours with human colonic crypts was found by HILIC-MS/MS to contain an ACh concentration of  $90 \pm 10$  nM (N = 5 subjects). Accordingly, crypt-conditioned media initiated a calcium signal in the stem cell niche of similar amplitude to that invoked by addition of exogenous ACh (100 nM, N = 3 subjects). Finally, exogenous ACh stimulated an increase in EdU positive nuclei from  $18 \pm 2\%$  to  $44 \pm 5\%$  ( $n > 5$  crypts in each group; representative of  $N > 5$  subjects) in the human colonic stem cell niche. **Conclusions:** Non-neuronal secretion of ACh by human colonic crypts triggers calcium signals in LGR5-positive intestinal stem cells and promotes crypt progenitor cell proliferation.

Humane Research Trust

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB116

**Gastric ulcer healing effects of virgin coconut oil in experimental animals**O.S. Adeniyi<sup>1</sup>, E.U. Eru<sup>1</sup>, J.J. Oloche<sup>2</sup> and R. Vhirtherhire<sup>3</sup><sup>1</sup>Physiology, Benue State University, Makurdi, Nigeria, Makurdi, Nigeria, <sup>2</sup>Pharmacology, Benue State University, Makurdi, Nigeria, Makurdi, Nigeria and <sup>3</sup>Anatomical Pathology, Benue State University, Makurdi, Nigeria, Makurdi, Nigeria

Gastric ulcer is the most prevalent gastrointestinal disease ever known, accounting for an estimated 15 mortality out of every 15,000 complications yearly worldwide. This disease is mainly treated using western medicine, but these drugs possess side effects and the ulcer often recurs. Therefore, the use of natural products has gained the interest of many researchers, and virgin coconut oil (VCO) is promoted as healthy oil with several health benefits. Consequently, this study was carried out to investigate the effects of VCO on the healing of gastric ulcer in experimental animals. Male albino Wistar rats weighing 150 – 200g were divided into 5 groups: control without ulcer, and the others groups were ulcerated rats treated with 1 mL/kg normal saline (NS), 5 mL/kg VCO, 10 mL/kg VCO and 20 mg/kg omeprazole respectively. Gastric ulcer was induced by application of acetic acid using the gastric kissing model in animals anaesthetized with 50mg/kg thiopentone sodium (i.p). All treatments were done daily using oral route for 9 days, and the assessment of ulcer healing was done on day 3 and 9 after ulcer induction by macroscopic measurement of ulcer area. Lipid peroxidation, superoxide dismutase activity and histological assessment of the stomach tissue were also carried out. Values were expressed as mean  $\pm$  SEM and compared by ANOVA with Bonferroni post hoc test,  $n = 5$  and difference was considered significant when  $P < 0.05$ . By day 3, there was no significant difference in the macroscopic area of ulcer among the rats with ulcer. By day 9 however, the percentage area healed in NS treated (66.15%) was significantly lower than in 5 mL/kg VCO treated (84.67%), 10 mL VCO treated (83.59%) and omeprazole treated (80.41%). By day 9, lipid peroxidation in NS treated rats ( $1.60 \pm 0.06 \mu\text{mol}/\text{MDA}/\text{mg protein}$ ) was significantly higher than in 5 mL/kg VCO treated ( $1.20 \pm 0.04 \mu\text{mol}/\text{MDA}/\text{mg protein}$ ) and 10 mL/kg VCO treated rats ( $1.13 \pm 0.02 \mu\text{mol}/\text{MDA}/\text{mg protein}$ ). Superoxide dismutase activity was significantly higher in VCO treated than in NS treated rats. Furthermore, re-epithelization and clearing of inflammatory cells was greater in VCO treated than in NS treated, omeprazole treated and control animals. We conclude that VCO accelerated gastric ulcer healing by facilitating mucosal re-epithelization, increasing antioxidant activity, and reducing oxidative stress.

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PCB117

**Gestational and developmental exposure to polychlorinated biphenyls (PCBs) in the maternal diet causes impaired intestinal physiology and altered host-microbe interactions in genetically susceptible mice**K. Rude<sup>1</sup>, J. Sladek<sup>1</sup>, M. Pusceddu<sup>1</sup>, S. Sethi<sup>2</sup>, K. Keil<sup>2</sup>, I. Pessah<sup>2</sup>, P. Lein<sup>2</sup> and M. Gareau<sup>1</sup><sup>1</sup>Anatomy, Physiology and Cell Biology, University of California Davis, Davis, CA, USA and <sup>2</sup>Molecular Biosciences, University of California Davis, Davis, CA, USA

The microbiota-gut-brain (MGB)-axis is increasingly being recognized as an important physiological pathway for maintaining health and homeostasis with disruptions identified in numerous disease states, including inflammatory bowel disease (IBD), major depressive disorders, and autism spectrum disorders (ASD). Early neonatal life is an important stage for normal colonization of the microbiota, development of gastrointestinal physiology and neuronal growth and maturation, which together form the MGB-axis. Exposure to pathophysiological stimuli during development can potentially impact the MGB axis detrimentally. Exposure of the developing brain to neurotoxins, including polychlorinated biphenyls (PCBs) and intestinal dysbiosis have independently been implicated in the origin of neurodevelopmental disorders (NDDs). Therefore, we hypothesized that PCB-induced dysbiosis causes intestinal pathophysiology, including mucosal barrier deficits and subsequent inflammation in a genetically susceptible host. Intestinal physiology and the composition of the microbiota were assessed following developmental exposure to PCBs during late gestational and early neonatal life in both neurotypic and mice expressing risk genes for neurodevelopmental disorders (NDD). Both wild type (WT - C57BL/6-SV129) and double mutant (DM - *Ryr1*[T4826I] *gain of function mutation and X-linked FMR1 premutation* [180-200 CGG repeats]) juvenile mice (P28-30) were used to assess host-microbe interactions and gene by environment interactions following PCB exposure. Dams were exposed to a mixture of PCB congeners starting 2 weeks prior to mating and continued until weaning. Developmental PCB exposure caused ileal and colonic mucosal barrier defects (increased secretory state and permeability deficits), alterations in the ileal and colonic inflammatory profile (increased pro-inflammatory and decreased anti-inflammatory cytokines) in DM mice at P29-P30. Dysbiosis, including decreased beta-diversity and changes in abundance at the phylum level, were also observed at P28-P30 in DM mice vs. WT controls. These PCB-induced changes were only observed in DM mice following administration of 1 mg/kg of PCBs but not 0.1 or 6 mg/kg, suggesting a non-linear dose effect in a genetically susceptible host. In conclusion, we demonstrated that maternal exposure to developmental neurotoxins can negatively impact both colonization of the gut microbiota and establishment of intestinal mucosal barrier function in weaning mice that are genetically susceptible to NDD.

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## PCB118

**Application of ethanol with fatty acids induces  $\text{Ca}^{2+}$  responses in pancreatic stellate cells *in vitro* and leads to activation of these cells *in vivo***

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Pancreatic fibrosis, a frequent complication of alcoholic pancreatitis, is a result of an excessive activation of pancreatic stellate cells (PSCs), which assume a myofibroblast-like phenotype and deposit collagen fibres in the tissue. The products of non-oxidative alcohol metabolism, fatty acid ethyl esters formed by ethanol and fatty acids, have long been known to cause pancreatic acinar cell (PAC) injury via induction of excessive  $\text{Ca}^{2+}$  responses, leading to premature intracellular activation of digestive enzymes (1). Although it has been suggested that ethanol may contribute to PSC activation (2), the exact mechanism of pathology induced by ethanol and its metabolites has not yet been elucidated in PSCs. Recently it has become clear that PACs and PSCs, despite their very close proximity in the tissue, respond differently, in terms of  $\text{Ca}^{2+}$  signals, to physiological and pathophysiological stimuli (3). Therefore it becomes essential to examine the effects caused by ethanol metabolites in PSCs and understand their role in PSC activation and the development of pancreatic fibrosis.

To achieve this, the effects of acute administration of ethanol and palmitoleic acid (POA) on intracellular  $\text{Ca}^{2+}$  were investigated in human PSCs. Also, the expression of fibrotic markers in PSCs was examined *in vivo* in a recently established mouse model of alcoholic acute pancreatitis and compared to models of bile-induced pancreatitis (procedures performed in accordance with UK Home Office regulations).

POA/ethanol (10  $\mu\text{M}$ /10mM to 200  $\mu\text{M}$ /200mM) induced global and sustained cytosolic  $\text{Ca}^{2+}$  rises in PSCs. Complete removal of extracellular  $\text{Ca}^{2+}$  reduced but did not abolish the POA/ethanol-induced  $\text{Ca}^{2+}$  elevation in PSCs, indicating that the initial  $\text{Ca}^{2+}$  release from the ER triggers further  $\text{Ca}^{2+}$  influx to the cytosol from the extracellular environment. After the ER store was depleted with cyclopiazonic acid (CPA), POA/ethanol failed to trigger  $\text{Ca}^{2+}$  responses in PSCs.

Further, POA and ethanol induced acute pancreatitis *in vivo*, which was characterised by mild to moderate pancreatic oedema, necrosis, immune cell infiltration and activity of myeloperoxidase. Importantly POA/ethanol increased the expression of  $\alpha$ -SMA in between pancreatic acini, which has been attributed to the increased activation of PSCs. In the model of alcoholic pancreatitis the  $\alpha$ -SMA expressing cells were uniformly distributed across the entire tissue, whereas in two models of bile-induced pancreatitis elevated expression of  $\alpha$ -SMA was limited only to the necrotic areas.

Our study sheds new light on the current understanding of mechanisms underlying alcohol metabolite-induced

pancreatic pathology, which involves not only signalling in PACs but also in PSCs, likely leading to their activation *in vivo*. Our results also suggest that despite a relatively mild severity, in alcoholic pancreatitis PSCs become activated uniformly throughout the pancreas, possibly even to a greater extent compared to bile-induced pancreatitis.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB119

**Selective inhibition of Bcl-2 by venetoclax (ABT-199) does not induce intracellular  $\text{Ca}^{2+}$  responses or cell death in pancreatic acinar cells**

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Many cancer cells depend on the mitochondrial function of anti-apoptotic B cell lymphoma 2 (Bcl-2) proteins for their survival. Therefore Bcl-2 antagonism through BH3 mimetics emerged as a precision anti-cancer therapy. We previously showed that early Bcl-2 inhibitors BH3I-2' and HA14-1 elicit sustained  $\text{Ca}^{2+}$  responses in pancreatic acinar cells (PACs) inducing not only apoptosis but also cell necrosis. Because of this, BH3 mimetics could potentially be toxic for the pancreas when used to treat cancer. Venetoclax (ABT-199) is a recently developed selective Bcl-2 inhibitor which was introduced into the clinic for the treatment of relapsed chronic lymphocytic leukaemia patients. Although Venetoclax was shown to kill Bcl-2-dependent cancer cells without affecting intracellular  $\text{Ca}^{2+}$  signalling, its effects on PACs have not yet been determined. Therefore, it becomes essential and timely to assess whether the recently approved anti-leukemic drug might have potentially pancreatotoxic effects.

In this study single-cell real-time  $\text{Ca}^{2+}$  measurements and cell death analysis were performed on freshly isolated mouse PACs. We show here that selective inhibition of Bcl-2 via ABT-199 neither elicited intracellular  $\text{Ca}^{2+}$  signalling on its own nor altered  $\text{Ca}^{2+}$  signalling induced by physiological/pathophysiological stimuli in PACs. Also, cell death was mostly unaffected by ABT-199. In contrast, selective inhibition of Bcl-X<sub>L</sub> or inhibition of multiple anti-apoptotic Bcl-2 family proteins potentiated pathophysiological  $\text{Ca}^{2+}$  responses in PACs, but without exacerbating cell death. Selective inhibition of either Bcl-2 or Bcl-X<sub>L</sub> provided a modest protective effect against cell death induced by menadione.

In conclusion, our results demonstrate that ABT-199 does not alter intracellular  $\text{Ca}^{2+}$  homeostasis in normal PACs and thus should be safe for the pancreas when used for treating cancer.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB120

**Taurodeoxycholate inhibits ion secretion via basolateral TGR5 receptors on L-cells in mouse ileal and colonic mucosae.**

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Bile acids (BAs) are released into the duodenum to facilitate absorption of dietary fat and reabsorbed via the enterocyte apical bile salt transporter, ASBT, primarily in the ileum but also in the colon. The BAs cell-surface receptor, TGR5 is expressed by L cells that are found throughout the intestinal tract and release postprandially the satiety and incretin hormones peptide YY (PYY) and glucagon-like peptide 1 (GLP-1), respectively (1). PYY and GLP-1 are released following intrarectal infusion of taurocholate in diabetic patients (2) and by taurodeoxycholate (TDCL) stimulation of isolated L cells (3), thus TGR5 is a putative target for antiobesity and type 2 diabetes agents. Recently we observed antisecretory responses to apically (ap) or basolaterally (bl) added TGR5 agonist in mouse colon mucosa, similar to glucose-dependent, PYY-mediated, GPR119 responses (4). However, unlike GPR119, a latency in ap responses was noted. Here we investigated the effect of TDCL on ion secretion in muscle-stripped terminal ileum or distal colon mucosa from C57BL/6J129Sv mice (>10 weeks, male and female), voltage-clamped in Ussing chambers as described previously (4). Mucosae were prestimulated with vasoactive intestinal polypeptide (10 nM) prior to ap or bl additions of TDCL (10  $\mu$ M -1 mM), with responses measured as changes in short-circuit current (Isc). Values were pooled from mucosal responses in different animals and expressed as means $\pm$ 1S.E.M., compared using Student's t test or ANOVA. TDCL (ap) responses were monophasic and reduced Isc whereas bl responses were biphasic, with primary increases followed by decreases in Isc. ASBT1 blockade with 10  $\mu$ M GSK2330672 (5) blunted ap TDCL (100  $\mu$ M) responses from  $-13.4\pm 1.0$   $\mu$ A.cm<sup>-2</sup> to  $-3.5\pm 1.0$   $\mu$ A.cm<sup>-2</sup> (n=4; p<0.001) in terminal ileum and from  $-21.0\pm 1.7$   $\mu$ A.cm<sup>-2</sup> to  $-8.3\pm 2.4$   $\mu$ A.cm<sup>-2</sup> (n=5; p<0.01) in distal colon. TDCL antisecretory responses were concentration-dependent in distal colon (ap and bl pEC50 values:  $4.0\pm 0.1$  and  $4.1\pm 0.1$ , with maximal Isc reductions of  $-32.2\pm 1.7$   $\mu$ A.cm<sup>-2</sup> and  $-49.1\pm 1.7$   $\mu$ A.cm<sup>-2</sup>, respectively), whereas no maximum response was reached for bl secretory responses. Additionally, bl responses were unaffected by TTX (100 nM) pretreatment; however, the PYY Y1 receptor blocker BIBO3304 (300 nM) potentiated the secretory phase (from  $12.7\pm 4.7$  to  $38.1\pm 5.8$   $\mu$ A.cm<sup>-2</sup>; n=5, p<0.01) and abolished the secondary phase (from  $-36.9\pm 7.2$  to  $-2.0\pm 0.9$   $\mu$ A.cm<sup>-2</sup>; n=5, p<0.001). Furthermore, replacement of apical glucose with mannitol attenuated the bl antisecretory phase (from  $-36.1\pm 1.6$  to  $-11.1\pm 3.7$   $\mu$ A.cm<sup>-2</sup>; n=5, p<0.001) in distal colon. We conclude that in the mouse intestine, ap transported BAs stimulate TGR5 on the bl membranes of L cells to cause glucose-dependent release of PYY, which subsequently has a paracrine antisecretory action via Y1 receptors. Symonds et al, (2015). *Gut* 64, 618-26.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB121

**Transformation of nuclear pores promotes human lung cancer malignancy**I. Luchtefeld<sup>1</sup>, I. Liashkovich<sup>1</sup>, I. Azzam<sup>1</sup>, G. Rosso<sup>2</sup> and V. Shahin<sup>1</sup><sup>1</sup>Institute of Physiology II, University of Münster, Münster, Germany and <sup>2</sup>BIOTEC, University of Dresden, Dresden, Germany

Nuclear pore complexes (NPCs) are of particular physiological importance. They form highly selective transport barrier for all molecules between the cytosol and the nucleus thereby safeguarding the enclosed nuclear DNA. Several NPC proteins are potent gene transcription regulators and are thus of substantial importance for development, differentiation and maintenance of cells. Their expression beyond or below physiological levels is linked to cancer onset and progression. These links, however, remain unclear. Exploring the links between NPCs and cancer has therefore become an attractive target in cancer research. Our goal is to shed light onto the relationship between NPCs and cancer. We therefore set out to study from multiple aspects the differences between NPCs in non-metastatic compared to highly aggressive non-small human lung cancer cell lines. Our findings demonstrate that transformation in NPCs barrier function, permeability and molecular composition promotes malignancy of lung cancer cells. Moreover, controlled breakdown of NPC barrier function following exposure to certain drugs prevents cancer cell migration. The observations made here present direct links between NPCs transformation and cancer malignancy and may usher in the design of new strategies for the treatment of cancer.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB122

**Neutrophil chemotaxis depends on extracellular pH and Na<sup>+</sup>/H<sup>+</sup>-exchanger activation**

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Neutrophils are the first immune cells to arrive at a site of inflammation. Acidification of the local environment is one of the major stress factors which immune cells face while approaching the core of the inflamed tissue. It is now well known, that most neutrophil functions such as ROS production, phagocytosis and apoptosis are influenced by extracellular pH (pH<sub>e</sub>). Since activated neutrophils often have to cope with harsh pH<sub>e</sub> conditions, they display multiple regulatory mechanisms to maintain their functions. In our project, we study the role of the Na<sup>+</sup>/H<sup>+</sup>-exchanger (NHE1) in murine (C57BL/6 strain) neutrophil migration and intracellular pH homeostasis upon chemoattractant stimulation in an acidic environment.

Neutrophil chemotaxis was monitored in a 3-dimensional collagen I matrix using Ibidi  $\mu$ -Slides. Live-cell imaging was performed for 30 min and images were acquired in 5s intervals. Chemotaxis was induced in gradients of different

concentrations of the complement component C5a in the absence or presence of the NHE1 inhibitor cariporide.  $pH_e$  in the chemotaxis chambers ranged from  $pH_e$  6.5 to  $pH_e$  7.6. Intracellular pH ( $pH_i$ ) of neutrophils was measured using the ratiometric pH indicator BCECF. Cells were seeded onto a fibronectin coating and superfused with Ringer's solution ( $pH$  7.4 or  $pH$  6.5) containing C5a without/with NHE1 inhibitor. All values are given as means  $\pm$  SEM, compared by ANOVA. Speed and directionality of migrating neutrophils are decreased upon NHE1 inhibition (mean speed [ $\mu m/min$ ]  $11.8 \pm 0.3$  vs control  $14.7 \pm 0.2$ ). However, the velocity in the direction of the C5a gradient is affected much stronger upon NHE1 inhibition: It is reduced by  $\sim 70\%$ . In  $pH_e$  6.5, the mean speed is reduced by  $\sim 27\%$ , whereas the velocity towards the C5a gradient is lowered by  $\sim 76\%$  (compared to values in  $pH_e$  7.0). Upon stimulation,  $pH_i$  rapidly decreases ( $\Delta pH_i$   $-0.21 \pm 0.015$ ), before stabilizing after  $\sim 2$ min. Simultaneous inhibition of NHE1 augments the acidification ( $\Delta pH_i$   $-0.51 \pm 0.04$ ). In  $pH_e$  6.5, no compensatory stabilization is detected, whereas inhibition of NHE1 again enhances the acidification upon stimulation.

Our results suggest a chemotaxis optimum at  $pH_e$  7.0, while NHE1 inhibition and a more acidic  $pH_e$  impair directed migration. Notably, acid  $pH_e$  and NHE1 inhibition affect primarily the directionality of migration, while the velocity is only slightly decreased.  $pH_i$  is similarly affected by an extracellular acidification and by NHE1 inhibition. We therefore propose that the acidification at a site of inflammation decreases speed and directionality of the migrating neutrophil by challenging the intracellular pH homeostasis. Possibly, these mechanisms imply an immunological control of neutrophil chemotaxis, helping the cells to approach the inflamed tissue, but causing a migration halt when the neutrophils arrive at the acidic core of the inflammation.

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PCB123

### Comparative electrophysiological analysis of the bile acid-sensitive ion channel (BASIC) from different species

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Despite the identification of cholangiocytes in the liver and unipolar brush cells in the cerebellum as sites of expression, the physiological function of the bile acid-sensitive ion channel (BASIC) remains unknown. Rat BASIC (rBASIC) and mouse BASIC (mBASIC) share 97% of their amino acid sequence but show strikingly different biophysical properties. rBASIC is inactive at rest while mBASIC is constitutively active, when expressed in *Xenopus* oocytes. This conundrum rendered the identification of the physiological function even more difficult. In this study we investigated the electrophysiological and pharmacological properties of BASIC from rat, mouse and human in Hek293 cells using the patch clamp technique. Surprisingly, in Hek293 cells rBASIC and mBASIC showed almost completely identical properties. Both are blocked by extracellular  $Ca^{2+}$  and thus are inactive at rest; both are selective for  $Na^+$ , show similar affinities for extracellular  $Ca^{2+}$ , were inhibited by diminazene and activated by various bile acids. This is in contrast to previous results derived from *Xenopus* oocytes as expression system and suggests that the cell type is important for shaping the biophysical properties of BASIC.

We speculate that the composition of the plasma membrane is crucial.

Furthermore, we compared hBASIC with rBASIC and mBASIC and observed similar properties between these channels with one exception: the bile acid-sensitivity profile of hBASIC is different from rBASIC and mBASIC; hBASIC is more sensitive to bile acids which are abundant in human bile but not in rodent bile. Taken together, these results might help to shed new light on the physiological role of BASIC in different tissues.

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PCB124

### Tracking the uptake of newly designed fluorescent-labeled chitosan-mRNA complexes and their influence on human bronchial epithelial cells

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Cystic fibrosis (CF) is the most lethal autosomal recessive disease due to mutations in the *cystic fibrosis transmembrane conductance regulator* (CFTR) gene. Non-viral vehicles like chitosan (CS) are of special interest for gene therapy purposes. Due to its polycationic character CS is able to form nanocomplexes with mRNA via self-assembly.

In previous studies we generated unlabeled CS-wtCFTR-mRNA complexes and successfully transfected human CF airway epithelial cells to restore CFTR function.

The aim of this study is to characterize labeled CS-wtCFTR-mRNA complexes, track their uptake and determine the impact on cell properties in human bronchial epithelial cells. Thus, wtCFTR-mRNA fused with GFP as well as wtCFTR-mRNA labeled with fluorescent UTPs were used to form complexes with CS. Complexes of varying charge ratios (+/-) were characterized for their physicochemical properties, e.g. size, polydispersity index and zeta potential.

Afterwards, using these optimized complexes, CF cells (CFBE41o-) were transfected at different time points.

Our results revealed that the complexes enter the cells after 2h of incubation in a lower amount compared to the positive control (*Lipofectamine*® 2000 transfection reagent), which lead to a slightly higher uptake of mRNA after the same time ( $n=6$ ), yet with a higher rate of cytotoxicity.

Also, checking the expression of the imported mRNA with CS as delivery system is part of the ongoing studies.

Furthermore, CFTR is directly as well as indirectly connected to the cytoskeleton and the tight junction (TJ) complex [1]. Restoration in CF cells using CS-wtCFTR-mRNA complexes may have an effect on its cellular assembly.

We compared 16HBE14o- (healthy human bronchial epithelial cells), CFBE41o- cells and transfected CFBE41o- cells using different approaches, e.g. immunofluorescence (IF) and Western Blotting (WB). These experiments revealed a different expression pattern of ZO-1 (zonula occludens-1), a major TJ protein, in 16HBE14o- compared to the CFBE41o- cells indicating a disorganization of the TJ complex in CF cells (IF:  $n=18$ , WB:  $n=3$ ). After transfection of CF cells with CS-wtCFTR-mRNA complexes we observed a reassembly of TJs similar to that of the 16HBE14o- control.

Based on the previous results we indicate an interaction between CFTR restoration and the TJ complex in human bronchial epithelial cells.



These findings demonstrate that the non-toxic CS transfection reagent is suitable to form proper nanocomplexes with wtCFTR-mRNA, restores CFTR function, reveals an impact on the cellular assembly and shows almost the same transfection efficiency as the conventional but cytotoxic transfection reagent *Lipofectamine®2000*.

Castellani S et al. (2012) *Lab Invest.* 92(11):1527-40.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB125

### **Proof of Phospholipid Scrambling activity by TMEM16E/ANOS: opposite effects of mutations causing bone dysplasia and muscular dystrophy.**

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TMEM16E is highly expressed in bone tissue and skeletal muscle and different mutations in the human TMEM16E (ANO5 or GDD1) gene are associated with the autosomal dominant bone disease gnathodiaphyseal dysplasia (GDD) or with different recessive forms of muscular dystrophy (LGMD2L, limb-girdle muscular dystrophy-2I and distal MMD3, miyoshi muscular dystrophy-3). At present, eight GDD-causing TMEM16E/Ano5 mutations have been identified leading to amino acid exchanges at six positions, while more than 70 frameshift, nonsense, insertion, deletion and aberrant splicing mutations have been reported in LGMD2L and MMD3 patients. The physiological function of TMEM16E and its role in the pathophysiology of these diseases are currently unclear. Members of the TMEM16 or anoctamin membrane protein family are involved in a variety of functions that include ion transport, phospholipid scrambling (movement of phospholipids, in particular of phosphatidylserine, between leaflets of the membrane bilayer). In particular, TMEM16A/Ano1, 16B/Ano2 and 16F/Ano6 have a clear plasma membrane localization but while TMEM16A and B work as ‘pure’ ion channels, TMEM16F shows both  $\text{Ca}^{2+}$ -dependent ion channel and scramblase activities. Recent data suggest that also TMEM16E/Ano5, the closest relative of TMEM16F, may as well work as a phospholipid scramblase.

We use patch-clamp experiments and annexin-V binding assays to address the function of human TMEM16E and the effects of mutations causing GDD and muscular dystrophy. Our data demonstrate that TMEM16E, when overexpressed in mammalian cell lines, displays partial plasma membrane localization and gives rise to phospholipid scrambling as well as non-selective ionic currents with slow time-dependent activation at highly depolarized membrane potentials and in the presence of elevated cytosolic  $\text{Ca}^{2+}$  concentrations (Di Zanni et al 2018).

Furthermore, TMEM16E proteins carrying the GDD-causing T513I exchange (Marconi et al 2013) show phospholipid scrambling activity and large time-dependent ion currents even at low cytosolic  $\text{Ca}^{2+}$  concentrations. Contrarily, the mutation associated to LGMD S555I (Savarese et al 2015), involving an amino acid located in the putative scrambling domain, causes a loss of both ionic current and scramblase activity at high intracellular  $\text{Ca}^{2+}$  concentrations.

Our data provide the first direct demonstration of  $\text{Ca}^{2+}$ -dependent phospholipid scrambling activity for TMEM16E wild type protein and suggest that a gain-of-function of this activity contributes to the pathophysiology of GDD in bone tissue, while a loss-of function leads to muscular dystrophy.

Di Zanni E, Gradogna A, Scholz-Starke J, Boccaccio A. *Gain of function of TMEM16E/ANO5 scrambling activity caused by a mutation associated with gnathodiaphyseal dysplasia.* *Cell Mol Life Sci.* 2018 May;75(9):1657-1670. doi: 10.1007/s00018-017-2704-9. Epub 2017 Nov 9.

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Di Zanni, Gradogna and Picco equally contributed

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB126

### **Inhibitor of the $\text{Ca}^{2+}$ -activated $\text{K}^+$ channel $\text{K}_{\text{Ca}}3.1$ has an impact on the extravasation of A549 lung cancer cells**

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Non-small cell lung cancer (NSCLC) is one of the leading causes of cancer related deaths worldwide. The prognosis of lung cancer patients is very poor with a 5-year survival rate that is only ~15 % in advanced stages. The early development of metastasis is one of the main reasons for this poor prognosis. Metastasis is a complex phenomenon in which the process of extravasation is of enormous importance. In principal, extravasation can be divided into the processes of adhesion to endothelial cells and transmigration through the endothelial layer. In our study, we were investigating whether the  $\text{Ca}^{2+}$  activated  $\text{K}^+$  channel  $\text{K}_{\text{Ca}}3.1$  plays a role in cell-cell adhesion of A549 NSCLC cells to HMEC-1 endothelial cells, and in the transmigration process of A549 cells. Applying single force spectroscopy, we estimated the adhesion force [nN] between A549 and HMEC-1 cells. The role of  $\text{K}_{\text{Ca}}3.1$  channels was assessed pharmacologically ( $\text{K}_{\text{Ca}}3.1$  channel blocker senicapoc) or by silencing of  $\text{K}_{\text{Ca}}3.1$  in A549 or HMEC-cells. In the presence of senicapoc the adhesion force increased to 49% ( $0.85 \pm 0.03$  nN in senicapoc vs  $0.43 \pm 0.02$  nN in DMSO). Silencing of  $\text{K}_{\text{Ca}}3.1$  in A549 cells increased the adhesion force from  $0.48 \pm 0.02$  nN (in control transfected cells) to  $0.62 \pm 0.03$  nN, in HMEC-1 cells from  $0.54 \pm 0.04$  nN to  $0.82 \pm 0.05$  nN. Western blotting ( $N = 3$ ) and immunofluorescence staining ( $N = 3$ ) reveals that the elevated adhesion force is due to increased expression of ICAM-1 in both cell lines when  $\text{K}_{\text{Ca}}3.1$  channels are silenced. Applying an anti-ICAM-1 blocking antibody abolishes the  $\text{K}_{\text{Ca}}3.1$ -dependent strengthening of cell-cell adhesion ( $N = 7$ ;  $p < 0.05$ ; One-Way ANOVA). Furthermore, using an *in vitro* approach for transendothelial migration, we found that senicapoc inhibits transendothelial migration of A549 cells by 50% ( $N = 6$ ;  $p < 0.05$ ; students t-test). This inhibitor in transendothelial migration was not found when  $\text{K}_{\text{Ca}}3.1$  channels

were silenced in A549 cells. The latter finding suggests that transendothelial migration depends predominantly on  $K_{Ca}3.1$  channels in endothelial cells.

In conclusion, our findings indicate that  $K_{Ca}3.1$  channels regulate ICAM-1 dependent cell-cell adhesion between A549 lung cancer and HMEC-1 endothelial cells and that also affects the transmigration step in the metastatic process.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB127

### Establishing an antibody to verify expression of the bovine TRPV3 channel by the rumen of cattle

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Detection of TRPV3 channels in the ruminal epithelium on the protein level is of considerable interest, since the bovine analogue of this non-selective cation channel, bTRPV3, has emerged from functional studies and mRNA data as a candidate protein for the uptake of various cations from the rumen of cattle. Among these is ammonium ( $NH_4^+$ ), the degradation product of dietary protein (1, 2). After absorption, excess ammonium needs detoxification via the liver and excretion via the kidney, with the resulting emissions of nitrogen by dairy cattle playing a considerable role in climate change. Additionally, bTRPV3 is the first known candidate suitable for mediating the uptake of calcium by the rumen (1,2), which is essential for meeting demand in dairy cattle. However, antibodies suitable for use in the bovine species are lacking.

In a first step, the epitopes of commercial antibodies against the human TRPV3 channel were aligned with the sequence of the bovine homologue. To confirm the efficiency of the antibody, standard expression systems were used. *Xenopus* oocytes were either injected with RNase-free water (control) or linearized strep-tagged bTRPV3-cRNA or hTRPV3-cRNA. Likewise, HEK-293 cells were either transiently transfected with a pIRES2-AcGFP1 vector (control) or with the same vector construct including a strep-tagged bTRPV3 or hTRPV3 sequence. Successful expression was confirmed by immunodetection (n = 8) of the strep tag. In a second step, the antibody was tested in western blots (n = 7) for both expression systems and native ruminal protein. To test the specificity of the antibody, immunohistological stainings of bTRPV3, hTRPV3, and controls in *Xenopus* oocytes (n = 3) and HEK-293 cells (n = 2) were performed. We could confirm the expression of the channel protein in the cell membrane. Finally, the commercial TRPV3 antibody revealed staining of the transporting layers of the ruminal epithelium.

In conclusion, we could establish an antibody useful for staining the bovine TRPV3 channel. Furthermore, we show that the bTRPV3 channel protein is highly expressed by the transporting syncytium of the ruminal epithelium. Additionally, successful expression of bovine and human TRPV3 was confirmed in the membrane of cellular models, which enables further functional experiments to characterize bTRPV3 and hTRPV3. Detection of TRPV3 on the protein level in the rumen is in line with functional data indicating a diverse role of the bTRPV3 channel in ruminal transport, exceeding its commonly known sensory function in other tissues.

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PCB128

### Proteolytic activation of the epithelial sodium channel: role of pro-protein convertases and prostasin

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The epithelial sodium channel (ENaC) is essential for regulating renal sodium excretion. Cleavage of the  $\gamma$ ENaC subunit increases ENaC activity both *in vitro* and *in vivo*; however, the involved endogenous proteases remain ambiguous. This study investigates the mechanistic roles of the pro-protein convertase (PCSK) family and the serine protease prostasin in proteolytic activation of ENaC.

We used the M-1 mouse collecting duct cell line that natively expresses functional ENaC channels for pharmacological inhibition studies with pro-protein convertase inhibitor decanoyl-RVKKR-chloromethyl ketone and serine protease inhibitor camostat mesilate. Knockout cell lines of prostasin and three PCSKs identified by mRNA expression were generated with the CRISPR/Cas9 system in M-1 cells. As an index of ENaC activity, the amiloride-sensitive short-circuit current ( $I_{SC}$ ) and transepithelial voltage ( $V_{TE}$ ) were measured on polarized cells on transwell filter supports in Ussing chambers.  $\gamma$ ENaC membrane expression and cleavage were tested by western blotting of surface biotinylated M-1 cells with an antibody against the C-terminal of  $\gamma$ ENaC. All values are means  $\pm$  SE, compared with students t-test or ANOVA as appropriate.

Pro-protein convertase inhibition decreased the amiloride-sensitive  $I_{SC}$  in M-1 cells compared with controls ( $25.9 \pm 1.9 \mu A$ , n=5, vs.  $45.7 \pm 7.0 \mu A$ , n=6, p<0.05). Similarly, serine protease inhibition decreased  $V_{TE}$  ( $-34.5 \pm 7.8 mV$ , n=3, vs.  $-74.2 \pm 0.9 mV$ , n=3, p<0.05) but  $I_{SC}$  was not significantly lower than controls ( $68.0 \pm 8.5 \mu A$ , n=3, vs.  $74.0 \pm 2.3 \mu A$ , n=3, n.s.). Both inhibitors decreased membrane expression of  $\gamma$ ENaC, but only serine protease inhibition reduced  $\gamma$ ENaC cleavage (n=1). Knockout of the *Pcsk3* (furin) gene completely abolished ENaC activity, while knockouts of the *Pcsk4*, *Pcsk7*, and *Prss8* (proctasin) genes all showed decreased ENaC activity compared with wild-type M-1 cells. Knockout of PCSKs reduced  $\gamma$ ENaC surface expression, but only knockout of prostasin decreased  $\gamma$ ENaC cleavage (n=1).

We propose that PCSKs are not directly involved in proteolytic ENaC activation. Both pharmacological inhibition and knockout of prostasin decreased ENaC activity and  $\gamma$ ENaC cleavage, thus suggesting a potential role in proteolytic ENaC activation.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB129

**Development of an enhanced Cystic Fibrosis Transmembrane Regulator gene for Homology-Independent Targeted Integration in Cystic Fibrosis cell lines.**

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The comparatively easy, non-invasive accessibility of the lungs to aerosols and the monogenic nature of the disease makes Cystic Fibrosis (CF) an attractive candidate for gene therapy and genetic editing. It is suggested that ~10% of endogenous cystic fibrosis transmembrane regulator (CFTR) expression within target cells *in vivo* is sufficient for prevention of lung disease<sup>[1]</sup>. This level of gene transfer/correction in patients has not yet been achieved. However, over expression of CFTR and/or the use of CFTR potentiators have been shown to restore some functions of CFTR *in vitro* more efficiently than endogenous expression alluding to treatments that may allow prevention of CF lung disease with a lower level of gene transfer/correction<sup>[2]</sup>.

We have therefore constructed an enhanced CFTR gene (CFTR<sub>enh</sub>) with codon optimization, for increased expression, and containing two mutations (K978C and K190C) which have been described to permit a >2 fold increase in CFTR Po<sup>[3]</sup>. We have shown that this construct was successfully processed into protein that translocated to the membrane when transfected into human cell lines HEK 293T and H441. We showed that CFTR<sub>enh</sub> produced ~14x more CFTR protein than CFTR WT (n=2) whilst the activity of the CFTR<sub>enh</sub> was observed to be up to ~9x greater as shown in YFP quenching assay (n=3). We have flanked the CFTR<sub>enh</sub> gene with guide RNA sites specific for Intron 1 of human CFTR and incorporated an EGFP marker for intended use in Homology-independent targeted integration. We plan to integrate the enhanced CFTR gene into primary CF bronchiole epithelial cells, downstream of the endogenous CFTR promoter. Given that the CFTR<sub>enh</sub> gene shows increased expression and activity than that of WT CFTR provides support that correcting CF cells with this form of CFTR may restore function with a lower (and achievable) rate of genetic correction.

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Cystic Fibrosis Trust

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## PCB130

**Protective Effect of NADPH Oxidase Inhibitor Apocynin on Ischemia-Reperfusion Injury In The Ovarian Tissue**

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Ovarian ischemic injury, although seen rarely, is one of the important gynecological emergency disorders, especially as a result of ovary torsion. Following ischemia, oxygen deficiency and free radical accumulation occur in the ovaries. For reestablished tissue viability, reperfusion is required, although abundant blood causes more toxic products and results in reperfusion injury. Apocynin (APO) is one anti-inflammatory, antioxidant agent that acts as a potent and a selective inhibitor of NADPH oxidase (NOX). To evaluate the role of APO in ischemia/reperfusion-induced ovarian injury, forty Wistar Albino female rats were subjected to Sham operation (S), ischemia/reperfusion (IR), IR plus dimethyl sulfoxide (IR-D), IR plus apocynin 20 mg/kg (APO20), and IR plus apocynin 50 mg/kg (APO50). In all IR groups, a bilateral adnexal 3-h period of ischemia was performed, followed by 3-h of reperfusion. A single dose of apocynin was given intraperitoneally 15 min before ischemia and just before reperfusion in APO20 and APO50 groups. MDA, TAS, TOS, SOD, and MPO levels were measured using biochemical methods. In IR, MPO, MDA, TOS and OSI increased in significant level, while TAS and SOD decreased. Significant difference in TAS and OSI were seen in APO20. APO50 caused significant induction in TAS and SOD and reduction in OSI and MDA levels in comparison to the IR. Our findings suggested that the apocynin, a NADPH inhibitor, reduces oxidative damage induced by IR in ovarian tissue and restores the oxidative balance.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## PCB131

**Control of cellular shape by actomyosin-dependent apical constriction**

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Autosomal dominant polycystic kidney disease (ADPKD) is the most common monogenic cause of end-stage renal disease affecting approximately 1 in 500 adults. Mutations in two genes cause ADPKD. *Polycystic kidney disease 1 (PKD1)* accounts for 85 % of patients and *PKD2* accounts for the remaining 15 % of patients. Elegant experiments in model organisms have uncovered three evolutionarily conserved core principles of PKD function: 1) the respective proteins, Polycystin-1 (PC1) and transient receptor potential Polycystin-2 (TRPP2), form a receptor-ion channel complex; 2) the PC1/TRPP2 complex



localizes to primary cilia; and 3) both genes are part of morphogenetic programs controlling elementary functions in vertebrates ranging from angiogenesis and kidney development to left-right organ patterning. However, the precise molecular relation between these loosely connected key insights has remained largely enigmatic. Or more specifically, the intracellular effectors translating PC1/TRPP2 receptor-ion channel activity into three-dimensional tissue organization connecting genetics, cellular output, and tissue mechanics are unknown. We have addressed this issue for the kidney with an unbiased high-resolution mass spectrometry-based screen for TRPP2-binding proteins in polarized renal epithelial cells followed by targeted analyses of PC1/TRPP2 function *in vitro* and *in vivo*. *In vitro* analyses of PC1/TRPP2 and candidate proteins were performed using CRISPR/Cas- and TALEN-based gene-specific knock-out and knock-in Madin-Darby canine kidney (MDCK) cell models. Wild-type and mutant zebrafish, mice, and human samples were used to validate results *in vivo*. Here we identify an apical junction associated multi protein signaling complex as functional PC1/TRPP2 effector and show: 1) how the PC1/TRPP2 complex controls cellular shape by actomyosin-dependent apical constriction; 2) that this cellular contraction translates on a tissue-scale into a coordinated fine-tuning of renal tubular diameter *in vivo*; and 3) that in human ADPKD kidneys, tubular shape is lost, because of impaired signal integration disconnecting mutant cells from their healthy surroundings.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCB132

##### Mineralocorticoid receptor deficiency in endothelial cells does not aggravate renal fibrosis during unilateral ureteral obstruction in mice

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Mineralocorticoid receptors (MR) are involved in inflammation and end-stage organ damage such as renal fibrosis in chronic kidney diseases (CKD) independent of their role in blood pressure regulation. Capillary rarefaction is a hallmark feature in CKD, correlating with declining renal function and development of renal fibrosis. MR is expressed in endothelial cells of the renal microvasculature, and we tested the hypothesis that loss of MR signaling in the endothelium attenuates the development of renal fibrosis through improved renal microvascular integrity. Mice with conditional knock out (KO) of MR in endothelial cells (EC) were generated by crossing mice with a floxed exon 3 in the *Nr3c2* gene encoding the MR with mice expressing *cre* recombinase driven by the Tie-2 promoter. Conditional EC-MR KO mice and littermate controls were anaesthetized using a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) and unilateral ureteral obstruction (UUO) or sham surgery was performed. 7 days after surgery, animals were sacrificed and kidneys snap frozen in liquid nitrogen or fixed by systemic perfusion with 4% paraformaldehyde with kidney tissue embedded in paraffin for morphological analyses. Renal fibrosis was examined by quantitative determination of extracellular matrix markers by quantitative PCR. The density of renal microvessels was investigated

by immunohistochemical staining of the endothelial cell marker CD34. Results showed no significant changes in body weight between genotypes. Kidney/body weight ratio of the obstructed kidneys was significantly increased in both EC-MR KO mice and littermate controls compared to sham operated controls (n=9). Moreover, kidney/body weight ratio of the contralateral kidney showed a strong tendency towards an increase in both genotypes but this did not reach statistical significance. Quantitative determination of extracellular matrix markers showed a significant increase of collagen I, collagen III, fibronectin, alpha-smooth muscle cell actin and fibroblast-specific protein 1 mRNA expression in both EC-MR KO mice and littermate controls after 7 days UUO. No difference was detected in between genotypes. No difference in renal microvessel density was observed in sham operated EC-MR KO mice and littermate controls. After 7 days UUO, capillary rarefaction was evident but no difference between genotypes was observed. Since Tie-2 is also expressed in a subgroup of macrophages, MR expression was determined in primary cultures of bone marrow derived macrophages (BMDM) isolated from both EC-MR KO mice and littermate controls. No significant difference in MR mRNA expression was detected between genotypes. In conclusion, MR signaling in endothelial cells does not play a role for renal fibrogenesis including capillary rarefaction in the UUO model of CKD.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCB133

##### High chloride silences the aldosterone - mineralocorticoid receptor - epithelial Na<sup>+</sup>-channel - pathway in the distal nephron

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As a long appreciated but still unresolved phenomenon, dietary potassium (K<sup>+</sup>) supplementation in the setting of high salt (NaCl) intake exerts a natriuretic effect, even though it simultaneously increases plasma aldosterone levels. Generally, aldosterone strongly enhances renal sodium (Na<sup>+</sup>) retention by binding to the mineralocorticoid receptor (MR) in the distal nephron, which increases the activity of the amiloride-sensitive epithelial Na<sup>+</sup>-channel ENaC. Even though recent studies suggest that deactivation of the thiazide-sensitive NaCl cotransporter (NCC) contributes to the natriuretic effect of high potassium intake, it remains unexplained why this effect is not counterbalanced by an amplified Na<sup>+</sup> reabsorption via the activated aldosterone-MR-ENaC pathway. We therefore questioned whether a further mechanism exists, which in the context of high salt and high potassium intake impedes the aldosterone-MR-ENaC pathway to prevent Na<sup>+</sup> reabsorption in the aldosterone-sensitive distal nephron. We analyzed the effect of normal or high potassium diet on MR localization, ENaC mRNA and protein expression, and ENaC activity in salt replete mice. The effects of high extracellular chloride concentration [Cl<sup>-</sup>] on MR localization and ENaC expression in mCCD cells were also determined.

We found that MR translocation to the nucleus, activation of ENaC, and ENaC-dependent Na<sup>+</sup> reabsorption were undetectable in Na<sup>+</sup>-replete mice receiving a high KCl diet in spite of high plasma aldosterone concentrations. Increasing extracellular [Cl<sup>-</sup>] also suppressed aldosterone-induced MR

translocation and ENaC mRNA and protein expression in mCCD cells in vitro. The aldosterone response was rescued at the biochemical as well as functional level in vivo when the increase in extracellular  $[Cl^-]$  was prevented during the high potassium diet.

These findings show that aldosterone signaling in the kidney is silenced by increased extracellular  $[Cl^-]$  and provide an explanation for the natriuretic effect of potassium in the presence of a high NaCl consumption, characteristic for the Western diet.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB134

### Correlation of proteomics and function in isolated perfused single cortical collecting ducts

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The understanding of the kidney's ability to regulate and maintain salt and water homeostasis is highly relevant in modern medicine: by this fine-tuning the kidney keeps blood volume (i.e. blood pressure) and plasma electrolyte composition in a narrow range. Pathological misbalancing easily leads to hypertension or endangers excitable tissues like the heart. On the other hand the kidney is action side of powerful pharmacotherapy to correct misbalancing. Especially with respect to hypertension the distal parts of the nephron, its salt-sensitivity and regulation have been in the focus of investigation.

Cortical collecting ducts (ccd) of 6-8 week old C57Bl6 mice have been mechanically isolated and immediately investigated electrophysiologically in a double barreled perfusion system. The length of the tubule was taken as a measure of protein amount. Transepithelial voltage ( $V_{te}$ ), transepithelial resistance and amiloride sensitive equivalent short-circuit current ( $\Delta I'_{sc_{amil}}$ ) were measured to estimate the functional state of the tubules. For every single tubule ultrasensitive mass spectrometry was then used to determine the expression level of a distinct set of tubular proteins.

CCDs were of 400-600  $\mu m$  of length, showed lumen negative  $V_{te}$  between -8.5 and -32.5 mV and an  $\Delta I'_{sc_{amil}}$  between 54 and 192  $\mu A/cm^2$ . The tubular length was correlated with actin and  $Na^+-K^+-ATPase$  expression.  $V_{te}$  correlated with the regulator protein NEDD4 and, intriguingly, the water channel AQP2.  $\Delta I'_{sc_{amil}}$  reflected the expression level of the b-subunit of the epithelial sodium channel.

In contrast to large-scale omic data sets generated from bulk samples which suffer from crude biochemical isolation procedures, single-tubule functional proteomics predicts physiological function of the respective individual nephron segment. It allows identification of novel players within regulatory networks and a better understanding of how different cell types and transport systems in this segment are interconnected and regulated in a concerted manner.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB135

### Permeability properties of the proximal tubular tight junction changes along its course and depends on claudin-2 and -10a

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In the renal proximal tubule (PT) the paracellular pathway is crucial for bulk reabsorption of water and solutes via the so-called solvent drag mechanism. The perception of this pathway has changed from an unregulated and passive transport route to being a highly regulated and adaptable. It is regulated by the tight junction (TJ) complex and its selectivity is determined by claudin expression. For PT TJ, claudin-2 and claudin-10a are considered to be the main components. Claudin-2 has been described as cation and water permeable and claudin-10a as anion selective. The aim of this study was to investigate paracellular properties along the proximal tubular axis and the contribution of claudin-10a by using isoform-specific claudin-10a knock-out mice (KO).

Micro-dissected PTs of KO and of wild-type controls (WT) were investigated in a double-barreled perfusion system. PT was sub-divided in early proximal convoluted tubule (early PCT), intermediate PCT (PCT), late PCT and proximal straight tubule (PST). To investigate paracellular permeability properties diffusion potentials (DPs) were generated by either replacing the basolateral solution by an isosmotic 30 mM NaCl solution or by replacing  $Cl^-$  by  $HCO_3^-$ . DP were used to calculate relative permeability (P) ratios for  $Na^+$ ,  $Cl^-$  and  $HCO_3^-$ . Immunofluorescence against claudin-10, claudin-2 and claudin-3 was performed on single isolated tubules.

In WT, early PCT showed anion selectivity ( $P_{Cl^-}/P_{Na^+} = 1.52 \pm 0.07$ ), the segments in between (PCT and late PCT) showed intermediate selectivity and in PST selectivity turned to cation selective ( $P_{Na^+}/P_{Cl^-} = 1.26 \pm 0.06$ ). PT TJ showed higher  $Cl^-$  than  $HCO_3^-$  permeability with the highest  $P_{Cl^-}/P_{HCO_3^-}$  in late PCT ( $1.86 \pm 0.25$ ). Early PCT TJ were highly meandering and showed high claudin-10a expression in the TJ. This pattern attenuated gradually along the course of the PT and PST TJ were shorter, straight and dominated by claudin-2 expression. In addition, in this last segment of the PT, the tightening claudin-3 was expressed and co-localized to the TJ. In KO all proximal convoluted parts of PT lost anion selectivity completely but displayed high cation selectivity ( $P_{Na^+}/P_{Cl^-} = 2.86 \pm 0.45$  in early PCT). In PST the differences were less pronounced but qualitative similar ( $P_{Na^+}/P_{Cl^-} = 2.00 \pm 0.14$ ). KO late PCT, however, now preferred bicarbonate over  $Cl^-$  ( $P_{HCO_3^-}/P_{Cl^-} = 1.37 \pm 0.25$ ). In all segments of KO, claudin-2 expression dominated TJ staining.

In summary, claudin-10a confers anion selectivity with preference of  $Cl^-$  over bicarbonate to the proximal tubular TJ with the exception of the slightly cation selective PST where claudin-2 dominated. Claudins 2 and 10a and their distribution along the course of the PT are responsible for the distinct properties of the paracellular pathway in this segment.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB136

**Contribution of the proton sensors OGR1 and GPR4 to renal disease progression in mice**J. Sprenger<sup>1,5</sup>, E.M. Pastor Arroyo<sup>1,5</sup>, T. Knöpfel<sup>1</sup>, G. Pellegrini<sup>2</sup>, N. Olsen Camara<sup>3,4</sup>, C.A. Wagner<sup>1,5</sup> and P. Imenez Silva<sup>1,5</sup><sup>1</sup>Institute of Physiology, University of Zurich, Zurich, Switzerland, <sup>2</sup>Institute of Veterinary Pathology, University of Zurich, Zurich, Switzerland, <sup>3</sup>Division of Nephrology, Federal University of Sao Paulo, Sao Paulo, Brazil, <sup>4</sup>Institute of Biomedical Sciences - Department of Immunology, University of Sao Paulo, Sao Paulo, Brazil and <sup>5</sup>National Center for Competence in Research NCCR Kidney.CH, Zurich, Switzerland

Chronic kidney disease (CKD) represents a major health problem and contributes to high economical costs. Local tissue inflammation and acidification already occur in early stages of CKD. Both local conditions may act synergistically, accelerating disease progression. Therefore, elucidating mechanisms linking extracellular pH, pH-sensing, and immune responses may provide relevant insights to better understand renal disease progression. Moreover, it may provide relevant therapeutic targets. This study aims to investigate the contribution of the pH sensing G protein coupled receptors (GPCRs) OGR1 and GPR4 in renal inflammation and fibrosis.

A ten-day high-oxalate diet (0.67% oxalate in calcium-free diet) was used to induce crystalline nephropathy in litter-mate wild-type (WT), OGR1 knock-out (KO) and GPR4 KO male C57BL/6 mice. Age, sex and genotype matched littermates received control diet (calcium-free). There was a three days adaptation period with calcium-free diet prior to treatment and a three days recovery period with standard diet (1.05% calcium) afterwards. At the end, blood and kidneys were collected from isoflurane-anesthetized mice. Plasma parameters were measured and RNA was extracted from kidneys to perform qPCR. In another set of experiments, the anesthetized mice were perfused with 4% PFA/PBS and kidneys were collected for histological evaluation. Data are presented as mean  $\pm$  SD, compared by ANOVA.

Plasma urea and creatinine values were elevated upon oxalate diet in all three genotypes. However GPR4 KO displayed significantly less elevated values (71.3 mg/dL  $\pm$  13.8 and 0.2 mg/dL  $\pm$  0.05, n=4) compared to WT (173.7 mg/dL  $\pm$  64.7 and 0.5 mg/dL  $\pm$  0.2, n=7). In situ RNAscope mRNA hybridization and qPCR showed that OGR1 and GPR4 mRNA expression were highly increased in WT mice subjected to the oxalate nephropathy model. Proinflammatory factors and cytokines including Nalp3, Tnf, Tgf $\beta$ , and Ccl5 were similarly upregulated in all oxalate treated mice. Only Il-1 $\beta$  values of OGR1 KO and GPR4 KO mice were significantly less elevated upon oxalate diet compared to WT (both GPR4 and OGR1 showed  $\sim$ 40% reduced increase compared to WT, n=4-7). Fibrosis markers Mmp9, Timp1, and Acta2 were attenuated in GPR4 KO. Taken together, GPR4 KO mice showed evidence of lower fibrosis and had better preserved kidney function when compared to WT mice challenged with high oxalate diet. Preliminary histological assessment of kidney tissue showed similar oxalate crystal count, collagen staining by van Gieson and epithelial damage between WT and OGR1 KO mice. Histological assessment of renal tissue from GPR4 mice is ongoing. GPR4 may be involved in the progression of oxalate nephropathy.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCB137

**Erythropoietin stimulates Fibroblast Growth Factor 23 (FGF23) in mice and men**A. Daryadel<sup>1,3</sup>, T. Haider<sup>2</sup>, P. Imenez Silva<sup>1,3</sup>, U. Schnitzbauer<sup>1,4</sup>, C. Bettoni<sup>1,3</sup>, E.M. Pastor Arroyo<sup>1,3</sup>, R. Wenger<sup>1,3</sup>, M. Gassmann<sup>2</sup> and C.A. Wagner<sup>1,3</sup><sup>1</sup>Institute of Physiology, Zürich, Switzerland, <sup>2</sup>Institute of Veterinary Physiology, Zürich, Switzerland, <sup>3</sup>National Center of Competence in Research NCCR Kidney.CH, Zürich, Switzerland and <sup>4</sup>National Center of Competence in Research NCCR Kidney.CH, Zürich, Switzerland

**Introduction:** Fibroblast growth factor 23 (FGF23) is a major endocrine regulator of phosphate and 1,25 (OH)<sub>2</sub> vitamin D<sub>3</sub> metabolism and is mainly produced by osteocytes. Its production is up-regulated by a variety of factors including 1,25 (OH)<sub>2</sub> vitamin D<sub>3</sub>, high dietary phosphate intake, and parathyroid hormone (PTH). Recently, iron deficiency and hypoxia have been suggested as additional regulators of FGF23 and a role of erythropoietin (EPO) was shown. However, the regulation of FGF23 by EPO and the impact on phosphate and 1,25(OH)<sub>2</sub> vitamin D<sub>3</sub> are not completely understood. Therefore, we aimed to investigate the regulatory effect of EPO via FGF23 on phosphate and 1,25 (OH)<sub>2</sub> vitamin D<sub>3</sub> metabolism.

**Methods:** Blood samples were collected from healthy 32 subjects (EPOPERF-Project, a randomized double-blinded, placebo-controlled, crossover clinical trial (<https://clinicaltrials.gov>, identifier code: NCT01889056) at 24 hours post treatment with a single high dose of recombinant erythropoietin (rhEPO). Additionally, C57BL/6 male mice were injected daily with rhEPO for 24 hours or 4days. Plasma intact FGF23 (iFGF23), C-terminal FGF23 fragment (cFGF23), PTH and 1,25 (OH)<sub>2</sub> D<sub>3</sub> levels were measured by Elisa and radioimmunoassay respectively. Abundance of FGF23 in bone marrow cells was analyzed by real time PCR and immunostaining assays. Furthermore, renal EPO mRNA levels were assessed by real time PCR and NaPilla and  $\alpha$ -klotho protein expression were tested by western blot.

**Results:** We demonstrate that acute administration of rhEPO to healthy humans increased the cFGF23, but not iFGF23. In mice, rhEPO stimulated acutely (24 hrs) cFGF23 but iFGF23 only after 4 days without effects on PTH and plasma phosphate. Systemic 1,25 (OH)<sub>2</sub> D<sub>3</sub> levels and  $\alpha$ -klotho and NaPilla expression in kidney decreased after 4 days. rhEPO induced FGF23 mRNA in bone marrow but not in bone with increased staining of FGF23 in CD71<sup>+</sup> erythroid precursors in bone marrow. Chronic elevation of EPO in transgenic mice (Tg6) increased iFGF23. Finally, acute injections of recombinant FGF23 reduced renal EPO mRNA expression.

**Conclusion:** Our data demonstrate stimulation of FGF23 levels in mice which impacts mostly on 1,25 (OH)<sub>2</sub> vitamin D<sub>3</sub> levels and metabolism. In humans, EPO caused elevation of cFGF23, in mice EPO has a time-dependent effect on both FGF23 forms. EPO and FGF23 may form a feedback loop controlling and linking erythropoiesis and mineral metabolism.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*



PCB138

**mCCD<sub>cl</sub> cells show Plasticity Consistent with the Ability to Transition between Principal and Intercalated Cells**A. Assmus, M.K. Mansley, L. Mullins, A. Peter and J.J. Mullins  
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The cortical collecting duct of the mammalian kidney plays a critical role in the regulation of body volume, sodium pH and osmolarity and is composed of two distinct cell types, principal cells and intercalated cells. Each cell type is detectable in the kidney by the localization of specific transport proteins such as Aqp2 and ENaC in principal cells and V-ATPase B1 and Cx30 in intercalated cells. The inter-relationship between PC and IC cells, both during development and in the adult, is complex and is yet to be fully elucidated. The ratio of the number of principal cells to intercalated cells (typically 70:30 in healthy mice) has been shown to be influenced by multiple factors involved in Notch signalling [1], [2].

mCCD<sub>cl</sub> cells have been widely used as a mouse principal cell line on the basis of their physiological characteristics. In this study, the mCCD<sub>cl</sub> parental cell line and three sub-lines cloned from isolated single cells (Ed1, Ed2, and Ed3) were grown on filters to assess their transepithelial resistance, transepithelial voltage, equivalent short circuit current and expression of the cell-specific markers Aqp2, ENaC, V-ATPaseB1 and Cx30, as well as precursor markers p63 and IC-specific  $\Delta$ Np63. The parental mCCD<sub>cl</sub> cell line presented typical [3] amiloride-sensitive electrogenic sodium transport indicative of principal cell function, however immunocytochemistry and RT-PCR showed that a subset of cells expressed the intercalated cell-specific markers V-ATPase B1 and Cx30, including cells also positive for Aqp2 and ENaC (intermediate cells). Intermediate cells represented ~42% of the mCCD<sub>cl</sub> cell population and were present in different proportions in the clonal sub-lines. Parental and clonal sub-lines all exhibited expression of collecting duct precursor markers p63 and  $\Delta$ Np63, and RNA sequencing showed the expression of a large range of other PC and IC specific markers such as ROMK or NKCC1.

The vertical transmission of both principal and intercalated cell characteristics via single cell cloning reveals the plasticity of mCCD<sub>cl</sub> cells, and a direct lineage relationship between these two physiologically important cell types, and is consistent with mCCD<sub>cl</sub> cells being precursor cells.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB139

**The role of the actin-sequestering protein, thymosin- $\beta$ 4, on renal podocyte function**W.J. Mason<sup>1</sup>, C. Peppiatt-Wildman<sup>2</sup>, D.A. Long<sup>3</sup> and E. Vasilopoulou<sup>1,3</sup>

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**Introduction**

End-stage renal disease (ESRD) is a condition that requires life-long dialysis or transplantation and is associated with increased risk for all-cause and cardiovascular mortality. A major cause of ESRD is damage of the glomerulus, the filtration unit of the kidney. Podocytes, epithelial cells located in the glomerulus, are responsible for the ultrafiltration of blood into urine. In glomerular disease, podocytes lose their morphology and glomerular filtration is disrupted. Thymosin- $\beta$ 4 (T $\beta$ 4) is a G-actin sequestering protein that regulates actin cytoskeleton assembly, cell morphology and motility. We have previously reported that downregulation of endogenous T $\beta$ 4 in podocytes *in vitro* increases migration and actin stress fibre formation. The aim of this study is to assess the effect of exogenous T $\beta$ 4 on healthy and injured podocytes.

**Methods**

To assess the effect of T $\beta$ 4 on healthy podocytes, differentiated immortalised mouse podocytes were treated with 0, 10, 100 or 1000 ng/ml T $\beta$ 4 (n=4-5). A range of Adriamycin (ADR) concentrations (0, 0.0125, 0.125, 0.25, 0.5, 1 or 2.5 mg/ml) was assessed for the induction of podocyte injury (n=2-4). Co-treatment with T $\beta$ 4 (0, 10, 100 or 1000 ng/ml) and ADR (0, 0.0125 or 0.125 mg/ml) was used to investigate the effect of T $\beta$ 4 on injured podocytes (n=2). Cell viability (MTT assay) was assessed 24, 48 and 72 hours post-treatment. Podocyte migration was analysed by a scratch wound assay at 6 and 24 hours post wound formation. Actin filaments were visualised 24 hours post-treatment with Acti-stain 488 phalloidin and cell area, F-actin density and stress fibre prevalence were assessed.

**Results**

Treatment of healthy podocytes with T $\beta$ 4 did not alter cell viability, migration, cell area, F-actin density, or stress fibre prevalence. ADR treatment reduced cell viability by >60% at doses higher than 0.125 mg/ml at all time points (p<0.05). Migration was unchanged after 6 hours, but after 24 hours 0.0125 mg/ml ADR increased migration (40%, p<0.05), whereas decreased migration and cell detachment was observed for the rest of the doses (p<0.05). Changes in the podocyte cytoskeleton were also observed with decreased cell area and F-actin density (0.125 and 1 mg/ml ADR; p<0.05) and increased prevalence of cytoplasmic stress fibres (0.0125-0.5 mg/ml ADR; p<0.05). Treatment with T $\beta$ 4 (100 ng/ml) significantly reduced the ADR-induced increase in cytoplasmic stress fibre prevalence (p<0.05) but did not modify the effects of ADR on cell viability and migration.

**Conclusion**

Exogenous T $\beta$ 4 has no effect on healthy podocytes *in vitro*. ADR injury results in pronounced cell death and changes in the podocyte cytoskeleton *in vitro* in a dose-dependent manner. Our data indicates that treatment with T $\beta$ 4 may rescue the ADR-induced changes on the podocyte cytoskeleton.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB140

### Adaptations to Pregnancy: Role of the Proteinase Activated Receptor 2 (PAR2)

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Pregnancy requires avid sodium retention to drive a massive plasma volume expansion (~40%), which occurs in the setting of reduced or normal blood pressure. This volume expansion is critical to the development of the growing uterus and fetus as inadequate plasma volume expansion and hypertension are associated with preeclampsia, the leading cause of maternal and fetal morbidity and mortality. The mechanisms permitting the decreased maternal blood pressure and increased renal sodium retention are still largely unknown. Research in non-pregnant rats has shown that activation of proteinase activated receptor type 2 (PAR2) can produce vasodilation and stimulate sodium chloride reabsorption by two collecting duct mediate mechanisms pendrin/NDCBE and ENaC. Here, we investigate PAR2 activation and inhibition in virgin (V) and normal late pregnant (LP) Sprague Dawley rats (Envigo). Catheters (PE-50) were implanted into the bladder, left femoral artery, and left femoral vein under 1-4% isoflurane anesthesia. We measured *in vivo* blood pressure (BP) and natriuretic responses to 0.1mg/kg, 0.3mg/kg, and 1 mg/kg doses of PAR2 agonist peptide (SLIGRL-NH2) in anesthetized V and LP rats. We then measured net natriuretic response to thiazide (HCTZ, 7.5mg/kg) and benzamil (Bz, 1.05mg/kg) with and without PAR2 inhibitory peptide (FSLRY-NH2) in LP rats to determine the transport mechanism. Values are presented as means  $\pm$  SEM. We found that LP rats had larger decreases in BP compared to baseline than V rats at 0.1 mg/kg ( $V=-3.67\pm0.92$  vs.  $LP=-6.93\pm0.53$  mmHg,  $p<0.05$  by two-way RM ANOVA,  $V$  n=7,  $LP$  n=11) and 0.3 mg/kg ( $V=-6.96\pm3.62$  vs.  $LP=-21.49\pm1.86$  mmHg,  $p<0.05$  by two-way RM ANOVA,  $V$  n=7,  $LP$  n=11) doses of agonist peptide. We also found that LP rats had increased net sodium retention compared to V rats following 0.3mg/kg ( $V=23\pm4$  vs.  $LP=106\pm33$   $\mu$ mol/hr,  $p<0.05$  by two-way RM-ANOVA,  $V$  n=7,  $LP$  n=11) and 1mg/kg ( $V=29\pm4$  vs.  $LP=125\pm29$   $\mu$ mol/hr,  $p<0.05$  by two-way RM-ANOVA,  $V$  n=7,  $LP$  n=11) agonist peptide. We found increased HCTZ-mediated sodium transport after PAR2 inhibition ( $Veh=621\pm41$  vs.  $Inh=728\pm52$   $\mu$ mol/hr,  $p<0.05$  by t-test,  $Veh$  n=5,  $Inh$  n=7), however there were no differences detected in Bz-mediated transport ( $Veh=581\pm67$  vs.  $Inh=551\pm75$ , N.S., n=8/group) in LP rats. These data taken together, demonstrate that activation of PAR2 causes larger decreases in BP and increases in sodium retention in LP compared to V controls. Furthermore, that the PAR2 mediated sodium reabsorption in LP rats is through the pendrin/NDCBE-mediated transport mechanism, not ENaC. These data suggest that PAR2 may play a role in the renal and vascular adaptations to pregnancy and thus this mechanism could potentially provide new targets for treatment of preeclampsia.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB141

### The role of the inwardly rectifying potassium channel Kir5.1 in the distal convoluted tubule

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The kidneys are essential for regulating the volume and electrolyte homeostasis. Potassium ( $K^+$ ) is the most abundant cation in intracellular fluid and plays an important role in maintaining membrane potential across the membrane. In the basolateral membrane of the distal convoluted tubule (DCT) KCNJ10 (Kir4.1) – a member of the inwardly rectifying  $K^+$  channel (Kir) family. Kir4.1 is able to form homotetramers or heterotetramers with its close homologous KCNJ16 (Kir5.1). The relevance of Kir4.1 in the DCT was highlighted by the loss-of-function mutations in KCNJ10 which result in the EAST/SeSAME-syndrome (acronym for epilepsy, ataxia, sensorineural deafness, and salt-wasting tubulopathy), a complex disease with renal salt wasting, hypokalemic metabolic alkalosis, hypomagnesemia and hypocalciuria. However, the role of Kir5.1 is relatively unknown and the Kir5.1<sup>-/-</sup> (C57BL/6J) mice exhibit a renal phenotype that is the opposite to the one of KCNJ10 except for the hypokalemia.(1) It has been suggested that Kir5.1 in combination with Kir4.1 might act as basolateral  $K^+$ -sensors in the DCT modifying  $K^+$  secretion in the late distal nephron by indirectly regulating of the activity of the apical sodium-chloride ( $Na^+/Cl^-$ ) symporter NCC.(2) Here, we reinvestigated the expression and distribution of KCNJ16 in the kidney by analyzing manually sorted tubular segments using quantitative real time PCR. Expression of KCNJ16 was highest in proximal tubules and DCT. This data was confirmed by KCNJ16-promotor-driven X-gal staining in Kir5.1<sup>-/-</sup> mouse kidneys. To test whether Kir5.1 might indeed be a  $K^+$  sensor in the DCT as hypothesized by Terker et al. (2), we isolated murine DCTs and exposed them to changes in basolateral and apical  $K^+$ . Fura-2 mediated  $Ca^{2+}$ -measurements revealed that only basolateral, but not apical, application of low  $K^+$  solution translated into increased intracellular  $Ca^{2+}$  levels. However, deletion of Kir5.1 did not alter the  $Ca^{2+}$  signals suggesting that Kir5.1 channels are not essential for this component of the  $K^+$  sensing mechanism. To further study the effect of KCNJ16 deletion in mice, we analyzed renal electrolyte handling in those mice. Under control diet, Kir5.1<sup>-/-</sup> mice revealed hypokalemia and hypermagnesuria and when challenged with a high  $Na^+$  diet they revealed a massive increased excretion of electrolytes which suggests an impaired adaptation capacity of electrolyte balance. Our analysis of the Kir5.1<sup>-/-</sup> mice indicates a crucial role for Kir5.1 in renal electrolyte balance although Kir5.1 seems nonessential for  $K^+$  sensing in the DCT.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB142

**Neutral amino acid transporter LAT1 (*Slc7a5*) in kidney and its role in compensatory growth**

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LAT1 is a System L amino acid exchanger translocating preferentially large neutral amino acids (AA) including aromatic or branched chain ones into the cell in exchange for non-essential AAs and

independently of sodium. Thereby LAT1 plays a pivotal role in mTORC1 activation, possibly also by sensing intracellular AA concentrations and thus orchestrating protein synthesis, cell growth and cell division. LAT1 has been shown to be expressed in the kidney but its role remains unclear. In the present study we investigated the localization of LAT1 within the murine kidney and explored its role in compensatory growth of the remnant kidney after unilateral nephrectomy (UNX).

To identify the expression of LAT1 and its localization within specific renal tubular segments, we used a combination of RNA *in situ* hybridization and immunofluorescence on kidney cryosections from 4% paraformaldehyde perfused mice. LAT1 expression pattern confirms that it is not part of the AA reabsorption machinery as it is present only at a low level in the proximal tubule. In contrast, we observed substantial LAT1 expression level in the thick ascending limb and the distal convoluted tubule.

To study the role of LAT1 in the renal tubule we generated a doxycycline inducible knockout mouse strain using the Pax8-rtTA /LC1 line (Traykova-Brauch M. et al, 2008) and crossed these mice with the previously generated floxed *Slc7a5* mouse strain (Poncet N. et al, 2014). Doxycycline treatment at 1mg/ml in 2% sucrose for 14 days resulted in a 73% (+/-5%) decrease in LAT1 mRNA expression in the kidney. Values are means  $\pm$  S.D., compared by ANOVA. As LAT1 is well described to be expressed in endothelial cells the residual mRNA expression levels might originate from the renal vasculature.

To investigate whether LAT1 is involved in renal growth control we performed unilateral nephrectomy (or sham surgery) after anesthesia by 3% isoflurane inhalation. Surgeries were performed on knockout (n=18) and control animals (n=18). We analyzed the growth of the remnant kidney using a MicroCT device. We will further analyze plasma and urine composition and investigate growth and proliferation of the remnant kidney using BrdU incorporation method, western blot and immunofluorescence to examine mTORC1 downstream targets activation.

With our study we demonstrate for the first time the localization of LAT1 in specific tubular segments of the murine kidney and investigate its role in compensatory growth of the remnant kidney after UNX.

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PCB143

**Sexual dimorphism in *Bmal1* regulation of blood pressure and diurnal sodium handling in the rat**

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Previous studies suggest that core clock genes generally function to facilitate sodium (Na) conservation such as *Per1* effects to mediate the renal effects of aldosterone. *Bmal1* gene knockout mice have multiple pathologies, but little is known about 1) *Bmal1* control of Na handling by the kidney, 2) *Bmal1* function in the rat, or 3) possible sex differences in *Bmal1* function in the kidney. Using Crispr/Cas9 methodology, we created a new whole-body *Bmal1* knockout rat (*Bmal1*<sup>-/-</sup>) to determine the impact of *Bmal1* gene deletion on the diurnal rhythms of urinary Na excretion (UNaV) in both acute and chronic salt loading conditions of male and female rats. Due to the importance of endothelin signaling in Na homeostasis, we also sought to determine the renal responses of our rats to endothelin B (ET<sub>B</sub>) receptor blockade for our chronic salt loading experiments. Male and female *Bmal1*<sup>-/-</sup> rats and littermate controls (*Bmal1*<sup>+/+</sup>) were implanted with telemetry transmitters to monitor mean arterial pressure (MAP). After a recovery period of at least one week, baseline urine samples were collected in 12hr light/dark intervals. The night-day difference in UNaV was evident in *Bmal1*<sup>+/+</sup>, but not *Bmal1*<sup>-/-</sup> male rats (404  $\pm$  77 and -188  $\pm$  56  $\mu$ Eq Na/12hr *Bmal1*<sup>+/+</sup> vs. *Bmal1*<sup>-/-</sup>, n=13 and 10, respectively; P<.05). This loss of diurnal rhythm was not observed in female *Bmal1*<sup>-/-</sup> rats (266  $\pm$  60 and 193  $\pm$  61  $\mu$ Eq Na/12hr *Bmal1*<sup>+/+</sup> vs. *Bmal1*<sup>-/-</sup>, n=10 and 9, respectively; P<.05). Unlike *Bmal1*<sup>-/-</sup> mice, *Bmal1*<sup>-/-</sup> rats displayed a normal circadian rhythm in MAP; however, MAP was lower in *Bmal1*<sup>-/-</sup> compared to *Bmal1*<sup>+/+</sup>, but was this difference was significant only in female rats. Rats given a single 900 $\mu$ Eq Na load (p.o.) at the beginning of their inactive (Zeitgeber Time (ZT) 0) or active (ZT12) period. Both male and female, *Bmal1*<sup>+/+</sup> and *Bmal1*<sup>-/-</sup> rats had a prompt natriuretic response to the salt load, excreting most of the salt in the first 12 hours at both ZT0 or ZT12. UNaV was significantly reduced in male *Bmal1*<sup>-/-</sup> rats on a normal or high salt diet during the active period compared to *Bmal1*<sup>+/+</sup> rats. Addition of the ET<sub>B</sub> receptor antagonist, A-192621, to rats of both genotypes on a high salt diet caused significant hypertension and had no significant effect on UNaV. These data demonstrate that rats lacking *Bmal1* expression display a blunted rhythm of diurnal UNaV in male but not female rats. Furthermore, this blunted rhythm may be due to a reduced amount of Na excretion during the active period.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*



PCB144

### Skeletal muscle unfolded protein response is different during muscle growth achieved by resistance training or its “mimetic” myostatin/activin blocker.

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Accumulation of misfolded proteins into the endoplasmic reticulum (ER) is termed ER stress. It activates unfolded protein response (UPR) (1) which has a role in muscle function regulation (2). Resistance exercise (RE) probably remains the best option to increase muscle hypertrophy and strength, but “exercise mimetics” may provide a pharmacological option to prevent muscle loss in the future. RE (3,4) and its “pharmacological mimetics” myostatin/activin blockers (5) both increase muscle protein synthesis and hypertrophy. However, the effect of these two different stimulus to induce muscle growth on UPR is not well known. The aim of this study was to elucidate from the previously collected muscle samples the effects of resistance exercise and training in untrained young men as well as administration of soluble activin receptor 2B (sACVR) to block myostatin and activins in healthy mice on UPR. Untrained healthy men (27±4 years, n=12) performed a resistance exercise bout in leg press (5 x 10 RM) and muscle biopsies were obtained 1 and 48 hours after the RE bout as well as after 21 weeks of resistance training (RT) from vastus lateralis (VL) muscle. Male, 6–7-week-old C57Bl/10Snj mice were administered with sACVR (produced in house; i.p. 10 mg/kg,) or placebo (PBS; n=6) and were euthanized by cervical dislocation under isoflurane anaesthesia 1 or 2 days after a single sACVR administration (n=6 in each group) or after 2 weeks of sACVR (n=12) or PBS (n=6) administration. UPR markers were analysed by western blot and RT-qPCR. Holm-Bonferroni corrected *t*-tests were used to analyse RE and RT effects. One-way Anova followed by Holm-Bonferroni corrected LSD test was used to analyse 1 and 2 d sACVR effects. Student's *t*-test was used to analyse 2 week effects of sACVR administration. Statistical significance was set at *p*<0.05.

Both resistance exercise/training and sACVR administration led to rather similarly increased mTOR-signaling and muscle hypertrophy (4,5). Now we showed that several UPR indicators (PERK, ATF4, GRP78 and *Xbp1s*) were increased (*p*<0.05) at 48 h after a single resistance exercise bout, but not at post 1 h or after the 21-week resistance training period in untrained young men. On the other hand, UPR indicators were unchanged 1 and 2 days after a single sACVR administration. However, after 2 weeks of sACVR administration, which is a time-point when muscle growth had occurred, the UPR indicators HSP47, p-eIF2α and GRP78 increased (*p*<0.05). Conclusions. Resistance exercise/training and myostatin/activin blocking have different effects on skeletal muscle unfolded protein response even though both increase muscle protein synthesis and hypertrophy.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB145

### Influence of VX-745, inhibitor of p38 MAPK, on the E3-ligases expression in rat soleus during hindlimb unloading

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Unloading causes rapid skeletal muscle atrophy mainly due to the increased protein degradation. Muscle proteolysis results from the activation of ubiquitin-proteasome system. The ubiquitination proteins is carried out by muscle-specific E3 ubiquitin ligases – MuRF-1 and MAFbx. It is known that MuRF-1 and MAFbx expression increases on the third day of muscle unloading. We tested the hypothesis that p38 MAPK participates in the regulation of E3 ligases expression and the development of skeletal muscle atrophy during unloading. To check this idea we inhibited p38 MAPK by VX-745. 21 male Wistar rats were divided into 3 groups (7 rats in each group): intact control (C), rats suspended for 3 days (HS) and rats suspended and injected i.p. with VX-745 (10 mg/kg/day) (VX). The hindlimb suspension was carried out according to Morey-Holton technique. The animals were anaesthetised with an i.p. injection of tribromoethanol (240 mg/kg). Under anesthesia, the m. soleus were excised, frozen in liquid nitrogen, and stored at -80°C. All procedures with the animals were approved by the Biomedicine Ethics Committee of the Institute of Biomedical Problems of the Russian Academy of Sciences/Physiology section of the Russian Bioethics Committee. mRNA expression was estimated by RT-PCR (3 repeats for each parameter). The statistical analysis was performed using the REST 2009 v.2.0.12 and Origin Pro programs at the significance level set at 0.05. The results are given as median in percent and interquartile range (0.25-0.75).

The muscle weight in HS group was reduced (72,3±2,5 mg) compared to C (83,0±3 mg), *p*<0.05, while the soleus weight of VX group didn't differ from the control (84.2±5 mg). The MuRF1 mRNA expression was elevated dramatically in HS group (165 (138-210) %) when compared with the C (100 (64.6-112.5) %), *p*<0.05. In the VX group the level of MuRF1 mRNA expression (127 (105-138) %) didn't differ from the control group. The MAFbx mRNA expression was observed to increase equally in both suspended groups (294 (265-342) % and (271 (239-309) %).) vs C (100 (91-106) %) so, VX-745 administration did not have any significant effect on its expression. We also found that the level of ubiquitin mRNA expression in the soleus of HS rats was higher (423 (325-485) %) in comparison with the C group (100 (78-166) %), *p*<0.05) while VX-745 injection prevented increasing the mRNA ubiquitin expression (200 (190-237) %). We discovered that the elevation of calpain-1 mRNA expression upon HS was prevented by VX-745 administration and its level didn't differ from the C group (C - 100 (97-105) %, HS - 120 (116-133) %, VX - 107 (100-115) %, *p*<0.05). Thus, the results indicate that the p38 MAPK signaling pathway takes part in the regulation of E3-ligase MuRF1 but not MAFbx expression. The p38 MAPK inhibition prevents muscle atrophy and the elevation of ubiquitin and calpain mRNA expression at the early stage of

hindlimb unloading. This work was supported by RFBR grant No.17-04-01838.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB146

**The role of HDACs in the regulation of E3-ligases MuRF-1 and MAFbx expression in rat soleus at the early stage of muscle unloading.**

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Muscle unloading leads to its atrophy development. The MuRF-1 and MAFbx E3-ligases expression is increasing under this condition. Moresi et al. have shown that the histone deacetylases 4 and 5 signaling cascade (HDAC4/5) may regulate the expression of MuRF1 and MAFbx through the myogenin activation upon the muscle's denervation. We hypothesized that the HDAC 4 and 5 may regulate the E3 ligases expression in the early stages of muscle unloading, and the myogenin may be involved in this process. We checked this hypothesis by trichostatin A (inhibitor of HDAC4/5) administration in male Wistar rats (180-200 g) upon 3-day hindlimb suspension. Russian Bioethics Committee (protocol no. 418, 28.03.2017). The method of hindlimb suspension was described in Morey-Holton E & Globus R (2002). 24 animals were divided into 3 groups (n=8 in each): C-control, HST-hindlimb suspension with Trichostatin A (i.p. 0.6 mg/kg), or placebo (HS group) administration. The animals were anaesthetized with an i.p. injection of tribromoethanol (240 mg kg<sup>-1</sup>), soleus muscles were surgically excised, frozen in liquid nitrogen (Vilchinskaya NA et al., 2017). The Western blot and RT-PCR analyzes were done (three replicates of each sample). The Statistical analysis was performed using REST 2009 v.2.0.12 and Bio-Rad CFX Manager programs at the significance level set at 0.05. The significant differences between groups were statistically analyzed using Mann-Whitney test. All PCR data are expressed as median and interquartile range (0.25-0.75); all other data are given as mean  $\pm$  standard error of the mean. HDAC4 protein content in soleus of HST group has decreased in nuclear fraction (in contrast to HS and C group by 88 $\pm$ 6% and 86 $\pm$ 7% respectively) and increased in cytoplasmic fraction in both suspended HST and HS groups by 33 $\pm$ 10% and 21 $\pm$ 9% respectively vs C group (p<0.05). On the contrary, the nuclear content of HDAC5 protein in the HST group didn't differ from those of C group, while this parameter in HS group was found to be significantly reduced by 41 $\pm$ 10.2% (p<0.05). The Myogenin content in the HST group did not differ from that of the C group, while its amount in the HS group was significantly higher by 30 $\pm$ 6% vs C (p<0.05). As for E3-lygases mRNA expression, MAFbx level in the HST group did not differ from those of control, while in the HS group it increased dramatically by 2.5 fold (1.75-3.5) (p<0.05). MuRF1 mRNA expression was equally elevated in both HS and HST suspended groups relative to the control (p<0.05). It could be concluded that The myogenin regulates the expression of MAFbx E3 ligase expression in the early stages of hindlimb unloading. The inhibition of HDAC 4 and 5 does not affect the regulation of MuRF1 E3 ligase expression. This work was supported by Russian Science Foundation (grant 18-15-00062).

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB147

**Effects of High Intensity Interval versus Continuous Moderate Intensity Training on Post Traumatic Stress Disorder Induced Cognitive Impairment in Rats**

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High intensity interval training (HIIT) has become popular recently<sup>1</sup>. Post-traumatic stress disorder (PTSD) declines cognitive capacity. This study aimed to compare the effect of HIIT and continuous moderate-intensity training (CMT) on cognitive functions and anxiety levels of PTSD induced rats and to investigate possible underlying oxidative and anti-oxidative mechanisms. Female Wistar Albino rats (n=44) were randomly divided into six groups: Sedantary (SED), CMT, HIIT groups and PTSD-induced SED, CMT, and HIIT groups (n=6-8). Exercise groups performed treadmill training for 6 weeks (5 days/week, 30 min/day). Following a 2 min warm-up period, HIIT groups exercised 4 min at 80-90% of maximum oxygen consumption (VO<sub>2</sub>max) and 3 min at 50-60% VO<sub>2</sub>max for 4 cycles and CMT groups exercised at 50-60% VO<sub>2</sub>max. On the fifth week of exercise sessions, a single prolonged stress protocol was used to induce PTSD. Following the last stress stimulation, cognitive functions were evaluated by object recognition test. Anxiety-like behavior was analyzed via Hole-board and elevated plus maze tests. The passive avoidance test was performed to evaluate fear conditioning. After the rats were decapitated, malondialdehyde (MDA) levels, glutathione (GSH) content, myeloperoxidase (MPO), superoxide dismutase (SOD) and catalase (CAT) activities, luminol and lucigenin levels were measured, and histopathological damage was evaluated. The data was analyzed by one-way ANOVA and student's t test. As an indicator of anxiety-like behavior, decreased proportion of time spent on the open arms, declined head-dipping frequency and rearing number in SED+PTSD group were increased in CMT+PTSD and HIIT+PTSD and the significant increase in freezing time was decreased only in HIIT+PTSD group (p<0.05-0.001). PTSD-related cognitive decline in SED+PTSD group was increased in CMT+PTSD and HIIT+PTSD groups (p<0.05-0.01). The luminol and lucigenin levels, MPO activity and histological damage were increased in SED+PTSD group, while the scores were depressed in CMT+PTSD and HIIT+PTSD groups (p<0.05-0.001). The increased brain MDA levels in SED+PTSD

group were suppressed in HIIT+PTSD group ( $p<0.01-0.001$ ). The decreased antioxidant GSH levels and CAT activity were significantly increased in CMT+PTSD and HIIT+PTSD groups, respectively ( $p<0.05-0.001$ ). Compared to SED group, the latency as a sign of fear conditioning decreased in SED+PTSD ( $p<0.05$ ). Microscopically, neuronal damage observed in the cortex and hippocampus of PTSD-SED rats was alleviated in both exercise groups. These findings demonstrate that impaired cognitive function in PTSD was improved by both of the exercise protocols but mainly by HIIT via decreasing oxidative stress, anxiety levels and neuronal damage. Gibala MJ et al. (2012). *J Physiol.* 1; 590, 1077–1084.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCB148

##### **Spectrum of esophageal motor abnormalities in patients with scleroderma on high resolution manometry**

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**Background/aim:** The classic manometric findings in scleroderma esophagus are ineffective peristalsis and reduced esophageal sphincter pressure. With implementation of high-resolution manometry (HRM), different patterns of esophageal dysmotility are seen. The aim of this study is to characterize esophageal motor function by HRM and to assess associations between demographic features and esophagoscopy results and HRM findings.

**Material and methods:** Patients with scleroderma who underwent HRM between January 2017 and May 2018 were identified. Esophageal motility disorders were classified according to the Chicago Classification, v3.0 criteria. Demographic data and esophagoscopy findings were collected via retrospective chart review.

##### **Results:**

A total of 28 patients (96.4% female) with a mean age of  $56.5 \pm 13$  years were included. HRM showed absent contractility ( $n=11$ ; 39.3%), ineffective esophageal motility ( $n=10$ ; 35.7%), esophagogastric junction outflow obstruction ( $n=1$ ; 3.6%) and normal motility ( $n=6$ ; 21.4%). Esophagoscopy showed abnormalities more frequently in patients with absent contractility than patients with other motility disorders (80% vs 8.3%,  $p=0.01$ ). In fact, patients with absent contractility were more likely to have erosive esophagitis than other patients with marginal statistical significance (40% vs 0%,  $p=0.07$ ). There was no association between age and frequency of erosive esophagitis. However, lower esophageal sphincter pressure was negatively and significantly correlated with age ( $r=-0.513$ ;  $p=0.007$ ).

##### **Conclusion:**

Absent contractility and ineffective esophageal motility are the most common motility disorders in patients with scleroderma. According to endoscopic findings, patients with absent contractility seem to have more severe reflux.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCB149

##### **Role of p-Coumaric acid in alleviating of the intestinal Ischemia/Reperfusion injury**

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Intestinal Ischemia/Reperfusion (I/R) injury is an undesirable consequence of a multitude pathologies such as small bowel transplantation, cardiopulmonary bypass, hernias, neonatal necrotizing enterocolitis and abdominal aortic aneurysm surgery. Various therapy procedures have been conducted to reduce or eliminate intestinal I/R injury in experimental models to date. This study was planned to investigate the protective role of p-coumaric acid against intestinal I/R injury. For this purpose, an experimental intestinal I/R model was established in our study. In the p-coumaric acid and I/R groups, superior mesenteric artery was clamped for 1 h. Then, the clamp was removed and reperfusion was allowed for 2 h. The beginning of bleeding of the tissue and the pinking of the color were accepted as reperfusion. At the end of 2h, rats were sacrificed and intestinal tissue samples were collected rapidly. It was determined that MPO activity, MDA levels, TOS and OSI values increased due to severe oxidative damage depending on I/R. In addition, TAC value and SOD activity decreased in the I/R group. TAC value and SOD activity increased while TOS, OSI values, MPO activity and MDA level were reducing in the I/R + p-coumaric acid (50 and 100 mg/kg bw.) groups. In the p-coumaric acid 100 mg/kg group, TAC value and SOD activity more increased and OSI, TOS values more decreased compared with p-coumaric acid 50 mg/kg group. Different doses of the p-coumaric acid demonstrated protective effects against intestinal I/R (1h: 2h) injury in experimental animals

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCB150

##### **The Efficiency of Sinapic acid on oxidative parameters in secondary lung and kidney damage in the peritonite model provoked by sepsis in rats**

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Sepsis, which requires urgent treatment, is a systemic disease caused by infectious reasons and it leads to shock, organ failure, acute respiratory syndrome and even death. In this study, we examined the possible beneficial effects of Sinapic acid on rat kidney and lung tissue in an experimental cecal ligation puncture model. The cecal ligation was applied to the rats and two through-and-through punctures were made



through the antimesenteric face of the cecum and the established CLP polymicrobial sepsis model was performed in the sepsis (CLP) group. 20 mg/kg of Sinapic acid was administered in the CLP-Sinapic acid group. At the end of the experiment, lung and kidney tissues were taken and biochemical analyzes were performed. Antioxidant levels decreased, oxidant levels increased in lung and kidney tissues due to CLP. Sinapic acid showed effective results against oxidative stress caused by CLP induction. The results of our study demonstrated that Sinapic acid has beneficial effects on the oxidative stress in the lung and kidney tissues.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## PCB151

### **Role of p-Coumaric Acid on Reproductive and Remote Organ Damages Created by Adnexial Torsion/Detorsion**

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The aim of this study is to investigate the possible beneficial effects of p-coumaric acid on ovary and lung injury generated by bilateral ovary torsion/detorsion model in rats. In our study, 24 Sprague-Dawley female rats were randomly divided into 3 groups. Groups planned as group I (sham), group II (T/D) and group III (p-coumaric acid+T/D). In group I, the abdomen was opened and closed again without T/D model. In group II, 3 h of torsion followed 3 h of detorsion were made. In the group III, p-coumaric acid was given orally 100 mg/kg dose for 15 days and T/D was performed as defined in group II. At the end of detorsion period, rats were sacrificed and removed the ovary and lung tissues. MDA level, MPO activity, TOS and OSI values increased significantly in group II according to group I. However, antioxidant enzyme activity and TAS value decreased in group II. Conversely, the antioxidant enzyme activity increased, while OSI value, MPO activity and MDA level decreased significantly due to p-coumaric acid in the group III. As a conclusion, p-coumaric acid was found to be effective in protection on the ovary and lung against damage that formed by experimental T/D in rats.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## PCB152

### **Hormonal contraceptive use might have an effect on health gains achieved after combined strength and endurance training in women: results from the pilot study**

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Hormonal contraceptives (HC) are the most frequently used type of birth control among young women. The use

of HC may affect inflammation status (Kluft et al. 2002). In addition, using HC has been suggested to modify fat metabolism during exercise (Isacco et al. 2015). Considering the increased number of women doing high-intensity training and using HC, it is important to investigate the combined effects on inflammatory state and overall health. This pilot study examined the effects of a 10-week period of high intensity combined strength and endurance training on body composition, and inflammation marker concentrations in physically active women using hormonal contraceptives (HC, n = 9) compared to those who had never used hormonal contraceptives (NHC, n = 9). Training consisted of two strength training sessions, and two high-intensity running interval sessions per week.

All the measurements were done pre and post training between days 1 - 5 of each subject's menstrual cycle. Whole body composition was estimated by Dual X-ray Absorptiometry (LUNAR Prodigy, GE Medical Systems, Madison, USA). Automatic analyses (Encore-software, version 14.10.022) provided total body fat mass and total body lean mass. Abdominal fat was calculated manually. Fasting venous blood samples were drawn from an antecubital vein in the morning after a 12 h overnight fast. Serum was kept at -80°C until analysed for circulating high-sensitivity C-reactive protein (hs-CRP), interleukin-6 (IL-6), interleukin-1beta (IL-1b) and tumor-necrosis factor-alpha (TNF-α). The primary analytical method for assessing the change in the variables will be repeated measures analysis of variance (ANOVA). The data is expressed in mean ± SD.

Total fat mass decreased significantly in NHC (-6.0 ± 6.6%, p = 0.032), where as in HC group no significant changes were observed (-6.8 ± 9.7%, p = 0.068). Circulating concentrations of hs-CRP decreased significantly in the NHC from pre to post (1.3 ± 1.2 mg/l vs. 0.7 ± 0.7 mg/l, p = 0.021), whereas no significant change was observed in HC from pre to post (1.3 ± 1.3 mg/l vs. 2.3 ± 1.9 mg/l, p = 0.40). In addition, CRP concentration was significantly higher in HC than in NHC after training (p = 0.036). Circulating TNF-α concentration significantly increased in both groups from pre to post, however no between-group differences were observed.

It appears that the high-intensity exercise training can modify the inflammation status of young women. The present study shows that inflammation status in terms of hs-CRP was higher post training in HC than NHC where favorable changes in body composition were observed.

Kluft, C. et al. (2002). Pro-inflammatory effects of oestrogens during use of oral contraceptives and hormone replacement treatment. *Vascular pharmacology*, 39(3), 149-154.

Isacco, L., et al. (2015). Maximal fat oxidation, but not aerobic capacity, is affected by oral contraceptive use in young healthy women. *European journal of applied physiology*, 115(5), 937-945

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCB153

### Effects of progressive resistance training targeting skeletal muscles rich in type 1 fibers on fat oxidation rate in young sedentary individuals

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Fat oxidation rate during submaximal exercise is one of the important parameters of fitness level and is expected to be low in sedentary individuals and could be improved with training. And there is some evidence that resistance training could improve fat oxidation rate at rest. The aim of this study was to investigate the effects of progressive resistance exercises (PRE) targeting the muscles, rich in type 1 fibers, on fat oxidation rate at submaximal work rates, in young sedentary individuals. Seven sedentary volunteers (university students) recruited for this study. Their rating of global physical activity questionnaire (GPAQ) demonstrated that they were indeed sedentary individuals. All of the voluntary participants underwent an incremental cardiopulmonary exercise testing (CPET) till exhaustion using cycle ergometer. Heart rate, work load, respiratory exchange ratio (RER) and respiratory gas analysis were determined by breath by breath method. RER was used as indice to determine the fat oxidation rate. Fat oxidation rate was expressed as a percentage of the ratio of fat usage to total substrate utilization (which were calculated automatically) at the %30, %40, %50 and %60 of maximal VO<sub>2</sub>. Then the individuals performed PRE specifically targeting five muscles (namely soleus, tibialis anterior, biceps femoris, vastus medialis and adductor magnus) which known to be rich in type 1 fibers. The PRE was performed three times a week and lasted 6 weeks, then the test was repeated. The Wilcoxon test was used to evaluate pre-post training values.

The basal median value of the fat oxidation rate at %30 VO<sub>2</sub> max was % 28,5 and rised up % 44,2 but the difference was not significant (p=0,237). The median value of %40 VO<sub>2</sub> max was %17 and rised up to %24,6 after the exercise but the difference was not significant (p=0,063). The median of fat oxidation rate at %50 VO<sub>2</sub> max was %14,1 and declined to %12,05 but the difference was not statistically significant (p=0,735). The fat oxidation rate at %60 VO<sub>2</sub> max was % 4,94 and declined to %3,85 (p=0,499).

Although the effect of PRE on fat oxidation at analysis points was not statistically signifanct, this results indicated that this kind of exercise could be beneficial for fat oxidation rate while people performing the low intensity exercise at intensities around about %40 VO<sub>2</sub> such as walking in low speed, engaging in housework. This results have some implications of hope for obese people to achieve better fat oxidation with PRE, and this topic deserve further comprehensive investigation

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB154

### Time course adaptations in oxygen uptake and muscle deoxygenation kinetics during submaximal exercise subsequent to a 12 week moderate-intensity continuous training or low-volume, high-intensity interval training intervention in type 2 diabetes.

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The dynamic response of pulmonary oxygen uptake ( $\tau\text{VO}_{2p}$ ) at the onset of moderate-intensity exercise is slowed in middle-aged individuals with type 2 diabetes (T2D) (O'Connor *et al.* 2012, 2015). This effect is attributed to progressive limitations of O<sub>2</sub> delivery and/or utilisation. While traditional moderate-intensity continuous training (MICT) is an established strategy which enhances  $\tau\text{VO}_{2p}$  (McAnaney *et al.* 2012), the effects of a time efficient low-volume, high-intensity interval training (LVHIIT) on  $\tau\text{VO}_{2p}$  as well as the time course and mechanisms of adaptations of these enhanced VO<sub>2</sub> kinetics following either MICT or LVHIIT are unknown. The aim of this study was to assess the rates of adjustment of VO<sub>2p</sub> and muscle deoxygenation (i.e., deoxygenated haemoglobin and myoglobin, [HHb+Mb]) during the on-transition to moderate-intensity cycling before training and at weeks 3, 6, 9 and 12 of MICT and LVHIIT. Twenty nine middle-aged individuals with T2D (17 men, 12 women; mean  $\pm$  SD: age 53  $\pm$  10 yr, body mass index 29.8  $\pm$  4.4 kg.m<sup>-2</sup>) were randomly assigned to MICT (n=11, 50 min of moderate-intensity cycling) or LVHIIT (n=9, 10 x 1 min cycling at ~90% maximal heart rate interspersed by 1 min of 'unloaded' cycling) or to a non-exercising control group (n=9). Exercising groups trained 3 times/week and every 3 weeks exercise intensity was adjusted. VO<sub>2</sub> kinetics was calculated from continuously measured breath-by-breath VO<sub>2</sub> data, whilst the rate of muscle deoxygenation was continuously measured by near-infrared spectroscopy at the *vastus lateralis* muscle. Time point analysis of VO<sub>2</sub> and [HHb+Mb] responses were performed using a two-way ANOVA with repeated-measures, and post-hoc Tukey tests performed when significant differences were returned. Pre-training  $\tau\text{VO}_{2p}$  decreased (P<0.05) by a similar magnitude at wk 3 of training in both MICT (from 44  $\pm$  12 to 32  $\pm$  5 s) and LVHIIT (from 42  $\pm$  8 to 32  $\pm$  4 s) with no further changes thereafter. No changes were reported in the control group. The pretraining overall adjustment of  $\Delta[\text{HHb+Mb}]$  was faster than  $\tau\text{VO}_{2p}$  in all groups, resulting in  $\Delta[\text{HHb+Mb}]/\text{VO}_{2p}$  showing an "overshoot" during the transient relative to the subsequent steady-state level. After 3 wks of training the  $\Delta[\text{HHb+Mb}]/\text{VO}_{2p}$  overshoot was attenuated in both MICT and LVHIIT so that the overall adjustment of  $\Delta[\text{HHb+Mb}]$  was similar to  $\tau\text{VO}_{2p}$  in both groups. The enhanced VO<sub>2</sub> kinetics response consequent to both MICT and LVHIIT in T2D is likely attributed to a training-induced better matching of O<sub>2</sub> delivery to utilisation.

O'Connor E, Kiely C, O'Shea D, Green S & Egaña M (2012). Similar level of impairment in exercise performance and oxygen uptake kinetics in middle-aged men and women with type 2 diabetes. *Am J Physiol Regul Integr Comp Physiol* 303, 70-76.

O'Connor E, Green S, Kiely C, O'Shea D & Egaña M (2015). Differential effects of age and type 2 diabetes on dynamic versus peak responses of pulmonary oxygen uptake during exercise. *J Appl Physiol* 118, 1031-1039.

MacAnaney O, O'Shea D, Warmington S, Green S & Egaña, M. (2012). Gymnasium-based unsupervised exercise maintains benefits in oxygen uptake kinetics obtained following supervised training in type 2 diabetes. *Appl Physiol Nutr Metab*, 37, 599-609

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB155

### Analysis of the Major Risk Factors and Genetic Variations of Bone Formation Signaling Pathways in Patients of Osteoarthritis

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Osteoarthritis (OA), the most common human arthritis, is considered as polygenic disease causing pain and disability in affected individuals, with very high incidence and prevalence across the globe. Several risk factors have been involved in OA development and progression including age, gender, obesity, injury, family history and genetic variations (Jeon et al. 2017). More than 80% of the adults with OA have knee OA (KOA) in which body weight plays a very crucial role. Moreover, genetic studies revealed that OA has a substantial hereditary component and the associated genes tend to be related to the process of synovial joint development (Reynard 2017). Therefore, the study was designed to identify the potential risk factors which include vulnerable age group, gender and genetic alterations in the development of OA. To execute the case control study, detailed history with clinical and radiographic examinations were recorded in patients' data collection form. Blood samples were collected with equal number of patients (n=400) and controls (n=400) having the age range from 40 – 60 years. Tetra-primer amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) was used for genotypic analyses (Medrano & Oliveira 2009). It was observed that KOA constituted a major disease burden in patients, especially in females. Obese and overweight individuals were at high risk of developing the disease. A SNP (T>C; rs1044122) of a member from disintegrin and metalloprotease (ADAM12) gene representing the synonymous polymorphism (Ala825Ala) showed significant association with OA in local population. For the observed genotype frequencies in control group, chi-square test was applied for the Hardy Weinberg equation (HWE). The odds ratio (OR) of ADAM12 polymorphisms rs1044122 and OA risk was evaluated and the strength of association was indicated as OR (95% CI). These data suggest that the genetic variant (rs1044122) of ADAM12 gene play an important role in the progression of OA.

| Clinical Characteristics  | Patients (400) | Controls (400) | p-value* |
|---------------------------|----------------|----------------|----------|
| Age (year)                | 50.84±11.4     | 48.43±11.43    | N.S      |
| Male/Female               | 42/358         | 101/299        | <0.001   |
| BMI (Kg/m <sup>2</sup> )  | 38.10±9.08     | 26.10±5.15     | <0.001   |
| VAS                       | 5.74±1.58      | –              | –        |
| WOMAC                     | 54.7±14.47     | –              | –        |
| Onset age (years)         | 48.39±11.5     | –              | –        |
| Duration of OA (years)    | 2.44±3.29      | –              | –        |
| Radiographic KL grade (%) |                |                |          |
| 2                         | 319 (79.8%)    | –              | –        |
| 3                         | 41 (10.3%)     | –              | –        |
| 4                         | 40 (10%)       | –              | –        |

Data were as mean ± SD. Difference between groups was calculated using independent t-test and chi-square test. The \*p-values were considered as not significant when they were >0.05. SD: Standard deviation

Jeon, O.H., Kim, C., Laberge, R.M., Demaria, M., Rathod, S., Vasserot, A.P., Chung, J.W., Kim, D.H., Poon, Y., David, N. and Baker, D.J., 2017. Local clearance of senescent cells attenuates the development of post-traumatic osteoarthritis and creates a pro-regenerative environment. *Nature medicine*, 23(6), p.775.

Medrano, R.F.V. and de Oliveira, C.A., 2014. Guidelines for the tetra-primer ARMS-PCR technique development. *Molecular biotechnology*, 56(7), pp.599-608.

Reynard, L.N., 2017, February. Analysis of genetics and DNA methylation in osteoarthritis: what have we learnt about the disease? In *Seminars in cell & developmental biology* (Vol. 62, pp. 57-66). Academic Press.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB156

### Does post-exercise hypokalemia increase risk of arrhythmias?

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Intense exercise induces pronounced hyperkalemia, where arterial plasma potassium concentration ( $[K^+]_a$ ) may reach as high as 7-8 mM, then followed by a rapid decline in recovery from exercise, inducing a transient hypokalemia, with  $[K^+]_a$  less than 3.5 mM. These substantial changes occur over a time-course of minutes. As  $[K^+]_a$  is a modulator of cardiac repolarization during exercise and recovery, we investigated whether the post-exercise decline in  $[K^+]_a$  and hypokalemia affected cardiac repolarization, via QT hysteresis and/or T-wave dispersion. Eleven healthy, normokalemic



adults performed 3 min intense rowing exercise, with  $[K^+]_a$  measured at rest, every 30 s during exercise and for 60 min recovery. The QT interval, QT hysteresis, Tpe and Tpe/QT ratio were calculated from ECG (n=8). The  $T_{peak}-T_{end}$  interval (Tpe) is thought to reflect dispersion in ventricular repolarization, and abnormalities in Tpe have been associated with increased risk of arrhythmia.  $[K^+]_a$  increased above baseline during exercise (rest,  $3.91 \pm 0.21$  vs end-exercise,  $7.21 \pm 0.57$  mM,  $P < 0.001$ , mean  $\pm$  SD) and decreased rapidly during early recovery to below baseline to nadir at 5 min ( $3.26 \pm 0.25$  mM,  $P < 0.05$ ); restoration was incomplete at 60 min post-exercise ( $3.70 \pm 0.32$  mM,  $P < 0.05$ ). The post-exercise decline in  $[K^+]_a$  was correlated with QT hysteresis ( $r = 0.326$ ,  $P < 0.05$ ). The decrease in  $[K^+]_a$  from end-exercise by  $\sim 4$  mM was associated with reduced QT hysteresis by  $\sim 75$  ms. The recovery-induced hypokalemia was also associated with a prolongation of Tpe compared to pre-exercise baseline ( $112 \pm 10$  ms vs.  $87 \pm 5$  ms,  $p < 0.05$ ) and an increase in Tpe/QT ratio ( $0.29 \pm 0.02$  vs.  $0.23 \pm 0.009$ ,  $p < 0.01$ ). Analyses of serial data revealed  $[K^+]_a$  as a predictor of Tpe (after logarithmic transformation) in these healthy subjects ( $\beta = -0.37 \pm 0.1$ ,  $p < 0.001$ ) after adjusting for heart rate and recovery state. The  $[K^+]_a$  was also a predictor of Tpe/QT ratio in these healthy participants. Thus post-exercise hypokalemia impaired cardiac repolarization and predicted both Tpe and the Tpe/QT ratio, which could unmask electrophysiological vulnerabilities to arrhythmias, thereby potentially causing sudden cardiac death in susceptible individuals with pre-existing hypokalemia and/or heart disease.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB157

### Resistance training upregulates skeletal muscle $Na^+, K^+$ -ATPase content, with elevations in both $\alpha_1$ and $\alpha_2$ but not $\beta$ isoforms

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The  $Na^+, K^+$ -ATPase (NKA) is a key protein involved in the maintenance of skeletal muscle excitability and contractility, and is expressed as multiple isoforms for catalytic ( $\alpha_{1-3}$ ) and regulatory ( $\beta_{1-3}$ ) subunits. Increases in muscle NKA are established for endurance and sprint training, but the effects of resistance exercise training are less clear. We investigated whether muscle  $[^3H]$ ouabain binding site content, which measures NKA content, was increased after resistance training and which NKA isoforms ( $\alpha_1$ - $\alpha_3$ , and  $\beta_1$ - $\beta_3$ ) accounted for any NKA upregulation. Twenty-one healthy, young adult males participated in the study (age,  $22.9 \pm 4.6$  yr; height,  $1.8 \pm 0.70$  m, mass:  $85.1 \pm 17.8$  kg, mean  $\pm$  SD) and underwent either 7 weeks of either resistance training (RT, n=16) or control (CON, n=5). Participants performed three sets of 10-12 repetitions of each exercise interspersed with 60 - 90 s of recovery between sets. To induce progressive overload, the training weight was incremented by  $\sim 5\%$  when a participant completed 12 repetitions of the three total sets. The training program involved upper and lower body exercises and comprised back squat, leg raise, leg press, bench press, incline bench press, lat pull-down, lunge, dumbbell shoulder press, dumbbell bicep curl, lying triceps extensions, bent-over row, upright row, barbell

biceps curl, dips and sit-ups. The training was conducted in a laboratory under supervision. Participants underwent a resting vastus lateralis biopsy prior to and following RT or CON, for measurement of NKA content and NKA  $\alpha_{1-3}$  and  $\beta_{1-3}$  isoform abundances. The one repetition maximum (1RM) leg press increased after RT from  $339 \pm 79$  kg to  $465 \pm 111$  kg (37%,  $p = 0.001$ ) whilst the 1RM bench press increased from  $80 \pm 17$  kg to  $86 \pm 21$  kg (8%,  $p = 0.01$ ). The muscle NKA content increased (student t-test) by 12% after RT ( $311 \pm 76$  vs  $349 \pm 76$  pmol.g wet weight<sup>-1</sup>,  $p = 0.012$ ), with no change in CON ( $316 \pm 86$  vs  $328 \pm 70$  pmol.g wet weight<sup>-1</sup>,  $p = 0.271$ ). The NKA  $\alpha_1$  isoform increased by 32% ( $p = 0.013$ ) and the  $\alpha_2$  isoform increased by  $\sim 10\%$  after RT ( $p = 0.001$ ), with no significant changes in CON ( $p = 0.174$  and  $p = 0.33$ , respectively). These increases in each of NKA content,  $\alpha_1$  and  $\alpha_2$  isoform abundances with RT were robust, observed in 14, 14 and 16 of the participants, respectively. However, no differences were found in the  $\beta_1$  or  $\beta_2$  isoform abundances following RT ( $p = 0.180$  and  $p = 0.221$ , respectively). The  $\alpha_3$  and  $\beta_3$  isoforms were not detected. Thus, an increase in muscle NKA content induced by resistance training comprised increases in both of the  $\alpha_1$  and  $\alpha_2$  NKA isoform abundances. The increased NKA  $\alpha_1$  may facilitate recovery of  $Na^+/K^+$  disturbances after sets within training sessions, and increases in NKA  $\alpha_2$  could contribute to minimising  $Na^+/K^+$  disturbances and thereby resisting fatigue during intense resistance training sessions.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB158

### Three-dimensional representation of two-dimensional sonography scans to look further into human skin *in vivo*

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Sonography is a well-known non-invasive technique for skin imaging, used primarily to assess skin lesions, such as tumours, prior to surgery, but also to monitor oedema and inflammatory processes. Commercially available scanners have limited image resolution which complicates analysis especially when very different echo-densities coexist. In this work we propose a three-dimensional (3D) analysis reconstruction of the skin bi-dimensional echo image to better define, compare and quantify different echo-density structures. Images from the volar forearm were taken using a 20-MHz ultrasonograph (Dermascan C, Cortex Technology, Denmark) in 6 young healthy volunteers (aged 18-30) of both sexes. We compared non-treated skin with skin hydrated with a 5% solution of glycerine in an occlusive patch for 24 hours. All procedures complied with the principles of the Helsinki declaration and subsequent amendments. The probe was placed on the skin in a fixed standard position, with the echo recorded at a velocity of 1580 m/s, to obtain a two dimensional-image.

The colour image was converted to a grey scale image for further analysis. From the scans, 3D length-thickness-echo intensity representations were created. These representations allowed the calculation of the surface area (SA, expressed in square arbitrary units) and the volume under the surface (VUS, expressed in cubic arbitrary units) of the epidermal and dermal layers, normalized for layer thickness. The more echogenic epidermal layer showed higher mean relative VUS and SA relative to the more homogenous dermal layer ( $VUS_{epidermis}$   $0.093 \pm 0.096$ ;  $VUS_{dermis}$   $0.042 \pm 0.033$ ;  $SA_{epidermis}$   $1.0 \pm 0.005$ ;  $SA_{dermis}$   $0.144 \pm 0.001$ ). A decrease in the echo was observed in hydrated skin, for both the epidermis and dermis. Consequently, the  $VUS_{epidermis}$  decreased from  $0.215 \pm 0.250$  to  $0.041 \pm 0.075$ ; and  $VUS_{dermis}$  from  $0.033 \pm 0.050$  to  $0.009 \pm 0.012$ . No changes were observed for the SA, however. Under these experimental conditions, higher echogenicity is better revealed by higher VUS, but not SA. These 3D representations and quantitative parameters seem to offer a useful potential to further explore sonography as a quantitative instrument in experimental dermatology.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCB159

### A Comparison of the Effects of Resistance Exercise Training on Muscle Mass and Function in South Asians and White Europeans

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People of South Asian origin have age-standardised rates of type 2 diabetes ~2-4 fold higher than white people of European descent (Sattar and Gill, 2015). South Asians also have lower levels of muscle mass and strength and our recent work has shown that this low muscle strength is associated with their higher diabetes risk (Ntuk et al., 2017). This indicates that South Asians in particular may benefit from increases in muscle mass and function. The most efficacious way to increase muscle mass and function is through resistance exercise but this has rarely been investigated in South Asians. The aim of the current study, therefore, was to compare the effects of resistance exercise training on muscle mass and function in South Asians and White Europeans. Participants were 17 South Asian ( $24 \pm 4$  years,  $24.58 \pm 4$  kg/m<sup>2</sup>) and 12 European ( $32 \pm 7$  years,  $25.19 \pm 2.2$  kg/m<sup>2</sup>) men. Body composition (via MRI scan), resting metabolic rate (RMR), fasting blood glucose and 1 repetition maximum (1RM), were measured before and after 12 weeks of twice weekly resistance exercise training (Leg Press, Leg Extension, Leg Flexion, Calf Press, Chest Press, Seated Row, Lat Pull Down, Bicep Curl and triceps Curl). Inter-ethnic differences in responses to resistance training were compared via two-way (time and ethnicity) repeated measures ANOVA using SPSS version 24. Thigh muscle mass increased significantly ( $P < 0.05$ ) with exercise training with no differences ( $P > 0.05$ ) in responses between ethnic groups (South Asians: baseline  $12.73 \pm 1.12$  Litres to  $13.19 \pm 1.23$  Litres at 12 weeks and White Europeans: baseline  $13.00 \pm 1.02$  Litres to  $13.23 \pm 0.92$  Litres at 12 weeks). The sum of the 1RM for both upper and lower body exercises significantly increased ( $P < 0.05$ ) for both groups (1RM for upper body at baseline was  $497 \pm 108$  lb (SA),  $536 \pm 59$  (WE) compared to  $626 \pm 131$  (SA),  $699 \pm 78$  lb (WE) at 12 weeks and 1RM for lower body

at the baseline  $628 \pm 149$  (SA),  $695 \pm 101$  (WE) lb compared to  $815 \pm 231$  (SA),  $914 \pm 123$  (WE) lb at 12 weeks), with no significant ( $P > 0.05$ ) differences in responses between ethnic groups. The 12 weeks of resistance exercise had no effect ( $P > 0.05$ ) on Fasting blood glucose, RMR, visceral fat or liver fat with no group or interaction effects observed either. The current study has shown that South Asian men respond to resistance exercise with increases in muscle mass and function and that these responses are of a similar magnitude as in White Europeans. Further work with a larger sample size is needed to confirm these findings and the wider metabolic effects of resistance exercise training in South Asians.

Faris ABA alkhayl, Ahmad Ismail, John Wilson, Naveed Sattar, Sasha Radjenovic, Carlos Celis-Morales, Jason MR Gill, Stuart R Gray

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCB160

### Light-induced ROS production by SuperNova activates Nrf2 response

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Reactive oxygen species (ROS) oxidize and damage DNA, lipids or proteins leading to oxidative stress which is considered a central feature of diseases such as cancer, diabetes and atherosclerosis. However, when produced at low concentrations and in a controlled manner, ROS can mediate redox signaling in various tissues in response to growth factors, cytokines and calcium signals. However, information on transient and local changes in ROS production are still missing. To acutely induce ROS production and study time-dependent changes in redox signaling, we have used an optogenetic tool called SuperNova. This is a GFP homolog which, upon irradiation with green light, produces singlet oxygen ( $^1O_2$ ) and superoxide ( $O_2^-$ ) as a product of the reaction of oxygen with the excited state of the chromophore. Thereby, SuperNova is able to inactivate target molecules (redox-targets) by a process named Chromophore-assisted light inactivation (CALI). HEK293 cells expressing SuperNova were illuminated for various times (15 seconds, 1 min, 5 min, 10 min and 15 min) and oxidation of peroxiredoxin 3 (Prx3) was analyzed as a footprint of acute SuperNova-dependent ROS production. However, no changes in the ratio of Prx3 dimers to its monomers (as compared to  $100 \mu\text{mol/L H}_2\text{O}_2$ ) were observed after lighting the cells. Alternatively, HEK293 cells expressing SuperNova were illuminated for one minute and Nrf2-dependent genes of the so called antioxidant response were evaluated by qPCR after 15, 30, 60 and 120 minutes of incubation. NQO1 (NAD(P)H Quinone Dehydrogenase 1) and Gclc (catalytic glutamate-cysteine ligase), were significantly increased after 15 minutes incubation and reduced back to basal levels after 120 minutes. With this novel light-inducible (optogenetic approach), we could produce transient amounts of ROS which trigger an Nrf2 response which is essential for redox homeostasis.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB161

**Acute aerobic or resistance exercise does not affect circulating levels of mitochondrial peptides Humanin and MOTS-C**

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**Introduction:**

The role of mitochondria in cellular metabolism (ATP production and thermogenesis) is well recognized. Yet, mitochondria are involved in intra-cellular signaling, apoptosis, and other cellular processes. Recently, the discovery of mitochondrial-derived peptides (MDPs) has drastically expanded the importance of mitochondria for whole body metabolism and longevity. Humanin (HN) and MOTS-C are encoded and secreted mitochondria-derived peptides (MDPs) linked to glucose metabolism and tissue protecting mechanisms. The aim of this study was to investigate how different exercise regimes influence serum levels of HN and MOTS-C.

**Methods:**

Healthy volunteers (n=30; 18 men and 12 women; 31 ± 8 yrs, 25 ± 4 kg/m<sup>2</sup>) were randomized to an acute bout of either endurance (45 min biking, 70% VO<sub>2</sub>max) or resistance exercise (4 x 7 x 2 exercises), or control. Blood samples were obtained before and at 30 min, 3 hrs, 8 hrs and 24 hrs after the exercise session, and serum MDPs were analyzed using ELISA. All subjects were characterized with respect to estimated VO<sub>2</sub>max (Eklom-Bak test) and 7-repetition maximum (7RM) for the knee extension and leg press exercise.

**Results:**

Estimated mean VO<sub>2</sub>max was 45.2 ± 9.7 ml/kg/min, knee extension 7RM was 68.3 ± 18.9 kg, and leg press 7RM was 148.0 ± 45.3 kg, with no differences across groups. Basal HN and MOTS-C concentration did not differ between groups, and was 583.6 ± 115.4 pg/mL, and 271.8 ± 69.3 ng/mL, respectively. No statistical differences were observed for neither HN nor MOTS-C across time points or exercise mode. Furthermore, no significant correlations were found between serum MDP levels and any of the physical parameters measured.

**Discussion**

Our data suggest that serum levels of mitochondrial peptides (HN and MOTS-C) do not change in response to neither acute endurance nor resistance exercise. Hypothetically, acute exercise stimulates production of MDPs, which are not released into the circulation but rather exerting a local paracrine/autocrine effect. During the experimental trial, skeletal muscle biopsies were also obtained and this hypothesis will be tested in the near future.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## PCB162

**Limitations to high-intensity exercise tolerance: implications of the task power**

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High-intensity exercise tolerance is described by the hyperbolic power-tolerable duration (P-tLIM) relationship. Tolerable duration is proposed to be defined by the expenditure of a fixed amount of work, reflected in the curvature constant parameter (W') of the hyperbola. However, it is unclear whether W' depletion at task failure represents the attainment of the physiological limits for producing the required task power in all high-intensity tests, or whether a 'reserve' in the capacity to generate power exists. Therefore, we investigated (1) whether the physiological limits were attained at task failure in four different high-intensity tests and (2) the relationship between fatigue and W' depletion, in tests with different W' depletion rates.

In nine healthy participants, maximal voluntary isokinetic cycling power (P<sub>ISO</sub>) at 80 rpm was measured before and immediately at task failure of four constant-power cycling tests of differing power and tolerable duration. The constant-power tests were used to characterise the P-tLIM relationship, while the reduction in P<sub>ISO</sub> from baseline provided a measure of fatigue and identified the presence of any reserve (task failure P<sub>ISO</sub> vs. task power). Subsequently, the power predicted to result in task failure at 6 min (WR6) and 12 min (WR12) was estimated from the P-tLIM relationship. During completion of these WR6 and WR12 tests we measured P<sub>ISO</sub> at baseline, 1/3<sup>rd</sup> and 2/3<sup>rd</sup> of expected W' depletion, and immediately at task failure.

Baseline P<sub>ISO</sub> was not different across the six constant-power tests (mean ± SD: 837 ± 108 W; p > 0.05). Similarly, VO<sub>2peak</sub> was not different across all six constant-power tests, despite the differences in task power (4.23 ± 0.52 L/min; p > 0.05). Fatigue developed to the extent that task failure P<sub>ISO</sub> was not greater than task power (i.e. no reserve) in the two higher-power, shorter duration tests (338 ± 53 vs. 325 ± 35 W and 348 ± 57 vs. 305 ± 34 W; both p > 0.05). A reserve was present, with fatigue insufficient to limit the task, in the two lower-power, longer duration tests (356 ± 85 vs. 282 ± 34 W and 420 ± 89 vs. 266 ± 37 W; both p < 0.05). During WR6 vs. WR12, P<sub>ISO</sub> was not different at 1/3<sup>rd</sup> (623 ± 119 vs. 606 ± 94 W; p > 0.05) and 2/3<sup>rd</sup> (485 ± 95 vs. 479 ± 111 W; p > 0.05) W' depletion. However, task failure P<sub>ISO</sub> in WR6 was less than WR12 (360 ± 75 vs. 413 ± 99; p < 0.05).

Reducing the task power and W' utilisation rate, thus increasing the exercise duration, led to task failure at VO<sub>2peak</sub> with a power reserve (task failure P<sub>ISO</sub> greater than task power). This suggests some tests are terminated at VO<sub>2peak</sub> with a reserve in the capacity to generate power, and mechanisms other than muscle fatigue are contributory. The time course of the fatigue-induced fall in P<sub>ISO</sub> during WR6 vs. WR12 suggests these alternative mechanisms of exercise limitation become more prevalent late in the exercise test.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*



## PCB163

**Differences in the cardiorespiratory and metabolic responses in endurance athletes, members of counter-terrorism center and senior athletes**E. Tóth<sup>2</sup>, A. Koller<sup>1,3</sup>, F. Ihász<sup>2</sup> and L. Kósa<sup>2</sup><sup>1</sup>Natural Sciences, University of Physical Education, Budapest, Hungary, <sup>2</sup>Sportsciences, Eötvös Loránd University, Szombathely, Hungary and <sup>3</sup>Faculty of Health Sciences, Semmelweis University, Budapest, Hungary

Exercise is a great challenge for cardiovascular system and metabolism and can achieve different adaptive responses depending on the frequency, intensity, and type of physical activity and also on the age of individuals. Thus we hypothesized that the adaptations of cardiovascular, respiratory, nervous and hormonal systems will be different to the increased energy and oxygen requirements in different groups of people exposed to different physical activities. Thus we aimed to carry out these investigations in endurance athletes (EA), members of Counter Terrorism Center (CTC) and senior athletes (SA) because their training methods and ages of the three the groups are different according to the requirements of their professions.

Endurance athletes (age:  $24 \pm 5.9$ ,  $n=8$ ), members of Counter Terrorism Centre (age:  $31 \pm 3.4$ ,  $n=14$ ) and senior athletes (age:  $52 \pm 3$ ,  $n=17$ ) participated in the study. The study was approved by the Research Ethics Committee of ELTE University. In all groups, the body weight, body height, and exercise load were measured. The cardiorespiratory fitness was measured by Marquette-2000 treadmill (Pittsburgh, PA, USA) to maximal fatigue. The resting heart rate ( $\text{beat}/\text{min}$ ,  $\text{HR}_{\text{rest}}$ ) and maximal heart rate ( $\text{HR}_{\text{max}}$ ), were measured by "Cardiosoft", (Milwaukee, USA), aerobic capacity ( $\text{VO}_{2\text{max}}$ ), ventilation (VE) ( $\text{BTPS L} \times \text{min}^{-1}$ ) and its components were measured by Sensor Medics "Vmax 29C" (Yorba Linda, CA, USA). Statistical analysis was performed by Stat. Soft. 2013. Data are presented as mean  $\pm$  SEM.

We have found a significant correlation between the average meanbodyweightofthegroups ( $\text{BW}_{\text{EA}}=72 \pm 7.5$ ;  $\text{BW}_{\text{CTC}}=84 \pm 7.8$ ;  $\text{BW}_{\text{SA}}=84 \pm 7.8 \text{ kg}$ ). The exercise performance of the endurance athletes was the greatest ( $451 \pm 49 \text{ W}$ ). The relative aerobic capacity measured at the peak of the exercise performance of endurance athletes was  $3 \text{ ml} \times \text{kg}^{-1} \times \text{min}^{-1}$  higher than that of the members of Counter Terrorism Centre ( $\text{RVO}_{2\text{maxEA}} = 59.1 \pm 9.2 \text{ mL} \times \text{kg}^{-1} \times \text{min}^{-1}$ ;  $\text{RVO}_{2\text{maxCTC}} = 56.4 \pm 6.6 \text{ mL} \times \text{kg}^{-1} \times \text{min}^{-1}$ . These values differed significantly from each other at the anaerobic threshold ( $\text{RVO}_{2\text{maxEA}} = 56 \pm 8.6$ ;  $\text{RVO}_{2\text{maxCTC}} = 46.02 \pm 5.4$ ,  $p < 0.05$ ). The maximum heart rate corresponded to the age characteristics ( $\text{HR}_{\text{maxEA}} = 190.8 \pm 9.2$ ;  $\text{HR}_{\text{maxCTC}} = 183.4 \pm 10.95$ ;  $\text{HR}_{\text{maxSA}} = 153.4 \pm 17.3 \text{ beat} \times \text{min}^{-1}$   $p < 0.05$ ). The volume of ventilation of the first two groups reached  $150 \text{ L} \times \text{min}^{-1}$ , whereas the normalized ventilation values to body mass showed significant differences ( $\text{RVE}_{\text{EA}} = 2.1 \pm 0.2$ ;  $\text{RVE}_{\text{CTC}} = 1.79 \pm 0.2$ ).

Thus the exercise performance of endurance athletes and senior athletes depend primarily on aerobic metabolism, whereas the members of Counter Terrorism Centre due to their special exercise training have greater anaerobic energy support to achieve their exercise performance. In contrast, their cardiorespiratory performance was lower than that of endurance athletes. Interestingly, however - in some individuals - extreme load-induced changes in physiological parameters were group-independent.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB164

**Effect of physical activity on plasma PCSK9 in prediabetic subjects.**K.A. Mäkelä<sup>1</sup>, J. Leppäluoto<sup>1</sup>, J. Jokelainen<sup>2</sup>, S. Keinänen-Kiukaanniemi<sup>2</sup> and K. Herzig<sup>1</sup><sup>1</sup>Research Unit of Biomedicine, University of Oulu, Oulu, Finland and <sup>2</sup>Center for Life Course Epidemiology and Systems Medicine, Faculty of Medicine, University of Oulu, Oulu, Finland

**Problem statement:** Proprotein convertase subtilisin-kexin type 9 (PCSK9) is a liver serine protease that regulates LDL cholesterol metabolism (Seidah et al. 2014). PCSK9 binds to LDL receptors, and guides them to lysosomes, where they will be degraded. In the absence of LDL receptors, liver cannot anymore bind the LDL, and the level of circulating LDL cholesterol increases. The aim of the study was to measure the plasma PCSK9 concentrations in prediabetic subjects with or without statin treatment and its association between PCSK9 and physical activity.

**Methods:** 67 subjects with high risk of type 2 diabetes were recruited to a 3-month intervention to achieve moderate physical activity registered by an accelerometer. Blood samples were collected at baseline and after intervention for analysis of PCSK9 and lipid biomarkers. Univariate analysis was used to assess the statistical significances.

**Results:** The mean number of daily steps was  $5870 \pm 3277$  in the intervention group and  $4034 \pm 3460$  in the control group at the acceleration levels 1.3-1.7 g ( $p < 0.029$ ). At baseline statin treated subjects had higher plasma PCSK9 levels and total cholesterol was positively correlated with plasma PCSK9 ( $R=0.320$ ,  $p=0.008$ ), while maximal oxygen uptake was negatively associated ( $R=-0.252$ ,  $p=0.044$ ). After the 3 months physical activity intervention plasma PCSK9 levels were positively correlated with HDL cholesterol ( $R=0.264$ ,  $p=0.030$ ), while maximal oxygen uptake was even stronger inversely associated ( $R=-0.410$ ,  $p=0.0008$ ). Interestingly, plasma PCSK9 levels were higher in April than in January.

**Conclusion:** The physical activity by our subjects with high risk for type 2 diabetes did not influence plasma PCSK9 levels. Higher physical activities might be more effective in reducing PCSK9 levels, but our prediabetic subjects were not able to increase their physical activity further.

Seidah, N.G., Awan, Z., Chrétien, M. & Mbikay, M. 2014, "PCSK9: A key modulator of cardiovascular health", *Circulation research*, vol. 114, no. 6, pp. 1022-1036.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB165

# **Tissue saturation index in human skeletal muscle during cycling at different exercise intensity and cadence.**

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Skeletal muscle oxygenation may be a factor that determines cadence during cycling exercise. It can be investigated non-invasively in humans via near-infrared spectroscopy, which determines degrees of oxygenated and deoxygenated haemoglobin, total haemoglobin and a tissue saturation index (TSI) (Grassi and Quaresima, 2016). For a given power output, increasing cadence is associated with a greater cardiopulmonary and metabolic response (Formenti et al., 2015). Previous studies presented an associated decrease in skeletal muscle oxygenation during cycling exercise (Skovereng et al 2016), when cadence was studied in an incremental sequence (from 60 to 110 revolutions per minute). We hypothesised that TSI would decrease both at a very low cadence (30 rpm) due to intermittent blood flow caused by the elevated intramuscular pressures, and at a high cadence (110 rpm) due to the associated increase in oxygen consumption.

We recorded cardiopulmonary and metabolic responses (heart rate, lactate, oxygen uptake and carbon dioxide production) to cycling at exercise intensities of 70% and 90% of the ventilatory threshold (Tvent), and used near-infrared spectroscopy to determine TSI as a measure of skeletal muscle (*vastus lateralis*) oxygenation. Twelve participants (7 male and 5 female) cycled at cadences of 30, 50, 70, 90 and 110 revolutions per minute (rpm), each for 4 min, in a randomised sequence, interspersed with periods of active recovery.

Cardiopulmonary and metabolic responses were greater at 90% than at 70% Tvent ( $p < 0.05$ ), and at 110 rpm compared with lower cadences ( $p < 0.05$ ). In contrast, most indices of *vastus lateralis* oxygenation were not different between the two exercise intensities and five cadences tested (Figure 1). The amplitude of the cyclical TSI trace, a dynamic index of skeletal muscle oxygenation, did not show physiologically important differences between 90% and 70% Tvent, and between cadences.

Our results indicate that skeletal muscle tissue saturation index is not substantially affected during cycling for short periods of time at constant, moderate exercise intensity at cadences between 30 and 110 rpm. We conclude that skeletal muscle oxygenation may not be an important negative feedback signal in the choice of self-selected cadence during cycling exercise.

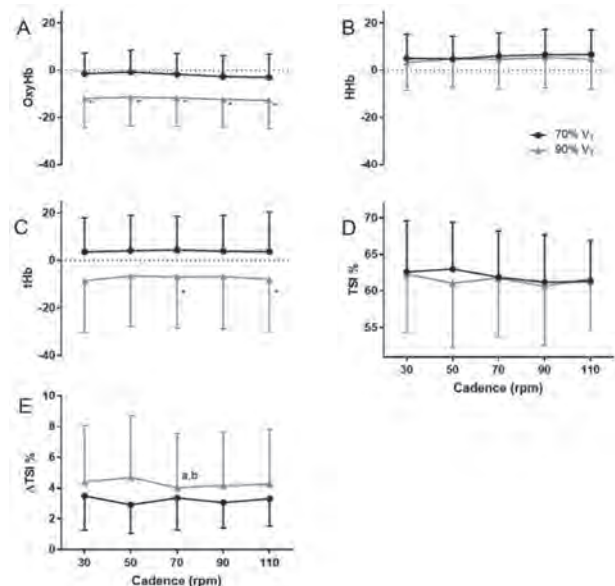


Figure 1: Indices of skeletal muscle oxygenation at different cycling exercise intensities and cadences.

Mean and standard deviation values: (A) OxyHb, (B) HHb, (C) tHb, (D) TSI and (E)  $\Delta$ TSI for each cadence (30, 50, 70, 90 and 110 rpm) during the 70% VT (black symbols) and 90% VT (grey symbols) tests.  $N = 12$ . TSI and  $\Delta$ TSI are presented as percentage values; OxyHb, HHb and tHb are presented in arbitrary units and shown as changes from resting values.

\*  $P < 0.05$  when compared to the same cadence at 70% VT.

a, b:  $P = 0.016$  and  $0.002$  when compared to 30 and 50 rpm at the same VT.

Abbreviations: TSI: Tissue Saturation Index;  $\Delta$ TSI: amplitude of TSI cyclical oscillation within pedal revolution; Hb: Haemoglobin; OxyHb: Oxygenated Hb; HHb: Deoxygenated Hb; tHb: total Hb; VT: Ventilatory threshold; rpm: revolutions per min.

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Formenti F, Minetti AE & Borrani F (2015). *Physiol Rep* 3, e12500.

Skovereng K et al. (2016). *Eur J Appl Physiol* 116, 1207-1217.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB166

# **NR4A3 is a contraction-responsive transcription factor that is induced by acute bouts of exercise and influences metabolism in human skeletal muscle**

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Perturbation of skeletal muscle homeostasis by exercise training promotes extracellular, contractile and metabolic modifications that increase the robustness of this tissue. These beneficial adaptations are a culmination of repeated, transient

changes in post-exercise gene expression – coordination of which is heavily regulated by the activity of exercise-induced transcription factors. *NR4A3* is one such transcription factor, identified as being responsive to aerobic exercise and recently hypothesised to be an important mediator of the adaptive response to exercise in rodents. Nevertheless, little is known regarding the role of *NR4A3* in human skeletal muscle.

A meta-analysis of publically available microarrays was performed to interrogate the response of *NR4A3* after acute exercise, exercise training and chronic unloading of human skeletal muscle and its association with *PPARGC1A* expression. The contractile response of *NR4A3* in cell culture was measured after electrical pulse stimulation (3 h at 40 V, 1 Hz and 2 ms) by RT-qPCR. The effect of siRNA silencing of *NR4A3* on substrate flux and metabolic regulatory genes, with and without salbutamol treatment (20  $\mu$ M), was quantified by Seahorse XF and RT-qPCR analysis, respectively. The impact of *PPARGC1A* viral overexpression on *NR4A3* was also assessed by RT-qPCR. Primary human skeletal muscle myotubes from healthy donors were the model of study for all *in vitro* experiments and data is presented as mean  $\pm$  SD.

*NR4A3* is upregulated in response to acute aerobic and resistance exercise but downregulated after chronic unloading in human skeletal muscle biopsies. Furthermore, *NR4A3* and *PPARGC1A* expressions correlate in the acute (3 h) post exercise period, in sedentary cohorts only ( $r = 0.65$ ,  $P = 0.003$ ). *In vitro*, electrical pulse stimulation significantly increases *NR4A3* relative to basal control ( $2.2 \pm 0.9$ ,  $P = 0.04$ ). ~80% silencing of *NR4A3* attenuates salbutamol-stimulated upregulation of glycolysis ( $3.7 \pm 1.1$  vs.  $5.3 \pm 1.1$  mpH.min<sup>-1</sup>,  $P < 0.001$ ). This effect may be associated with greater *PDK4* in knockdown conditions ( $7.4 \pm 6.1$  vs.  $1.7 \pm 0.6$  relative mRNA,  $P = 0.06$ ). Viral overexpression of *PPARGC1A* results in ~1-fold increase of *NR4A3* relative to GFP control ( $2.1 \pm 1.5$ ,  $P = 0.03$ ), whilst *PPARGC1A* is unchanged by *NR4A3* silencing.

These data establish *NR4A3* as a contraction-responsive, exercise-inducible transcription factor that influences metabolism in human skeletal muscle. *NR4A3* may also operate downstream of *PPARGC1A*.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB167

### Effect of supplementation with fish oil rich in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on motor nerve function after eccentric contractions

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We have previously shown that eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) supplementation ameliorated reductions in muscle strength, the development of delayed onset muscle soreness (DOMS), and limited range of motion (ROM) following eccentric contractions (ECC) [1]. We have hypothesized that, as one of the mechanisms underlying this phenomenon, EPA and DHA play an important role related to nerve structure and/or function. However, as yet no study has investigated the effect of EPA and DHA supplementation on ECC-induced nerve damage. Therefore, this study investigated the effect of supplementation with fish oil rich in EPA and DHA on the M-wave latency of biceps brachii

and muscle damage after a single session of maximal elbow flexor ECC. The study was conducted in accordance with the principles of the Declaration of Helsinki; it was approved by the Ethics Committee for Human Experiments at Juntendo University (ID: 27-66) and has been registered at the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR, identifier: UMIN000018285). Twenty-one men were completed the randomized, double-blind, placebo-controlled, and parallel-design study. The subjects were randomly assigned to the fish oil group ( $n = 10$ ) or control group ( $n = 11$ ). The fish oil group consumed eight 300-mg EPA-rich fish oil softgel capsules (containing, in total, 600 mg EPA and 260 mg DHA) per day for 8 weeks before the exercise, and continued this for a further 5 days. The control group consumed an equivalent number of placebo capsules. The subjects performed six sets of ten eccentric contractions of the elbow flexors using a dumbbell set at 40% of their one repetition maximum. M-wave latency was assessed as the time taken from electrical stimulation applied to Erb's point to the onset of M-wave of the biceps brachii. This was measured before and immediately after exercise, and then after 1, 2, 3, and 5 days. Values are expressed as means  $\pm$  standard deviation (SD). Measurements over time were compared between the CON and EPA groups by two-way repeated-measure analysis of variance (ANOVA). When a significant main effect or interaction was found, Bonferroni's correction was performed for the post-hoc testing. A  $p$ -value of  $<0.05$  was considered statistically significant. Compared with the control group, M-wave latency was significantly shorter in the fish oil group immediately after exercise (CON,  $133.0 \pm 32.1\%$  vs. EPA,  $113.3 \pm 26.5\%$ ;  $p = 0.040$ ). MVC torque was significantly higher at 1 day after exercise ( $p = 0.049$ ), ROM was significantly greater at post and 2 days after exercise (post;  $p = 0.006$ , day 2;  $p = 0.014$ ), and there was significantly less delayed onset muscle soreness at 1 and 2 days after exercise (day 1;  $p = 0.049$ , day 2;  $p = 0.023$ ). In summary, eight weeks of EPA and DHA supplementation may play a protective role against motor nerve function and may attenuate muscle damage after eccentric contractions.

Tsuchiya Y et al. (2016). *Eur J Appl Physiol*. 116, 1179-1188.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB168

### A comparison of the effect of high and low intensity intermittent and continuous exercise on gastric emptying rate, appetite and substrate utilisation in healthy males

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Recent studies have suggested high-intensity intermittent exercise reduces body weight and increases cardiovascular fitness and insulin sensitivity in untrained individuals [1-3]. Little is known whether intermittent compared to continuous exercise differentially affects subsequent gastrointestinal and appetite response as well as substrate utilisation. This study aimed to investigate the effect of high intensity and low intensity intermittent and continuous cycling on gastric emptying rate, appetite, blood glucose and substrate utilisation in the hours following exercise and the consumption of a standardised semi-solid meal. Twelve healthy males (Mean  $\pm$  SD;



age  $30 \pm 6$  years; height  $179 \pm 8$  cm; body mass  $79 \pm 9$  kg;  $VO_{2peak}$   $38 \pm 6$  ml/kg/min) completed four 60 min cycles. These were either at 60%  $VO_{2peak}$  (HIGH) or 40%  $VO_{2peak}$  (LOW) in a continuous (CON) or intermittent (INT) manner. INT consisted of 20 X 1 min exercise bouts followed by 2 min rest. INT and CON at each intensity were matched for total work output and were completed in a randomised order after an overnight fast. Gastric emptying rate was measured for 2 h post-meal using the  $^{13}C$ -breath method. Blood glucose was measured at baseline, mid-exercise, post-exercise, pre-meal and at 30 min intervals following ingestion. Appetite was assessed at regular intervals throughout the trials and substrate utilisation was measured at baseline and every 30 min after exercise. No differences in fat and carbohydrate oxidation ( $P>0.05$ ) between trials were observed. Blood glucose was greater on LOW-CON compared to LOW-INT post-exercise ( $4.8 \pm 0.5$  v  $4.4 \pm 0.5$  mmol/L;  $P=0.006$ ) and 30min post-meal ( $7.6 \pm 1.0$  v  $7.0 \pm 1.0$  mmol/L;  $P=0.013$ ). Blood glucose was greater on HIGH-INT compared to HIGH-CON mid-exercise ( $4.9 \pm 0.4$  v  $4.3 \pm 0.5$  mmol/L;  $P=0.027$ ), but lower 30 min ( $6.5 \pm 0.9$  v  $7.5 \pm 0.9$  mmol/L) and 60 min post-meal ( $4.8 \pm 0.4$  v  $5.4 \pm 0.7$  mmol/L;  $P=0.011$ ). No differences for gastric emptying lag-phase and half-emptying time of the test meal were observed ( $P<0.05$ ), however, Delta Over Baseline (DOB) values were lower ( $P < 0.05$ ) on HIGH-INT compared to HIGH-CON from 30-120 min after ingestion (Figure 1). Hunger and Prospective Food Consumption were lower between 60-75 min during the HIGH-INT compared to HIGH-CON ( $P<0.05$ ). The results of this study demonstrate that high intensity intermittent cycling may delay subsequent gastric emptying rate of a semi-solid meal and reduce blood glucose compared to high intensity continuous cycling without significantly increasing appetite or effecting substrate utilisation within a 2 h recovery period. The mode of exercise appears to have little impact on these markers when performed at a low intensity.

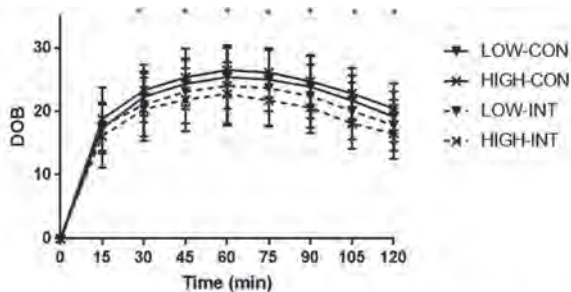


Figure 1: Gastric emptying rate of the standardised semi-solid meal and 100mg  $^{13}C$  sodium acetate. Delta over baseline (DOB) values were mean  $\pm$  SD ( $n = 12$ ). \* HIGH-INT values were significantly lower from HIGH-CON values, determined by Bonferroni adjusted paired  $t$  test,  $P < 0.05$ .

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## Circadian influences on gastric emptying rate, blood glucose and substrate utilisation during fasted and non-fasted brisk walking in healthy males

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Recent studies have highlighted the importance of the biological clock as a modulator of energy balance and metabolism<sup>[1,2]</sup>. Intermittent fasting has become an increasingly popular intervention for metabolic health and combining intermittent fasting with exercise may lead to benefits for weight management<sup>[3]</sup>. However, little is known about the diurnal variation of fasted exercise and energy balance. Therefore, the aim of this study was to investigate the circadian influences on gastric emptying rate, blood glucose, and substrate utilisation during fasted and non-fasted brisk walking. Twelve healthy males (Mean  $\pm$  SD; age  $25 \pm 3$  years; height  $178 \pm 6$  cm; body mass  $83 \pm 12$  kg;  $VO_{2peak}$   $39 \pm 4$  ml/kg/min) completed four 45 min treadmill walks in a randomised order. Walks were completed in the morning (AM) and evening (PM) and either fasted (FAST) or after consumption of a standardised breakfast meal (FED). Gastric emptying (GE) rate of a standardised semi-solid lunch meal was measured for 2 h using the  $^{13}C$ -breath method. Blood glucose concentration was measured at baseline, pre- and post-breakfast period, pre- and post-exercise, plus pre-lunch meal and at 30 min intervals following ingestion for 2 h. Substrate utilisation was measured every 30 min and continuously throughout exercise. Appetite was assessed at 15 min intervals throughout and 24 h energy intake post trials was assessed by diet diaries. No difference in GE half-emptying time was observed but lag phase was slower in PM-FAST compared to AM-FAST and PM-FED ( $75 \pm 18$  vs.  $63 \pm 14$  min,  $P=0.006$  and vs.  $67 \pm 16$  min,  $P=0.040$ ). Blood glucose concentration pre-exercise was greater in AM-FED compared to PM-FAST ( $5.80 \pm 1.30$  vs.  $4.34 \pm 0.31$  mmol/L  $P=0.014$ ), and greater in PM-FED compared to AM-FAST and PM-FAST ( $6.38 \pm 1.15$  vs.  $4.69 \pm 0.58$ ,  $P=0.005$  and  $4.34 \pm 0.31$  mmol/L,  $P=0.001$ ). No differences between trials were seen post-exercise ( $P>0.05$ ), however, glucose concentration was greater pre-lunch in AM-FED compared to AM-FAST ( $5.28 \pm 0.63$  vs.  $4.71 \pm 0.40$  mmol/L;  $P=0.042$ ). Fat oxidation was greater pre-exercise in PM-FAST compared to PM-FED ( $0.11 \pm 0.04$  vs.  $0.06 \pm 0.02$  g/min;  $P=0.003$ ), greater throughout exercise for both FAST trials compared to FED (all  $P<0.05$ ) and greater at pre-lunch in AM-FAST and PM-FAST than PM-FED ( $P=0.018$ ;  $P=0.021$ ). No differences between trials were observed for appetite post-lunch ( $P>0.05$ ) or 24 h post-energy intake ( $P=0.476$ ). These results suggest that appetite does not follow a diurnal variation following low intensity fasted exercise and that regardless of the time of day fasted exercise favours fat metabolism and may help induce a negative energy balance without a subsequent compensatory response in energy intake. Further studies on the implications of fasted exercise for energy balance and metabolic health are required.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB170

### Diving-related microparticle responses following imposed oscillatory shear stress

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Diving with self-contained underwater breathing apparatus (SCUBA) leads to endothelial dysfunction and the elevation of circulating microparticles (MPs) that could initiate a systemic inflammatory process after resurfacing. An impaired endothelial function has also been observed following acute periods of oscillatory shear stress in the conduit arteries. The aim of this study was to assess whether the vascular endothelium is more susceptible to induced oscillatory shear stress following SCUBA dive. Twelve male divers (age 39.3±9.2 years, height 186±6 cm, weight 88.0±11.6 kg) completed a single dive to a depth of 18m sea water with a 47-min bottom time. Subgroups of endothelium-derived (CD31+/CD41b-), platelet specific (CD41a+) and platelet-endothelium adhesion molecule (CD31+) MPs were assessed before and after an intervention of imposed oscillatory shear stress by a 20-min cuff inflation to 75 mmHg over the forearm, pre- and post-dive. Endothelium derived CD31+/CD41b- MPs remained unchanged pre- and post-dive and intervention. Diving induced an increase in CD31+ and CD41a+ (p<0.05). Oscillatory shear stress increased CD31+ and CD41a+ pre-dive (p<0.05), but not post-dive (p>0.05). Some markers of endothelial function and platelet activation seem to be susceptible to oscillatory shear stress pre-dive, but resistant post-dive.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB171

### High intensity exercise training prior to major elective surgery is well tolerated and associated with impressive cardiopulmonary improvement.

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Impaired cardiorespiratory fitness (CRF) is associated with poor post-operative outcome following major surgery (Moran et al., 2016). Given its importance, approximately 30,000 preoperative cardiopulmonary exercise tests (CPET) are conducted in the UK each year to assess patient risk and plan care (Levett et al, 2018). Exercise training can improve CRF prior to surgery (West et al. 2015), however the current evidence base is lacking. Thus, we conducted a clinical case study to address feasibility and benefits of high intensity interval training (HIIT).

A 70 year-old female who underwent an oesophagectomy for oesophageal cancer developed ischaemia of the gastric conduit and was left with a pharyngostomy, and feeding jejunostomy. Further surgery for restoration of the upper gastrointestinal tract with a colonic interposition was considered, however the patient's fitness for surgery was stratified as high-risk. The medical history also included a myocardial infarction and coronary artery bypass graft. Following CPET to determine baseline fitness, a 10-week supervised HIIT intervention was conducted. Three exercise sessions of 40 minutes duration separated by 48 hours recovery were completed each week using cycle ergometry. Sessions comprised six, two-minute bouts of heavy exercise (50% difference between power output at peak exercise and anaerobic threshold (AT)) interspersed by three minutes of moderate exercise (80% power at AT) based on previous research (West et al. 2015). Heart rate (12-lead ECG) and blood pressure was monitored during exercise. A CPET was conducted every two weeks and HIIT intensity adjusted accordingly.

The HIIT intervention was well tolerated with no adverse events occurring, and 29 of 30 sessions completed. After 10 weeks, pulmonary oxygen uptake at peak exercise and AT increased by 36 and 27% respectively (18.6 vs 13.7 and 10.5 vs 8.3 ml.kg<sup>-1</sup>.min<sup>-1</sup>) and traversed a fitness stratification threshold of 15 ml.kg<sup>-1</sup>.min<sup>-1</sup> for peak oxygen uptake. The patient was referred for surgery.

Despite the high intensity exercise, HIIT proved feasible, safe, and well tolerated. Impressive cardiopulmonary adaptation occurred in excess of variation typical of repeated measures (Rose et al., 2018). Improved CRF over a short period of time enabled the patient to be classified as "fit" for surgery, and thus supports investigating HIIT in future trials as an adjunct prior to major surgery.

Table 1. Cardiorespiratory fitness response to a high intensity interval training intervention prior to major surgery.

|                                                                                     | Baseline | 2 weeks HIIT | 5 weeks HIIT | 7 weeks HIIT | 10 weeks HIIT | % change from baseline |
|-------------------------------------------------------------------------------------|----------|--------------|--------------|--------------|---------------|------------------------|
| Peak oxygen consumption (mL.kg <sup>-1</sup> .min <sup>-1</sup> )                   | 13.7     | 13.8         | 17.5         | 16.1         | 18.6          | +36%                   |
| Peak ventilation (L.min <sup>-1</sup> )                                             | 35.8     | 32.7         | 49.1         | 51.4         | 57.7          | +61%                   |
| Respiratory exchange ratio at peak exercise                                         | 1.38     | 1.42         | 1.38         | 1.60         | 1.55          | +12%                   |
| Heart rate peak (b.min <sup>-1</sup> )                                              | 100      | 120          | 118          | 120          | 133           | +33%                   |
| Power at peak oxygen consumption (W)                                                | 70       | 76           | 91           | 95           | 102           | +46%                   |
| Oxygen consumption at anaerobic threshold (mL.kg <sup>-1</sup> .min <sup>-1</sup> ) | 8.3      | 8.5          | 8.8          | 8.1          | 10.5          | +27%                   |
| Ventilatory equivalent for carbon dioxide at anaerobic threshold                    | 30       | 28           | 30           | 28           | 28            | -7%                    |
| Power at anaerobic threshold (W)                                                    | 40       | 36           | 40           | 40           | 52            | +30%                   |

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB172

### South Asian people have reduced exercise capacity unexplained by type-2 diabetes or impaired skeletal muscle oxidative capacity

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#### Background and aims

South Asian people are at increased risk of type-2 diabetes (T2DM) compared to Europeans. Further, the health risks associated with T2DM are elevated in South Asians, and this may adversely influence exercise capacity. T2DM is associated with reduced exercise capacity and metabolic disturbances to skeletal muscle. We hypothesised that, in South Asian people, poor exercise capacity is associated with impaired skeletal muscle oxidative capacity.

#### Materials and methods

Participants in the Southall and Brent Revisited Study, a population-based cohort resident in UK comprised of Europeans and first generation South Asian and African-Caribbean migrants undertook a 6-minute stepper test. Near Infrared Spectroscopy (NIRS) was used to non-invasively assess skeletal muscle oxidative capacity using changes in oxygenated and deoxygenated haemoglobin concentrations during arterial occlusions at rest and post-exercise. Recovery time constants (T) were calculated; longer T represents poorer muscle oxidative capacity. Analysis was by multiple linear regression adjusted for age, gender and ethnicity.

#### Results

183 participants (mean age=72±6 years, male=137) undertook the test. In multivariable models age, sex, T2DM and South Asian ethnicity were independent predictors of fewer steps. Participants with T2DM (n=34) completed fewer steps than people without (-48 steps, 95%CI=-72, -24, p<0.001). Compared to Europeans, South Asians achieved fewer steps (-32.4 steps, 95%CI=-53, -12, p=0.002) whereas African-Caribbeans achieved a similar number (-5.6 steps, 95%CI=-33, 22, p=0.7) after adjustment for presence of T2DM.

In multivariable models only South Asian ethnicity was independently associated with T ( $\Delta$ =10.1s, 95%CI=2.3, 17.9, p=0.011 after adjustment). Differences in exercise capacity associated with T2DM and ethnicity were not attenuated when muscle oxidative capacity was included in models.

#### Conclusion

Exercise capacity is impaired in South Asian people independently of T2DM. Skeletal muscle oxidative capacity is impaired in South Asian people but this does not explain the reduction in exercise capacity.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB173

### High-Intensity Exercise Does Not Activate Coagulation

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**Background:** Current guidelines for cardiac rehabilitation advocate moderate-intensity continuous exercise training (MICT) at ~60% peak oxygen uptake (VO<sub>2</sub>peak). High-intensity interval training (HIIT), with repeated bouts of exercise at >80% VO<sub>2</sub>peak appears to be an effective alternative, resulting in greater improvements in VO<sub>2</sub>peak (1), but concern remains about its link with thromboembolic events (2). Exercise activates coagulation in an intensity-dependent way, potentially leading to thrombophilia (3). We therefore conducted the first study to examine in-vivo coagulation in maximal exercise, HIIT and MICT using Fourier transformation rheology.

**Methods:** Sixteen active men (age: 21±3 years, stature: 1.80m ± 11.6cm and mass: 85.8kg ± 3.9kg) were recruited and after random assignment into either a HIIT or MICT group, undertook a cycling challenge to volitional exhaustion on a mechanically braked ergometer (Monark, Varberg, Sweden). VO<sub>2</sub> was sampled using an online metabolic cart (Medgraphics UK, Gloucester, UK) and power output corresponding to 40% and 80% VO<sub>2</sub>peak (HIIT) or 60% (MICT) was noted. Participants returned 3 days later and performed an acute, isovolumic bout of HIIT or MICT. Pre/post exercise, blood was sampled from an intravenous cannula. 10 mL of blood was drawn and discarded, then exactly 7mL of fresh whole blood was injected into a double walled concentric rheometer (Discovery Hybrid 2, TA Instruments, DE, USA), for analysis of blood fractal dimension (D<sub>f</sub>) using previously described methods (4). Data were analysed using a 2-way repeated measures ANOVA and Bonferroni corrected paired sample t-tests and independent sample t-tests. Significance was established at P<0.05 and data were expressed as mean ± SD

**Results:** There were no differences in D<sub>f</sub> prior to exercise between groups (1.81±0.01, HIIT vs. 1.74±0.11, MICT). D<sub>f</sub> increased in both groups after maximal exercise (1.95±0.08, HIIT vs. 1.87±0.10, MICT, P<0.05). Acute HIIT did not alter D<sub>f</sub> (1.82±0.07) but acute MICT increased D<sub>f</sub> vs. pre-exercise (1.83±0.16, P<0.05).

**Conclusion:** An acute bout of MICT at the current recommended training intensity for cardiac rehabilitation increased D<sub>f</sub> (figure 1, B). The reference range for D<sub>f</sub> is 1.74±0.07 (5), which suggests MICT activated coagulation and increased the density and cross-linkage of the insipient clot outside the reference range. Conversely, an acute bout of HIIT did not increase D<sub>f</sub>. Our data conflict with the present literature that used less sensitive in-vitro markers of coagulation (6,7) and suggest that HIIT is a safe, viable exercise regimen and deserving of further examination in patients.



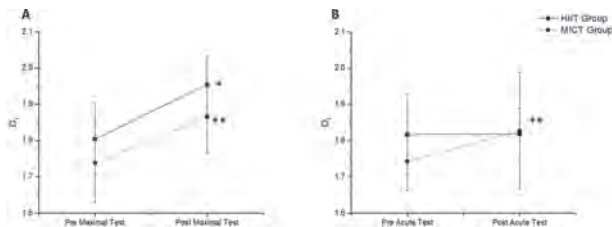


Figure 1: Effect of maximal exercise, acute high-intensity interval training (HIIT) or moderate intensity continuous training on blood fractal dimension ( $D_f$ ). Data are means  $\pm$  standard deviation, \* = different to pre-exercise HIIT \*\* = different to pre-exercise MICT,  $P < 0.05$

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB174

### Mechanisms by which isometric handgrip (IHG) training of one arm may improve systemic endothelial dilator function in young healthy men

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Isometric (IHG) handgrip training can reduce resting arterial blood pressure (ABP), especially in hypertensives, but the mechanisms remain unclear (1). We recently showed that IHG training in healthy young men augmented peak exercise hyperaemia and reactive hyperaemia in the *contralateral* arm by  $\sim 30\%$  (2,3). This suggested that IHG training of one arm improves endothelial dilator function remotely. The aim of the present study was to determine the changes in blood flow induced in the contralateral arm (FBF) and ipsilateral calf (CBF) during a single bout of IHG training, which might serve as a stimulus for improving endothelial function, and to test whether endothelium-dependent cyclooxygenase (COX) products contribute. Two different series of experiments, each on 10 men (aged 19–23 years) who were recreationally active and normotensive (SP/DP  $< 140/90$  mmHg). FBF or CBF were recorded by venous occlusion plethysmography at 15 s intervals before and during IHG contractions of the dominant arm at 30% maximum voluntary contraction (5 x 3min at 5 min intervals) on 2 different days in the absence or presence of the COX inhibitor aspirin (600mg p.o.). Mean arterial pressure (MABP), recorded by finger photoplethysmography, increased from  $79.3 \pm 2.1$  to  $90.0 \pm 2.4$  mmHg in the FBF series and from  $89.3 \pm 4.1$  to  $114.8 \pm 5.4$  mmHg in the CBF series. FBF increased by an average of  $30.1 \pm 2.6^*$  and  $37.0 \pm 2.8^*$  ml/min/100g in the first and last IHG respectively (\*; RMANOVA, IHG vs baseline:  $P < 0.05$ ). Similarly, CBF increased by  $8.62 \pm 1.42^*$  and  $10.02 \pm 2.38^*$  ml/min/100g in the first and last IHG. COX

inhibition had no effect on baseline values of MABP, FBF or CBF. However, increases in FBF were attenuated to  $11.7 \pm 1.9$  and  $11.6 \pm 1.9^{\S}$  ml/min/100g in the first and last IHG ( $^{\S}$ : before vs after aspirin:  $P < 0.05$ ) and the increases in CBF were attenuated to  $0.43 \pm 0.17^{\S}$  and  $0.72 \pm 0.24^{\S}$  ml/min/100g respectively. EMG activity recorded over major muscles of forearm and calf and visual observation of the limbs suggested negligible muscle contraction in forearm or calf, without or with aspirin, indicating the increases in FBF and CBF were unlikely to be exercise hyperaemia.

We propose that the increase in ABP and consequently in FBF and CBF evoked by each period of IHG training leads to increased shear stress and generation of vasodilator COX products that facilitate vasodilatation in both limbs. When repeated in sessions over 4–5 weeks, IHG training may therefore act as a shear stress-dependent stimulus to improve endothelium-dependent dilator function not only in the trained arm, but also in the contralateral arm and legs and possibly other tissues. Such effects might be expected to reduce resting ABP and reduce risk of cardiovascular disease.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB175

### Modeling the oxyhemoglobin dissociation curve at the cerebral capillary level in humans

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Hitherto, changes in the configuration of the oxyhemoglobin dissociation curve triggered by the increase in  $PCO_2$  and reduction in pH that occurs, as the red blood cell passes through target tissue capillaries, have largely been ignored in most human-experimental studies of the oxygen transfer from capillary blood to brain tissue mitochondria. In the present study, we developed a method that permits the calculation of the mean capillary  $P_{50}$  and the Hill slope,  $h$ , from paired arterial-jugular venous  $PO_2$  and  $SO_2$ -values. By mathematical formalism, and by assuming a linear relationship between the oxygen tension and saturation in the Hill plot within the capillary range, we derived the following equation for the Hill slope (Figure 1):  $h = (\ln(S_{artO_2}/(1-S_{artO_2})) - \ln(S_{jugO_2}/(1-S_{jugO_2}))) /$

$(\ln(P_{artO_2}) - \ln(P_{jugO_2}))$ . The corresponding  $P_{50}$  was found using the Hill equation:  $P_{50} = P_{artO_2} ((1 - S_{artO_2}) / S_{artO_2})^{1/h}$ . From this, we found that the  $P_{50}$  was 3.7 (0.2) kPa and the Hill slope was 2.4 (0.2) in 30 healthy volunteers during resting breathing. These values differed from the standard arterial values of 3.5 kPa and 2.8 ( $p < 0.001$  for both), which are often used when modeling the cerebral capillary transfer of oxygen in humans. The use of the calculated capillary  $P_{50}$  and Hill slope resulted in systematically higher capillary,  $P_{capO_2}$ , and mitochondrial,  $P_{mitO_2}$ , oxygen tensions than when using the standard arterial values, with a bias of 1.4 kPa and limits of agreement of 0.5 kPa to 2.3 kPa for both  $P_{capO_2}$  (Figure 2) and  $P_{mitO_2}$ . The method presented here for modeling the oxyhemoglobin dissociation curve at the cerebral capillary level provides physiologically relevant estimates of  $P_{capO_2}$  and  $P_{mitO_2}$ , which are systematically higher than when assuming a 'fixed' oxyhemoglobin curve.

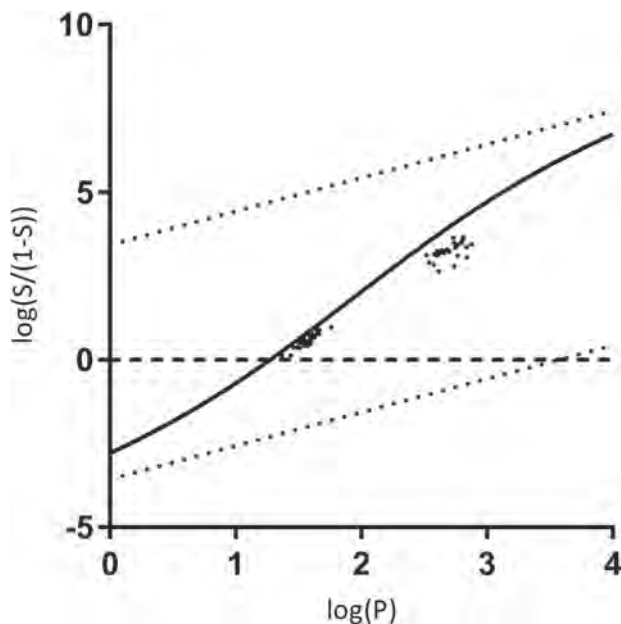


Figure 1. Theoretical relationship between the oxygen tension and oxygen saturation illustrated in the Hill plot (black line). Experimentally determined arterial and jugular venous values are shown for 30 healthy volunteers.

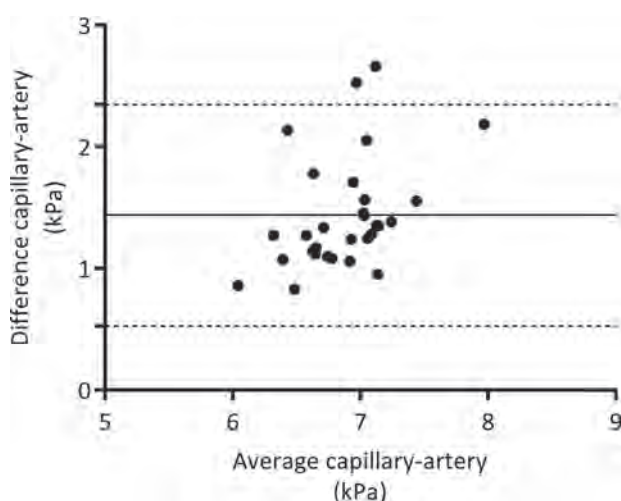


Figure 2. Bland-Altman plot of the capillary oxygen tension calculated from the  $P_{50}$ -value and Hill slope using either calculated mean capillary values or arterial standard values in 30 healthy volunteers. The mean difference (solid line) and the limits of agreement (hatched line) are shown.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## Changes in the oxyhemoglobin dissociation curve between the arterial and cerebral capillary level during normo- and hyperventilation in healthy human volunteers

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We have developed a new method to model the configuration of the oxyhemoglobin dissociation curve at the cerebral capillary level, which may be used to calculate the capillary,  $P_{capO_2}$ , and mitochondrial,  $P_{mitO_2}$ , oxygen tension in humans. In the present study, we sought to examine whether the estimated mean capillary  $P_{50}$  and mean Hill slope differ from measured arterial values during normo- and hyperventilation in healthy volunteers, and whether the use of the respective values results in physiologically relevant differences in  $P_{capO_2}$  and  $P_{mitO_2}$ .

We obtained paired arterial-jugular venous blood samples during resting breathing and following 15 minutes of voluntary hyperventilation in 8 healthy volunteers. A Hill plot was used to estimate the mean capillary  $P_{50}$  and Hill slope. Global cerebral blood flow and the cerebral metabolic rate of oxygen,  $J_{O_2}$ , were measured by the Kety-Schmidt-technique using  $^{133}\text{Xe}$  as the tracer.  $P_{mitO_2}$  was found using a standard value for the cerebral oxygen diffusion capacity,  $L_{O_2}$ , of 33.0  $\mu\text{mol}/100\text{g}/\text{min}/\text{kPa}$ :  $J_{O_2} = L_{O_2} (P_{capO_2} - P_{mitO_2})$ . During normoventilation, neither  $P_{50}$  (arterial: 3.7 (0.3) kPa; capillary: 3.7 (0.1) kPa; arterial vs. capillary:  $p = 0.14$ ) nor the Hill slope (arterial: 2.5 (0.1); capillary: 2.4 (0.1); arterial vs. capillary:  $p = 0.14$ ) changed significantly from artery to capillary. In contrast, both  $P_{50}$  (arterial: 2.9 (0.1) kPa; capillary: 3.4 (0.2) kPa; arterial vs. capillary:  $p < 0.001$ ) and the Hill slope (arterial: 2.0 (0.1); capillary: 2.2 (0.1); arterial vs. capillary:  $p < 0.001$ ) increased significantly from artery to capillary during hyperventilation. Neither  $P_{capO_2}$  nor  $P_{mitO_2}$  differed when the measured arterial rather than capillary  $P_{50}$  and Hill slope were used during resting breathing (bias of -0.17 kPa and limits of agreement of -0.71 kPa to 0.37 kPa) (Figure 1). By contrast, the use of the capillary  $P_{50}$  and Hill slope resulted in systematically higher  $P_{capO_2}$  and  $P_{mitO_2}$  than when using the measured arterial values, with a bias of 0.45 kPa and limits of agreement of 0.35 kPa to 0.54 kPa (Figure 1).

In conclusion, measured arterial  $P_{50}$  and Hill slope values may be used to provide reasonable estimates of  $P_{capO_2}$  and  $P_{mitO_2}$  during resting breathing, but are probably not reliable for this purpose when the configuration of the arterial oxyhemoglobin curve is physiologically modulated, as exemplified by voluntary hyperventilation.

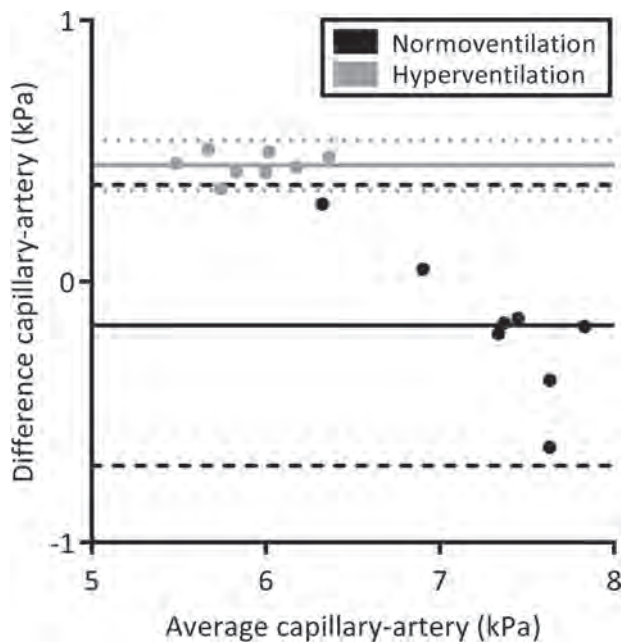


Figure 1. The capillary oxygen tension estimated from the  $P_{50}$  and Hill slope using either the mean capillary or the arterial standard values during normoventilation (black lines) and hyperventilation (gray lines). The mean difference (solid lines) and the limits of agreement (hatched lines) are shown.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB177

### Metabolic alteration and muscle dysfunction in mice with breast cancer

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Breast cancer accounts for ~25% of diagnosed cancer types in woman [1]. Decreased physical fitness and muscle weakness are common complications in patients with breast cancer. In cancer, muscle weakness has traditionally been linked to muscle wasting and significant weight loss (cachexia) [2]. However, muscle weakness is present in non-cachectic, weight-stable patients with breast cancer [3]. In fact, cancer-induced muscle dysfunction is a broad clinical challenge that is not restricted to palliative or advanced stage patients, but also observed in newly diagnosed patients with low tumor burden [4]. Further, with the breast cancer treatment improving, it is important to take a look on the patients quality of life [5]. However, little is known about the features underlying breast cancer-induced muscle impairments and no drug preventing cancer-induced muscle weakness is clinically proven. Here we aim at characterizing the metabolic status and the muscle function in mice with breast cancer.

The breast cancer mouse-model MMTV-PyMT (PyMT) used here, is characterized by an early onset of mammary cancer (from 5 weeks of age) and follows a similar progression pattern as the one observed in human patients [6]. Soleus muscle from PyMT mice exhibited ~30% lower specific force (kN/m<sup>2</sup>) than soleus muscle from wildtype (WT) mice (n=28-29,  $p \leq 0.05$ , mice were 12 week old at sacrifice). There were no significant differences in muscle mass, fiber

size or fiber type distribution between PyMT and WT muscle. Furthermore, there were no differences in glycogen content ( $\mu\text{g/g}$  muscle) in soleus muscle from PyMT and WT mice. Simultaneous measurement of numerous parameters (e.g. oxygen consumption ( $\text{VO}_2$ ), carbon dioxide production ( $\text{VCO}_2$ ), and food and water intake) was carried out using comprehensive lab animal monitoring system (CLAMS) to gain insight into the metabolic status of the mice. The mice were monitored over a week and the average respiratory exchange ratio ( $\text{RER} = \text{CO}_2$  production:  $\text{O}_2$  uptake) were significantly differed between PyMT and WT mice, with mean PyMT RER of  $0.95 \pm 0.01$  and WT RER of  $1.0 \pm 0.01$  (mean data  $\pm$  SEM,  $n=8$ ,  $p < 0.001$ ). Thus, indicative that PyMT have an altered metabolism towards fatty acid utilization.

In summary, soleus muscles are weaker and the whole-body metabolism appears altered in mice with breast cancer as compared with healthy control mice. Gene and molecular analysis are currently being performed to further assess mitochondrial and glucose metabolism. Nevertheless, further studies are needed to gain insight into cancer-derived factors that contributes to skeletal muscle dysfunction and altered metabolism.

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Johns, N., N.A. Stephens, and K.C. Fearon, *Muscle wasting in cancer*. Int J Biochem Cell Biol, 2013. 45(10): p. 2215-29.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB178

### Skeletal muscle capillarisation and myoglobin are higher but mitochondrial content is similar in elite breath-hold divers versus non-divers.

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Hypoxia is a condition of reduced oxygen concentration. Elite breath-hold divers (EBHD) during their habitual training are regularly exposed to acute transient hypoxia combined with skeletal muscle activation, interspersed with periods of normal breathing. Diving mammals and high-altitude natives who are also exposed to hypoxic conditions/environments have a higher capillary density, type I fibre distribution and myoglobin content in their most active skeletal muscles but a lower mitochondrial content than mammals and humans that live habitually in normoxic environments (1;2). Currently, little is known about the skeletal muscle characteristics of EBHD. Therefore, this study made the first investigations into the skeletal muscle phenotype of male EBHD. Resting percutaneous skeletal muscle biopsies were obtained from the



*m. vastus lateralis* of 18 healthy anthropometry-matched male participants, of which 9 were EBHD (national team members) and 9 were non-divers (ND). Cryosections were stained with anti-lectin UEA-I to identify the capillaries and anti-myosin heavy chain type I to identify type I skeletal muscle fibres leaving type II fibres unstained. The fibre type stain was also co-incubated with either anti-myoglobin or anti-cytochrome C oxidase IV for determination of myoglobin and mitochondrial content, respectively. Images were captured and quantitated using immunofluorescence microscopy and analysed by ANOVAs. Data are means $\pm$ SD. Capillary density was greater (19%) in the EBHD (605.1 $\pm$ 87.1cap/mm<sup>2</sup>) compared to the ND group (490.2 $\pm$ 98.9cap/mm<sup>2</sup>; p=0.019). For both groups the diffusion distance ( $R_{95}$ ) was lower in type I fibres (EBHD 27.59 $\pm$ 3.1 $\mu$ m<sup>2</sup>; ND 31.16 $\pm$ 3.8 $\mu$ m<sup>2</sup>, p<0.05) versus type II fibres (EBHD 33.5 $\pm$ 4.4 $\mu$ m<sup>2</sup>; ND 37 $\pm$ 6.1 $\mu$ m<sup>2</sup>), with a lower  $R_{95}$  for type I fibres in the EBHD versus ND (13%, p=0.045). Type I fibres had a higher myoglobin content (27%) than type II fibres in EBHD (p=0.002), whereas no fibre type differences were observed in the ND group (p=0.879). Type I fibre myoglobin content was significantly higher (27%) in EBHD than ND (p=0.025), but type II fibre myoglobin content was not different between groups. Type I fibres had a higher mitochondrial content (35%) than type II fibres in EBHD (p=0.015), but no fibre type differences were observed for ND (p=0.635) or between groups (p=1). Fibre type distribution (EBHD type I 56 $\pm$ 12% vs ND type I 55 $\pm$ 13%) and mean fibre area (EBHD 5575 $\pm$ 806 $\mu$ m<sup>2</sup> vs ND 5163 $\pm$ 804 $\mu$ m<sup>2</sup>) were not different between groups (p>0.05). This is the first study to report the skeletal muscle phenotype of male EBHD. The novel findings suggest a greater capillary density, improved diffusion distance and myoglobin content in the skeletal muscle of EBHD and these supply a mitochondrial content that is not significantly different to ND.

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Ponganis, P. J. (2011). 'Diving mammals.' *Comprehensive Physiology*, 1(1), pp:447-465.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCB179

### Investigation of Sportive Capacities of Elite Mountain Skiers with Physiological Parameters of Cardiopulmonary Exercise Test

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The extreme environments of high altitude and cold, and movement complexity makes mountain ski racing a difficult sport and athletes involves in this activity as physiologically demanding. Cardiopulmonary exercise test (CPET) is a procedure that provide information for diagnostic and prognostic purposes that assess the individual's capacity for dynamic exercise and being increasingly used in broad spectrum of sportive and clinical applications in order to objectively determine the functional capacity.

The aim of this study was to investigate sportive capacity-related physiological parameters [(maximal oxygen consumption:  $VO_{2max}$ , respiratory Exchange ratio (RER), heart rate (HR), heart rate reserve (HRR) and anaerobic threshold)] of elite mountain skiers using CPET.

A total of 15 elite mountain skiers (age of 16-21 years, body mass index: 21.2 $\pm$ 2.9 kg/m<sup>2</sup>) licensed by Turkish Mountaineering Federation was involved and their physiological parameters reflecting sportive capacity was assessed. The elite mountain skiers performed an incremental (5 Watt/20 sec) CPET, pedaling at 60 rpm, up to their maximum and  $VO_{2max}$ , RER, HR, HRR, spirometric parameters (FVC, FEV1, MEF) and anaerobic threshold levels determined by the software of the cardiopulmonary exercise testing system (equipped with 12 lead ECG gas analyzer and ergospirometry).

The results regarding  $VO_{2max}$  of the cohort was (57.2 $\pm$ 10.9 ml/kg/min), slightly over than % predicted value for their age, gender, and weight (128 $\pm$ 20% of predicted).  $VO_2$  at anaerobic threshold (V slope) was 41 $\pm$ 11 ml/kg/min. The mean maximal pulmonary ventilation was 121.9 $\pm$ 28 L, (109 $\pm$ 16 % predicted value for their age, gender, and weight). They also had a large vital capacity. The mean highest power for the group was calculated to be 230 $\pm$ 56 Watts (100 $\pm$ 21 % of their predicted values). They achieved 97 $\pm$ 16 beats/min of their predicted heart rate at exhaustion. By measuring physiological parameters of elite mountain skier team of the Turkish team, for the first time, and found that they have a relatively good level of physiological capacity as reflected by  $VO_{2max}$  and. Their capacities are compatible with the requirements of this extreme sport and the values are comparable with published relevant data for international athletes from other countries, despite their relative short sport and biological age.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCB180

### Effect of catecholamines on phagocytosis, oxidative burst and the cytokine-producing activity of neutrophils in athletes

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It is known that exercise through endogenous catecholamines such as adrenaline (Adr) and noradrenaline (Nor) can have a significant effect on number and activity of immune cells, including the neutrophils. The mechanisms of these influences have not been studied sufficiently. The aim of the present study was to investigate *in vitro* effect of Adr, Nor and selective agonist of  $\beta$ -adrenergic receptor isoproterenol (Iso) on neutrophil functional activity and cytokine production in athletes. Ethical approval for the study of neutrophils from 29 healthy athletes of both sexes (19.5 $\pm$ 1.14 years old) was granted by the University Ethics Committee. The functional activity of neutrophils was determined in EDTA-stabilized venous blood. We defined the capacity of neutrophils to ingest zymosan in phagocytosis assay, oxidative burst in spontaneous and stimulated NBT-test. Before the tests, the blood was incubated (37°C, 1 h) with 100  $\mu$ l of DPBS or Adr (0.01, 0.1, 1  $\mu$ M), Nor (0.001, 0.1, 1  $\mu$ M), Iso (0.1  $\mu$ M). To analyse how catecholamines effect on cytokine secreting activity, neutrophils were isolated by two different methods: (1) by density gradient centrifugation of whole blood over medium Lympholyte-H, dextran sedimentation and red cells osmotic lysis (*Neu1*); (2) similarly to *Neu1*, but with an additional immunomagnetic negative selection step, using the EasySep Human Neutrophil Enrichment Kit (StemCell Technologies) (*Neu2*). Purity of the isolated neutrophils was assessed by Romanowsky-Giemsa staining and flow cytometry using markers CD66b-FITC/CD16-PE.

The purity of neutrophil suspension averaged 92-98% for *Neu1* and >99% for *Neu2*. Neutrophils were resuspended in RPMI 1640 medium and incubated (37°C, 5% CO<sub>2</sub>, 20 h) at a concentration of 1×10<sup>6</sup> cells/ml/well with addition of 100 µl DPBS or 100 µl of different agonists of adrenoreceptors. Concentrations IL-1b, IL-6, IL-8, TNF-α and MIP-1b in cell-free supernatants were measured by ELISA kits. Statistical analysis was carried out using non-parametric models. We found that Adr and Nor in the pharmacological concentration (1 µM) inhibited neutrophil phagocytic and fMLP-induced oxidative activity (P<0.05). Adr in all doses stimulated the secretion of IL-1b by *Neu1* (P<0.05). Stimulation of IL-1b secretion was also observed after the incubation of *Neu1* with Iso (P<0.05). Nor did not effect on neutrophil cytokine release. A basal secretion level of IL-1b and MIP-1b was not detected in supernatants from non-stimulated neutrophils after additional immunomagnetic negative selection (*Neu2*). Unlike *Neu1* Adr and Iso did not stimulate release IL-1b by *Neu2*. Taken together, our results indicate that Adr and Nor in the pharmacological concentration have anti-inflammatory effect on neutrophil phagocytic and oxidative activity, but they have limited effects on the secretion of cytokines by neutrophils in athletes.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB181

### Differential effects on lower and upper body muscle mass following 7 days unloading on a hyperbuoyancy floatation bed

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Exposure to a micro-gravity (µG) environment, particularly in the absence of counter measures, is known to induce a loss of skeletal muscle mass and function, with the postural muscles of the lower limb most affected (Narici & De Boer 2011). We have recently used supine whole-body unloading on a hyper-saline filled water bed (hyper buoyancy floatation, HBF) as an analogue of µG and demonstrated an average ~1kg loss of total muscle mass after 7 days of HBF unloading (Morris-Patterson et al. 2018). The aim of the present study was to identify changes in muscle mass in specific regions of the body following this 7-day unloading period using magnetic resonance imaging (MRI).

Twelve male subjects aged (27.3±4.2 yrs) completed the study. Six weeks prior to unloading each subject underwent a one-week control period during which time their diet was controlled. Pre and post the control period and at standardised time of day subjects undertook an MRI (Siemens MAGNETOM Verio 3T, Siemens Healthcare, Erlangen, Germany). For the intervention period the subjects were asked to lie supine on the HBF for 7 days, during which time they were also fed a

controlled diet. Subjects were allowed a maximum of 15 mins per day when they were not on the HBF (for personal hygiene etc). One day prior to and 1.5-3hrs post-unloading, further scans were performed. Up to 400 images were taken along the length of the body, with every 10<sup>th</sup> image used in the derivation of skeletal muscle mass.

All 12 subjects who were entered in the intervention arm of the study, successfully completed the 7-day period of unloading. No significant changes were observed over the control period. However, the results showed that the unloading period induced muscle loss which was proportionally greatest in the lower leg (Pre 3.81 ± 0.68 vs Post 3.65 ± 0.67kg, 4.2%, mean±bSD, ANOVA with repeated measures, p<0.05), followed by the upper leg (10.37 ± 1.83 vs 10.02kg, 3.4%, p<0.05) and then the trunk (12.56 ± 2.01 vs 12.22 ± 1.99 kg, 2.8%, p<0.05). No changes were observed in the lower arm (1.54 ± 0.3 vs 1.49 ± 0.32), the upper arm (3.46 ± 0.79 3.41 ± 0.79 kg) or the neck (kg) 0.45 ± 0.13 vs 0.46 ± 0.11 kg) as a result of the unloading period.

The results showed that HBF unloading was able to induce regional changes in skeletal muscle mass in a manner comparable to that observed in space flight, supporting its utility as an analogue of µG.

Morris-Paterson TE, Jones EJ, Tsai C-C, Hasegawa H, Carmichael O, Van Someren KA, Puthuchearry ZA, Green DA, Zanello SB, Rosenzweig I, Harridge SDR (2018) The effects of 7 days of whole body unloading using a hyperbuoyancy (HBF) floatation bed on lean body mass and total skeletal muscle mass. Proceedings of the Physiological Society In press

Narici MV & De Boer MD (2011) Disuse of the musculo-skeletal system in space and on earth European Journal of Applied Physiology. 111; 03-420

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<sup>2</sup> Sleep and Brain Plasticity Centre, Department of Neuroimaging, IoPPN, King's College London, UK; <sup>3</sup> Sleep Disorders Centre, Guy's & St Thomas' Hospital, London, UK; <sup>4</sup>Pennington Biomedical Research Center, LA, USA; <sup>5</sup>Department of Sport, Exercise & Rehabilitation, Northumbria University, UK; <sup>6</sup>KBRwyle, European Astronaut Centre, Linder Höhe, D-51147, Cologne, Germany; <sup>7</sup>Institute of Health and Human Performance, University College London, UK

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB182

### The effects of low-volume high-intensity interval training on pulmonary oxygen uptake and muscle deoxygentaion kinetics during heavy intensity exercise in type 2 diabetes.

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Aaron Nevin<sup>1</sup>, Norita Gildea<sup>1</sup>, Adam McDermott<sup>1</sup>, Joel Rocha<sup>2</sup>, Domenico Crognale<sup>1</sup>, Simon Green<sup>3</sup>, Donal O'Shea<sup>4</sup>, Mikel Egana<sup>1</sup>

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We tested the hypothesis that a 12 week intervention involving low-volume high-intensity interval training (LVHIIT) would increase the speed of the adjustment of the primary phase ( $\tau_p$ ,  $\tau_p$ ) of pulmonary oxygen uptake ( $\dot{V}O_2$ ) and/or reduce the amplitude of the slow component of  $\dot{V}O_2$  during high-intensity cycling in type 2 diabetes (T2D) to a similar extent as a traditional moderate-intensity continuous training (MICT) intervention.

Twenty eight middle-aged participants with T2D (17 men, mean  $\pm$  SD: age  $53 \pm 10$  yr, body mass index  $30.0 \pm 4.0$  kg.m<sup>-2</sup>) were randomly assigned to MICT ( $n=10$ , 50 min of moderate-intensity cycling) or LVHIIT ( $n=9$ , 10 x 1 min at  $\sim 90\%$  maximal heart rate interspersed by 1 min of 'unloaded' cycling) or to a non-exercising control group ( $n=9$ ). Exercising groups trained 3 times/week and every 3 weeks exercise intensity was adjusted. Before and after the intervention participants completed two bouts of constant-load cycling corresponding to 50% between their ventilatory threshold and peak  $\dot{V}O_2$  power outputs (i.e. 50% $\Delta$ ) previously established during a ramp incremental test.  $\dot{V}O_2$  kinetics were calculated from continuously measured breath-by-breath  $\dot{V}O_2$  data, whilst the rates of muscle deoxygenation (i.e., deoxygenated haemoglobin and myoglobin, [HHb+Mb]) were continuously measured by near-infrared spectroscopy at the *vastus lateralis* muscle.  $\dot{V}O_2$  and  $\Delta$ [HHb+Mb] responses were modelled using a bi-exponential model. Time point analysis of  $\dot{V}O_2$  and [HHb+Mb] responses were performed using a two-way ANOVA. Values are mean  $\pm$  SD.

The  $\dot{V}O_2\tau_p$  were speeded by a similar magnitude in both MICT (Pre:  $33.6 \pm 5.1$ , Post:  $24.7 \pm 5.1$  s;  $P < 0.05$ ) and LVHIIT (Pre:  $30.7 \pm 5.3$ , Post:  $26.4 \pm 2.7$  s;  $P < 0.05$ ) but no effect was observed for the same parameter for the control group. Similarly, the overall mean response time of the  $\dot{V}O_2$  response (fitted by a mono-exponential model) in both training groups was significantly speeded, but not in the control group. The amplitude of the  $\dot{V}O_2$  slow component ( $A_s$ ) was reduced in the MICT group only. There were no changes between or within group pre and post intervention for muscle deoxygenation parameters. In middle-aged individuals with T2D, MICT and LVHIIT enhanced  $\dot{V}O_2$  kinetics responses during high intensity exercise likely consequent to a training-induced increase in muscle blood flow and/or improved muscle perfusion to metabolic rate.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB183

### Examining the behaviour of reactive oxygen species in specific intracellular locations of skeletal muscle fibres in inflamed and non-inflamed conditions.

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Skeletal muscle weakness is a feature of diseases associated with inflammation, such as cancer and rheumatoid arthritis.

Our previous work proposed that peroxynitrite-mediated 3-nitrotyrosine accumulation on actin contributes to muscle weakness in rodent models of rheumatoid arthritis (Yamada et al., 2014). Furthermore, an increased protein expression of NADPH oxidase 2 (NOX2) in a rat model of arthritis suggested that reactive oxygen species (ROS) from NOX2 could be increased in inflammatory conditions, contributing to peroxynitrite formation (Yamada et al., 2015). Genetically encoded, redox sensitive green fluorescent proteins (roGFPs) enable the measurement of changes in ROS levels in specific intracellular locations, in live cells, in real time. Our aim was to use roGFPs to determine whether the ROS production from known intracellular sources differs between skeletal muscle fibres from inflammatory and healthy conditions. The sources of ROS assessed were the mitochondria (Mito-roGFP), NOX2 (p47-roGFP), and the cytosolic environment (PLPCX-Orp1). A protocol was developed in order to demonstrate that the fluorescent roGFP signals were reversible and could be measured and quantified based on a calibration, and that the impact of muscle contraction could be tested. Mouse flexor digitorum brevis (FDB) muscles were transfected by *in vivo* electroporation (mice were anaesthetised with 4.8% isoflurane) with one of three roGFPs: p47-roGFP, Mito-roGFP, or PLPCX-roGFP. FDB muscles were enzymatically dissociated into single fibres using collagenase type I. Confocal images were taken while fibres were perfused with standard Tyrode solution. Perfusion with dithiothreitol (DTT) and then hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) enabled measurement of maximum and minimum fluorescent signals, respectively. Muscle contraction was induced via electrical stimulation. Our pilot data clearly demonstrate that roGFPs respond dynamically to changes in intracellular ROS concentrations in real time, which can be quantified relative to the minimum and maximum signals. Furthermore, ROS concentrations were different in different intracellular sources in resting skeletal muscle fibres, being  $\sim 8$  and  $\sim 5$  fold higher in the mitochondrial networks compared to NOX2 located near the t-tubules, and the cytosolic environment, respectively. At the time of writing this abstract, the aforementioned protocol is being applied in an adjuvant-induced arthritis model, in order to study ROS behaviour in an inflamed and non-inflamed muscle condition at rest and following muscle contraction. A more precise understanding of the intracellular sources of ROS in inflammatory conditions will aid in the identification of a therapeutic target.

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Yamada, T., Abe, M., Lee, J., Tatebayashi, D., Himori, K., Kanzaki, K., Wada, M., Bruton, J.D., Westerblad, H. and Lanner, J.T., 2015. Muscle dysfunction associated with adjuvant-induced arthritis is prevented by antioxidant treatment. *Skeletal muscle*, 5(1), p.20.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.



PCB184

**Peroxynitrite-induced modifications results in increased open probability of RyR1 in skeletal muscle**M.M. Steinz<sup>1</sup>, N. Beard<sup>2</sup> and L.T. Johanna<sup>1</sup><sup>1</sup>Department of Physiology and Pharmacology, Karolinska Institutet, Solna, Sweden and <sup>2</sup>Faculty of Science and Technology, University of Canberra, Canberra, ACT, Australia

Skeletal muscle weakness contributes to decreased quality of life for patients with chronic inflammatory conditions, such as rheumatoid arthritis (RA). We have previously reported that muscle weakness in mice with arthritis (collagen-induced arthritis) was accompanied by increased peroxynitrite-derived nitrosative stress (3-nitrotyrosine, 3-NT) on the ryanodine receptor (RyR1) complex (Yamada T, et al 2015). RyR1 is the major intracellular Ca<sup>2+</sup> release channel and essential for apt excitation-contraction coupling in skeletal muscle. Moreover, altered RyR1 Ca<sup>2+</sup> release has been linked to muscle dysfunction in several clinical aspects, e.g. in muscle dystrophies (Bellinger et al., 2009; Hernández-Ochoa et al., 2015; Lanner et al., 2012). However, the functional effects of peroxynitrite-induced RyR1 modifications are still unclear. The aim of this study was to induce peroxynitrite-induced RyR1 modifications using 5-amino-3-(4-morpholinyl)-1,2,3-oxadiazolium chloride (SIN-1) to identify modified amino acid residues with mass spectrometry and examine RyR1 gating properties using single channel recordings.

SIN-1 [1mM, 15 min] introduced a significant 1.6-fold increase in 3-NT levels on RyR1 as identified by immunoblotting (t-test,  $p < 0.05$ ,  $n = 4$ ,  $SE = 0.05$ ). In the presence of SIN-1, RyR1 single channel recordings in planar bilayers showed a 2.7-fold increase in open probability (t-test,  $p < 0.05$ ,  $n = 6$ ,  $SEM = 0.12$ ). Mass spectrometry analysis of SIN-1 treated RyR1 revealed 28 specific amino acid residues with oxidative modifications (15 tyrosine residues carried a 3-NT modification and malondialdehyde (MDA) adducts were identified on 13 residues). The oxidative modifications were located in the linker regions as well as in the pore domain of RyR1.

Here we have specifically identified RyR1 residues that undergo peroxynitrite-induced protein modification (3-NT and MDA), which were directly linked to increased open probability of RyR1. The identified 3-NT and MDA-modified RyR1 residues appear to be clustered in regions of RyR1 that may serve as drug targets in the search for novel therapeutics to counteract muscle dysfunction associated with increased oxidative stress and altered RyR1 function.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB185

**Tissue oxygenation: a novel modulator of compression-induced hyperaemia**A. Messere<sup>1</sup>, M.E. Tschakovsky<sup>2</sup>, S. Seddone<sup>1</sup>, W. Franco<sup>3</sup>, D. Maffiordio<sup>3</sup>, C. Ferraresi<sup>3</sup> and S. Roatta<sup>1</sup><sup>1</sup>Neuroscience, University of Torino, Torino, Italy, <sup>2</sup>School of Kinesiology, Queen's University, Kingston, ON, Canada and <sup>3</sup>Mechanical and aerospace engineering, Politecnico di Torino, Torino, Italy

Skeletal muscle vasculature exhibits a rapid dilatation in response to mechanical compression (MC), that leads to transient hyperaemia. This hyperaemia is markedly attenuated in response to a second stimulus delivered within a short time (2 min) from the first (Messere et al., 2017). Indirect evidence suggests that the increase in tissue oxygenation developing during the first hyperaemia could be responsible for attenuating further hyperaemic responses. Alternatively, the effect could be mediated by transient inactivation of the mechano-sensitive structures underlying the rapid dilatation. Aim of this study was to test the hypothesis that a prior hyperaemia and ensuing increase in tissue oxygenation is necessary for the attenuation of MC-induced hyperaemia.

In 8 healthy subjects two MCs separated by a 25-s interval were delivered to the forearm by means of a pneumatic cuff. The sequence was performed a second time after a resting interval of at least 5 min. In one of the sequences (randomly selected) the hyperaemic response to the first MC was prevented by partial occlusion of the axillary artery. Tissue oxygenation from forearm muscles and brachial artery blood flow were continuously monitored by means of Near Infrared Spectroscopy (NIRO 200, Hamamatsu) and Doppler-echography (MyLab 25, Esaote), respectively. The magnitude of the hyperaemic response was quantified as the excess blood (in ml) supplied to the forearm during the hyperaemia.

In the absence of blood flow restriction, the hyperaemic response to the first MC was  $14.8 \pm 3.9$  ml ( $P < 0.05$ ), which was followed by a slower increase in tissue oxygenation from  $65.2 \pm 4.7$  % to  $76.5 \pm 3.8$  % ( $P < 0.01$ ), remaining significantly elevated at the time of delivery of the second MC ( $75.3 \pm 4.2$  %,  $P < 0.05$ ). In this condition the hyperaemic response to the second MC was markedly attenuated:  $5.7 \pm 3.3$  ml ( $P < 0.05$ ). When the hyperaemic response to the first MC was prevented, tissue oxygenation did not increase ( $64.9 \pm 4.9$  %) and the magnitude of the hyperaemic response to the second MC did not exhibit attenuation ( $14.4 \pm 3.9$  ml).

In conclusion, the present study demonstrates that the putative inactivation of mechano-sensitive structures upon the first MC is not responsible for the attenuation of the rapid hyperaemia in response to the second MC. Conversely the development of prior hyperaemia is a necessary condition for the attenuation to take place and tissue oxygenation appears to be a major modulator of the rapid dilatation. In addition, these results support the concept that tissue hyper-oxygenation is a key signal in the regulation of muscle blood, activating a prompt and effective action against hyper-perfusion (Golub & Pittman 2013) and may have relevance for investigations and treatments in which mechanical stimuli are delivered to improve muscle perfusion.

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Messere A, Ceravolo G, Franco W, Maffiolo D, Ferraresi C & Roatta S. (2017a). Increased tissue oxygenation explains the attenuation of hyperemia upon repetitive pneumatic compression of the lower leg. *J Appl Physiol* (1985) 123, 1451-1460.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB186

### Maximum Inspiratory Pressure association with Countermovement Jump with regard to age and gender

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Once verified large similarities in the influence of gender on jumping ability and inspiratory capacity in a group of active elderly (1), this section of the study aims to analyse the association between their Maximum Inspiratory Pressure (MIP), and their Height (H) and Relative Power (RP) (2) in a Countermovement Jump (CMJ). Whether these strength capacities evolve together or not with age, it is important for their preservation and improvement, and remains unknown. With this aim, 24 non-smokers healthy elderly (15 women: 71.44±4.31 years; 61.87±7.53 Kg; 1.54±0.05 cm and 9 men: 69.81±4.18 years; 80.67±9.12 Kg; 1.66±0.05 cm), undergoing a multicomponent exercise training program (MCTP), were assessed of body composition, blood pressure, MIP (3) and CMJ(4). The measurements were carried out for two days separated by one week. After testing for normality, Pearson correlation analysis were conducted, with and without the covariates age, gender, and age+gender. Scatter plot and linear regression where added considering the whole sample and selecting men and women separately. Cero Pearson's Coefficient showed a high correlation between both strength capacities, whatever the jumping variable (MIP-CMJ<sub>H</sub>:  $r=0.711$ ,  $p=0.000$ ; MIP-CMJ<sub>RP</sub>:  $r=0.716$ ;  $p=0.000$ ); an association which was slightly reduced when considering the covariate age (MIP-CMJ<sub>H</sub>:  $r=0.647$ ,  $p=0.001$ ; MIP-CMJ<sub>RP</sub>:  $r=0.616$ ,  $p=0.002$ ). However, the covariate gender reduced the correlation (MIP-CMJ<sub>H</sub>:  $r=0.425$ ,  $p=0.043$ ) or even lost it when adjusting the performance to body composition (MIP-CMJ<sub>RP</sub>:  $r=0.353$ ,  $p=0.098$ ). This gender influence was confirmed when controlling for both, age and gender together, since the correlation totally disappeared in both associations (MIP-CMJ<sub>H</sub>:  $r=0.356$ ,  $p=0.103$ ; MIP-CMJ<sub>RP</sub>:  $r=0.180$ ,  $p=0.422$ ). Important changes and the reduction in the linear regression ( $R^2$ ) also evidenced this large gender influence (Figures 1&2). According to our results, mainly gender explains that higher CMJ height or relative power does not involve higher inspiratory muscle strength in active and functionally trained elderly, although age must be also considered. In fact, MIP formulae already considered these two influences on inspiratory capacity (5). Lower limbs and inspiratory

muscle strength seem to respond in a different manner to exercise training, since the MCTP was more neuromuscular than respiratory or metabolic. In conclusion, the improvement of lower limb functional capacities does not ensure the enhancement or even preservation of the inspiratory musculature, suggesting the inclusion of specific respiratory training exercises in MCTP, especially for older women.

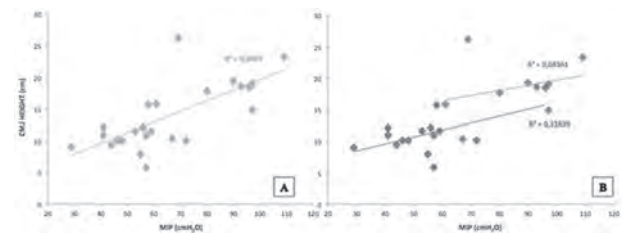


Figure 1. Scatter plot and linear regression for MIP and CMJ Height (left: whole sample; right: considering gender)

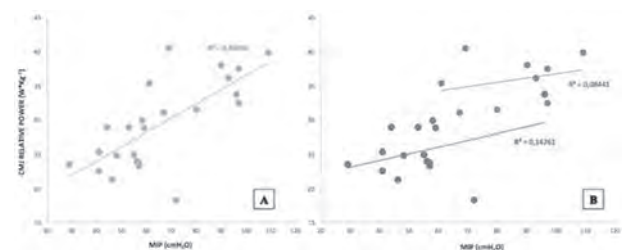


Figure 2. Scatter plot and linear regression for MIP and CMJ relative power (left: whole sample; right: considering gender).

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB187

### The role of septins in skeletal muscle

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Septins are 30-65 kDa, evolutionarily highly conserved GTP-binding proteins that now deserve recognition as the fourth cytoskeletal component. The encoded 13 septins in humans are either ubiquitous or tissue specific, and they are classified into four homology groups (SEPT2, SEPT3, SEPT6 and SEPT7). Septins control different cellular processes by polymerizing into hetero-oligomeric protein complexes that can further form filaments, bundles or rings. All septin

filaments include SEPT7, which is the only member of its group and it occupies the ends of hexameric building blocks which assemble into non-polarized filaments. The function of the ubiquitously expressed SEPT7 is diverse, it has a role in actin dynamics, axon growth, chromosome segregation, cytokinesis and dendrite formation, but also regulates cell shape, membrane trafficking, microtubule regulation, cell motility and DNA repair. Septin assemblies have been shown to act as scaffolds at the plasma membrane to regulate the distribution of membrane-bound proteins.

So far there are limited information about the expression and function of septins in skeletal muscle. We have identified several septin isoforms at mRNA and protein levels both in skeletal muscle samples from mice and human, and in cultured C2C12 cell line. We observed ontogenesis- and differentiation-dependent septin 7 expression in mice and C2C12 cells, respectively. To provide more information about the role of septin 7 in skeletal muscle either gene expression was modified using shRNA gene silencing *in vitro* (Lipofectamine evaluated cell transfection) and *in vivo* (electroporation of intact muscles). Because *SEPT7* knockout is embryonically lethal, we are in the process of generating an *in vitro* *SEPT7* knockout cell line with Crispr-Cas9 technique and an *in vivo* skeletal muscle-specific septin 7 knockout mouse using the Cre/lox system.

In cultured C2C12 cell line stable septin 7 knockdown (KD) clones were generated and marked changes in cell shape and size were observed. The average area and perimeter of the cells increased in septin 7 KD clones, and cells appeared more circular/round following the gene silencing. In control cells the presence of septin 7 was present as a long, filamentous structure throughout the cytoplasm mostly co-localized with actin filaments, while in septin 7 KD cells this well organized structure was broken, the immunopositivity showed pointwise pattern indicating an shRNA-induced inhibition of translation and/or protein degradation. In enzymatically isolated skeletal muscle fibres marked septin 7 immunostaining was detected representing strong co-localization with RyR1.

In conclusion, septin 7 has a crucial role in skeletal muscle physiology, and it could have a potential function in muscle regeneration and/or in different muscle diseases.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB188

### Glycogenolytic derived ATP is essential for skeletal muscle fibre excitability and Na,K-ATPase activity in the transverse tubular system

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Despite the clearly documented importance of glycogen in muscle cell function, the precise mechanism whereby muscle glycogen affects basic cellular events, is far from understood (Ørtenblad et al., 2013). This study examined the effect of glycogen phosphorylase (GP) inhibitors DAB and CP-316,819, omitting the use of glycogen derived energy, on muscle force (F) and fatigue development. Further, we examined whether

the Na,K-ATPase function in the T-system is dependent on ATP derived via the glycogenolytic-glycolytic pathway, under conditions with normal cytoplasmic ATP concentration.

Whole rat EDL muscles from male sprague dawley rats ( $n = 9$ ,  $134 \pm 21$  g (mean  $\pm$  SD)) where incubated in a Krebs-Ringer bicarbonate buffer, with 5 mM glucose, for 3 hrs in presence of 100  $\mu$ M (CP-316,81) or a vehicle (Control). Muscles then underwent a fatiguing stimulation protocol (Fig. 1). In order to test the effect of glycogenolytic derived ATP on t-tubular system Na,K-ATPase function, single fibers from rat fast-twitch muscle were mechanically skinned, sealing off the t-system and thereby permitting action potential triggered twitch and tetanic force (F) responses. The T-system can then be depolarized and/or the GP activity inhibited by DAB (2 mM) or CP-316,819 (10  $\mu$ M).

In whole muscle GP inhibition caused a  $38 \pm 20\%$  decrease in F in fresh muscle and a pronounced faster fatigue development, with half  $F_{max}$  obtained after 109 (CP-316,81) and 201 (control) stimulations (Fig. 1). In the mechanically skinned fibres, tetanic F decreased by  $28 \pm 3\%$  when depolarized to -67 mV. In the fully polarized fibres, inhibition of GP, decreased tetanic F by  $33 \pm 3\%$  (CP,  $P < 0.001$ ) and  $12 \pm 4\%$  (DAB,  $P = 0.004$ ), which was further pronounced in the depolarized fibre (CP  $86 \pm 3\%$  and DAB  $61 \pm 10\%$ ,  $P < 0.0001$ ). Similar results were obtained with twitch responses. To evaluate the Na,K-ATPase function one can quantify the membrane ability to respond to two closely spaced AP (elicited with 1-20 ms space). If second pulse generate an AP, it will produce a larger twitch response and this interpulse space is dependent on the membrane repriming time, which in turn is dependent on the Na,K-ATPase function (Dutka and Lamb, 2007). In a fully polarized fibre, the repriming time was  $4 \pm 0.2$  ms (20 fibres), which was increased to  $6 \pm 0.5$  ms (19 fibres,  $P < 0.001$ ) in the depolarized fibre, indicative of a longer time for the Na,K-ATPase to repolarize the fibre (Fig. 2). There was no effect of GP inhibition on RP in the fully polarized fibre. However, when the T-system was depolarized and GP inhibited, the RP increased to  $8 \pm 1.2$  ms ( $P < 0.001$ ) (13 fibres).

These findings demonstrate that glycogenolytic derived ATP is vital for normal muscle function and fatigue resistance. This may be fully or partly explained by the observation that glycogenolytic resynthesis of ATP is critical to t-system Na,K-ATPase function, and maintain muscle excitability, even in the presence of high global muscle ATP.

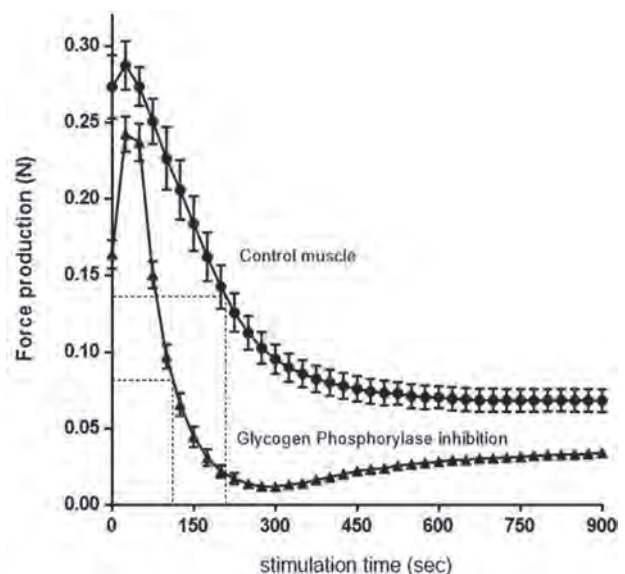


Fig. 1. Whole EDL muscle fatigue protocol (0.4 s trains of 60 Hz, 0.1 ms, every 5 s for 900 s) in presence of GP inhibitor or Control.



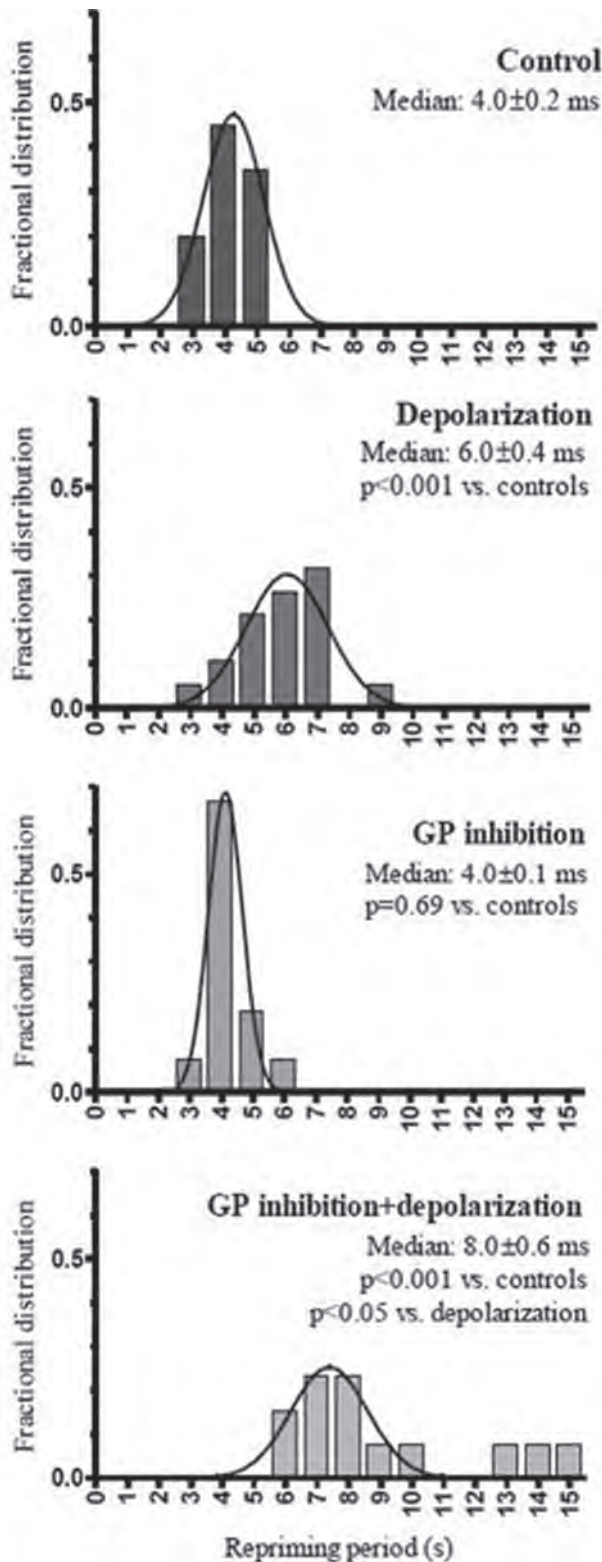


Fig. 2. Fractional distribution of the repriming period in fibers with and without depolarization and GP inhibition. The plotted lines indicate the normal distributions. Increased repriming period indicates decreased Na,K-ATPase function.

Ørtenblad et al. (2013) J Physiol 591, 4405-4413

Dutka and Lamb (2007) Am J Physiol 293, C967-C977

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

# **The effects of antenatal hypothyroidism and voluntary exercise training on oxidative capacity and gene expression in soleus muscle of adult rats**

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Thyroid hormones are important regulators of skeletal muscle growth and development. Therefore, thyroid deficiency during early development may have long-lasting consequences for muscle tissue characteristics in adulthood. The effects of antenatal hypothyroidism (AH) may depend on muscle fibers type and be important for muscle adaptation to aerobic exercise training. *The aim* of this work was to explore the effects of AH and voluntary exercise training in the soleus muscle from rats with AH compared to control rats. We studied: 1) the oxidative potential of muscle tissue (the contents of the mitochondrial respiratory chain oxidative phosphorylation (OXPHOS) complexes and citrate synthase mRNA), 2) myosin phenotype (MHCs gene expression), 3) deiodinase 2 and thyroid hormone receptor  $\alpha 1$  genes, 4) activation and differentiation of satellite cells (FOXO3A, MyoD and myogenin genes), 5) the activity of catabolic pathways (MAFbx and MuRF-1 genes). To induce AH, Wistar dams were given a solution of propylthiouracil PTU (7 ppm) during the whole pregnancy and two weeks post-partum. Six-week-old male offspring of control (CON) and PTU dams were divided into sedentary control (CON, PTU) and trained (CON-Tr; PTU-Tr) groups. The trained groups had 24-hour access to the running wheels for 8 weeks (until 14-week age). T4 and T3 blood contents (ELISA) were reduced in PTU vs CON at the age of 2 weeks, but no intergroup differences were observed at 6 and 14 weeks. Total 8-week run distance did not differ between CON-Tr and PTU-Tr groups. The samples of soleus muscle were taken from 14-week old rats and studied by Western blotting (for contents of OXPHOS complexes) and by real-time PCR (gene expression). The contents of OXPHOS complexes and MHC IIa mRNA were less in sedentary PTU group than in sedentary CON. In control rats, exercise training increased the contents of all OXPHOS complexes, but only complex I was increased in PTU-Tr. Similarly, in CON-Tr group, but not PTU-Tr group, we observed the increases in citrate synthase, deiodinase 2, thyroid hormone receptor  $\alpha 1$  and MyoD mRNA expression levels. Along with that, MHCI mRNA content was increased by training in PTU-Tr but not changed in CON-Tr. The expression levels of myogenin, FOXO3A, MAFbx and MuRF-1 were not affected by training in either group of rats. Our data show that AH reduces oxidative potential and fast MHC expression in soleus muscle of adult rats. Importantly, the effects of training on the oxidative potential and gene expression in soleus muscle were generally suppressed by AH, in spite of unchanged level of running activity. This study was supported by the Russian Science Foundation (grant N14-15-00704).

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB190

**Plasma oxytocin level is enhanced by acute high-intensity interval exercise in men**

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Oxytocin (OT) is a neuropeptide released from neurohypophysis and promotes social cognition and brain-related health. Plasma OT is enhanced by acute very high-volume exercise but not by low- to moderate-volume exercise (Hew-Butler et al. 2008). However, acute very high-volume exercise impairs brain function (Grego et al. 2005) whereas cognitive function is improved by acute high-intensity interval exercise (HIIE) (Tsukamoto et al. 2016). Yet, it is unknown whether HIIE affects OT secretion. Arginine vasopressin is structurally similar to OT and is also released from the neurohypophysis and both plasma OT and arginine vasopressin increase in parallel in response to very high-volume exercise (Hew-Butler et al. 2008). Arginine vasopressin is released by high lactate concentration in the hypophysis (Ohbuchi et al. 2010). Previously, we found that repeated HIIE diminishes cerebral lactate uptake by muscle glycogen depletion-induced low lactate production (Hashimoto et al. 2018). Thus, we hypothesized that plasma OT would be elevated in response to HIIE by increased brain OT release and evaluated internal jugular venous-arterial differences across the brain for OT ( $v\text{-a diff}_{OT}$ ) at rest and end of repeated HIIE in healthy young men. In addition, we also aimed to determine the relationship between OT and lactate across the brain. Subjects ( $n = 14$ , age  $24 \pm 2$  years) performed a HIIE protocol twice (warm-up at 50-60% of maximal workload ( $W_{max}$ ) for 5 min and four 4 min bouts of exercise at 80-90%  $W_{max}$  interspaced by four 3 min bouts at 50-60%  $W_{max}$  for a total of 28 min) separated by a 60 min resting period because very high-volume induced plasma OT enhancement is returned to the resting level within 60 min after exercise. Blood samples were obtained from the bulb of the right internal jugular vein and a brachial artery to determine  $v\text{-a diff}_{OT}$ . Transcranial Doppler determined middle cerebral artery mean blood velocity ( $MCA V_{mean}$ ), and the global cerebral blood flow (gCBF) was estimated from changes in  $MCA V_{mean}$ . Brain OT release/uptake balance was estimated as  $v\text{-a diff}_{OT} \times gCBF$ . Jugular venous and arterial plasma OT increased similarly in response to both HIIEs (both  $P < 0.01$ ), but brain OT release/uptake balance was not affected by HIIE. Nevertheless, the exercise-induced increase in jugular venous OT level correlated to brain OT release/uptake balance (1<sup>st</sup> HIIE  $r = 0.75$   $P < 0.01$ , 2<sup>nd</sup> HIIE  $r = 0.80$   $P < 0.01$ ). In addition to this, there were significant inverse correlations between high arterial plasma OT level and decreased brain OT release/uptake balance (1<sup>st</sup> HIIE  $r = -0.64$   $P < 0.05$ , 2<sup>nd</sup> HIIE  $r = -0.66$   $P < 0.01$ ). There were no correlations between brain OT release/uptake balance and cerebral lactate uptake. These results indicate that HIIE enhances plasma OT level regardless exercise condition with low cerebral lactate and probably increases both brain OT release and uptake.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB191

**GLP-2 inhibits food intake in DMH and NTS**

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GLP-2, expressed in the hypothalamus (the dorsomedial hypothalamic nucleus, DMH and arcuate nucleus, ARC) and in the brain stem (the dorsal motor nucleus of vagus nerve, DMV and the parabrachial nucleus, PBN) and produced from PPG neurons in the brain stem the nucleus tractus solitarius (NTS), is the key signal for the brain and pancreas to control energy balance (1, 2). Previous studies show that GLP-2 inhibits food intake in CNS by GLP-2 infusion into lateral ventricle and 4th ventricle (3). However, which exact central nerve nuclei involves GLP-2 central effects on food intake is currently unknown. In this study, we examined the effect of GLP-2 on food intake in DMH and NTS at free- or fasted-feeding (16h) rats. Male SD rats, from the Medical Experimental Animal Center of Xi'an Jiaotong University, with stainless steel cannulas implanted unilaterally into the DMH and NTS were used. All protocols were complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH No. 80-23). Rats were anaesthetized by pentobarbital sodium and isoflurane before the surgical operation and then given injection GLP-2. After 7 days recovery, experiments began and lasted for 11 days under the count-balance design. Food intake were recorded at different time point. Values are means  $\pm$  S.E.M., compared by two-way ANOVA. Subsequent comparisons between groups used post-hoc Bonferroni tests. Our data showed that compared to the control (0ug/0.5ul), 10ug/0.5ul GLP-2 injection into DMH after 4h at free-feeding could inhibit food intake about 19.6% ( $3.73 \pm 0.66$  g vs  $3.00 \pm 0.66$  g,  $n=8$ ,  $p < 0.05$ ), and GLP-2 injection with same concentration into DMH after 6h could inhibit food intake by 46.1%

at fasted-feeding ( $14.1 \pm 1.6$  g vs  $7.6 \pm 1.6$  g,  $n=8$ ,  $p < 0.05$ ). These food inhibiting effects can be blocked by 10ug/0.5ul Extendin 9-39 after GLP-2 injection 6h (Saline + GLP-2 vs Extendin + GLP-2:  $7.3 \pm 2.3$  g vs  $10.1 \pm 0.9$  g,  $n=8$ ,  $p < 0.05$ ) or by 0.5nmol/0.5ul SHU9119 after GLP-2 injection 24h (Saline + GLP-2 vs SHU9119 + GLP-2:  $12.65 \pm 3.7$  g vs  $24.2 \pm 3.7$  g,  $n=8$ ,  $p < 0.05$ ).

While 10ug/0.5ul GLP-2 injection into NTS could also inhibit food intake by 32.3% after injection 6h at fasted-feeding (0ug/0.5ul vs 10ug/0.5ul:  $11.23 \pm 3.6$  g vs  $7.6 \pm 2.6$  g,  $n=6$ ,  $p < 0.05$ ) but could not inhibit food intake at free-feeding status (0ug/0.5ul vs 10ug/0.5ul:  $1.43 \pm 0.8$  vs  $1.16 \pm 0.26$ ,  $p > 0.05$ ). These food inhibiting effects can also be blocked by Extendin 9-39 with same concentration after GLP-2 injection 24h (Saline + GLP-2 vs Extendin + GLP-2 :  $14.73 \pm 2.5$  g vs  $22.10 \pm 2.8$  g,  $n=6$ ,  $p < 0.05$ ) but not by 0.5nmol/0.5ul SHU9119 after GLP-2 injection 24h (Saline + GLP-2 vs SHU9119 + GLP-2:  $22.2 \pm 3.75$  vs  $18.45 \pm 2.28$  g,  $p > 0.05$ ).

These data suggested that GLP-2 food inhibiting effect can happen in both NTS and DMH. Future works will focus on the underlying mechanism of the GLP-2 on feeding behavior between DMH and NTS circle.

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## PCB192

### Dominant effect of experimental cancer over blocking activin receptor ligands on skeletal muscle metabolome

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Cancer cachexia is a life-threatening disease associated with skeletal muscle atrophy, decreased anti-cancer treatment tolerance and reduced survival<sup>1</sup>. We have shown that continued reversal of skeletal muscle loss by soluble activin receptor type IIB (sACVR2B-Fc) has improved survival in experimental cancer<sup>2</sup>. Currently, there is no clear explanation for mechanisms behind this phenomenon highlighting the complexity of cachexia. This study aimed to investigate whether the alternations in skeletal muscle metabolism could be associated with improved survival of the C26 tumour-bearing mice administered with sACVR2B-Fc.

BALB/c mice were anaesthetized with a mixture of ketamine (60–70 mg/kg, i.p.) and xylazine (9 mg/kg, i.p.) and subcutaneously inoculated with C26 cancer cells ( $n = 33$ ) or

vehicle control ( $n = 15$ ). Tumour-bearing mice were treated with vehicle (C26+PBS) or sACVR2B-Fc either only before (C26+ACVR/b) or both before and after (C26+ACVR/c) C26 tumour formation. Gastrocnemius (GA) muscles were collected 11 or 13 days post cancer cell inoculation depending on the experiment. Muscle metabolome was analysed by gas chromatography–mass spectrometry (GC-MS) and further bioinformatics searches were performed with Ingenuity software (IPA) after the false discovery rate (FDR) corrections. Statistical significance in metabolomics was set at FDR < 0.05 and fold change (FC) > 1.2, and in other analyses at  $P < 0.05$ . In total, 219 metabolites were detected from the skeletal muscle. In accordance with previous studies<sup>3</sup>, the most altered metabolites (42 in total) in cancer were distributed among: 1) amino acids, 2) energy sources, 3) glycolysis components and 4) nucleotide related intermediates. An increase in the aromatic amino acids in tumour-bearing mice indicates enhanced proteolysis while decrease in glycolytic amino acids supports their usage in the tricarboxylic acid (TCA) cycle. From nucleotide metabolism, a decrease in RNA yield was observed together with decreased ribosome biogenesis markers ( $n = 7–9$  per group). Only a few metabolites tended to be reversed by continued sACVR2B-Fc treatment towards healthy control mice.

Although blocking activin receptor ligands reverses cachexia in the skeletal muscle by increasing muscle size, the effect of experimental cancer on skeletal metabolome is more pronounced. The results suggest that changes in the skeletal muscle metabolome are not strongly associated with the improved survival in the sACVR2B group. The decreased protein synthesis capacity in tumour-bearing mice may in part explain our previous findings of decreased protein synthesis<sup>2</sup>. Fearon K et al. (2011). *Lancet Oncol*. 12 (5):489-495

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB193

### Detection of intracellular cysteine modification with green fluorescent protein variants

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Post-translational protein modifications at cysteine residues play key roles in regulatory processes, and excess cysteine oxidation may induce protein damage and contributes to degenerative phenomena. Commonly applied methods for the detection of thiol modification in intracellular proteins involve their biochemical workup. Genetically encoded redox-sensitive fluorescence indicators hold promise as real-time sensors for live-cell imaging with minimal impact on the natural cellular state (1). The redox-sensitive green fluorescent protein (GFP) variant roGFP2 is a powerful tool for studying protein formation and breakage of a disulphide bridge in living cells (2). In roGFP2 one endogenous cysteine is eliminated (C48S), and two are introduced in the neighbourhood of the chromophore (S147C, Q204C), such that they are accessible and close enough to form a disulphide bridge upon oxidation.



Subsequent structural rearrangement leads to an increase of fluorescence intensity at 400 nm and a decrease in intensity at 470 nm, thus providing a ratiometric signal related to the redox state ( $F400/F470 = r$ ). A roGFP2 signal, however, does not provide information on the type of modification that occurred at either one of the two cysteine residues. We therefore examined the properties of the individual cysteine mutants (S147C, Q204C) by performing real-time fluorescence imaging 24 h after transfection in HEK293T cells (21–24 °C). In resting cells, the following  $F400/F470$  were determined (mean  $\pm$  sem,  $n$  = number of cells): roGFP2,  $0.024 \pm 0.0005$  ( $n = 208$ ); S147C,  $0.027 \pm 0.0004$  ( $n = 175$ ); Q204C,  $0.036 \pm 0.0003$  ( $n = 140$ ). The time course of  $r$  was recorded in response to extracellularly applied cysteine modifying agents. 2,2'-Dithio-bis(5-Nitropyridine) (DTNP, 20  $\mu$ M), a specific Cys modifier, elicited an increase in  $F400/F470$  by  $0.09 \pm 0.0005$  with an exponential time constant of  $46.7 \pm 1.12$  s ( $n = 24$ ). The ratio for mutant S147C increased by  $0.02 \pm 0.004$  ( $\tau = 995 \pm 227$  s,  $n = 28$ ) whereas mutant Q204C responded with a decrease in  $r$ :  $\Delta r = -0.01 \pm 0.0002$  ( $\tau = 119 \pm 4.4$  s,  $n = 20$ ). Exposure of the cells to diamide (100  $\mu$ M), which is expected to mediate glutathionylation of cysteines (3), resulted in the following changes: roGFP2,  $\Delta r = 0.10 \pm 0.002$ ,  $\tau = 31.2 \pm 1.12$  s, ( $n = 24$ ); S147C,  $\Delta r = 0.01 \pm 0.0007$ ,  $\tau = 319 \pm 47$  s, ( $n = 24$ ); Q204C,  $\Delta r = -0.01 \pm 0.00009$ ,  $\tau = 252 \pm 6.3$  s, ( $n = 18$ ). Application of  $Na_2S_4$  (10  $\mu$ M), a polysulfide donor that is expected to induce cysteine sulphydration, also affected  $r$  for roGFP2:  $\Delta r = 0.06 \pm 0.001$ ,  $\tau = 148 \pm 7.4$  s ( $n = 23$ ), while mutant S147C ( $n = 21$ ) only showed a slight increase in  $r$ , and no response of Q204C ( $n = 16$ ) was detected.

Cysteines at positions 147 and 204 in GFP have the potential of reporting on intracellular thiol modification by providing ratiometric fluorescence signals.

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PCB194

### **The effect of R547, a cyclin dependent kinase inhibitor, on hepatocellular carcinoma cell death**

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Cancer is a disease that has high mortality rate in the world. Hepatocellular carcinoma (HCC) is derived from hepatocytes and it is the 3<sup>rd</sup> cause of cancer related death type. Therefore, there are many studies about developing new drugs and new therapy methods. Cyclin-dependent kinases (CDKs) and their cyclin partners regulate the cell cycle and check points. Since deregulation of CDKs is found with high frequency in many human cancer cells, inhibition of CDKs gives some guiding ideas for cancer studies (1, 2). In this study, we aimed to determine the possible effect of R547 which is a member of CDKs inhibitor family, on the growth and apoptosis of Hep G2,

derived from human and H4IIE, derived from rat HCC. Both cell lines were exposed to 0, 1, 10, 50, 100  $\mu$ M of R547 for 24 and 48 hours. We determined in vitro survival rate with MTT assay, apoptosis with flow cytometry, statistical significance by one way analysis of variance followed by Tukey's multiple comparison tests. Cisplatin was used as a positive control. At least three independent experiments were carried out for each group. At 0.1, 1, 10, 50, 100  $\mu$ M doses of R547, the corresponding percentages of live Hep G2 cells were 101, 94, 93, 89 and 79 ( $p < 0.001$ ), respectively after 24 hours. The corresponding percentages of alive Hep G2 cells were 92, 101, 53.6 ( $p < 0.01$ ), 47.4 ( $p < 0.001$ ), and 41 ( $p < 0.001$ ) %, respectively, for 48 hours. After 24 hours culturing at the same doses of R547, the corresponding percentages of live H-4-IIE cells were 90, 80 ( $p < 0.01$ ), 63 ( $p < 0.001$ ), 47 ( $p < 0.001$ ) and 43 ( $p < 0.001$ ) % respectively. The corresponding percentages of alive H4IIE cells were 96, 85 ( $p < 0.01$ ), 46 ( $p < 0.001$ ), 44 ( $p < 0.001$ ) and 45 ( $p < 0.01$ ) %, respectively, for 48 hours. Because R547 was not affect the cell survival of Hep G2 cells for 24 hours, flow cytometry experiments were continued with H4IIE cells. The rates of early apoptotic H-4-IIE cells were about 38 and 45 ( $p < 0.05$  for both) % after applications of 10 and 25  $\mu$ M R547 after 24 hours (control: 4.1%), respectively. According to our results, it is clear that R547 has stronger growth inhibitory action when compared to cisplatin. Additionally, R547 has apoptotic effect on H-4-IIE cells for 24 hours. Diaz-Padilla I, Siu LL, Duran I. Cyclin-dependent kinase inhibitors as potential targeted. Invest New Drugs (2009), 27: 586-594.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB195

### **Hesperidin attenuates oxidative ovarian damage injured by ischemia-reperfusion**

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The purpose of this research is to examine the possible protective effects of Hesperidin as antioxidants on ovary injured by the ischemia reperfusion. We used 24 Wistar-type female rats within the scope of our study. The rats were randomly divided into 3 groups. Our groups are planned as follows; sham-operated group (only laparotomy was performed without ischemia reperfusion), ischemia reperfusion group (laparotomy was performed and the ovaries were exposed to torsion 360 degrees for 3 hours followed by detorsion for 3 hours for reperfusion but no medication was given), ischemia reperfusion+hesperidin group (first, ischemia was performed, then hesperidin 100 mg/kg i.p was administered and finally reperfusion was performed). Using the enzyme-linked immunosorbent assay kits, total antioxidant level (TAS), superoxide dismutase (SOD) were measured as antioxidant; total oxidant level (TOS), myeloperoxidase (MPO) and malondialdehyde (MDA) levels were measured as oxidant parameters in ovarian tissue. One way ANOVA and Tukey test were performed.

When our results of biochemical analysis were evaluated, it was determined that the oxidant parameters increased in the ischemia-reperfusion group but the antioxidant activity decreased significantly ( $p < 0.05$ ). Malondialdehyde level and total oxidant status value were decreased in the group treated with hesperidin, but superoxide-dismutase activity was found to be increased. These results have shown us that single dose administration of hesperidin is highly effective against oxidative ovarian damage caused by ischemia reperfusion.

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PCB196

### **Aspirin working with platelet for its anti-cancer role on metastatic breast cancer**

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Aspirin has recently been considered as potential treatment against cancer. Some studies showed that aspirin has great effect in the primary prevention, secondary prevention of cancer (especially in colorectal cancer etc.) [1, 2] but the specific molecular mechanism is still unknown, particularly its interactive role with platelet in treating cancer.

Breast cancer is a malignant disease with extremely high morbidity and mortality. One study [3] summarized the literature on the relationship between breast cancer and aspirin from 2000 to 2012 and indicated aspirin has a preventive effect for breast cancer when the dose exceeds 100 mg/d and the drug is regularly used for more than 3 years or longer. In addition, another study [4] showed that thrombocytosis is positively associated with tumour incidence.

In this study, we investigated effects of aspirin against metastatic breast cancer. We used human breast cancer cells 231 and mouse breast cancer cells 4T1 cell lines to test its anti-cancer effects along with human platelet-rich plasma and mouse platelet-rich plasma by proliferation assay, scratch test in vitro. We then used Balb/c mouse inoculated tumors subcutaneously to test the survival period of mice, tumor size, and some physiological and biochemical indicators in vivo.

Our results showed that aspirin has an inhibitory effect on the proliferation of tumor cells in vitro. Elevated concentrations of platelet-rich plasma could significantly inhibit cell proliferation after a long time; possibly due to the fibrinogen-formed film prevents tumor cells contact the external environment. Low concentrations of platelet-rich plasma had no effect on tumor cell proliferation. Platelet-rich plasma can advance the invasion of metastatic breast cancer cells, and this effect could be inhibited by aspirin.

In vivo results showed that aspirin can significantly prolong the survival time of tumor-bearing mouse, and mouse inoculated with tumor cells co-cultured with platelet-rich plasma in vitro have a higher hazard ratio. Tumor HE staining showed that mouse in the aspirin group had less metastasis cancer cells during the same period, suggesting that platelets play a catalytic role in tumor metastasis and this effect can be inhibited by aspirin. In addition, in the aspirin group the mouse tumor tissue mass exterior was better without wear and ulceration, suggesting that aspirin can reduce the pain in the late stage of cancer and extend the survival time. Biochemical indicators indicated that there is no significant relationship between

platelet index and the degree of cancer deterioration, which may be due to the small sample size.

In conclusion, our results indicated that the ability of aspirin against clinically advanced breast cancer is relatively limited, and that aspirin, platelets, and tumors may interact with each other through specific pathways, which need further verification.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

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PCB197

### **Cellular Effects of Silver Nanoparticles (AgNPs) on Human Keratinocyte Cell Line (HaCaT) in Vitro**

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Due to their intensive antibacterial properties, AgNPs have been widely used in many commercially products designed to direct contact with the skin [1]. This brings a growing concern on their potential risks to human health [2]. Previous studies show that AgNPs can induce oxidative damage in the primary HaCaT cells responded to modulation of antioxidant capacity. AgNPs can generate elevated intracellular reactive oxygen species (ROS) and down-regulation of antioxidant enzymes such as GPX and SOD, resulting in the formation of DNA adducts [3]. The mechanisms involved in the endogenous antioxidants effect appear to be primarily independent on increased ROS production, but superoxide anions levels are presently under investigation. Further studies on the shape dependency of AgNPs in antimicrobial preparations could help to design an agent with the highest efficacy and low systemic toxicity [4].

This study aimed to investigate the mechanism of cytotoxic and genotoxic effects of AgNPs on human skin keratinocytes (HaCaT) cells. Primary cultures of HaCaT cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin-streptomycin and supplement mix of fetal calf serum at 37 °C in a 5% CO<sub>2</sub> atmosphere. They were then treated with in the presence and absences of 15 μM of AgNPs for 2, 6 and 24 h. Genocytotoxic effects of AgNPs was assessed by the changes in various cellular parameters of HaCaT cells such as viability, superoxide anion radical production, lactate dehydrogenase release and the levels of the antioxidant enzymes, namely, catalase (CAT), glutathione peroxidase (GPX) and superoxide dismutase (SOD). Superoxide anion was detected using nitroblue tetrazolium NBT reduction assay. An LDH level was evaluated using the standard LDH kit, and activity of antioxidant enzymes such as CAT, GPX and SOD-1 were quantified using qPCR.

Our results showed that AgNPs exposure can cause cellular oxidative damage accompanied by excessive production of superoxide anion and a significant reduction in antioxidant, but the low cytotoxic effect was observed. This suggested that AgNPs may have a potential toxic effect on the skin level only after long-term exposure.

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## PCB198

### the Epigenetic landscape of two phenotypic extreme skeletal muscles - Soleus and EDL

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#### Introduction

By varying the relative composition of different fibre-types within a muscle, an organism has the ability to fine-tune muscle function according to physiological demands. Skeletal muscle tissue is composed of approximately 40 - 60 % myonuclei, while the remaining are non-muscle cells with a different origin such as stromal and hematopoietic. Therefore, it is important to isolate the subpopulation of interest from the whole tissue, as a mixed-cell population are prone to increase the number of false-positive results.

In this study, we present a novel method for isolating myonuclei from whole native muscle.

The resulting pure myonuclear fraction allowed us to explore the epigenetic landscape for the two phenotypic extremes the oxidative Soleus and the glycolytic Extensor Digitorum Longus muscle (EDL).

#### Methods

Animals were sedated with gas anaesthesia with 2 % isoflurane. Surgically were initiated following the loss of the metatarsus reflex. A small incision where made on the posture side of the hind limb and the Soleus and EDL muscles were surgically removed and frozen in liquid nitrogen (n=6 Female NMRI mice, weighing 25-30 g). The myonuclei population was biochemically isolated and used to create the epigenetic map by Chromatin Immunoprecipitation coupled with next-generation sequencing (ChIP-Seq) and bioinformatic analyses.

#### Results

Using our novel method, we show that that the myonuclei epigenetic environment is significantly different compared to the whole tissue. Global analysis of the epigenetic landscape in the Soleus and EDL muscles show that there are large dynamic differences between the two muscle types which

correlate with the myo-specific transcriptome and the properties of the muscles.

#### Conclusion

We have developed a novel method to isolate the myo-specific nuclei from skeletal muscle. We use the method to generate a global map of the epigenetic landscape of the two extremes, the oxidative Soleus and the glycolytic EDL muscles and find that their different physiological properties are reflected in their epigenome.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## PCB199

### DAG enriched oil activates Nrf2 pathway and impedes NF-κB translocation to mitigate renal injury in Cadmium Chloride (CdCl<sub>2</sub>) induced hypertensive rats

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#### Motivation:

The scope of the present work entailed to design a novel nutraceutical product of bioactive lipid (a diacylglycerol oil comprising caprylic acid, C<sub>8:0</sub>) and investigate its' subsequent ameliorative effect by targeting the molecular nodes of abnormality of CdCl<sub>2</sub> induced *in vivo* hypertensive model.

#### Methodology:

Male wistar rats (120-140g weight) were randomly divided into four groups with six number of animals (i.e., n=6) in each group: (1) control; (2) C<sub>8:0</sub>-DAG oil-treated group (2.0 g/day DAG oil); (3) hypertensive group (1.0 mg/kg body weight of Cadmium Chloride; CdCl<sub>2</sub>, single dose i. p); and (4) hypertensive (1.0 mg/kg body weight of CdCl<sub>2</sub>, single dose i. p) /DAG oil-treated group (2.0 g/day DAG oil). During the course of the experiment prior to animal sacrifice, non-invasive blood pressure (NIBP) measurements were performed and recorded by tail-cuff method at regular intervals. Further, following animal sacrifice, biochemical assays, immuno-blotting, immunofluorescence, reactive oxygen species (ROS) analysis and protein expression studies by flow cytometry analysis (vis-à-vis apt control groups) were employed to evaluate the protective efficacy of C<sub>8:0</sub>-DAG enriched oil against CdCl<sub>2</sub>-induced hypertension. All expressed results are means ± S.E.M., compared by ANOVA.

#### Results:

CdCl<sub>2</sub> administration showed significant elevation in the systolic blood pressure for the positive control groups 3 (162.29±1.37mmHg) and 4 (151.89±0.66mmHg) respectively as compared to the normal control group (125.65±0.68mmHg) and C<sub>8:0</sub>-DAG treated group (117.34±.75mmHg). Further, CdCl<sub>2</sub> had significantly increased renal oxidative stress markers and decreased the levels of renal enzymatic and non-enzymatic antioxidants and lipid peroxidation. An elevated level of cellular TBARS was found in CdCl<sub>2</sub>-treated renal tissue (3.66-fold) which was significantly (p < 0.05) inhibited with the co-treatment of C<sub>8:0</sub>-DAG oil (1.96-fold). Further, CdCl<sub>2</sub> significantly suppressed the level of non-enzymatic antioxidant, GSH and enzymatic antioxidants, SOD with respect to the control group. These abnormal conditions were significantly restored in pre-treated C<sub>8:0</sub>-DAG enriched oil group. In addition, C<sub>8:0</sub>-DAG oil reduced CdCl<sub>2</sub>-induced elevation of



pro-inflammatory cytokines: TNF- $\alpha$ , and IL-6. Further, it was found that C<sub>8:0</sub>-DAG enriched oil co-treatment normalized the renal expression of Nrf2/Keap1 and its downstream regulatory proteins in CdCl<sub>2</sub>-treated rat kidney. C<sub>8:0</sub>-DAG administration had significantly modulated the abnormal phosphorylation conditions of key regulatory proteins induced by CdCl<sub>2</sub>, i.e. PI3K, AKT, MAPK, and ERK1/2 and attenuated NF- $\kappa$ B translocation conditions in group 4 as compared to group 3. Histological and immunohistochemical observations provided further evidence that showed C<sub>8:0</sub>-DAG oil effectively counters CdCl<sub>2</sub>-mediated oxidative damage.

#### Conclusion:

The accrued empirical evidences support the designed product as a promising nutraceutical for clinical applications.

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### PCB200

#### Down expression of onco-suppressive CHEK-2, HOXB-13 genes and Up-regulation of Proto-onco AR gene in prostate cancer

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Cancer is group of interconnected diseases in which cells of the body starts growing and their growth go uncontrolled. It is 2<sup>nd</sup> main cause of death around the world after cardiovascular diseases being on number 1. Prostate gland is the main male accessory gland involved in sperm growth and lubrication. Among all the cancers, prostate cancer is the 2<sup>nd</sup> most diagnosed cancer in men across the globe. Molecular and genetic players of prostate cancer have been studied extensively in modern research era. Role of some genes such as BRCA1, BRCA2, PTEN, HOXB13, AR and CHECK2 is evident as tumor suppressor in progression of prostate cancer. Objective of this study was to observe the down expression of HOXB13, CHEK2 genes and Up-regulation of AR gene in male prostate carcinogenesis and understand the back stage signaling pathways involved in prostate cell aberration. Biopsy samples of prostate gland of affected patients was collected locally from PINUM health care facility with permission of ethical review committee and samples were kept in solution containing 0.9% normal saline and 10% formalin. After qRT-PCR analysis, it was observed that CHEK2 and HOXB13 genes were down expressed while Ar gene over expressed in patients samples which in turn favor the causation of cancer. The variance of data was analyzed through ANOVA test and DMR test was used to observe significance of data.

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### PCB201

#### Role of inflammation in papillary thyroid carcinoma (PTC) development and progression

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It is accepted that frequency of PTC is higher among patients with autoimmune thyroiditis. However, it is still unclear what mechanisms determine this relationship. The aim of the study was to assess possible immune-mediated mechanisms of PTC development and progression. 87 patients with histologically confirmed PTC were enrolled in the study, 32 of them had concurred Hashimoto's (HT) or focal thyroiditis (FT). Immunohistochemistry to the following markers was performed: CD68, CD163, MCT, CD138 and CD8 was performed. In addition, expression of VEGF and STAT6 was estimated. PTC with concurred HT or FT was associated with high number of macrophages, mast cells (MC), CD8 and plasma cells (P<0.0001). The tight association between high M2-macrophages number and lymph node metastasis was found (OR=8.56; 95%CI 2.46-28.22; P=0.0004). However, number of tumor associated CD8+ cells was not related with metastasis risk (P=0.821) that could be explained by potential PD-L1 expression. In addition, PTC with concurred HT or FT was accompanied with accumulation of numerous plasma cells, that were distributed inside the tumor foci and in lymphoid follicles around. Number of M2 macrophages tightly correlated with count of CD138+ cells, reflecting an association of M2 macrophages polarization with humoral immunity activation. M2-macrophages and MC count was related with high microvascular density (P=0.006) and VEGF expression (P<0.0001), intrathyroid invasion (P=0.03) and lymph node dissemination (P=0.005). Expression of VEGF was found in tumor cells and in stroma. VEGF expression in tumor cells varied from low to high and correlated with degree of infiltration (r=0.677; P<0.001) and number of M2 macrophages (r=0.812; P<0.0001). Thus, PTC progression is associated with humoral immunity acceleration, M2-macrophages polarization, accumulation of MC and high VEGF expression. As far as humoral immunity is mediated by IL-4, we supposed that PTC progression could be associated with changes in STAT6 expression. Normal thyroid tissue demonstrated lack of STAT6 expression. In PTC we found that in addition to numerous STAT6 positive stromal cells, there was middle to high cytoplasmic expression of STAT6 in tumor cells. Its intensity correlated with inflammatory infiltration (r=0.793; P<0.001) and was significantly higher in patients with concurred HT or FT (P=0.02). In addition, we found expression of STAT6 around tumor foci associated with infiltration and features of active thyroid tissue remodeling and cellular atypia. The similar pattern was found in patients with PTC and concurred HT – in zones of intensive infiltration and around lymphoid follicles. In conclusion, chronic inflammation accompanied with humoral immunity activation was associated with PTC progression and metastasis. One of the possible links between immune reaction and PTC could be IL-4 mediated activation of STAT6, associated with tissue remodeling and M2-macrophages polarization.

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PCB202

### Investigation of the Effect of Nifedipine on Kidney Catalase of Ischemia/Reperfusion Injured Rat

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**AIM:** Catalase (CAT) is a common enzyme found in nearly all organisms exposed to oxygen it catalyzes the decomposition of hydrogen peroxide to water and oxygen. The purpose of this study is to investigate the effect of nifedipine drug on rat kidney tissue CAT enzyme in ischemia / reperfusion (I / R) and I / R injured rats.

**METHODS:** Control, sham, I / R (1:24 hours) and nifedipine + I / R are as follows: A total of 24 male Wistar albino rats weighing 250-300 g were equally and randomly divided into four groups. In the last group, nifedipine was administered at the 4 mg/kg dose before intraperitoneal ischemia period. Kidney tissues were removed after bilateral I/R process. Tissue levels of enzyme activity of CAT were measured. CAT activity was determined by Aebi method which H<sub>2</sub>O<sub>2</sub> at 240 nm.

**RESULTS:** Specific activity values were determined for CAT enzyme at four different experimental groups. Groups were determined as follows: Control group 0.652 EU/mg protein, sham group 0.668 EU/mg protein, I/R group 1.027 EU/mg protein, and nifedipine + I/R group 1.714 EU/mg protein.

**CONCLUSION:** As a result, the activity of the CAT enzyme was determined to be mostly activated in nifedipine + I/R group among all applications. This demonstrates that the secretion is further increased in each administration compared to the control.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB203

### Ghrelin's effects on Levels of Metalloproteinases and Inflammatory parameters in septic intestinal tissue

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**Motivation/problem statement:** Sepsis is a multisystemic organ dysfunction, lead to tissue injury and organ failure by excessive activation of complement components and neutrophil actions against to bacterial infection (1). Lipopolysaccharide has been reported to induce cytokine release and production of reactive oxygen species, leading to inflammation, oxidative stress and tissue damage (2). Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs)

are biomarkers that include prognosis of sepsis (3). MMP-9 and TIMP-1 groups were associated with higher mortality in the onset of severe sepsis. YKL-40 is a 40 kd glycoprotein as known glycoprotein 39, chitinase 3Y like protein 1, involving in remodelling of extracellular matrix (4). YKL-40 probably participates in inflammation and remodeling/degradation of the extracellular matrix/damaged tissue. Ghrelin is known that an endogen ligand of growth hormone and it shows anti-inflammatory and anti-oxidant effect (5). In this study, we aimed to investigate the effects of exogenous Ghrelin on YKL-40, MMP-2, MMP-9, TIMP-1 immunoreactivities in the small intestine tissue in LPS induced septic rats.

**Methods/procedure/approach:** The approvals were obtained from Istanbul University Animal Experiments Local Ethics Committee for our study. Wistar albino male rats (180-230g, for each of group n=6) were divided into 4 groups: control, LPS, Ghrelin, LPS + Ghrelin. LPS were given twice dose first, 5 mg / kg i.v. and second dose 5 mg / kg i.p. Ghrelin 10 nmol / kg i.v. was administered in a single dose. Twenty-four hours after the first injections, the rats were decapitated. Intestinal tissue and blood samples were taken. MMP-2, MMP-9, YKL-40, and TIMP-1 immunoreactivities were detected by immunohistochemical staining with using proper antibodies on small intestinal tissue sections.

**Results:** In the histological examination; it was found higher immunoreactivities of TNF-a, MMP-2, MMP-9, and YKL-40 in the LPS group. However, IL-10 immunoreactivity was found lower in the LPS group.

**Conclusion/implications:** We may proposed that Ghrelin is improved on metalloproteinases and inflammatory parameters in damaged small intestinal tissue in LPS induced septic rats.

Ghrelin decreased proinflammatory cytokine level and protective on elevated matrix metalloproteinases and YKL-40 levels in damaged small intestinal tissue in LPS induced septic rats.

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Our study was granted by Istanbul University Research Projects (Projects No: 21440).

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## PCB204

**Effect of aqueous extract of *Tamarindus indica* Linn. (Leguminosae) pulp on basal and stimulated gastric acid secretion due to histamine, pentagastrin and carbachol in rats**

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Local folk claims that *Tamarindus indica* Linn. (Leguminosae) has a gastric beneficial role in humans (Pankaj et al., 2011)). This study was carried out to examine the effects of tamarind on basal and stimulated gastric acid secretion (GAS) in the Wistar rats. Each group of rats consisting of 6 rats (180 - 210 g) was treated with aqueous extract of tamarind pulp (1.5 mg/kg, 2.5 mg/kg and 4 mg/kg) orally for 21 days. The ethical guidelines in accordance with NIH Guide for care and use of laboratory animals were followed in the handling of the animals during the studies. At the last day of the treatment, the rats were fasted for 24 hours, anaesthetized with ketamine and cannulated through the femoral vein. GAS (mEq/l<sup>-1</sup>) induced by histamine, pentagastrin and carbachol administration were assessed by the continuous perfusion technique method of Ghosh and Schild (1958) and its acidity determined by titration method.

Data were expressed as mean  $\pm$  SEM. Student's t-test and one way ANOVA were used to determine levels of significance at  $p=0.05$ .

Data were analyzed using student's t- test and ANOVA at  $p=0.05$ .

The aqueous extract of tamarind significantly decreased ( $p<0.05$ ) GAS induced by the histamine, pentagastrin and carbachol at small and medium doses, but not at higher doses over the 80 minute's period in all the treated groups. The gastroprotective action of tamarind may be multifactorial that may include anti-secretory action (Flemstrong and Garner, 1982).

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## PCB205

**The role of endogenous oxytocin on the anti-stress effect of exercise**S. Arabaci Tamer<sup>1</sup>, S. Ucem<sup>2</sup>, M. Guner<sup>2</sup>, B. Buke<sup>2</sup>, A. Karakucuk<sup>2</sup>, N. Yigit<sup>2</sup>, O. Cevik<sup>3</sup>, B. Coskunlu<sup>4</sup>, F. Ercan<sup>4</sup>, S. SIRVANI<sup>4</sup> and B. Yegen<sup>1</sup>

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Exogenously administered oxytocin, which results in suppression of the hypothalamopituitary-adrenal (HPA) axis<sup>1</sup>, was proven to possess anti-ulcer effects<sup>2</sup>. Regular exercise protects gastric mucosa by alleviating stress<sup>3</sup>, suggesting that the anti-ulcer effects of exercise training might be mediated by endogenous oxytocin via the inhibition of HPA axis. The aim was to investigate the role of endogenous oxytocin in the anti-ulcer impact of exercise on gastric ulcerogenesis. Following the ethical approval, Wistar albino rats were randomly divided as exercise (30 min/day, 5 days/week swimming for 6 weeks) and sedentary groups. On 7<sup>th</sup> week, under ketamine and chlorpromazine anesthesia (100 and 10 mg/kg, intraperitoneally, ip) rats underwent a laparotomy, and acetic acid (80%; ulcer, n=28) or saline (control; n=28) was applied on serosal surface of stomachs for 1 min. Oxytocin-receptor-antagonist atosiban (0.1 mg/kg/day) or saline was injected ip on the last 4 days before surgery and on 3 days after surgery. On postsurgical 3<sup>rd</sup> day, anxiety levels were determined using hole-board. Then, on the 4<sup>th</sup> day gastric serosal blood flow was measured under anesthesia via laser Doppler flowmetry. The rats were euthanised following the withdrawal of blood by cardiac puncture. Using ELISA, serum cortisol levels and gastric levels of 8-hydroxy-2'-deoxyguanosine (8OHdG) and caspase-3 activity were determined. Gastric injury was assessed histologically (hematoxylin & eosin) and immunohistochemical expression of oxytocin was evaluated in the supraoptic nuclei. Statistical analyses were made using ANOVA and Student's t-tests. Compared to sedentary control group, serum cortisol level was increased in the sedentary-ulcer groups ( $p<0.05$ ), while head-dipping numbers on holeboard were reduced ( $p<0.05$ ), indicating increased anxiety. Regular exercise depressed cortisol and increased head-dipping number, showing decreased anxiety ( $p<0.05$ ). When compared with sedentary control group, caspase-3 activity, 8-OHDG levels and gastric damage scores showed significant increases in the sedentary ulcer group ( $p<0.001$ ), while serosal blood flow was significantly decreased ( $p<0.05$ ). Swimming exercise elevated serosal blood flow and attenuated ulcer-induced gastric damage score ( $p<0.05$ ), while atosiban treatment reversed exercise-induced reduction in gastric damage score ( $p<0.05$ ). Oxytocin expression in supraoptic nucleus showed a tendency to decline in the sedentary ulcer groups (control:  $1.3 \pm 0.4$ , ulcer:  $0.3 \pm 0.3$ ), but exercise increased expression above control level ( $2.5 \pm 0.5$ ,  $p<0.05$ ), while atosiban blunted exercise-induced increase in oxytocin expression ( $0.7 \pm 0.2$ ;  $p<0.05$ ). These data suggest that exercise reduces anxiety, protects gastric mucosa and supports gastric blood flow by suppressing HPA activity, which appears to involve the upregulation of hypothalamic oxytocin synthesis. Gibbs DM. (1984). *Life Sci* 35.5, 487-491.



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## PCB206

### Vagal afferents modulate high fat diet induced visceral obesity and dysbiosis

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Obesity has been characterized by an abnormal microbiota composition or dysbiosis (1). Chronic consumption of high fat diet promotes dysbiosis before any changes in body weight in rats (2). Diet-driven microbiota dysbiosis is associated with decreased vagal outputs to the central nervous system (3). The aim was to determinate to the interaction the gut microbiota and the vagal afferents on sulphate-producing *Desulphovibrio piger* (D. piger). Sprague-Dawley rats (male, 10 weeks old) were randomly divided as control (n=5), vagal denervation (Vx) (n=10), sham operated Vx (n=13) groups. Vx was induced perivagal 1% capsaicin administration, vehicle (10% Tween 80 in oil) was applied to make sham Vx. After 3 weeks of recovery, we have performed blink reflex to elucidate succession of the Vx. The rats were fed with chow diet (fat 2-7%) for the first 4 weeks and fed the high fat diet (HFD, % 45 fat) for the last 4 weeks until decapitation. Food intake was monitored daily and body weight was measured weekly. Food and water intake were measured after 16 hrs. of food and 24 hrs. of water deprivation in the last week. Weight of faeces for 16 hrs. and small intestinal transit (SIT) time with charcoal were measured. Fecal samples were taken at 0-3-7<sup>th</sup> weeks under sterile conditions to detect *D. piger* by qPCR. At the end of the 8 weeks, rats were decapitated and epididymal fat and liver was weighted and calculated as gr/ 100gr body weight. Data was expressed as S.E.M, comparison between groups were done with ANOVA. The blink number was decreased in Vx group as compared to control group (Vx:9.6±1.3, control: 12.6±2.4, p<0.01). Weight gain was similar during normal feeding, final weight gain was also comparable among study groups. The epididymal fat weight was significantly decreased in the Vx group (0.96±0.11, p<0.05) compared to sham-Vx (1.41±0.13), while liver weight and SIT time did not differ between the groups. Food consumption was higher calorically in HFD Vx group than HFD control group after 16 hrs. of food deprivation (Vx:15.7±1, control: 12.5±1, p<0.05). Water consumption after deprivation was similar in all study groups. During the normal diet or HFD period, Vx did not induce any alteration in *D. piger* as compared to control, but *D.piger* raised in HFD Vx (p<0.01) and HFD sham Vx (p<0.05) groups compared to normal diet Vx and sham Vx, respectively. Vagal afferents denervation ameliorates HFD associated visceral obesity. Vagal afferent nerves and HFD affect *D. piger* content of faeces. But further studies are needed to clarify interaction between vagal afferents and dysbiosis to control obesity.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB207

### Beneficial effect of oat β-glucans on the inflammation-related gene expression in colitis

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β-glucans are polysaccharides belonging to the soluble fiber fraction, extractable from e.g. cereals and classified as a physiologically active. The positive impact of β-glucans on organisms result from their immune-stimulating properties and ability to binding to immune cells receptors, immune cells activation, and regulation of humoral and cell-mediated immunity.

The aim of the study was to determine the effects of the dietary supplementation with chemically pure high- and low-molecular-weight oat β-glucans on the gene expression of inflammatory cytokines and receptors in rats colon tissue with colitis.

Adult male Sprague Dawley rats (380±20g, n=54) were divided into two groups: control (n=27) and with experimentally-induced colitis (n=27, single administration of 2,4,6-trinitrobenzenesulfonic acid (TNBS), per rectum). In the control groups 0.9% NaCl was administered in the same way. Both experimental and control groups were divided into three subgroups fed during 21-day AIN-93M feed supplemented with 3% (w/w) of (1) low molecular weight β-glucan (βGI+, n=9), (2) high molecular weight β-glucan (βGH+, n=9) or (3) feed without β-glucan (βG-, n=9). At the end of the feeding period under Isoflurane anesthesia colon samples were collected. RNA was then isolated and reverse transcribed into cDNA (Qiagen). Targeted RT-qPCR (RT2 Profiler PCR Array Rat Inflammatory Cytokines & Receptors, Qiagen) was performed to analyze inflammatory-related gene expression. Data analysis was performed using the online Qiagen analysis software (RT2 profiler PCR array data analysis V3.5) with >2 fold-change and p<0.05 considered to be statistically significant.

Result indicate that out of 84 genes TNBS (in colitis (βG-) group) compared to control (βG-) induced up-regulation of 32 genes (including chemokines and its receptor genes: *Ccl3*, *Ccl4*, *Ccl12*, *Ccl17*, *Ccl19*, *Ccl22*, *Ccr3*, *Ccr4*, *Ccr5*, *Ccr6*, *Ccr8*, *Cxcr1*, *Cxcr2*, *Cxcr3*, *Cxcr5*; interleukin and its receptor genes: *Il10ra*, *Il13*, *Il16*, *Il1rn*, *Il21*, *Il2rb*, *Il2rg*, *Il5ra*, *Il6r* and other genes involved in inflammation: *Faslg*, *Lta*, *Ltb*, *Osm*, *Spp1*, *Tnf*, *Tnfsf11*, *Tnfsf14*). The addition of β-glucan to animal feed significantly altered gene expression level in the rats with colitis (in colitis (βGI+) up-regulation of: *Cxcl1*, *Il17a*, *Cxcr1*, *Spp1* and down-regulation of: *Ccl19*, *Cd40lg*, *Cxcr5*, *Il10ra*, *Il16*, *Il21*, *Il2rg*, *Il5ra*, *Lta*, *Ltb*, *Osm*, *Tnf*, *Tnfsf11*, *Tnfsf14*) (in colitis (βGH+) up-regulation of: *Cxcl1*, *Cxcl2*, *Cxcr2*, *Il17a*, *Il1a*, *Spp1* and down-regulation of: *Ccl19*, *Cd40lg*, *Cxcr5*, *Lta*, *Ltb*, *Tnfsf11*) with stronger protective effect demonstrated by low molecular weight β-glucan.

The obtained results indicate the protective effect of  $\beta$ -glucan on the colon, which in consequence may reduce the negative effects of inflammation in the large intestine.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB208

### Effects of Gum Arabic (*Acacia Senegal*) on visceral adiposity index and blood pressure in Type 2 Diabetes Mellitus as indicators of cardiovascular disease: a randomized and placebo-controlled clinical study

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#### Background:

There is a strong association between cardiometabolic risk and adipose tissue dysfunction with great consequences on type 2 diabetic patients. Visceral Adiposity Index (VAI) is an indirect clinical marker of adipose tissue dysfunction. Gum Arabic (GA) is a safe dietary fiber resultant of exudates from *Acacia Senegal*. GA has shown lipid lowering effect in both humans and animals. Therefore, the aim of this study is to determine the effect of (GA) on anthropometric obesity marker, Visceral Adiposity Index (VAI) and blood pressure in patients with Type 2 diabetes.

**Methods:** A total of 91 Type 2 diabetic patients, aged 50.09  $\pm$  9.3 with HA1c more than 7% and on hypoglycemic agents were randomly assigned to receive either 30g of GA or 5g pectin as a placebo daily for 3 months. Waist circumference (WC), systolic (SBP) and diastolic (DBP) blood pressure were measured, VAI, body mass index (BMI), waist-hip ratio (WHR), body adiposity index (BAI), lipid accumulation product (LAP) and deep-abdominal-adipose-tissue (DAAT) accumulation were calculated and high-density lipoprotein cholesterol (HDL-C) and triglycerides (TG) were determined in fasting blood samples at the start and end of the study period. This study was conducted according to the guidelines by the Declaration of Helsinki and procedures were approved by the Research Ethics Committee - Khartoum State Ministry of Health and the

Institutional Review Board at University of Medical Science and Technology (UMST); SUM 116-IRB number: 00008867. The trial is registered under PACTR201403000785219.

**Results:** After intervention, BMI and VAI were decreased in GA group significantly ( $P < 0.05$ ). BAI and LAP significantly decreased in GA group while no significant difference in WHR or DAAT. Systolic blood pressure significantly decreased ( $P < 0.05$ ) in the GA group and placebo group with no difference in diastolic blood pressure in both groups.

**Conclusion:** Gum Arabic at a dose of 30g /d for 3 months may play an effective role in preventing weight gain and modulating adipose tissue dysfunction in type 2 diabetic patients.

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We acknowledge Dar Savanna Ltd. Khartoum, Sudan for providing Gum Arabic powder as a gift.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB209

### Effects of intermittent fasting on overall activity and anxiety level and autonomic nervous system balance in male rats.

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Obesity becomes one of the biggest modern society problem: the number of excessively overweight people has tripled since 1975 (1). Changes in diet and intermittent fasting are effective ways for weight normalization (2). However, these changes in food consumption may cause stress for an organism (3), and this condition can lead to development of mental illness in the prolonged period, such as anxiety disorders (4) and an increase in the blood norepinephrine level (5). At the same time, an autonomic nervous system balance is crucial for the state of an organism indication.

There is a lack of data about intermittent fasting influence on the level of anxiety, a chronic increase in which may lead to the development of mental disease. The purpose of this study was to analyze the effects of intermittent fasting on anxiety level and overall activity, the balance of autonomic regulation and the blood norepinephrine level in male rats.

Male Wistar rats (220-270 g) were used. We divided animals into control (n = 15) and experimental (n = 15) groups. Control animals were fed *ad libitum* whole time. Intermittent fasting protocol in conditions of free access to water (23h without food and 1h of free access) was used for experimental animals. After 4 days experimental rats were placed in *ad libitum* conditions. One day after the food deprivation, blood was taken to analyze the norepinephrine level. ECG registration and "Open Field" test were performed 24h and 7 d after deprivation. According to the "Open field" test, level of anxiety and overall activity were assessed. Using ECG data, parameters of heart rate variability and the balance of autonomic regulation were investigated. Parametric, non-parametric t-test and two-way ANOVA test were used for a statistical analysis.

Control and experimental groups' results in the "Open field" test (the number of racks, acts of grooming, traversed sectors and outlets to the center) showed no differences in comparison with each other.

Intermittent fasting strengthen the influence of the parasympathetic nervous system (RMSSD increased by 56.9%,  $p < 0.05$  and SD by 44.4%,  $p < 0.05$ , SI decreased by 55.8%,  $p < 0.05$ ), but this effect vanished after the recovery period. Maybe activity and anxiety level did not change due to the compensatory shift on the nervous system balance toward the parasympathetic.

In the experimental group the significant lower blood norepinephrine level was detected (95.8%,  $p < 0.0001$ ), suppression of the sympathetic system may be the cause of this effect. Low norepinephrine level could lead to lipolysis inhibition. In this way, animals try to support their energy balance and store fat for later usage.

Thus, intermittent fasting did not cause changes in overall activity or anxiety level, but lead to shifting in the balance of nervous system toward the parasympathetic in male rats. World Health Organization: <http://www.who.int>

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB210

### **The effects of quercetin and fenofibrate supplementation on serum insulin and adiponectin levels in male Sprague Dawley rats fed a high fructose diet, post-weaning**

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Fructose-rich diets are implicated in obesity and Type II diabetes development, associated with insulin resistance and metabolic syndrome (MetS). Insulin and adiponectin are important in energy metabolism. Hypoadiponectinemia associated with obesity is central to insulin resistance, Type II diabetes and MetS development. Increased adiponectin levels are protective against MetS as a result of its' anti-inflammatory, antidiabetic and cardioprotective effects. Phytotherapies are being investigated for treatment of risk factors associated with MetS. Quercetin, a flavonoid abundant in fruits and vegetables, decreases insulin and increases adiponectin levels in animal models of fructose-induced MetS, improving the insulin resistance and pro-inflammatory status associated with MetS. Quercetin is usually administered to adult rats as a form of treatment for MetS, not as a preventative measure. We investigated the effects of quercetin on serum insulin and adiponectin levels of growing male Sprague Dawley rats fed a high fructose diet, post weaning. Thirty-eight, male rats (21 days old) were randomly divided into five groups. Group 1 (n = 8) received normal rat chow (NRC), plain gelatine cubes (GC) and tap water (TW); Group 2 (n = 8) received NRC, GC with quercetin (100 mg/kg body mass daily) and TW; Group 3 (n = 8) received NRC, plain GC and a 20% fructose solution (FS); Group 4 (n = 7) received NRC, GC with quercetin (100 mg/kg body mass daily) and a 20% FS; Group 5 (n = 7) received NRC, GC with fenofibrate (100 mg/kg body mass daily) and a 20% FS. Rats were fed *ad libitum* for ten weeks after which blood samples were collected. Serum insulin and adiponectin levels were determined using ELISA. Values are means  $\pm$  SD, compared using ANOVA. Insulin levels were significantly increased ( $p < 0.0001$ ) in rats receiving fructose only (Group 3:  $4.8 \pm 1.2$  ng. $\text{ml}^{-1}$  vs Group 1:  $2.2 \pm 1.0$  ng. $\text{ml}^{-1}$  and Group 2:  $2.9 \pm 1.0$  ng. $\text{ml}^{-1}$ ). This increase was not prevented by the administration of quercetin (Group 4:  $5.0 \pm 0.6$  ng. $\text{ml}^{-1}$ ) or fenofibrate (Group 5:  $5.7 \pm 0.7$  ng. $\text{ml}^{-1}$ ), together with the fructose. Inclusion of quercetin in the diets of the rats receiving fructose resulted in significantly increased ( $p < 0.01$ ) adiponectin levels (Group 4:  $102.8 \pm 20.5$  pg. $\text{ml}^{-1}$ ) compared to the negative control groups (Group 1:  $26.0 \pm 6.4$  pg. $\text{ml}^{-1}$ ;

Group 2:  $42.0 \pm 19.1$  pg. $\text{ml}^{-1}$ ), however the adiponectin levels were not significantly different ( $p > 0.05$ ) to the rats receiving fructose only (Group 3:  $72.1 \pm 24.0$  pg. $\text{ml}^{-1}$ ). Adiponectin levels were significantly increased ( $p < 0.01$ ) in the group receiving fructose and fenofibrate (Group 5:  $169.3 \pm 66.3$  pg. $\text{ml}^{-1}$ ) compared to all other treatment groups. In the fructose-fed rats, that are prone to metabolic dysfunction, the increase in adiponectin levels observed following fenofibrate administration could result in protective effects in the rats with regards to metabolism. However, this effect was not as pronounced in the rats receiving quercetin together with fructose.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB211

### **Exposure to kerosene fumes deteriorates reproductive functions in male Sprague Dawley rats.**

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Daily exposure to crude oil and its products as air pollutants can affect the body functions. The implications of exposure to kerosene fumes on reproductive parameters and oxidative stress markers were therefore investigated in male rats.

Male albino rats (n=20) weighing 150-200 g were distributed into 2 groups of control (exposed to normal atmospheric air) and kerosene (KERO) (exposed to kerosene fumes) daily for 3hours in the exposure chamber for a period of 3 weeks. Serum level of testosterone, Luteinizing hormone (LH) and follicle stimulating hormone (FSH) were assayed using Enzyme-linked Immuno Assay (ELISA) kit. Epididymal sperm count, motility and morphology were assessed, activities of antioxidant enzymes; catalase (CAT), glutathione reductase (GSH), superoxide dismutase (SOD) and lipid peroxidase; malondialdehyde (MDA) in the serum and testis were measured. Testicular histopathology was also investigated. Rats were sedate with chloroform and sacrificed following cervical dislocation, values are represented as  $\pm$ SEM and compared with ANOVA. Results from the study showed a lower ( $p < 0.05$ ) sperm count ( $58.00 \pm 2.08 \times 10^6$  sp/ml vs  $71.00 \pm 2.12 \times 10^6$  sp/ml), motility ( $85.75 \pm 3.57\%$  vs  $98.50 \pm 0.96\%$ ) and higher number of abnormal sperm cells ( $13.00 \pm 2.31\%$  vs  $11.33 \pm 1.67\%$ ) in KERO when compared with control. Serum level of testosterone ( $1.25 \pm 0.21$  mg/ml vs  $2.40 \pm 0.29$  mg/ml) was significantly lower in the KERO group while the reduced serum level of LH and FSH in KERO was not significant when compared with control. The CAT ( $19.56 \pm 0.66$  mg/ml vs  $23.73 \pm 0.45$  mg/ml) and GSH ( $49.31 \pm 3.16$   $\mu\text{mol/ml}$  vs  $62.86 \pm 2.13$   $\mu\text{mol/ml}$ ) activities in the serum were also significantly lower while MDA ( $6.20 \pm 0.27$  nmol/ml vs  $4.26 \pm 0.27$  nmol/ml) was significantly higher in the KERO than in control. In the testis, GSH ( $22.85 \pm 3.15$   $\mu\text{mol/ml}$  vs  $35.13 \pm 3.49$   $\mu\text{mol/ml}$ ) was lower ( $p < 0.05$ ) while MDA ( $6.35 \pm 0.86$  nmol/ml vs  $3.55 \pm 0.64$  nmol/ml) was higher ( $p < 0.05$ ) in the KERO than in control. Histology of the testis showed alterations of the sperm cells and reduction of interstitial cells in the KERO when compared with the control.



In conclusion, frequent inhalation of kerosene fumes produces oxidative stress which deteriorates male reproductive functions in albino rats.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB212

### Involvement of endocannabinoid and vanilloid pathways in periodontal disease in rats

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Periodontal disease (PD) is an infectious illness characterized by alveolar bone loss (ABL) due, to a large extent, to an inflammatory response in the tissues surrounding teeth. Endogenous ligands and receptors of the endocannabinoid system have been associated with both bone metabolism and inflammation. Likewise, transient receptor potential vanilloid 1 (TRPV1) has been extensively linked to pain and inflammation. The aim of this study was to evaluate the effects of, on the one hand, the blockage of TRPV1 and, on the other hand, the increase of the bioavailability of anandamide (AEA), a CB1r and CB2r endogenous agonist, on the experimental PD in rats. Methods: male Wistar rats (300g) were used (n=6 per group). The groups performed were: 1) control (C); 2) injected with 20ul of *Escherichia coli* lipopolisaccharide (LPS) (1mg/ml) in upper and lower first molar gum, three days a week (days 1, 3 and 5 of each week) during six weeks; 3) injected with LPS as described for group 2) and treated daily with a topical solution of capsazepine (Cap) (2ug/ml saline), a TRPV1 antagonist, in the injection sites; 4) injected with LPS as described for group 2) and treated daily with topical solution of URB 597 (20ug/ml saline), an inhibitor of AEA degradation, in the injection sites. Anesthesia was induced by isoflurane inhalation for injections and topical applications procedures. After the rats' decapitation euthanasia, ABL of the upper and lower first molar was measured through morphometric and histomorphometric techniques. The content of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), an inflammatory and bone resorption mediator, was evaluated by radioimmunoassay in gums of the first molar. Statistical analysis: two-way ANOVA. In the interradicular bone examination of the first lower molar, the increase in the periodontal space height caused by LPS-induced PD (270.7um±33.5) with respect to C (217.1um±20.4) resulted significantly attenuated in animals that underwent Cap treatment (216.4 um±19.9) (p<0.05 vs. LPS). On the other hand, animals treated with URB 597 evidenced lesser periodontal space height (233.7um±18.5) than those only submitted to LPS (281.7um±18.5), which showed an increase in this value with respect to C (204.5um±30.3) (p<0.05 vs. LPS). Concordant results were obtained from the ABL analysis of first upper

molar. PGE<sub>2</sub> content in gingival tissue, increased by LPS-induced PD (54.55pg±16.86) with regard to C (32.93pg±15.83), also resulted attenuated in animals treated with URB 597 (27.72pg±10.77) (p<0.05 vs. LPS). These results indicate that both the TRPV1 blockage and the rise in the bioavailability of AEA are effective mechanisms to prevent local damage associated with inflammation and bone resorption, suggesting that vanilloid as well as cannabinoid pathways could integrate a homeostatic mechanism that plays a critical role in the pathophysiology of the periodontal disease.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB213

### Cholesterol metabolism and cancers: Toward new perspectives in breast cancer treatment

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Breast cancer (BC) is the most common cancer in women worldwide and still many of them either fail to respond or develop resistance to existing therapies, stressing the need for alternatives. We have recently shown that cholesterol-5,6-epoxide (5,6-EC) metabolism is deregulated in BC in favor of cholestane-3β,5α,6β-triol (CT) production, which is then further transformed into 6-oxo-cholestan-3β,5α-diol (OCDO), a novel oncometabolite stimulating tumor growth by binding to the glucocorticoid receptor. Inhibition of this pathway may thus lead to new perspectives in BC treatment. The enzyme producing OCDO was identified as 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), known to regulate glucocorticoid metabolism by converting active cortisol into inactive cortisone. The aim of the present study was to determine the enzymatic parameters of the enzyme for its oxysterol substrate and to determine the pharmacological profiles of 11β-HSD2 toward OCDO and cortisone production. The enzymatic reaction of the conversion of [<sup>14</sup>C]-CT into [<sup>14</sup>C]-OCDO was studied in HEK293T cells transfected with either a control plasmid or a plasmid encoding 11β-HSD2, as well as in different BC cell lines in comparison to its enzymatic activity for the conversion of [<sup>3</sup>H]-cortisol into [<sup>3</sup>H]-cortisone. Enzymatic measurements of 11β-HSD2 in cell lysates revealed an apparent K<sub>m</sub> value of 5±1 μM for CT, which is in the range of the intratumor CT concentrations measured in BC. Cortisol inhibited OCDO production with a K<sub>i</sub> of 259±73 nM, while 10 μM CT did not inhibit cortisol conversion. The Michaelis constant (K<sub>m</sub>) and inhibitory constants (K<sub>i</sub>) ± S.E. were determined in at least three individual experiments by nonlinear regression analysis using GraphPad Prism version 6. The inhibition of 11β-HSD2 for both OCDO and cortisone production was further tested with a series of molecules including cholesterol metabolites, oxysterols, pentacyclic triterpenoids, steroid hormones and their synthetic analogues, bile acids as well as various pharmacologically active agents. Triterpenoids were found to strongly inhibit both reactions, as did 18β-glycyrrhetic acid and its derivative carbenoxolone. Dexamethasone, a synthetic steroid used in the treatment of many pathologies including cancer, inhibits OCDO production with a K<sub>i</sub> of 1.6±0.5 μM, thus likely affecting this pathway in treated patients. Interestingly, OCDO production, but not cortisone production, was specifically inhibited by two compounds, indicating differences in the binding site of 11β-HSD2 for CT and cortisol. This shows

that 11 $\beta$ -HSD2 can be specifically targeted to inhibit the production of the tumor-promoting oncometabolite OCDO, representing new therapeutic opportunities for BC treatment.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCB214

##### Retinal oxygen levels in the eye are different in macular area of the retina compared to periphery of the retina

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Ischemic eye retinopathies have shown topographical patterns and the macula is preferentially affected in many eye diseases such as in diabetic macular edema and age related macular degeneration. We examine topographical aspects of retinal oxygen metabolism in the hope that these may underlie the topographical expression of ischemic retinal disease. The purpose of the study was therefore to evaluate the macular oxygen metabolism and compare to other, peripheral parts of the retina. Retinal vessel oxygen saturation and vessel diameter was measured with a retinal oximeter (Oxymap T1, Oxymap ehf., Reykjavik, Iceland) in healthy individuals. Analyzed vessels were categorized as either macular vessels or peripheral vessels (all in the temporal side of the retina, see Figure). In all, 16 arterioles and 21 venules were analysed. Statistical analysis was performed with a paired t-test. The results show that retinal oxygen saturation was higher in macular venous branches ( $72.1 \pm 4.5\%$ , mean  $\pm$  standard deviation) compared to peripheral branches ( $61.0 \pm 9.1\%$ ,  $p < 0.0001$ ). Arteriovenous difference was lower in macular vessels ( $19.0 \pm 6.5\%$ ) compared to peripheral vessels ( $29.1 \pm 8.3\%$ ,  $p < 0.002$ ). There was no difference between areas in oxygen saturation in arterioles ( $p = 0.3$ ). There was also no difference in vessel diameter between areas ( $p = 0.5-0.7$ ). According to the data, oxygen saturation is dramatically different in macular vessels compared to peripheral branches. This suggests substantial differences in oxygen metabolism between the macula and peripheral parts of the retina. The next step is to see whether these differences play a role in disease patterns and why the macula is so susceptible to diseases.



Figure demonstrates how a single arteriole (denoted with red color) or venule (denoted with green color) from the macula were compared to a single arteriole or venule from peripheral areas.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCB215

##### Association of serum Leptin level with body mass index and lipid profile in Diacetylmorphine (Heroin) addicts versus healthy controls.

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Background: Diacetylmorphine (DAM) addiction commonly known as heroin addiction, is a common socioeconomic problem markedly affecting the nutritional and metabolic status of the individuals. The circulating adipose tissue derived hormone Leptin is an important marker in regulating body weight and lipid metabolism. Previous reports suggest derangement of Leptin in DAM addicts. In this study our study was to compare circulating concentration leptin and its relationship to body mass index and lipid profile in DAM addicts in comparison to normal healthy individuals.

Methodology: This was a cross sectional analytical study including 41 male DAM addicts and 41 male healthy controls between 25 to 46 years of age. Detail history and physical examination along with baseline laboratory tests for exclusion of infection were performed before recruiting the subjects for participation in the study. Standard method was used to calculate the anthropometric measures. Blood samples were corrected to calculate serum lipid profile, complete blood picture. Serum leptin was calculated using enzyme linked immunosorbent assay (ELISA) technique. All statistical analysis was done using SPSS version 22. Numerical data was presented as mean and standard deviation. Independent sample T test was applied to compare mean of the two groups. Ethical approval was obtained from the ethical review committee of Khyber Medical University, Peshawar, Pakistan. Results: Basal serum leptin levels in DAM addicts were significantly decreased compared to controls ( $3.08 \pm 0.13$  pg/ml vs  $3.24 \pm 0.24$  pg/ml;  $p < 0.001$ ). Furthermore, LDL and hemoglobin were also significantly reduced in the addicts compared to controls ( $71.8 \pm 25.99$  vs  $105.39 \pm 39.09$ ;  $p < 0.001$  and  $13.44 \pm 0.78$  vs  $14.73 \pm 0.91$ ;  $p < 0.001$ ) respectively. The difference between other parameters were no significant. Univariate and Multivariate regression did not find any significant association of Leptin with BMI and lipid profile in DAM addicts ( $p = 0.198$ ), however, significant association of Leptin with BMI was observed in normal subjects ( $p = 0.027$ ).

Conclusion: In conclusion, circulating concentrations of leptin are decreased in heroin addiction. Moreover, positive correlation of serum leptin levels with body mass index and lipid profile is lost in addicts in contrast to control group.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB216

**Dose-Dependent Hemorheological Impairment in Patients with Differentiated Thyroid Cancer Following I-131 Ablation / Metastasis Treatment**

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Therapy of differential thyroid cancer (DTC) is total thyroidectomy followed by radioactive iodine therapy with Iodine-131 (RAIT) (1). Although RAIT is thought to affect blood cells and oxidative stress, hemorheological alterations following dose-dependent RAIT remains unknown. The aim of this study was to determine the effects of three different doses of RAIT on hemorheological parameters in patients with DTC. Serum total oxidant (TOS), total antioxidant (TAS) levels of these patients were also determined in order to explain the reasons of possible rheological alterations. Totally 31 DTC patients (mean age 46.32 ± 11.15 years) and 26 healthy controls (mean age 50.50 ± 6.22 years) were included. Of the 31 patients; 7 (%22.6) were treated with a low (30 mCi) RAIT dose, 19 (%61.3) with ablation (100 mCi) dose and 5 (%16.1) with higher dose (>100 mCi). Venous blood samples were collected from each patient before and after treatment (7 days, 1 month and 6 months). RBC aggregation and deformability were determined by an ektacytometer. TOS and TAS were measured by a commercial kit and the oxidative stress index (OSI) was calculated. Data were analyzed with the SPSS 21.0 program. Analysis of variance in repeated measures was used when parametric test assumptions were provided; and the Friedman test was used when parametric test assumptions were not provided. p < 0.05 was considered as statistically significant. RBC deformability of the patients determined at 16.87 and 30 Pascal (Pa) were lower than healthy individuals. RBC aggregation index (AI) of the patients was higher, whereas erythrocyte aggregation half-time (t<sub>1/2</sub>) was lower compared to control (p<0.05). These alterations indicate increased RBC aggregation in patients. Administration of higher dose of RAIT resulted in a further decrease in RBC deformability at 16.87 and 30 Pa 1 week after treatment compared to lower and ablation dose administrations (Table 1). TOS of ablation dose group measured on the first week following treatment was higher than low dose group (p<0.05) (Table 1). Patients were in a worse hemorheological condition compared to healthy individuals. The issue that high dose of RAIT results in further decrement in RBC deformability should be kept in mind especially for patients who are not hemodynamically stable.

Relationship between ablation doses in EI and oxidative stress values on the 7th day after the exercise

|                             | 30 mCi<br>(n=7) | 100 mCi<br>(n=19) | High doses (>100 mCi)<br>(n=5) |
|-----------------------------|-----------------|-------------------|--------------------------------|
| Shear stress (Pa)           |                 |                   |                                |
| 16.87                       | 0.57 ± 0.01     | 0.57 ± 0.01       | 0.53 ± 0.05*#                  |
| 30.0                        | 0.59 ± 0.02     | 0.58 ± 0.02       | 0.54 ± 0.05*#                  |
| Oxidative stress parameters |                 |                   |                                |
| TAS                         | 2.90 ± 1.42     | 2.52 ± 1.84       | 3.46 ± 1.22                    |
| TOS                         | 3.76 ± 1.02     | 6.78 ± 2.53*      | 4.05 ± 8.72                    |
| OSI                         | 0.15 ± 0.09     | 0.37 ± 0.24       | 0.14 ± 0.11                    |

Values are expressed as means±SD; EI: elongation index; Pa: pascal; mCi: millicurie; \*p<0.05: statistically significant from 30 mci, #p<0.05: statistically significant from 100 mci

DeGroot LJ, Kaplan EL, McCormick M, Straus FH. Natural history, treatment and course of papillary thyroid carcinoma. J Clin Endocrinol Metab 1990;71:414.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB217

**I-131 ablation/metastasis treatment may lead protective effects on lymphocyte DNA by activating repair mechanisms in patients with differentiated thyroid cancer**

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Thyroidectomy followed by administration of large activities of I-131-iodine (I-131) is the treatment of choice for differentiated thyroid carcinoma (DTC) (1). Although I-131 was suggested to form chain fractures in DNA of thyroid cells, the effect of RAIT on peripheral blood cell DNA remains controversial. The aim of this study was to determine the effect of RAIT on DNA damage in lymphocytes in patients with DTC. Totally 21 DTC patients were included in our study (mean age 45.09±10.02 years). Of the 21 patients; 6 (%28.6) were treated with a low (30 mCi) RAIT dose, 11 (%52.4) with ablation (100 mCi) dose and 4 (%19) with higher dose (>100 mCi). Venous blood samples were taken from each patient before treatment, 7 days and 6 months after the treatment. DNA damage was assessed using COMET assay following lymphocyte isolation from venous whole blood. Seventy-five cells per slide per sample were scored to evaluate DNA damage. Head length, tail length, head intensity, tail intensity, tail moment were evaluated for quantitative analysis of DNA damage. Data were analyzed with the SPSS 24.0 program. Repeated Measures Analysis of Variance was used when parametric test assumptions were provided; and the Friedman test was used when parametric test assumptions were not provided. p<0.05 was considered statistically significant. Tail intensity values were significantly lower after treatment (1<sup>st</sup> week and 6 months) (18.30±6.48 and 18.06±5.58) compared to before treatment (23.83±9.82) (p=0.002). The head intensity values were statistically significantly higher after treatment (1<sup>st</sup> week and 6 months) (81.69±6.48 and 81.93±5.58) than before treatment (76.16±9.82) (p=0.002). The tail moment was lower at 6 months following treatment compared to before treatment (5.24±2.51 vs. 8.04±3.86) (p=0.005) (Table 1). The increase in head intensity and decrease in tail intensity and moment followed by treatment have shown that RAIT does not cause significant DNA damage in peripheral blood cells. RAIT may have activated protective effects on lymphocyte DNA by activating repair mechanisms.



Table 1. DNA damage assay parameters of the patients

| COMET assay values | Before treatment<br>(n=21)<br>(mean±SD) | 7th day after treatment<br>(n=21)<br>(mean±SD) | 6th month after treatment<br>(n=21)<br>(mean±SD) |
|--------------------|-----------------------------------------|------------------------------------------------|--------------------------------------------------|
| Tail Intensity (%) | 23.83±9.82                              | 18.30±6.48                                     | 18.06±5.58*.#                                    |
| Head Intensity (%) | 76.16±9.82                              | 81.69±6.48                                     | 81.93±5.58*.#                                    |
| Tail Moment (µm)   | 8.04±3.86                               | 6.02±2.15                                      | 5.24±2.51*                                       |

Values are expressed as means±SD; \*p<0.05: the difference from before treatment; #p<0.05: statistically significant from 7th day after treatment

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB218

### Assessing cardiorespiratory and metabolic parameters responses to a mixed meal vs a glucose challenge- a pilot study in healthy volunteers

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The carotid bodies (CB) have been recently implicated in the genesis of metabolic diseases in animal models (1) however, in humans, this mechanism is not well characterized. Acutely, the CBs respond to circulating insulin to increase heart rate, respiratory rate and blood glucose levels (2). Early detection of disturbed insulin action by recording cardiorespiratory and metabolic responses to stimuli like a meal ingestion, is, therefore, a methodology with predictive value potential for metabolic dysfunction. Our aim was to assess physiological changes in CB-related cardiorespiratory and metabolic parameters induced either by ingestion of a mixed meal, or by a glucose challenge, in healthy volunteers. All procedures accorded with current European legislation.

Pilot-study tests were performed in five healthy volunteers recruited at Polytechnic of Leiria Health Sciences School. To assess CB activity the volunteers were fed either a mixed meal or a standard glucose tolerance test (OGTT) after a 12 hour fasting period, in different days. The mixed meal had a macronutrient distribution of 65% of carbohydrates, 23% of protein and 12% of lipids. The OGTT consisted of 200 ml of water and 75 g of sugar. Both meals were ingested during a period of 10 minutes. Heart rate (HR), oxygen saturation (SpO2) and respiratory rate (RR) were monitored by means of a real time biosignal acquisition system. Interstitial glucose was monitored continuously. CBView, a Matlab based software, was developed to perform integrated recordings of physiological responses assessed.

Results: The plasma glucose excursion curve maximum value was smaller after mixed meal ( $\Delta$ glucose= 20,0 ±10,7mg/dl glucose, latency to steady state= 49± 1min) than OGTT ( $\Delta$ glucose= 29,1±15,1 mg/dl glucose, latency to steady state = 54± 5min) and the slope of the linear regression on glucose excursion curves were significantly different (1,03±0,14 mg/dl/min vs 2,34±0,1 mg/dl/min). Heart rate varied

after OGTT but not after mixed meal in healthy volunteers ( $\Delta$ HR= 5,1 ± 0,4 bpm vs -1,3±0,32 bpm, respectively). There were no changes in SpO2 either after mixed meal ingestion or after OGTT. Respiratory Rate increased after OGTT ( $\Delta$ RR= 3,7 ± 0,2cpm) but not after mixed meal ingestion ( $\Delta$ RR= 0,55 ± 0,15cpm).

In conclusion, in healthy volunteers, ingestion of a mixed meal tested caused detectable, though minor, variations in cardiorespiratory and metabolic parameters compared to the OGTT. Data suggest that meal ingestion may be used as a challenge teste to assess early changes in physiological parameters that may reflect altered CB activity and an increased risk for metabolic dysfunction.

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2. Conde, Silvia V., et al. "Insulin resistance: a new consequence of altered carotid body chemoreflex?" The Journal of physiology 595.1 (2017): 31-41.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB219

### Impact of feeding cycle upon the biophysics of sodium and calcium currents of rat hippocampal CA1 neurones.

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Feeding behaviour is regulated by the central nervous system, through a concerted endeavour of different brain areas. The hippocampus, historically regarded as a substrate for learning and memory processes, has also been implicated in such energy regulation (1, 2). In recent years, researchers have established that hippocampal neurones form a memory of a meal and act to delay meal initiation during the postprandial period (3, 4). By assessing the impact of feeding cycle upon the sodium and calcium currents of Wistar rat hippocampal CA1 neurones, the present study identifies possible neuronal mechanisms by which hippocampus processes satiety and meal termination. Two classes of neurones were used: those obtained from animals that fasted overnight ('fasted neurones') and those from animals that, after such period, were fed ('fed neurones').

Voltage-gated Na<sup>+</sup> currents were recorded using whole-cell (WC) and excised inside-out configurations. Fed neurones, in comparison to fasted neurones, showed increased mean maximum macroscopic Na<sup>+</sup> current density (1.5±0.12mA.cm<sup>-2</sup>, n=18 vs. 1±0.10mA.cm<sup>-2</sup>, n=28) and a greater mean single-channel conductance (16.7 ± 0.76pS, n=8 vs. 12.6 ± 1.30pS, n=8). Furthermore, the larger amplitude of the 'window current' obtained in fed neurones, derived from hyperpolarized activation curves and depolarized steady-state of inactivation curves (h<sub>∞</sub>), indicates a greater Na<sup>+</sup> channel availability to respond to activation. Such variation is supported by the western blotting results that indicate a higher concentration of Na<sub>v</sub>1.2 isoform at the plasma membrane-enriched fractions of hippocampus of fed animals.

Voltage-gated Ca<sup>2+</sup> currents were analysed with WC recordings. It was observed heterogeneity in Ca<sup>2+</sup> currents, here sorted into three categories – 'A', 'B', and 'C' currents. The differential distribution of these currents between fed and fasted neurones determined significant alterations on the

inactivation properties of  $\text{Ca}^{2+}$  currents. The increased values of the time-constant of inactivation, observed upon feeding, can be ascribed to a conspicuous slowly-inactivating current mainly assigned to fed neurones (current 'A'), as oppose to the fastest kinetics of inactivation, solely seen in fasted neurones (current 'C').

Overall, the results indicate a) a variation in the biophysics and expression of voltage gated  $\text{Na}^+$  channels and b) a facilitated entry of  $\text{Ca}^{2+}$  into the soma of fed neurones. Altogether, these observations suggest impact of feeding on neuronal excitability and  $\text{Ca}^{2+}$ -dependent intracellular events, which may lead to a subsequent increase of neuronal synaptic performance. Hence, a positive relationship between feeding and higher levels of synaptic plasticity-related phenomena (formation and consolidation of memories) is suggested, which could help to explain the role of hippocampus on the regulation of energy intake, mainly due to its role on meal-related episodic memories.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB220

## The plasma membrane of Rat sub-cutaneous white adipocytes express a long $\text{Ca}_v1.2$ channel isoform

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White fat adipocytes possess a myriad of physiological processes controlled by intracellular calcium ions (Smith et al., 2014). Although protein expression of  $\text{Ca}_v1.3$  voltage-gated calcium channels (VGCCs) has been identified in white fat adipocytes (Smith et al., 2014), the expression of other VGCCS isoforms are poorly characterised. To address this question, the cytosolic and membrane fractions of Wistar rat sub-cutaneous white adipocytes was explored by western blotting. Primary adipocytes were isolated from male Wistar rat (260-360g) sub-cutaneous white adipose tissue (SC-WAT) by methods similar to those described by Bentley et al. (2014). Cytosolic and crude plasma membrane proteins were prepared by modification of the method of McKeel and Jarett (1970). Protein concentration was determined by Lowry assay and 12 - 20  $\mu\text{g}$  of protein was resolved on 4 - 20% gradient precast minigels and blotted onto a nitrocellulose membrane. Samples were probed with primary antibodies (anti  $\text{Ca}_v1.2$  and anti  $\text{Ca}_v3.1$  - 1:500 dilution, anti  $\beta$ -actin - 1:5,000) and appropriate infra-red dye labelled secondary antibodies. Blots were imaged and bands expressed as the signal intensity of protein bands normalized relative to  $\beta$ -actin expression for each sample.

Only a  $\text{Ca}_v1.2$  channel isoform of > 250 kDa was detected in both cytosolic and membrane fraction of adipocytes from rat SC-WAT (Figure 1A). The expression level of this large molecular weight  $\text{Ca}_v1.2$  protein was higher in the crude plasma membrane than in the cytosolic fraction (Figures 1A & C). For  $\text{Ca}_v3.1$ , three distinct bands of approximately 130 kDa, 70 - 75 kDa and < 70 kDa were detected, all with a slightly greater abundance in the membrane fraction relative to the cytosol (Figures 1B & D).

Our data suggests not only the presence of  $\text{Ca}_v1.2$  and  $\text{Ca}_v3.1$  channels in rat sub-cutaneous white adipocytes, but that these exist as a variety of isoforms.  $\text{Ca}_v1.2$  is a long isoform (Shistik et al., 1999, Kramer et al., 2012), preferentially expressed in the plasma membrane fraction of these cells. Whereas at least three  $\text{Ca}_v3.1$  isoforms exist, whether these are proteolytic products or represent three unique proteins is not known. The functional role of these different channel isoforms in adipocytes have yet to be identified.

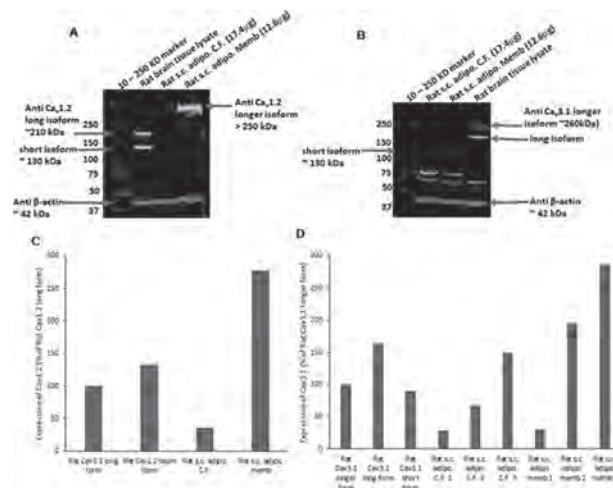


Figure 1: Protein expression of  $\text{Ca}_v1.2$  and  $\text{Ca}_v3.1$  channels in male Wistar rat sub-cutaneous adipocytes. 20 $\mu\text{g}$  of protein was loaded, unless otherwise specified.

(A) Representative western blot of  $\text{Ca}_v1.2$  and  $\beta$ -actin (B)  $\text{Ca}_v3.1$  and  $\beta$ -actin expression in cytosolic (C.F.) and membrane (memb.) fractions of male Wistar rat subcutaneous (s.c) adipocytes (C) Normalized signal intensity of  $\text{Ca}_v1.2$  and (D)  $\text{Ca}_v3.1$  expression for data shown in (A) and (B).

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB221

### Dysfunctional insulin secretion in the *Cftr<sup>EURtm1</sup>/F508del* CFTR mouse model due to defective processing of insulin and reduced exocytosis.

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**Background and Problem statement:** The most common complication in cystic fibrosis (CF) is CF-related diabetes (CFRD) yet the etiology of CFRD is largely unknown. CF patients with the F508del mutation have the highest risk for CFRD. The *CFTR<sup>EURtm1</sup>* mouse model of CF carries the human F508del CFTR mutation. The presence of CFTR in islets cells is not fully elucidated. Our previous data show presence of CFTR in both human and mouse pancreatic alpha- and beta-cells. In beta cells CFTR regulates priming and exocytosis of insulin-containing granules (Edlund et al., BMC Medicine, 2014), whereas in alpha cells it is involved in the control of the electrical activity (Edlund et al., Sci Rep, 2017). Here we aimed to investigate the regulation of insulin secretion in the F508del mouse model (F508del).

**Methods:** *In vivo* serum basal insulin and glucagon levels were measured in F508del and corresponding controls. Pancreatic hormone secretion was measured on isolated islets analyzed with RIA or ELISA. Exocytosis was investigated using the patch-clamp technique. CFTR was detected in beta cells using confocal microscopy, and islet and beta cell morphology was investigated with immunohistochemistry and transmission electron microscopy, respectively.

**Results:** Pancreatic islets from F508del mice had reduced beta cell mass ( $n=21$  pancreatic sections from  $N=7$  mice of each genotype,  $p<0.01$ ). This was not due to reduced beta cell number rather to reduced beta cell size. The F508del islets had an increased proinsulin secretion especially pronounced in the presence of cAMP ( $N_{WT}=4$ ;  $N_{F508del}=4$ ,  $p<0.01$ ). Proinsulin is cleaved into insulin and c-peptide in the granule and the increase in proinsulin secretion coincided with a decrease in c-peptide secretion ( $N_{WT}=4$ ;  $N_{F508del}=4$ ,  $p<0.05$ ). The conversion of proinsulin-to-insulin requires an acidic pH. Priming is a process important for granular acidification and for  $Ca^{2+}$ -dependent exocytosis. Effects on priming and exocytosis in single F508del beta cells measured as changes in membrane capacitance were reduced ( $n_{WT}=16$ ,  $n_{F508del}=16$ ;  $p<0.05$ ), indicating reduced exocytosis and defective priming in this mouse model. Moreover, the beta cells had a reduced number of docked granules ( $n_{F508del}=33$ ,  $n_{WT}=29$ ,  $p<0.01$ ). Serum levels of insulin were not different but the F508del had three-fold increase in serum glucagon ( $N_{WT}=14$ ,  $N_{F508del}=13$ ,  $p<0.005$ ). In addition, glucagon secretion measured on isolated islets was increased compared to WT ( $n=4$ ,  $p<0.05$ ).

**Conclusions:** We conclude that the *Cftr<sup>EURtm1</sup>/F508del* CFTR mouse model has reduced insulin secretion mainly due to impaired priming causing defective processing of proinsulin to insulin and reduced  $Ca^{2+}$ -dependent exocytosis. In line with previous investigations, we hypothesize that impaired insulin secretion contributes to the development of CFRD.

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Edlund A et al., Sci Rep. 2017 Dec;7(1):90

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB222

### T-type $Ca^{2+}$ channel blocker mibefradil inhibits ORAI store-operated channels

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#### Introduction

ORAI channels have been regarded as the molecular basis of  $Ca^{2+}$ -release activated  $Ca^{2+}$  (CRAC) channels, the highly  $Ca^{2+}$  selective store-operated channels (SOCs). The channels can be activated by the depletion of endoplasmic reticulum (ER)  $Ca^{2+}$  stores via the activation of G protein-coupled receptors (GPCR) and/or protein tyrosine kinase (PTK) coupled receptors (1). There are three isoforms of ORAI channels (ORAI1-3). These channels may act as therapeutic targets for immune disorders, cardiovascular diseases and diabetic complications (2, 3). However, potent and selective inhibitors or activators for these channels are still lacking. Here we examined the effect of mibefradil (Mib), a T-type calcium channel blocker(4), on ORAI channels overexpressing in T-REx cells.

#### Methods

The plasmid cDNAs of ORAIs tagged with cyan (CFP) or red (mCherry) fluorescent protein genes were stably co-expressed with STIM1-EYFP in HEK-293 T-REx cells. The expression of ORAIs was induced by  $1 \mu\text{g ml}^{-1}$  tetracycline for 24–72 h before patch clamp recording or  $Ca^{2+}$  imaging. The non-induced cells without addition of tetracycline or the non-transfected cells were used as controls. Cells were grown in DMEM-F12 medium containing 10% fetal calf serum (FCS). Cells were maintained at  $37^{\circ}\text{C}$  under 95% air and 5%  $\text{CO}_2$  and seeded on coverslips for experiments. The whole cell or excised membrane patch current was amplified using Axopatch 200B amplifier and the software pClamp 10.  $Ca^{2+}$  release and influx was measured by FlexStation 3 using the  $Ca^{2+}$  dye Fura-PE3AM. Values are means  $\pm$  S.E.M., compared by ANOVA or *t*-test where appropriate.

#### Results

Mibefradil (Mib, 0.1–100  $\mu\text{M}$ ) inhibited ORAI1, ORAI2 and ORAI3 currents in a dose-dependent manner. The IC<sub>50</sub> for ORAI1, ORAI2 and ORAI3 was 39.3  $\mu\text{M}$ , 24.8  $\mu\text{M}$  and 4.2  $\mu\text{M}$ , respectively ( $n=2-10$ ). Mib at 10  $\mu\text{M}$  inhibited ORAI1, ORAI2 and ORAI3 currents by  $21.82 \pm 2.49$  ( $n=10$ ),  $37.10 \pm 3.51$  ( $n=8$ ) and  $62.05 \pm 3.49\%$  ( $n=8$ ), respectively. To investigate whether Mib might act on the channels directly via an intracellular or extracellular site, higher concentrations (10–100  $\mu\text{M}$ ) of Mib were applied in patch pipette, and no significant inhibition was found on 2-APB (100  $\mu\text{M}$ ) evoked currents (the fold change of 2-APB activated current in pipette without Mib and with Mib were  $11.75 \pm 2.47$  vs.  $10.83 \pm 2.11$ , respectively,  $n=5$ ,  $P>0.05$ ). Outside-out patch on ORAI3 currents further



demonstrated that bath application of 10  $\mu$ M of Mib ( $n=4$ ) to the extracellular surface completely blocked the 2-APB (100  $\mu$ M) activated currents. Additionally, Mib at lower concentration (<50  $\mu$ M) has no significant effect on intracellular  $Ca^{2+}$  release from endoplasmic reticulum (ER), while higher concentrations of Mib (>50  $\mu$ M) inhibited  $Ca^{2+}$  release from ER store by  $35.0 \pm 4.9\%$ .

#### Conclusions

We concluded that Mib not only acts as a T-type calcium channel blocker, but also shows a potent inhibitory effect on ORAI channels, which provides a new pharmacological profile for ORAI channel blockers.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

### PCB223

#### Role of glycolysis in regulation of endothelial barrier integrity in inflammation

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**Background:** The integrity of the endothelial cell (EC) barrier of the microvasculature is compromised in inflammation and increased vascular permeability is a surrogate marker for the development of atherosclerotic lesions. In recent years, considerable advances have been made in the understanding of signalling mechanisms regulating the endothelial barrier integrity. The role of endothelial metabolism as a modulator of endothelial barrier integrity is not yet well-studied particularly in the setting of lung vascular injury. The aim of the present study was to investigate the role of glycolysis regulatory enzymes PFK1 and PFK-FB3 in the maintenance of endothelial barrier integrity.

**Methods:** The study was carried out on cultured human umbilical vein endothelial cells (ECs) and rat coronary microvascular ECs. Endothelial barrier function was analysed by measuring the flux of albumen through EC monolayers cultured on filter membranes. Gene expression was analysed by qPCR-based assays.

**Results:** Under inflammatory conditions (TNF $\alpha$ ; 1 ng/mL for 24h), an upregulation of glycolysis regulatory enzymes PFK1 and PFK-FB3 was observed in endothelial cells ( $n=5$ ;  $p<0.05$ ) at mRNA level. This was accompanied by an increase in total cellular ATP levels. Pharmacological inhibition or genetic depletion of either PFK1 or PFK-FB3 resulted in reduction of cellular ATP content and worsened TNF $\alpha$ -induced endothelial hyperpermeability. On the other hand, over expression of PFK-FB3 ameliorated TNF $\alpha$ -induced endothelial hyperpermeability. These data suggest that upregulation of glycolysis may serve as an endogenous metabolic adaptation to inflammatory vascular injury and protect endothelial barrier integrity.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

### PCB224

#### Study of Uterine Contractions in Pregnant Diabetic Rats: Role of Oxidative Stress Faten M. A. Diab, Aziza S.El-Naser, Mona A. Ahmed, Doaa M. Abdel Wahed, Manal S Abd-El hamid and Faiza A. El-Hamdy\*

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**Background:** Uterine contractility plays a major role in human reproduction. Dysfunctional uterine contractility, especially met with gestational diabetes mellitus (GDM), increases perinatal morbidity and mortality. GDM impairs the function of most smooth muscle containing organs, as the uterus; and the oxidative stress mechanism underlying organ damage has been implicated.

**Objective:** This work was conducted to study the changes imposed on rat's myometrium contractility, and the effect of vitamin E as a supplement with insulin treatment in modulating the diabetic-induced complications that develop in experimental gestational diabetes.

**Materials & Methods:** 80 time-dated pregnant Albino rats were studied. GDM was induced by intra-peritoneal injection of 40 mg/kg streptozotocin at gestational day 5. Rats were allocated into five groups: control pregnant (group 1), untreated diabetic (group 2), insulin-treated diabetic (group 3) vitamin E-treated diabetic (group 4) and combined insulin & vitamin-E treated (group 5). On gestation day 18, all rats were subjected for recording of spontaneous basal uterine muscle activity in isolated organ bath, and its responses to the uterotonic agent; oxytocin and to the tocolytic agent; atosiban. The plasma glucose, insulin and HbA1c levels, together with serum lipid profile; total cholesterol, triglycerides, HDL-c, LDL-c were assayed. The HOMA-IR and atherogenic index were calculated. Besides, serum malondialdehyde and total antioxidant capacity levels were estimated.

**Results:** Untreated diabetic (group 2) established significant reduction in uterine motility, with insulin resistance, dyslipidemia, enhanced oxidative stress state. Following insulin treatment alone, (group 3) demonstrated significant decrease in HbA1c, persistence of insulin resistance, with relative improvement in lipid profile and uterine motility, with still of altered oxidative stress status. With vitamin E treatment alone, (group 4) showed significant improvement of uterine motility, and amelioration of oxidative stress state but still altered hyperglycaemia and dyslipidemia. The best results were observed in the combined treatment (group5), where significant improvement in uterine motility, together with full correction to the glycaemic, lipidemic and oxidative stress states.

**Conclusion:** Vitamin E, as a supplement with insulin treatment in gestational diabetes, offers beneficial role in improving the contractile machinery state of the myometrium via restoring the normal redox balance.

**Key Words:** Gestational diabetes mellitus- Myometrium contractility – Vitamin-E.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB225

# Skeletal muscle atrophy before hibernation and recovery after hibernation in a mammalian hibernator, Syrian hamster

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Hibernation (HIB) is a strategy to survive harsh winter with a little or no food in immobility state. Such immobility induces severe muscle atrophy, whereas mammalian hibernators can manage to maintain skeletal muscle mass during HIB. However, molecular mechanisms how mammalian hibernators preserve skeletal muscles during HIB remain to be elucidated. A mammalian hibernator, Syrian golden hamsters (*Mesocricetus auratus*), undergo hibernation when exposed to chronic short photoperiod and cold ambient temperature (SD-Cold; Light:Dark=8:16 hours at 4°C cold room) (Chayama et al. 2016). In this study, female hamsters raised in long day photoperiod and warm ambient temperature (LD-warm; Light:Dark=16:8 hours at 23°C) were subjected to prolonged SD-Cold over 2 months or more to induce hibernation. The animals were anesthetized with inhalation of 4% isoflurane and then sacrificed by decapitation to collect tissues of interest. We found that mass of their skeletal muscles was significantly decreased by the onset of hibernation during the pre-HIB period, while its ratio to body mass was relatively preserved during HIB. Quantitative RT-PCR and immunostaining for fiber-type specific myosin heavy chains revealed that skeletal muscle fiber-composition was dramatically remodeled from fast-type to slow-type during HIB periods (n=5 or more per each group, p<0.01). The observed fiber-type changes began after over 2 months of SD-Cold before HIB. Along with these changes, proteasome components and atrophic markers MuRF1 and MAFbx were significantly up-regulated (n=5 or more per each group, p<0.01). Simultaneously, expression of myostatin, an inhibitor of myogenesis and muscle hypertrophy, was significantly reduced during HIB (n=5 or more per each group, p<0.01). Remarkably, these changes were cancelled in animals that spontaneously quit HIB even under SD-Cold condition. These results suggest that muscle-fiber type shift and suppression of myostatin may operate as a mechanism for preserving skeletal muscles during HIB in this species.

Chayama Y et al. (2016). R Soc Open Sci 3, 160002.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB226

# Effects of ouabain and/or salbutamol on subcellular glycogen distribution in isolated rat soleus muscle

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In skeletal muscle, glycogen is essential for sustaining prolonged work, however the underlying mechanism(s) remains unknown (Allen et al. 2008; Ørtenblad et al. 2013). One hypothesis is that compartmentalized energy transfer is required from the breakdown of specific glycogen particles located in a juxtaposition to specific energy-dependent

functions creating a spatially separate and efficient energy transfer network (Dzeja et al. 2007; Nielsen et al. 2009). Here, we aimed to investigate how salbutamol (a  $\beta_2$ -agonist, which stimulates glycogenolysis) and ouabain (a cardiac glycoside, which inhibits  $\text{Na}^+, \text{K}^+$  pumps) influenced spatially distinct depots of glycogen particles in skeletal muscle fibres. Soleus muscles from male sprague dawley rats (n = 26,  $164 \pm 17$  g (mean  $\pm$  SD)) were incubated in a Krebs-Ringer solution with 5 mM glucose (pH 7.4) at 25 °C, and continuously gassed with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ . Paired muscles (from left and right leg) were randomly allocated to two of the four groups: control (C; n = 11), salbutamol (S; 10  $\mu\text{M}$ , n = 15), ouabain (O; 1 mM; n = 11) or ouabain + salbutamol (OS; n = 15). Transmission electron microscopy was conducted to quantitate the volumetric content of glycogen particles in three distinct locations (Figure 1): 1) intermyofibrillar (IMF, 70% of total), 2) intramyofibrillar (Intra, 25% of total), and 3) subsarcolemmal (SS, 5% of total). Data are presented as medians and interquartile range. Main and interaction effects were tested using a linear mixed-effects model. In IMF glycogen, salbutamol mediated a 40% decrease (S: 3.44 (2.77:4.62) vs C: 5.76 (4.82:6.91), which was not affected (a 42% decrease) by pre-treatment with ouabain (OS: 4.48 (3.30:6.14) vs O: 7.71 (4.62:9.13), main effect:  $P < 0.0001$ , interaction:  $P = 0.24$ ). Ouabain-treatment alone increased IMF glycogen by 10% (OS+O: 5.01 (3.28:7.83) vs C+S: 4.54 (3.15:6.02),  $P = 0.009$ ). In Intra glycogen, salbutamol mediated a 55% decrease (S: 1.45 (0.71:2.34) vs C: 3.21 (2.06:4.77), which was slightly attenuated (to a 48% decrease) by pre-treatment with ouabain (OS: 1.92 (1.49:2.52) vs O: 3.67 (2.00:5.18), main effect:  $P = 0.0001$ , interaction:  $P = 0.096$ ). Ouabain-treatment alone did not significantly affect Intra glycogen (OS+O: 2.39 (1.50:3.70) vs C+S: 2.06 (1.09:3.21),  $P = 0.23$ ). Neither salbutamol ( $P = 0.31$ ), ouabain ( $P = 0.97$ ) nor both combined ( $P = 0.65$ ) influenced SS glycogen (S: 3.60 (2.03:5.85) vs C: 4.47 (2.74:7.21) and (OS: 4.31 (2.79:6.17) vs O: 4.81 (2.86:6.59). In conclusion, stimulation of glycogenolysis by salbutamol mediates a compartmentalized utilization of glycogen depots as characterized by a decrease in both IMF and Intra glycogen, but not SS glycogen. Blocking  $\text{Na}^+, \text{K}^+$  pumps with ouabain, tended to attenuate the utilization of Intra glycogen. The present data suggest that  $\text{Na}, \text{K}$ -pumps preferentially use this small depot of Intra glycogen, located in the sarcomeric I-band near the t-system.

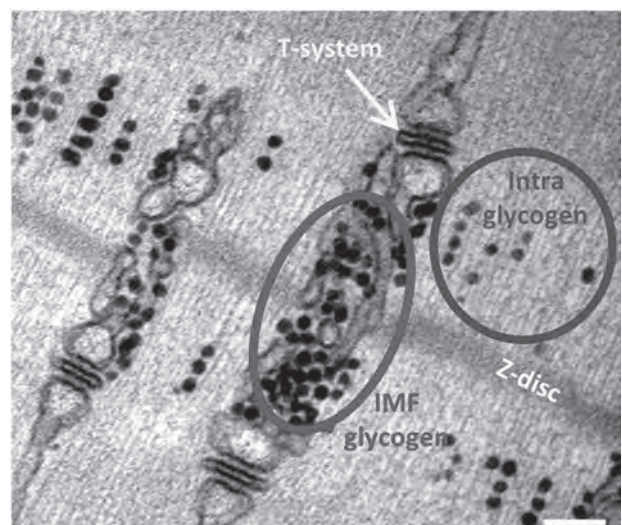


**Figure 1. Glycogen for the  $\text{Na}^+, \text{K}^+$  pumps: Where does it come from?** Transmission electron microscopy reveals the subcellular localization of glycogen (black dots). The  $\text{Na}^+, \text{K}^+$  pumps of the t-system (yellow arrow) are located distant both to glycogen particles located within the myofibrils (Intra glycogen, blue circle) and to those located close to sarcoplasmic reticulum (IMF glycogen, red circle). Scale bar = 100 nm.

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This study was supported by grants from the Danish Ministry of Culture Committee on Sports Research. Transmission electron microscopy was performed at the Department of Pathology, Odense University Hospital, Denmark.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB227

### Effect of Vitamin C on glucose metabolism in partial and total sleep-deprived male Wistar rat

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Chronic sleep deprivation is associated with elevations in oxidative stress and impaired glucose homeostasis. Vitamin C (a well-known antioxidant) prevented sleep-deprived induced memory impairment, however it is not known if Vitamin C has any effect on impaired glucose metabolism during sleep deprivation. This study investigated the effect of vitamin C administration on glucose homeostasis in 7 days sleep deprived animals.

Thirty male Wistar rats weighing 150-200 gram were divided into six (6) groups as Control; control + Vitamin C; partial sleep deprived; partial sleep deprived + Vitamin C; total sleep deprived; and total sleep deprived + Vitamin C. The animals were sleep deprived for 7 days using the modified multiple platform chambers. Partial sleep deprivation was achieved by sleep depriving the animals for 20 hours daily and allowed to rest for the remaining 4 hours while total sleep deprivation is when the animals were not allowed the daily 4 hours rest. Groups 2,4 and 6 were daily administered Vitamin C (300mg/kg) by oral gavage. At the end of the experiment, the animals were anaesthetized using ketamine (100mg/kg body weight, *i.p.*). Blood samples were obtained for fasting blood glucose level and lactate determination. Muscle and liver samples were obtained for glycogen determination while hexokinase and glucose-6-phosphatase activities were determined in the liver only. Differences in means were compared using student's t-test and ANOVA (Analysis of Variance) and p value was set as  $p < 0.05$ .

There was significant ( $p < 0.05$ ) decrease in body weight of the total sleep deprived rats compared to the control, the rate of the decrease body weight was however minimized in the total sleep deprived that were administered vitamin C. The fasting blood glucose level and plasma lactate were significantly reduced in both the total sleep deprived and total sleep deprived + Vitamin C groups compared to the control. Muscle glycogen was significantly depleted in total sleep deprived and the total sleep deprived + Vitamin C groups however, hepatic glycogen was increased in the total sleep deprived animals but decreased significantly in the total sleep deprived + Vitamin C animals. Glucose 6-phosphatase activity was significantly decreased in the partial sleep deprived animals while treatment with Vitamin C had no significant effect in the partial sleep deprived + Vitamin C group; it was however, increased in the total sleep deprived group but decreased significantly

in the total sleep deprived + Vitamin C group. Hexokinase activity was decreased in the total sleep deprived animals but increased in the total sleep deprived + Vitamin C. It was concluded that partial sleep deprivation had no effect on glucose metabolism but total sleep deprivation caused derailment in glucose metabolism which was not reversed by treatment with vitamin C.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB228

### Anti-oxidant supplementation ameliorates noradrenaline and serotonin contents in perimenopausal female rats following acute restraint stress.

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The decreased antioxidant defense characteristic of perimenopause leads to a decrease in stress resilience upon exposure to external stressors. Female 28-day old Sprague-Dawley rats were divided into 3 groups; Control group (injected s.c., Corn oil 2.5 µl/g BW); VCD group (injected s.c., 4-vinylcyclohexene diepoxide 160 mg/kg BW diluted in Corn oil) both for 15 days; and Aged group allowed to age naturally till 150 days. Seven weeks after VCD/corn oil administration, and 150 days in Aged group, rats were further divided into 3 sub-groups: L-Arginine (oral, 100mg/kg BW), D-ribose (oral, 200mg/kg BW) and a third group received neither for additional 30 days. At 130 days in Control and VCD groups, and 180 days in Aged group on diestrus morning, animals were subjected to acute restraint stress for 30 minutes. Blood samples were drawn before and after stress from the retro-orbital sinus for measurement of serum noradrenaline and serotonin concentrations using HPLC technique. Ovaries were processed through HPLC for assessment of noradrenaline and serotonin contents. Values are means  $\pm$  S.E.M., compared by ANOVA. In unstressed and stressed animals, D-ribose significantly decreased ( $p < 0.05$ ) serum noradrenaline and serotonin in Aged (Noradrenaline:  $44.90 \pm 3.54 \mu\text{g/ml}$ ,  $34.85 \pm 9.45 \mu\text{g/ml}$  respectively; Serotonin:  $3.49 \pm 0.26 \mu\text{g/ml}$ ,  $4.43 \pm 0.97 \mu\text{g/ml}$  respectively) and VCD (Noradrenaline:  $29.05 \pm 2.24 \mu\text{g/ml}$ ,  $13.13 \pm 3.94 \mu\text{g/ml}$  respectively; Serotonin:  $4.35 \pm 0.33 \mu\text{g/ml}$ ,  $3.78 \pm 0.55 \mu\text{g/ml}$  respectively) groups compared to Control group (Noradrenaline:  $63.66 \pm 3.46 \mu\text{g/ml}$ ,  $59.95 \pm 5.45 \mu\text{g/ml}$  respectively; Serotonin:  $6.73 \pm 0.51 \mu\text{g/ml}$ ,  $6.64 \pm 0.62 \mu\text{g/ml}$  respectively). It also significantly increased ( $p < 0.05$ ) ovarian noradrenaline and serotonin of stressed animals in Aged ( $5.34 \pm 0.23 \mu\text{g/g}$ ,  $24.58 \pm 0.13 \mu\text{g/g}$  respectively) and VCD ( $4.12 \pm 0.10 \mu\text{g/g}$ ,  $14.54 \pm 2.10 \mu\text{g/g}$  respectively) groups compared to Control group ( $2.43 \pm 0.14 \mu\text{g/g}$ ,  $8.51 \pm 2.04 \mu\text{g/g}$  respectively). L-Arginine significantly decreased ( $p < 0.05$ ) serum serotonin of unstressed and stressed animals in Aged ( $4.13 \pm 0.42 \mu\text{g/ml}$ ,  $3.30 \pm 0.30 \mu\text{g/ml}$  respectively) and VCD



( $3.13 \pm 0.18 \mu\text{g/ml}$ ,  $2.49 \pm 0.16 \mu\text{g/ml}$  respectively) groups compared to Control group ( $6.70 \pm 0.13 \mu\text{g/ml}$ ,  $5.30 \pm 0.28 \mu\text{g/ml}$  respectively). In both unstressed and stressed animals, L-Arginine significantly increased ( $p < 0.05$ ) ovarian noradrenaline and serotonin in Aged (Noradrenaline:  $6.38 \pm 0.94 \mu\text{g/g}$ ,  $2.01 \pm 0.15 \mu\text{g/g}$  respectively; Serotonin:  $12.78 \pm 0.92 \mu\text{g/g}$ ,  $15.56 \pm 0.65 \mu\text{g/g}$  respectively) and VCD (Noradrenaline:  $6.63 \pm 0.95 \mu\text{g/g}$ ,  $2.22 \pm 0.36 \mu\text{g/g}$  respectively; Serotonin:  $14.14 \pm 0.98 \mu\text{g/g}$ ,  $6.26 \pm 0.57 \mu\text{g/g}$  respectively) groups compared to Control group (Noradrenaline:  $3.25 \pm 0.31 \mu\text{g/g}$ ,  $1.11 \pm 0.26 \mu\text{g/g}$  respectively; Serotonin:  $8.31 \pm 0.94 \mu\text{g/g}$ ,  $2.22 \pm 0.38 \mu\text{g/g}$  respectively). These results show that L-Arginine and D-Ribose supplementation helped to prevent an exacerbation of stress hormonal response.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB229

### ***Ocimum gratissimum* Leaf Extract May Precipitate Infertility in Male Diabetic Wistar Rats**

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This study was designed to investigate the effect of *Ocimum gratissimum* (OG) on sperm quality and testicular cytoarchitecture in alloxan-induced diabetic rats.

Twenty male Wistar rats (150-200 g) were assigned into 4 groups (n=5) as A (control), B (OG), C (Dia) and D (Dia+OG). Groups A and B were normal animals administered distilled water or OG (400 mg/kg), respectively while diabetes was induced by alloxan monohydrate (100 mg/kg) in groups C and D followed by administration of distilled water or OG, respectively for 28 days. Blood samples were obtained for fasting blood glucose (FBG) and fructosamine determination while, epididymis and testes were obtained for sperm quality assessment using computer-assisted sperm analyser and testicular histomorphometry, respectively. Seminiferous tubule diameter and interstitial space distance were quantified in hematoxylin and eosin stained slides. Statistical analysis was done using ANOVA and student t test at  $\alpha_{0.05}$ .

Fructosamine and FBG were reduced in Dia+OG ( $80.11 \pm 3.80 \mu\text{mol/L}$  and  $132.0 \pm 8.41 \text{ mg/dl}$ , respectively) compared with Dia ( $139.66 \pm 4.29 \mu\text{mol/L}$  and  $285.6 \pm 26.69 \text{ mg/dl}$ , respectively). Sperm count was unchanged in Dia but decreased in OG and Dia+OG; abnormal sperm cells increased in OG, Dia and Dia+OG. Mild vacuolation in the seminiferous tubule, disorganized germinal cells layer, arrested sperm maturation with empty spermatozoa in lumen, decreased seminiferous tubule diameter and increased interstitial space were observed in the testes of OG, Dia and Dia+OG compared with control.

it was concluded that diabetes mellitus induces sperm impairments and distortions in testicular cytoarchitecture which was aggravated by OG leaf extract in male Wistar rats.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB230

### **Effects of Maternal Tobacco Smoke or Alpha Lipoic Acid on Sexual Behavior in Female Rats**

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Sexual behavior plays a main role in reproduction and may be influenced by a number of factors<sup>(1)</sup>. However, the effects of maternal tobacco smoke or alpha lipoic acid on sexual behavior is exactly unknown in female rats. In the literature, it has been suggested that exposed to tobacco components may affect female reproductive system<sup>(2)</sup>. For example, nicotine administration affects adversely ovarian function and fertility in female rats<sup>(3)</sup>. On the other hand, studies have shown that alpha lipoic acid may be positive effective on reproductive system, such as development of follicles in mice<sup>(4)</sup>. In this study, we aimed to explore the effects of maternal tobacco smoke or alpha lipoic acid on sexual behavior in female rats. For the experimental studies, adult female Sprague-Dawley rats exposed to maternal tobacco smoke or alpha lipoic acid were used. Depending on tobacco smoke or alpha lipoic acid treatment (for 8 week before pregnancy and throughout pregnancy), 4 groups were formed [control, alpha lipoic acid (ALA), tobacco smoke (TS) and tobacco smoke+alpha lipoic acid (TS+ALA), n=7 for each group]. All rats in the estrus phase were tested for female sexual behavior with a sexually experienced male. In non-paced mating tests, female and male rats were placed together in arena for 10 min or until the female received 10 mounts to quantify proceptive (anogenital investigation, sniffing the genital area of the stimulus male) and receptive behaviors (lordosis quotient and lordosis quality). Lordosis quotient was measured as the lordosis/mount ratio  $\times 100$ , and lordosis quality was computed according to the intensity of the lordosis responses scored on a 4-point scale (0-3)<sup>(5)</sup>. Results were analyzed using one-way ANOVA and values were expressed as mean  $\pm$  SE. There was no any significant change in the frequency of anogenital investigations, lordosis quotient and lordosis quality in all treatment groups compared to control group. However, the mean number of anogenital investigations was lower in ALA, TS and TS+ALA groups compared to control group ( $2.75 \pm 0.59$ ,  $2.5 \pm 0.81$ ,  $3.66 \pm 0.76$  and  $4.33 \pm 0.42$ , respectively). Although the lordosis quotient was similar in all groups (ALA, TS and control groups were 100%, TS+ALA group was  $97.62 \pm 2.38\%$ ), there was a decline in lordosis quality scores in all treatment groups compared to control group ( $1.97 \pm 0.2$ ,  $2.01 \pm 0.1$ ,  $2.07 \pm 0.27$  and  $2.41 \pm 0.1$  for ALA, TS, TS+ALA and control groups, respectively). These results indicate that maternal tobacco smoke or alpha lipoic acid may influence anticipatory phase (sexual motivation and/or arousal) or consummatory phase (sexual performance) of sexual behavior in female rats.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB231

### THE EFFECTS OF PAROXETINE, BUPROPION AND AGOMELATINE ADMINISTRATION ON SEMINAL VESICLE FLUID IN MALE RATS

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**Introduction and Objective:** Antidepressants are commonly used drugs in the treatment of psychiatric disorders. There are limited studies on the effects of paroxetine, bupropion and agomelatine on the reproductive system in male rats. It has also been noted that sperm parameters are adversely affected in selective serotonin reuptake inhibitor (SSRI) treatments as a significant side effect of these drugs. It is known that seminal vesicle fluid contains abundant amounts of fructose, citric acid, nutrients, prostaglandins and fibrinogen. Fructose and other nutrients provide nutrition and motility of the sperm in the ejaculate for a period of time until the fertilization of the ovum. The aim of this study is to investigate the possible effects of these antidepressants on the levels of certain components of the seminal vesicle fluid.

**Methods:** In the study, a total of 32 male rats were used and divided into 4 groups. The rats were given oral paroxetine (0.36 mg/100 g), bupropion (17 mg/100 g) and agomelatine (1 mg/100 g) from postnatal day 21 to 90; and only saline was administered to the control group. At the end of the experimental period, the animals were decapitated under anesthesia, seminal vesicle fluids were taken and evaluated. **Results:** Seminal vesicle fluid fructose levels were significantly decreased in paroxetine (74.9±3 mg/dL) and bupropion (29.3±5 mg/dL) groups ( $p<0.01$ ); whereas there was no significant difference in the agomelatine group (120.8±12 mg/dL) compared to the control group (169.5±11 mg/dL). Vitamin A levels were significantly increased in bupropion (0.031±0.003 mg/dL) and agomelatine (0.031±0.002 mg/dL) groups, compared to the control group (0.020±0.001 mg/dL) ( $p<0.05$ ). Vitamin E levels were significantly higher in the agomelatine group (0.152±0.013 mg/dL) than the other groups ( $p<0.05$ ). Vitamin D levels did not differ between groups. There were no significant differences in seminal vesicle fluid Na, K and Cl levels between groups.

**Conclusion:** Decrease of fructose levels in the seminal fluid due to paroxetine and bupropion may result in decreased vitality and motility of the spermatozoa. This result suggests that reduction in sperm fertilizing ability can be seen in long-term antidepressant use. However, agomelatine may be considered as a more preferable antidepressant with no effect on fructose levels and positive effects on Vit A and Vit E levels.

**Key Words:** Seminal Vesicle, Fructose, Antidepressant, Vitamin.

This study was supported by TUBITAK 113S193

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB232

### Diet-induced Obesity in Wistar rat: Influence of Normal diet Re-feeding on Altered Levels of Salivary Leptin and Ghrelin

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Leptin and ghrelin are two appetite hormones involved in the regulation of food intake and energy homeostasis. Although, altered circulating and salivary levels of these hormones have been reported in obese humans and animals, it is not known if re-feeding with normal diet will reverse these impairments. This study evaluated the effects of diet-induced obesity as well as re-feeding with normal diet on serum and salivary leptin and ghrelin in male Wistar rats. Weaning rats weighing 40 to 50 g were randomly divided into 3 groups, (control, obese and re-fed obese) of 7 rats each. Control animals were fed standard rat chow while obesity was induced with 20% fat diet for 15 weeks. In addition, re-fed obese group received normal diet for another 15 weeks following the 15 weeks of fat diet. Rats were anaesthetized with a mixture of ketamine (65mg kg<sup>-1</sup>) and xylazine (0.5mg kg<sup>-1</sup>). Blood and stimulated saliva (using pilocarpine 10mg kg<sup>-1</sup>) were collected for the analysis of leptin and ghrelin using ELISA. Values are means ± S.E.M., and compared by ANOVA with Tukey's HSD post hoc test. Body weight was significantly lower in re-fed obese group compared with obese group (290±1.5 vs. 317.86±2.2 g,  $p<0.05$ ). Serum and salivary levels of leptin were significantly higher in the obese group (882±23.5 and 239±68 pg/mL, respectively) compared to control (47.67±9.07 and 119.67±25.67 pg/mL, respectively). However, serum and salivary levels of leptin were significantly lower in the re-fed obese group (38±0.73 and 79±1.24 pg/mL, respectively) compared to obese group. While serum ghrelin was higher, salivary ghrelin was lower in the obese group (801.66±1.93 and 183.85±37.37 ng/mL, respectively) compared to control (689.37±9.51 and 348.83±10.01 ng/mL, respectively). However, serum ghrelin was lower while salivary ghrelin was higher in the re-fed obese group (752.15±2.41 and 282±10.6 ng/mL, respectively) compared to obese group. These findings suggest that levels of salivary leptin reflect their circulating levels in diet-induced obese rats. Importantly, re-feeding with normal diet reversed elevated levels of serum and salivary leptin as well as serum ghrelin. Further studies on the mechanisms that underlie these observations are needed.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB233

**MELATONIN AMELIORATES INFLAMMATION IN KIDNEY AND LIVER OF THE RATS WITH TYPE 2 DIABETES MELLITUS**H. Yapıslar<sup>1</sup>, E. haciosmanoglu<sup>3</sup> and T. Sarioglu<sup>2</sup><sup>1</sup>Physiology, Acibadem Mehmet Ali Aydinlar University, Istanbul, Turkey, <sup>2</sup>Histology and Embriology, Capa School of Medicine, Istanbul, Turkey and <sup>3</sup>Physiology, Istanbul Bilim University, Istanbul, Turkey

The diagnosed diabetes cases are mostly consist of 90% Type 2 diabetes mellitus all over the world. Inflammation is one of the main causes of complications observed in Type 2 diabetes mellitus.

Melatonin is a hormone released from pineal gland with circadian rhythm. It is known that melatonin has anticarcinogenic, antiapoptotic and antioxidant effects (Sainz et al, 2003). Melatonin is also related to inflammation (Chen et al, 2015). Earlier it was reported that melatonin release inhibited mice had suppressed humoral and cellular immune response. Also human studies show that insulin secretion is related to circadian rhythm in diabetic patients had altered diurnal melatonin secretion (Peschke et al, 2006).

Inflammation that occurs by different pathways and mechanisms causes a progress in diabetic microvascular complications.

In this study, we tried to evaluate the effect of melatonin on inflammation in kidney and liver tissues of rats with Type 2 diabetes mellitus. 2,5 months old Sprague Dawley male rats were used for this experiment. Type 2 diabetes was induced with nicotinamide (NA 100mg/kg) and streptozotocin (STZ 50 mg/kg). We had 3 experimental groups consisted of 10 rats each; control group, type 2 diabetes mellitus groups and type 2 diabetes mellitus group treated with melatonin (500 µg/day) during 4 weeks.

We studied inflammation markers, IL-1beta, TNF-alpha, IL-6 and NFkB protein levels both with Western Blot and immunohistochemistry in the liver and also we evaluated stated markers in kidney with immunohistochemistry. Protein densitometric analysis were performed with Image J programme. We found out that all inflammation marker levels were increased significantly in type 2 diabetes group than the control group ( $p < 0.05$ ). Our data showed that inflammation markers of melatonin treated groups decreased significantly when compared to type 2 diabetes group ( $p < 0.05$ ) both in kidney and in liver tissues.

As we know type 2 diabetes has microvascular complications. It is a metabolic disease affects all the tissues and the systems in the body through these complications. Liver and kidney are one of the tissues most affected of the disease due to their roles in the body. Earlier increased levels of inflammation markers were reported in diabetes (Donath et al, 2016). The main cause of this inflammation in diabetes is keeping its mystery. Also inflammation is being blamed for the microvascular complications of type 2 diabetes. Melatonin is a hormone well known by its anticarcinogenic and antiapoptotic effects. Lately its been reported that it has also antiinflammatory effects either. In our previous studies we observed that melatonin decreases inflammation in cell culture in vitro. Now in this study we observed that melatonin decreased inflammation in kidney and liver tissue of diabetic rats. Melatonin might be an important agent to prevent microvascular complications by decreasing inflammation in type 2 diabetes mellitus.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB234

**Intracerebroventricular MOTS-c infusion increases food consumption of obese rats**S. Tekin<sup>1</sup>, M. Durmus<sup>2</sup> and S. sandal<sup>1</sup><sup>1</sup>Physiology, Inonu University, Malatya, Turkey and <sup>2</sup>Physiology, Sanko University, Gaziantep, Turkey

Obesity is a chronic condition defined by an excess amount of body fat. If a person's body weight is at least 20% higher than it should be, he or she is considered obese. Obesity has also been increasing rapidly throughout the World. Obesity and overweight have in the last decade become a global problem. The Mitochondrial-Derived Peptide (MOTS-c) is one of the new hormones discovered in 2015. The first findings of the preliminary studies on the physiological roles of MOTS-c suggest that it prevents the formation of insulin resistance. The study was designed to evaluate effects of central infusion of MOTS-c on food consumption, water consumption and body weight in the rats.

40 Wistar Albino male rats were used in the study. The rats were separated into 4 groups as the control, sham, 10 µM MOTS-c and 100 µM MOTS-c infusion (n=10). The rats were fed with high-fat diet feed (Researchdiet, D12451) as of the 21th day of birth for 12 weeks. After 12 weeks, it was determined that obesity occurred in the rats by scoring according to the Lee Index. Then, the rats were anesthetized (except for the control group), and osmotic mini pumps (Alzet 2ML2) were placed in the lateral ventricle. With the help of the osmotic mini pumps, infusion of artificial cerebrospinal fluid (solvent) was performed to the sham group at a 120 µl volume (5 µl/hour) daily; and the infusion of 10 and 100 µM MOTS-c were performed to the study groups. Throughout the experimental period, the rats were kept in individual cages, and food consumption and body weight of the animals were daily recorded. During the analyses, IBM SPSS Statistics 22.0 for Windows package program was used. Multiple comparisons were performed through Bonferroni Corrected Mann Whitney U test. The value  $p < 0.05$  was accepted as statistically significant. At the end of the study, chronic infusion of both concentrations of the MOTS-c caused to significantly increases in food consumption ( $p < 0.05$ ), but in the body weights and water consumption of obese rats were not found any significant difference. Results indicates that long-term MOTS-c treatment increases food intake without increasing body weight on obese rats.



The study results support that MOTS-c can play important roles in hypothalamus on regulation of feeding behavior and appetite mechanism on obese rats.

This study was supported by TUBITAK (Project no: 116S744).

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB235

### **Effect of antioxidants supplementation on corticosterone induced changes in testosterone release by leydig cell**

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**Objectives:** To evaluate the effects of antioxidants, ascorbic acid and alpha tocopherol on corticosterone induced changes in testicular release by Leydig cells in relation to lipid peroxidation in terms of malondialdehyde levels.

**Methods:** Enzymatic dispersion of Leydig cells was done and purification of Leydig cells was obtained using Percoll Gradient. About 85000 cells were taken per tube. After removal of endogenous testosterone by preincubation, rat Leydig cells were incubated with varying concentrations of corticosterone alone and in combination with ascorbic acid and/or alpha tocopherol in the presence of Luteinizing hormone.

**Results:** Corticosterone decreased the testosterone production by Leydig cells ( $p < 0.001$ ). However there was insignificant effect of corticosterone on testicular production in the presence of ascorbic acid and alpha tocopherol, both separately and in combination. It was accompanied by significant reduction in malondialdehyde levels ( $p < 0.01$ ).

**Conclusion:** Both antioxidants, ascorbic acid and alpha tocopherol prevent the corticosterone induced decline in testosterone release from Leydig cells. It may be due to decrease in lipid peroxidation as suggested by fall in malondialdehyde levels.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB236

### **Effects of noopept on glucose, insulin, insulin resistance and pancreas of streptozotocin-induced diabetic prepubertal rats**

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The main purpose of type 1 diabetes mellitus (T1DM) management is blood glucose control. Since insulin resistance is higher than normal in puberty, problems arise in regulating insulin dosage in treatment. Usage of higher doses of insulin in treatment causes insulin resistance to rise and this leads higher daily insulin requirements. Because of this, new approaches to T1DM treatment are needed. Noopept is a synthetic nootropic dipeptide that is used as a cognitive regulator. Studies with noopept have suggested it to have anti-diabetic properties. In one study noopept has been shown to have incretin effect in glucose regulation, by inhibiting  $\beta$  cell degeneration. In our study, we tried to determine effects of noopept on;

blood glucose-insulin levels, insulin resistance, and pancreas in prepubertal DM rats.

In the study 60 prepubertal, 28 day-old, male, *Sprague Dawley* rats were divided into 6 groups (n=10/group). i) control, ii) DM control, iii) noopept, iv) DM+noopept, v) DM+insulin, vi) DM+insulin+noopept. Diabetes model was formed by 50 mg/kg streptozotocin on postnatal 28th day. At the end of the third day after streptozotocin administration, rats with blood glucose levels above 200 mg/dl were considered to have diabetes. Intraperitoneally 0.5 mg/kg noopept and 1 unit insulin was administered for 14 days in required groups. Blood glucose levels were measured at days 0, 3, 7 and 14 of the experiment. The research ended on the 46th day. Insulin levels were determined from sera by ELISA test and insulin resistance was determined by HOMA-IR formula. Pancreatic tissues were also histologically evaluated.

After diabetes formation, blood glucose levels were found to be low in the noopept treated groups ( $p < 0.05$ ). Insulin levels of DM control group were significantly higher than DM+ Noopept+insulin group ( $p = 0.05$ ). Insulin resistance of DM control group was higher than all other groups. DM + Noopept+Insulin group's insulin resistance was lower than DM+Insulin group ( $p < 0.05$ ). When pancreas was evaluated histologically, DM control group was found to have cellular distribution loss, vacuolization, vascular congestion, cellular infiltration. Islet cells of DM control group had dense small nuclei, vacuolated cytoplasm. Also edemas in vessels, degeneration of Langerhans islets were observed in DM control group. These changes were observed less frequently in the treated groups. Modifications were minimal in DM+Noopept+Insulin group.

At the end of the research we conclude that, noopept administration has positive effects on blood glucose-insulin levels, insulin resistance and pancreas tissue in T1DM prepubertal rats. Immunohistochemical studies about pancreas and noopept usage may be useful for future studies.

This study is supported by TÜBİTAK as 1002 project with 215S629 file number.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB237

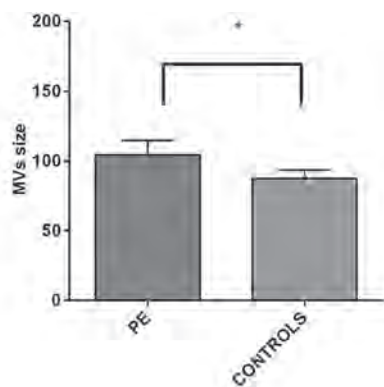
### **The pattern of serum microvesicles changes in preeclamptic women in comparison with controls. Analysis of their effects on renal glomerular endothelial cells and podocytes, cultured alone or in co-stimulation.**

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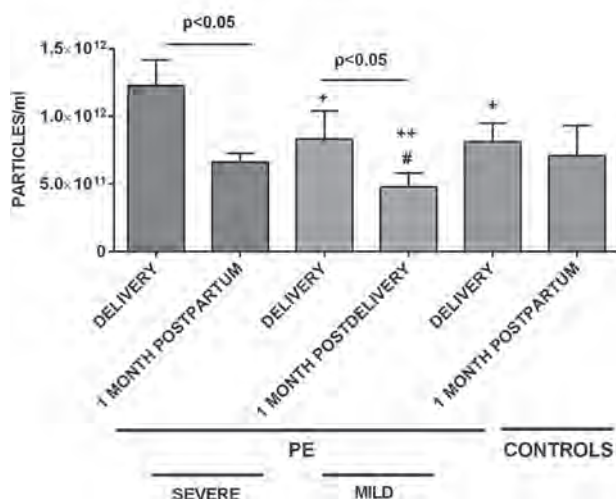
<sup>1</sup>University East Piedmont, Novara, Italy and <sup>2</sup>University East Piedmont, Novara, Italy

In preeclampsia (PE), the release of vasoconstrictive factors and cytokines by ischemic placental tissue would be involved in endothelial dysfunction and tissue damage in many organs among which the kidney (Timofeeva et al, 2018). Also, microvesicles (MVs), that are crucial in the processes of pregnancy, could play a role in the physiopathology of PE by modulating the crosstalk between different cell types (Adam et al, 2017). About this issue, however, the exact mechanisms have not been clarified, yet. For this reason, we aimed to analyze the pattern of MVs taken from serum of PE patients and to evaluate their role as biomarkers of disease activity and biological function on renal glomerular endothelial cells (GEC) and

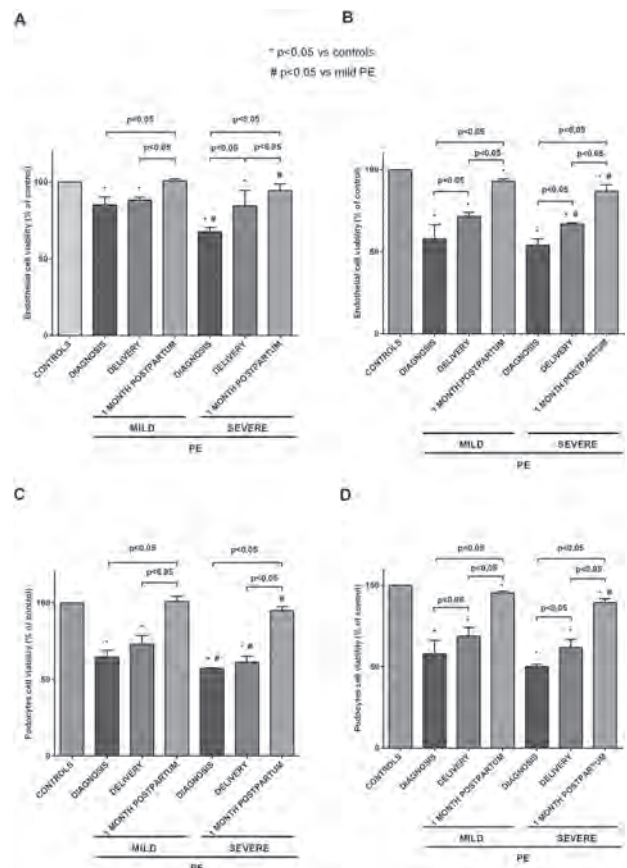
podocytes cultured alone and in co-stimulation. MVs dimensions, concentrations and profile pattern from 20 PE patients at diagnosis, delivery and 1 month postpartum were characterized and compared with those of 20 controls. Moreover, a correlation with uricemia, proteinuria, transaminases, angiogenic factors and arterial blood pressure was performed. The effects of serum and MVs on cell viability of GEC and podocytes cultured alone or in co-stimulation were examined, too. In vitro experiments were repeated in 5 different cell samples, at least. Statistical analysis was performed through one-way ANOVA followed by Bonferroni post hoc test or Student's t test for paired or unpaired data. For statistical significance a  $p < 0.05$  was considered. The results obtained have shown that in PE patients, MVs had higher dimensions in comparison with controls at delivery (Fig. 1). Moreover, the MVs concentration was higher in severe PE than in mild PE at delivery and at 1 month after delivery (Fig. 1). At diagnosis most MVs were PLAP+, CD24+, CD42b+ and CD105+. Both serum and MVs of PE patients caused a decrease of cell viability of GEC and podocytes, an effect which was more evident in severe PE (Fig. 2). Also podocytes treated with conditioning medium taken from GEC shown reduced cell viability, in particular when GEC were stimulated with MVs. In conclusion, in PE, serum MVs could represent a mechanism of kidney damage by modulation of the cross talk between GEC and podocytes. Our findings would be of clinical relevance and would open interesting diagnostic and therapeutic perspectives in the management of PE.



+  $p < 0.05$  vs severe PE delivery #  $p < 0.05$  vs 1 month PE  
++  $p < 0.05$  vs 1 month severe PE



MVs size and concentration in PE and controls



Effects of serum (A, C) and MVs (B, D) of PE patients on renal endothelial cells and podocytes viability

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PCB238

## Salt overload during gestation increases oxidative stress and impairs fetal survival ability in Sprague Dawley rats

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The increase exposure to high salt diet in populations during gestation despite reported complication in studies has made it compelling for further studies on fetal survival. This study is therefore aimed at investigating the effects of high salt diet at varying stages of gestation.

Fifty six female rats (150-200g) with regular estrus cycle were fed either normal diet of 0.3% sodium chloride (NaCl) or a high salt diet (HSD) of 8% NaCl for 6 weeks. Female rats were housed with male at ratio 2:1 on the evening of proestrus phase. The presence of spermatozoa in the vaginal smear of the rats the next day indicated Day 1 of pregnancy.

The pregnant rats were selected for the study and were distributed into 2 groups (n=20) of control (0.3% NaCl) and HSD (8% NaCl) and were fed the diet throughout gestation. On days 8, 12 and 19 of pregnancy, caudal blood pressure (BP) was measured; serum and urine electrolytes (Na<sup>+</sup>, Cl<sup>-</sup>, K<sup>+</sup>) were determined using SFRI ISE 6000 electrolyte analyzer. Serum progesterone and estrogen were also measured using an Enzyme-linked Immuno Assay (ELISA) kit. Implantation sites (IS) were counted on day 8 while fetal number and placenta weights were recorded on days 12 and 19. Litter numbers and birth weight were also documented. Malondialdehyde (MDA) and the activities of the antioxidants enzymes; catalase (CAT), glutathione reductase (GSH) and superoxide dismutase were measured in both serum and placenta homogenate. Rats were anaesthetized with chloroform and sacrificed following cervical dislocation. Values are represented as  $\pm$ SEM and compared with ANOVA.

Mean arterial BP (100.90 $\pm$ 2.55 mmHg vs. 89.41 $\pm$ 1.94 mmHg), serum Na<sup>+</sup> (146.2 $\pm$ 5.43 mmol/L vs. 133.3 $\pm$ 2.80 mmol/L) and Cl<sup>-</sup> (117.20 $\pm$ 2.14 mmol/L vs 96.55 $\pm$ 4.31 mmol/L) were significantly increased in the HSD when compared with control. Serum estrogen and progesterone was lower (p<0.05) in HSD (16.60 $\pm$ 1.04pg/ml vs 20.13 $\pm$ 0.61 pg/ml; 38.17 $\pm$ 2.75ng/ml vs 49.13 $\pm$ 2.46ng/ml) than control. Urine Na<sup>+</sup> in HSD was higher (p<0.05) on day 8 (60.83 $\pm$ 2.481mmol/L vs 51.25 $\pm$ 5.11mmol/L) than control. IS was reduced (11.00 $\pm$ 1.16 vs 14.00 $\pm$ 0.78, p<0.05) and fetal number was reduced (p<0.05) on day 12(9.33 $\pm$ 1.022 vs 12.83 $\pm$ 0.75) and 19 (6.50 $\pm$ 0.89 vs 9.67 $\pm$ 0.49) in the HSD when compared with control. In the serum and placenta homogenate, CAT (2.61 $\pm$ 0.53 mg/ml vs5.30 $\pm$ 0.58mg/ml; 3.64 $\pm$ 0.69mg/ml vs6.61 $\pm$ 0.72mg/ml), GSH (2.62 $\pm$ 0.39 $\mu$ mol/ml vs4.54 $\pm$ 0.41 $\mu$ mol/ml; 3.24 $\pm$ 0.51 $\mu$ mol/ml vs5.36 $\pm$ 0.47 $\mu$ mol/ml) and SOD (2.92 $\pm$ 0.08mg/ml 6.72 $\pm$ 0.073mg/ml;2.35 $\pm$ 0.33mg/ml vs6.64 $\pm$ 0.93mg/ml) were lower (p<0.05) while MDA (0.82 $\pm$ 0.04nmol/ml vs0.44 $\pm$ 0.01nmol/ml; 1.03 $\pm$ 0.27nmol/ml vs0.26 $\pm$ 0.09) was higher (p<0.05) in HSD than the control. In conclusion, high salt intake during pregnancy increases oxidative stress which affects fetal survival in rats.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB239

### Effects of some selected cooking oils on testosterone-induced benign prostatic hyperplasia in wistar rats

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Benign prostatic hyperplasia (BPH) is the benign uncontrolled growth of the prostate gland, leading to difficulty with urination. Anti-androgenic drugs, mainly prostate 5 $\alpha$ -reductase inhibitors, are indicated for the treatment of BPH although

their impact on BPH symptoms is modest. There is the quest for the search of better alternatives. This study sought to examine the effect of some cooking oils on testosterone-induced hyperplasia of the prostate in rats. Animals were distributed into eighteen groups (10 rats each). A negative control group was injected with soya oil; sixteen groups were injected with testosterone (3mg/kg) to induce hyperplasia: a positive control group, and fifteen groups treated orally at 800mg/kg with coconut oil, castor oil, canola oil, cottonseed oil, pomegranate oil, blackseed oil, shea butter, olive oil, cord liver oil, sardine oil, palm oil, repeatedly-heated palm oil, vegetable oil, repeatedly-heated vegetable oil, sesame oil, groundnut oil. Treatments were given for 14days. Rats were weighed before treatment and weekly thereafter. Animals were treated humanely. Rats were euthanized by cervical dislocation, and prostates were removed and weighed. The oils significantly reduced the increase in prostate weight (PW) and PW: body weight (BW) ratio induced by testosterone (% inhibition 77.96%; 67.55%, 86.51%;94.55%, 78.95%;98.28%, 51.64%;86.00%, 69.74%;82.88%, 76.32%; 96.75%, 63.82%; 71.85%, 77.96%;87.46%, 84.54%;93.23%, 61.51%;92.64%, 89.80%;79.94, 74.67%;80.42%). The oils contain appreciable concentrations of lauric, myristic, and oleic acids, these results could be attributed to this fact. In conclusion, this study shows that the oils reduced the markers of testosterone-induced prostatic hyperplasia in rats.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB240

### Normobaric hyperoxia treatment ameliorates renal injury following ischemia reperfusion in rats

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Introduction Acute kidney injury (AKI) represents an ever increasing clinical problem with high morbidity and mortality. The kidney, due to its inhomogeneous blood flow, is particularly susceptible to changes in oxygen delivery and renal hypoxia constitutes a common hallmark of AKI, and in the the progression to chronic kidney disease (CKD). However, the role of hypoxia *per se* in the acute phase of renal injury remains largely unclear. The present study was therefore designed to investigate 1) the role of systemic hypoxia in the acute progression and recovery of AKI, and 2) if normobaric hyperoxia treatment following AKI improves renal.

Methods Anaesthetized (thiobutabarbital 120mg/kg i.p.) adult male Sprague Dawley rats were subjected to unilateral complete warm renal ischemia for 45 minutes followed by 2 hours of reperfusion under normoxic (21% inspired oxygen, n=10), hypoxic (10% inspired oxygen, n=10), and hyperoxic (60% inspired oxygen n=3) conditions. Kidney function, haemodynamics, tissue PO<sub>2</sub>, and renal oxygen consumption (QO<sub>2</sub>) was estimated before and after ischemia reperfusion (IR).

Results All data are presented as mean  $\pm$ SEM. Statistical significance was determined by 2x2 ANOVA unless otherwise stated. All investigated parameters were equal between groups at baseline.

IR *per se* caused a steep decrease in GFR in all groups (-97 $\pm$ 1, -81 $\pm$ 7, and -52 $\pm$ 34% for hypoxic, normoxic and hyperoxic animals respectively) which did not differ significantly between groups. Renal blood flow (RBF) and renal vascular



resistance (RVR) were not affected by IR and did not differ between groups.

All animals subjected to hypoxic gas mixture developed cortical ( $24.4 \pm 1.7$  vs  $41.4 \pm 1.2$ ,  $p < 0.05$ ) and medullary ( $14.9 \pm 1.8$  vs  $23.1 \pm 1.4$ ,  $p < 0.05$ ) hypoxia versus normoxic animals following IR. Animals treated with normobaric hyperoxia had markedly increased cortical and medullary  $PO_2$  vs normoxic and hypoxic animals following IR ( $59.0 \pm 6.3$  and  $34.9 \pm 2.1$  mmHg in cortex and medulla respectively).

GFR at baseline were independent of renal tissue oxygenation. However, following IR there was a linear relationship between GFR and cortical  $PO_2$  (Fig 1, lin regression  $p < 0.05$ ).

**Conclusion** This study demonstrates that the reduced renal function following IR is dependent on reduced renal oxygenation levels. Normobaric hyperoxia treatment ameliorates renal injury following IR.

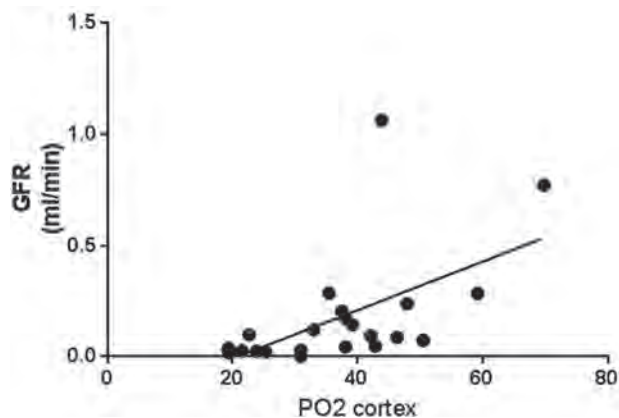


Fig 1 GFR in relation to cortical oxygen availability following ischemia reperfusion.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB241

### Gum Arabic (Acacia Senegal) supplementation alleviate oxidative stress and increases High density lipoprotein (HDL) level without affecting other lipid profile parameters among hemodialysis patients

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**Introduction:** Chronic renal failure prevalence and incidence is increasing worldwide and Sudan is not an exception. Several studies suggested that oxidative processes may be increased in patients with renal failure. Oxidative stress increased risk of atherosclerosis and cardiovascular complication -the major cause of mortality in renal failure patients. Clinical studies have confirmed that hemodialysis (HD) is associated with the development of oxidative stress and disturbance in the enzyme systems that protect against oxygen free radicals. Increased lipid peroxidation and depletion of chain-breaking antioxidants may contribute to increased risk of atherosclerosis and cardiovascular complication.

Gum Arabic (GA) is soluble dietary fibers and prebiotic agent. Has been claimed to act as an anti-oxidant and cytoprotective agent, protecting against experimental hepatic, renal and

cardiac toxicities in rats. This study was conducted to investigate the effect of Gum Arabic on Oxidative Stress and lipid profile among patients on regular hemodialysis.

**Materials and Methods:** this study was conducted among patients on regular hemodialysis in Military hospital. Forty End Stage Renal Disease patients age (18–80 years) who met the study inclusion criteria were recruited. Patients received 30 g/day GA for 12 weeks. Lipid profile, Total anti-oxidant capacity (TAC) and malondialdehyde (MDA) levels were measured by Colorimetric methods before and after GA intake. Blood samples were taken before and after Gum Arabic intake. The study was ethically approved from National Medicine and Poisons Board.

ClinicalTrials.gov Identifier:NCT03214692 Registered 11 July 2017 Prospective

**Results:** 40 patients were enrolled. Age ( $45.5 \pm 17.22$ ). Mean duration of hemodialysis is ( $59.7 \pm 38.38$ ) months. Gum Arabic significantly increase TAC level ( $P < 0.001$ ) (95%CI 0.408 to 0.625). And decreased the oxidative markers MDA ( $P < 0.001$ ) (95%CI -3.664 to -1.68). In addition increased HDL level ( $P = 0.002$ ) (95%CI -13.9 to -4.73). No significant difference was found in Total cholesterol, triglyceride and LDL level before and after Gum Arabic oral ingestion.

**Conclusions:** GA revealed potent anti-oxidative properties in hemodialysis patients. Daily supplementation of GA (30 g/day) significantly reduced the oxidative stress in hemodialysis patients and increase level on HDL, may be protective form atherosclerosis and cardiovascular related mortality among patients on regular hemodialysis.

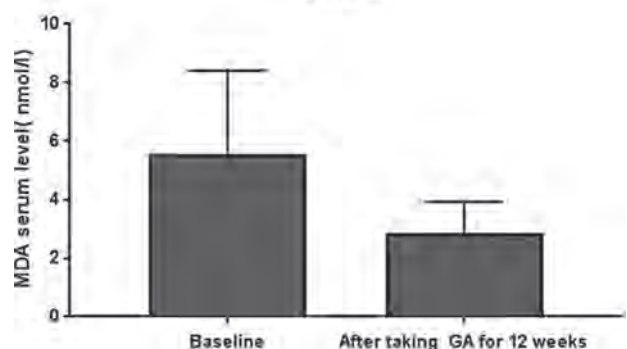


Figure I: Effect of GA intake on Total Antioxidant Capacity TAC Level ( $P < 0.001$ )

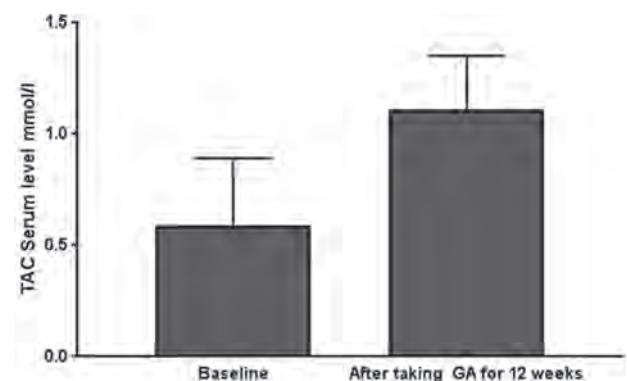


Figure II: Effect of GA intake on MDA Level ( $P < 0.0001$ )

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We would like to Gum Arabic council for providing gum Arabic as gift for the study. We also thank department of clinical chemistry in Central laboratory Military hospital. For their valuable help in measuring serum lipid profile.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB242

### Estrogen and Estrogen Receptor Agonists Protect Against Acetaminophen-induced Hepatorenal Toxicity in Rats

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Acetaminophen is the most widely used analgesic agent in the world. When taken at higher-than-recommended doses, acetaminophen has toxic effects on the liver and kidneys. Although clinical data reveal that women have a greater risk of experiencing drug-induced acute liver failure than men<sup>1</sup>, animal studies have reported that female mice showed a lower acetaminophen hepatotoxicity<sup>2</sup>. The aim of this study was to compare the impact of sex and ovarian hormones on acetaminophen-induced hepatorenal toxicity in rats and to elucidate the protective effects of estrogen and estrogen receptor agonists on the liver and kidneys. Experimental protocols were approved by the Marmara University Animal Ethics Committee. Under anesthesia (ketamine 100 mg/kg and xylazine 10 mg/kg, intraperitoneally) female Wistar Albino rats (n=49) had either ovariectomy (OVX) or sham-OVX. OVX groups received subcutaneously 17β-estradiol (1 mg/kg/day), ERβ-agonist diarylpropionitrile (1 mg/kg/day), ERα-agonist propyl pyrazole triol (1 mg/kg/day) or vehicle for 10 days starting at postsurgical 40<sup>th</sup> day. Male Wistar Albino rats (n=16) and sham-OVX groups were injected subcutaneously with vehicle for 10 days. At the end of the 7<sup>th</sup> week, rats received either acetaminophen (3 g/kg) or saline by orogastric gavage and 24 h later they were decapitated. Blood samples were obtained to measure serum alanine aminotransferase (ALT), aspartate transaminase (AST), blood urea nitrogen (BUN) and creatinine levels. Liver and kidney samples were obtained for histopathologic examination and for analyzing levels of tissue glutathione and myeloperoxidase activity. Data were analyzed using Mann Whitney-U and Student's t-tests. Histologically, renal and hepatic damage scores were elevated in all acetaminophen-treated female and male rats. Compared to their control groups, serum levels of AST, ALT, BUN, creatinine, renal myeloperoxidase activity were increased and hepatic glutathione level was decreased in vehicle plus acetaminophen-treated female and male rats (p<0.001), while hepatic myeloperoxidase activity was increased only in males (p<0.05). ERα-agonist reversed all parameters in liver and kidney. Pretreatment with ERβ-agonist or 17β-estradiol

elevated hepatic glutathione levels and diminished creatinine and renal myeloperoxidase activity (p<0.05-0.01). Except for hepatic neutrophil infiltration, severity of acute acetaminophen-induced hepatorenal toxicity was not affected by sex difference or by the absence of ovarian hormones. On the other hand, pretreatment with estrogen or estrogen receptor agonists, via their antioxidant actions, provided protective effects on acetaminophen-induced hepatorenal toxicity.

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Du K et al. (2014). *Toxicol Appl Pharmacol*. 15; 281(1), 58–66.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB243

### The Effect of Dietary Amino Acids on Chronic Kidney Disease Progression in Rats

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Chronic Kidney Disease (CKD) affects over 10% of the population (1). However, the mechanisms by which proteins/amino acids cause an increase in the Glomerular Filtration Rate (GFR) and kidney function deterioration are not well understood.

Our study aims to unravel the differential impact of specific dietary amino acids/groups of amino acids on the progression of CKD.

We used the well-established 5/6<sup>th</sup> Nephrectomy (5/6<sup>th</sup> Nx) model to induce CKD. 5/6<sup>th</sup> Nx Wistar Han rats were randomly divided 4 weeks after surgery, into groups receiving either the control diet (18% protein, n=10) or one of different diets, in each case containing 8% protein supplemented with a 10% mix of free amino acids (AAs): Essential- (EAAs n=15), Non-Essential- (NEAAs n=10), Branched Chain- (BCAAs n=14), Aromatic- (AAAs n=10), or all AAs in the same proportion as in the protein mix (8+10 n=18). Also, we had a group that was fed a diet of 18% protein, supplemented with 1.82% L-arginine (n=10). Both GFR and RPF were measured in free moving animals: GFR transcutaneously using FITC-sinistrin, and RPF using radiolabelled para-aminohippurate (PAH).

We found that none of the modified diets caused any decrease in body weight, food and water consumption in the different groups up to 9 weeks after surgery. However, the pace of RPF and GFR alteration was diet-dependent. Animals receiving AAAs and EAAs showed the slowest progression, whereas the most dramatic reduction was in the case of animals on the BCAA diet (from 1.5 +/-0.1 ml/min/100g BW to 0.55 +/-0.033 ml/min/100g BW, p<0.05, values expressed as means ± S.E.M., compared by ANOVA with Tukey post test). We also tested GFR changes in animals who were on the different diets but did not undergo 5/6<sup>th</sup> Nx and therefore, had no CKD. They showed no change in GFR 9 weeks after mock surgery (1.42 +/-0.11 and 1.42 +/-0.13 ml/min/100g BW). The kidneys of the BCAAs-receiving 5/6<sup>th</sup> Nx rats also showed the strongest increase in smooth muscle actin (2-fold) and collagen (more than 3-fold) mRNA expression, as expected for a higher level of inflammation and fibrosis compared to animals on the control diet. Towards deciphering the mechanisms underlying these differential effects of the various amino acid diets on CKD progression, we tested the activity of signalling pathways, specifically changes in phospho and total Stat3, Akt and S6k levels. No changes were observed at the phospho/total kidney

protein level in such cases (using Western blots, repeated 3 times) for the diet groups.

We found that different amino acid diets given for 5 weeks exert no impact on healthy kidneys, but they suggest that in CKD, high levels of dietary BCAAs exert a deleterious effect on progression, whereas high levels of AAAs surprisingly display a protective effect.

1) Troidle L (2014) The Chronic Kidney Disease Patient in the Acute Hospital Environment. *Advances in Chronic Kidney Disease*, 21(4) 355 – 359.

This study is supported by the Swiss National Centre of Competence in Research (NCCR), Kidney Control of Homeostasis (Kidney.CH).

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB244

### Renal Nox4 contributes to systemic redox homeostasis by controlling glutathione, methionine and cysteine metabolism

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The NADPH oxidase isoform Nox4 produces  $H_2O_2$  and is highly expressed in the kidney. It has a protective role in chronic renal disease and Nox4 knockout mice show increased albuminuria in diabetes. Interestingly, Nox4 expression is reduced in renal pathology, suggesting a potential role in normal kidney function.

To uncover the physiological role of Nox4 in the kidney, we first defined its localization. As demonstrated by *in situ* hybridization (RNAscope) combined with immunofluorescence, Nox4 is selectively expressed in the proximal tubule. As this part of the kidney mediates mass reabsorption and secretion, WT (wild type) and Nox4<sup>\*/</sup> (tamoxifen-inducible Nox4 knockout mice) were put on a sugar/fat diet, with low sodium (130mg/kg chow), low micro nutrients and no protein. Urine samples (day 0, 3, 14), renal cortex and plasma (day 14) were analyzed by global untargeted LC/MS for metabolites. In the urine, the knockout of Nox4 significantly increased the excretion of food components under normal chow (day 0). However, at day 3 of the diet, Nox4<sup>\*/</sup> had an increased excretion of xenobiotic metabolites. At day 14, the excretion of cysteine and cysteinylglycine disulfide was significantly reduced in Nox4<sup>\*/</sup>.

Systematic differences in methionine, cysteine and glutathione metabolites which are controlled by the redox-sensitive transcription factor Nrf2 were observed in plasma and renal cortex of Nox4<sup>\*/</sup> as compared to WT animals. In the renal cortex, Nox4<sup>\*/</sup> show reduced methionine, methionine sulfoxide, S-adenosylhomocysteine and S-methylcysteine. Plasma of Nox4<sup>\*/</sup> showed a reduction in cystathionine, cysteine s-sulfate and cysteine, indicating a reduced synthesis and therefore, attenuated reabsorption of these glutathione metabolites. Moreover, gamma-glutamyl amino acids (-glutamine, glycine, threonine and valine) were increased in plasma of Nox4<sup>\*/</sup> indicating a high activity of gamma glutamyl transferase during catalysis of glutathione in Nox4<sup>\*/</sup>.

The results using metabolomics indicate that by activating Nrf2, Nox4 controls the biosynthesis of glutathione as part of antioxidant response. By maintaining proper synthesis and reabsorption of metabolites, renal Nox4 contributes to systemic redox homeostasis.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB245

### Quercetin Prevents the Suppression of Epo by TNF-alpha

M. OZKURT, R. ÖZYURT, A. KARADAĞ and N. erkasap

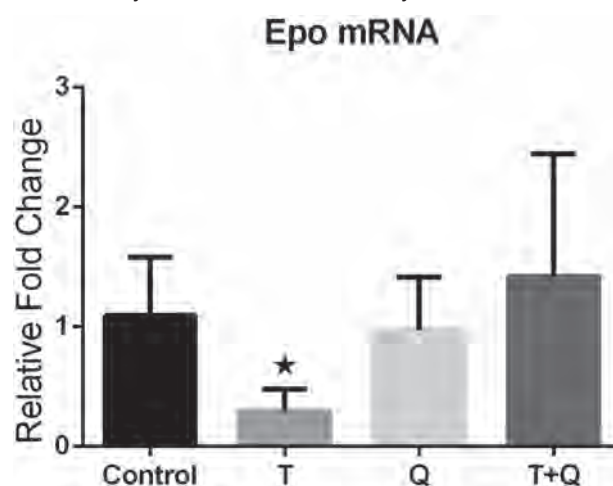
Eskisehir Osmangazi University, Eskisehir, Turkey

In-vitro studies have shown that TNF-alpha significantly lower the Epo synthesis in a dose dependent manner in HEPG2 cells (1). Quercetin is a flavanoid found in many fruits and vegetables such as oranges, grapefruits and onions which has been shown to have anti-oxidant and anti-inflammatory cell protective effect. Novel studies has shown that Quercetin increases the amount of Epo in-vitro (2). We aimed to try to remove the inhibition of TNF-alpha on Epo in with quercetin in HEPG2 cells.

After detecting the non-toxic doses of TNF-alpha and Quercetin with MTT, we seeded  $5 \times 10^5$  HEPG2 cells in 6 well plates. Each 6 well plates were a group and the groups were; no treatment group (control), 10 ng/ml TNF-alpha treated group (T), 10µM/ml Quercetin treated group (Q), TNF-alpha and Quercetin (T+Q) together treated group. After 24 hour of incubation we collected the cells and isolated total RNA, synthesised cDNA and performed Q-PCR for Epo. Data were analyzed by Kruskal-Wallis test and Dunn's test for post-hoc.

TNF-alpha significantly decreased ( $p < 0,05$ ) the Epo mRNA amount according to control group. In Quercetin alone treated group and the combination treatment group, Epo mRNA amount were same about the control group.

More detailed studies has to be done but this initial study shows that Quercetin may prevent the Epo suppression by TNF-alpha. We will try to explain the mechanism to understand the relation between Epo, TNF-alpha and Quercetin. Quercetin may be a source of treatment for anemia in chronic inflammatory diseases, as well as many other known effects.



Quercetin increased the Epo mRNA amount suppressed by TNF-alpha

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This study is supported by a grant from the Eskisehir Osmangazi University Scientific Research Projects Committee (no: 2018-2004), Turkey.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCB246

### Analysis of Micro-RNAs Participating the Inhibition of Erythropoietin Synthesis by TNF-alpha via Microarray Technology

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<sup>1</sup>Eskisehir Osmangazi University, Eskisehir, Turkey and <sup>2</sup>Luebeck Physiology Institute, Luebeck, Germany

**Aim:** In chronic inflammatory diseases pro-inflammatory cytokines such as TNF-alpha are high amount in circulation and are associated with anemia in most cases. Experimental studies have shown that TNF-alpha which plays a major role in the inflammation inhibits the synthesis of Erythropoietin (Epo), the main stimulant of hematopoiesis, and the mechanism of this raid has not been announced yet (1). In this study, we aimed to find out which micro-RNAs (miRNA) are involved in inhibition of Epo by TNF-alpha.

**Material-Methods:** Non-cytotoxic TNF-alpha doses were determined by MTT assay. Epo-targeted ELISA and qRT-PCR analyzes were performed to find the optimal dose of TNF-alpha that suppressed Epo in HEPG2 cells. Statistical significance was determined by One-Way Anova and Tukey test for post-hoc. After detecting the TNF-alpha dose as 20 ng/ml, we performed miRNA array (Affymetrix Genechip miRNA 4.0 Array) with untreated (n=6) and treated with 20 ng/ml dose of TNF-alpha HEPG2 cells (n=6).

**Results:** Of the 30,404 miRNAs, 99 human miRNAs showed 2 or more fold changes. According to data base, 8 of them had a potential for binding to Epo mRNA: hsa-miR-4459, hsa-miR-663a, hsa-miR-1908-5p, hsa-miR-4433-3p, hsa-miR-149-3p, hsa-miR-4739, hsa-miR-6805-5p, hsa-miR-122-5p.

**Conclusion:** The detection of miRNAs that play a role in inhibition of Epo by TNF-alpha may describe a physiological mechanism deeper. In the next months, silencing studies of the miRNAs that we will perform, may show new treatment opportunities for the anemia, seen in chronic inflammatory diseases.

Jelkmann, W. (1998). Proinflammatory cytokines lowering erythropoietin production. *J Interferon Cytokine Res*, 18(8), 555-559. doi:10.1089/jir.1998.18.555

This study is supported by a grant from The Scientific And Technological Research Council Of Turkey (TUBITAK, no: 216S729) and from the Eskisehir Osmangazi University Scientific Research Projects Committee (no: 2017-1635), Turkey.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCB247

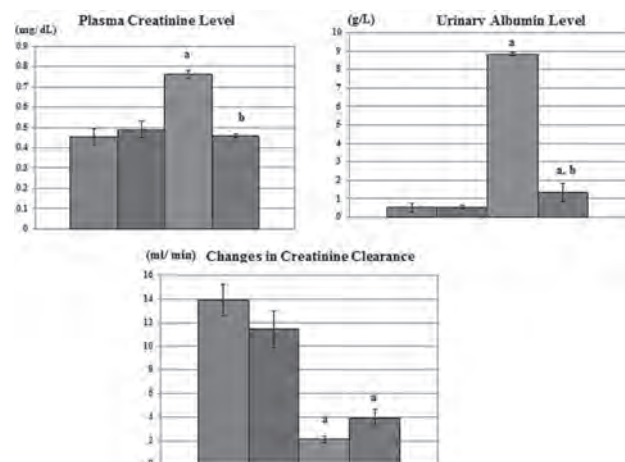
### Possible Role of Garlic Oil in Ameliorating Renal Injury after Liver Ischemia/Reperfusion in Rats

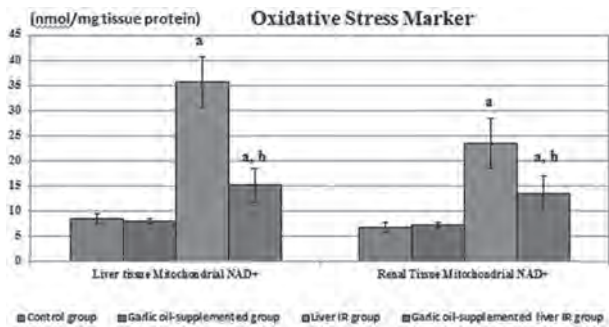
N. Lasheen<sup>1,2</sup>, W. Elayat<sup>3</sup>, M. Fathy<sup>4</sup>, R. El Sheikh<sup>5</sup>, D. Abo Raya<sup>5</sup> and F. Gad<sup>5</sup>

<sup>1</sup>Physiology, Faculty of Medicine Ain Shams University, Cairo, Egypt, <sup>2</sup>Physiology, New Giza University, School of Medicine, Giza, Egypt, <sup>3</sup>Biochemistry, Faculty of Medicine, Ain Shams University, Cairo, Egypt, <sup>4</sup>Anatomy, Faculty of Medicine, Ain Shams University, Cairo, Egypt and <sup>5</sup>Medical student, Faculty of Medicine, Ain Shams University, Cairo, Egypt

Acute liver failure induces kidney injury by triggering inflammation and oxidative stress. The heme oxygenase system has a preventive role against reperfusion injury, while garlic oil has antioxidants and anti-inflammatory effects. This study investigated the protective effects of garlic oil supplementation on renal functions after liver ischemia/reperfusion (I/R). Forty adult female Wistar rats were randomly and equally divided into control, garlic oil-supplemented (positive controls), liver I/R, and garlic oil-supplemented I/R groups. Liver ischemia was performed in anesthetized rats (by i.p. injection of pentobarbitone) for 45 min. Reperfusion was allowed for 24 hours in metabolic cages. Garlic oil was administered by gavage in a dose of 5 ml/kg/ day for 2 weeks prior to I/R. Plasma samples were used for determination of liver enzymes and creatinine levels. 24 hour urinary samples were assayed for albumin, volume and creatinine concentration to calculate glomerular filtration rate (GFR). Right liver lobe and right kidney specimens were used for determination of mitochondrial NAD<sup>+</sup> (colorimetrically) and hemoxegenase1 (HO1) by real time PCR. Left lobe of liver and left kidney specimens were used for histopathological studies. Statistical analysis by One way ANOVA was performed. Compared to control group, liver I/R group exhibited significantly elevated liver enzymes, creatinine in plasma, urinary albumin mitochondrial NAD<sup>+</sup> in liver and kidney tissues and HO1 gene expression in renal tissues; also, they had significant reduction in GFR. Garlic oil-treated I/R group had significantly lowered plasma levels of liver enzymes, urinary albumin and mitochondrial NAD<sup>+</sup> in liver and kidney tissues compared to liver I/R group in addition to higher HO1 gene expression in renal tissues.

Therefore, Liver I/R caused renal impairment through inducing oxidative stress, while garlic oil supplementation partially improved such impairment by limiting oxidative stress and inducing more gene expression of HO1 in renal tissues.





Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB248

### Effects of Salmon Calcitonin and Omega-3 Fatty Acids on some Biochemical Parameters in Induced Diabetic-Knee Osteoarthritic Rats

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Early evidence on the association between type 2 diabetes (T2D) and osteoarthritis (OA) dated back to the 1960s (Waine *et al.* 1961). Studies revealed that diabetes has an independent role in the pathogenesis and advancement, while there was an increased risk of diabetes among OA patients (King *et al.* 2013; Rahman *et al.* 2014). However, there are controversies on the optimum therapy in diabetic condition. The study was carried out to determine the effects of salmon calcitonin (Sct) and/or omega-3 fatty acids (N-3) in induced diabetic-knee osteoarthritic rats. The 35 male Wistar rats that were used for this study were randomly divided into 7 groups (n=5 rats) which include: Group 1 - Normal control; Group 2 - Diabetic-Osteoarthritic (D + OA) control; Group 3 - D + OA + N-3 (200mg/kg, *p.o.* once daily); Group 4 - D + OA + Low Salmon (Sct.Lw - 2.5IU/kg, *i.m.* once daily); Group 5 - D + OA + High Salmon (Sct.Hi - 5.0IU/kg, *i.m.* once daily); Group 6 - D + OA + N-3 + Sct.Lw; and Group 7 - D + OA + N-3 + Sct.Hi. Diabetes was induced in overnight fasted rats with streptozotocin (65mg/kg, *i.p.*) in citrate buffer (pH - 4.5). This was administered 15 minutes after the administration of nicotinamide (110mg/kg, *i.p.*) in normal saline (Satheesh & Pari, 2008). Knee OA was induced with 4mg monosodium iodoacetate in 40µl of sterile saline. The resultant solution was injected (using a 27-gauge needle) intra-articularly (*i.a.*) through the patellar ligament of the rats' left knee joint, while the rats in the normal control group were injected with citrate buffer (0.1ml, *i.p.*) and sterile saline (40µl, *i.a.*), all under sodium pentobarbital (40mg/kg, *i.p.*) anaesthesia (Orita *et al.* 2011). OA was induced about 12 hours after the induction of diabetic condition. Treatments with Sct and N-3 started 9 days after the induction of the diabetic and osteoarthritic states, and they lasted for 28 days. The induced diabetic-knee osteoarthritic featured significant elevations in total alkaline phosphatase (TALP), malondialdehyde, c-telopeptide of type I collagen (CTX-1), and collagen type 2 alpha 1 (C2M) levels; but, significant decreases in catalase, glutathione peroxidase (GPX), total bilirubin (TB), and total antioxidant capacity (TAC). The hyperglycaemic effect of Sct was significantly abated when it was co-administered with N-3. Moreover, Sct had a dose-specific effect, and an additive action with N-3 in reducing malondialdehyde and LDH, and in elevating TB and TAC. However, Sct demonstrated non-dose graded effects, and non-additive actions with N-3

in increasing SOD, catalase and GPX, and in decreasing TALP, CTX-1 and C2M. In this study, there was evidence for the additive, non-additive and antagonistic effects of both Sct and N-3, and the combined administration of Sct and N-3 could offer better therapeutic actions in diabetic-knee osteoarthritis.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB249

### SALTY BRAIN; NEW INSIGHTS IN SALT-SENSITIVE HYPERTENSION

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Salt sensitivity, described as association between salt intake and blood pressure (BP), varies among individuals. Traditional views on salt induced hypertension has been focused on the kidney and extracellular volume expansion. Recent studies have provided new insights linking dietary salt, inflammation and defective sodium sensing in brain. The hypothalamus in particular are intricately involved in the pathogenesis of sympathetic mediated hypertension. Ion channel function has been proposed to contribute to hypothalamic-driven sympathetic activity.

Our aim was to examine if modest BP elevation observed in normotensive male Wistar rats after exposure to high salt diet could be at least in part mediated by alterations in the availability and/or action of sodium (Na<sup>+</sup>) in the hypothalamus. Blood pressure (BP) was recorded in male Wistar rats (3 months of age) after 2 weeks of low salt diet (LSD, 0.1% NaCl, n=6) or high salt diet (HSD, 8% NaCl, n=6) using radio-telemetry (DSI Instruments). Osmolality and electrolyte content were measured in the cerebrospinal fluid (CSF) and hypothalamus using osmometer and HPLC respectively. Endogenous Ouabain (soluble inhibitor of the sodium pump Na<sup>+</sup>/K<sup>+</sup> ATPase) was measured in plasma, CSF and hypothalamus using ELISA (Lifeome). Hypothalamic ion channels including the voltage-gated sodium channel (Nax-SCN7a) and epithelial sodium channel (ENaC) were identified using immunohistochemistry (anti-rabbit ENaC, anti-rabbit Nax, anti-mouse Abcam; GFAP, anti-mouse NeuN Novus) and semi-quantified using QPCR

Primers: NaxF-AACTCGAGAGGACCTGAACC, NaxR-TTTTATCAAAGCTCTCAGAA CCAC; ENaCF-ACTGTCTGCACCCTTAATCCTT ENaCR-GTGATGCGGTCCAGCTCT.

Only a modest increase in systolic BP (HSD, 130±2 versus LSD, 126±3 mmHg, P=0.07, n=6) was noted after two weeks of HSD. The HSD rats demonstrated increased (Na<sup>+</sup>) and Ouabain in the CSF (P<0.05) and hypothalamus (P<0.05) with no effect on CSF osmolality or plasma Ouabain. Nax immunostaining in the PVN (hypothalamus) showed no effect on the cellular phenotype between HSD and LSD. Nax immunostaining was not co-localized with NeuN (neuron-marker) or GFAP

(astrocyte marker) immunostaining. Relative quantitative PCR demonstrated a 2 fold increase in hypothalamic Nax mRNA and ENAc mRNA in HSD versus LSD. Nax mRNA expression was positive correlated with the systolic BP ( $r=0.62$ ,  $P<0.05$ ). In conclusion, hypothalamic sodium channels may be involved in salt sensitivity, highlighting recent finding that HSD cause sodium imbalance in the hypothalamus which long term recruit pro-hypertensive via sympathoexcitatory neural mechanism.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB250

### Investigating the role of the apelinergic system in the central control of blood pressure

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The neuropeptide apelin and its G-protein coupled receptor, APJ, are expressed in numerous autonomic brain regions, including the parvocellular nucleus of the hypothalamus (PVN) and rostral ventrolateral medulla (RVLM), and are thought to play a role in modulating arterial blood pressure (ABP) by altering sympathetic nerve activity (SNA). Exogenous application of apelin to the PVN and RVLM of anaesthetised rats elevates ABP and SNA, while increased apelin gene expression is observed in the RVLM of spontaneously hypertensive rats (SHRs). This suggests that dysfunction in the central apelinergic system could contribute to the pathophysiology of hypertension. We investigated the role that central APJ plays in the basal control of ABP in the normotensive Wistar Kyoto (WKY) and in the progression of hypertension in the SHR. Values are mean  $\pm$  SEM, compared by unpaired t-test or one-way ANOVA. Relative expression of the gene for APJ (*aplnr*) was elevated in the RVLM of SHR compared to WKY ( $4.2 \pm 1.1$  vs  $1.0 \pm 0.2$ ;  $p<0.05$ ;  $n=8$ ) and exogenous application of [Pyr<sup>1</sup>]apelin-13 to the RVLM of sodium pentobarbital ( $50\text{mg.kg}^{-1}$  i.p.) anaesthetised rats caused a greater increase in mean ABP (MABP), recorded via femoral artery catheter, in SHR compared to WKY ( $20 \pm 2\text{mmHg}$  vs  $10 \pm 2\text{mmHg}$ ;  $p<0.01$ ;  $n=8$ ) thus suggesting an enhancement of the apelinergic system in hypertensive rats. To further investigate this WKY ( $n=12$ ) and SHR ( $n=12$ ) were chronically implanted with blood pressure radiotelemeters under isoflurane anaesthesia (2% in O<sub>2</sub>). After a 5-day baseline period rats were anaesthetised with a mixture of ketamine ( $60\text{mg.kg}^{-1}$ ) and medetomidine ( $250\mu\text{g.kg}^{-1}$ , both i.m.) prior to bilateral RVLM microinjection with a lentiviral APJ-specific-shRNA construct. 25 days after lentivirus injection, RVLM *aplnr* expression was decreased in APJ-shRNA-injected compared to scrambled-shRNA-injected rats ( $0.5 \pm 0.1$  vs  $1.1 \pm 0.1$ ;  $p<0.001$ ;  $n=24$ ) and increases in MABP associated with exogenous injection of [Pyr<sup>1</sup>]apelin-13 to the RVLM were abolished ( $10 \pm 1\text{mmHg}$  vs  $-2 \pm 1\text{mmHg}$ ;  $p<0.001$ ). 25 days after virus injection MABP was unchanged relative to baseline in scrambled- and APJ-shRNA-injected WKY ( $2 \pm 1$  vs  $3 \pm 7\text{mmHg}$ ) and SHR ( $10 \pm 4$  vs  $7 \pm 8\text{mmHg}$ ). Additionally, spectral analysis of BP revealed no changes in low frequency systolic BP variability between scrambled- or APJ-shRNA-injected WKY or SHR, thus suggesting that sympathetic vasomotor tone was

unaffected by RVLM *aplnr* knock-down. Therefore, these data imply that endogenous apelin, acting via APJ in the RVLM, does not play a role in maintaining basal BP in WKY rats or in the genesis of hypertension in the SHR.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB251

### The role of the alanine-Serine-Cysteine-1 transporter (SLC7A10, Asc-1) in glycinergic transmission in the pre-Bötzinger Complex

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Mice, which lack the Alanine-Serine-Cysteine-1 transporter (SLC7A10, Asc-1), show reduced glycinergic synaptic transmission although the exact mechanism remains unclear. We conducted whole-cell voltage-clamp recordings and biochemical experiments to test the role of Asc-1 in glycinergic transmission in the pre-Bötzinger complex, the medullary core network that is critical for breathing. Using both, a transportable Asc-1 inhibitor (D-isoleucine) and the non-transportable antagonist (Lu AE00527), we found that D-isoleucine reduces glycinergic transmission and increases glycine release, whereas Lu AE00527 has no acute effect. Furthermore, D-isoleucine increases the frequency and reduces amplitude of the phrenic nerve activity in arterially perfused working heart brainstem preparation. Based on these results, we propose that Asc-1 is modulating the presynaptic levels of glycine and other small amino acids like D-serine using its hetero-exchange antiporter activity.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB252

### Synaptic expression of interleukin-6 receptors and dystrophin may indicate a role in cognitive deficits associated with loss of dystrophin.

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The symptoms of Duchenne Muscular Dystrophy (DMD), a heritable X-linked recessive disorder which affects approximately 1 in 3500 live male births, are due to loss of the cytoskeletal protein, dystrophin. Dystrophin has a crucial role in protecting skeletal muscle from contraction-induced damage but is also expressed in neurons of some brain regions, such as the hippocampus. An absence of dystrophin can result in dysfunctional synapses in both DMD boys and the dystrophin-deficient *mdx* mouse, and DMD patients exhibit significant deficits in cognitive function (1). Chronic inflammation in DMD is associated with elevated circulating levels of the pro-inflammatory cytokine, interleukin-6. Although typically found at low levels in the healthy brain, IL-6 can be elevated in diseased brains where cognitive function is also impaired (2, 3). The aim of this study was therefore to determine if



IL-6 receptors (IL-6Rs) are co-expressed with dystrophin at hippocampal synapses. If so, this may indicate a role for IL-6 in dysregulated synaptic transmission associated with loss of dystrophin.

Dissociated hippocampal neurons (3-5 day old) from dystrophin-expressing C57BL/6 mice were cultured for 10-12 days in culture medium with or without supplemental recombinant IL-6 (1nM). Immunofluorescence and confocal microscopy were used to visualise fixed hippocampal neurons labelled with antibodies against IL-6 receptors, synaptophysin and dystrophin (n=3 cultures). RT- qPCR was used to compare gene expression of IL-6 and IL-6R in WT and *mdx* hippocampal tissue (n=10 per group).

IL-6R expression was present in cell bodies and processes of cultured hippocampal neurons and was co-localised with the pre-synaptic marker, synaptophysin. Incubation with supplemental IL-6 resulted in clustering of IL-6Rs, particularly within neuronal somata. Dystrophin expression was also evident in synaptophysin-expressing clusters but was unaffected by incubation with IL-6. Although peripheral IL-6 is elevated in both DMD patients and *mdx* mice, we did not find any differences in the gene expression of IL-6 ( $p>0.05$ ) or IL-6R ( $p>0.05$ ) between our WT or *mdx* hippocampal tissue.

These findings indicate that pre-synaptic IL-6Rs may interact with dystrophin and contribute to synaptic transmission in hippocampal neurons. Although we did not detect differences in overall hippocampal IL-6Rs in WT and *mdx* tissue using RT-qPCR, further studies examining synaptic expression of IL-6Rs in dystrophin-deficient *mdx* mouse hippocampal neurons may help to elucidate the possible role of this cytokine in dystrophin-deficient associated cognitive dysfunction.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

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PCB253

### **Role of the $\text{Ca}_v\beta$ and the actin cytoskeleton on synaptic transmission in mouse hippocampal neurons**

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Activation of voltage-gated calcium channels ( $\text{Ca}_v$ ) initiates neurotransmitter release. The neuronal  $\text{Ca}_v$  core complex is formed by the  $\text{Ca}_v\alpha_1$  ion-conducting and the  $\text{Ca}_v\beta$  subunits. It is well established that the association of  $\text{Ca}_v\beta$  with  $\text{Ca}_v\alpha_1$  up-regulates calcium currents and that  $\text{Ca}_v\beta$  interacts with several other partners fulfilling  $\text{Ca}_v$ -independent functions. Most recently, we reported that  $\text{Ca}_v\beta$  associates directly with actin filaments that in synaptic terminals appears to organize synaptic vesicle clusters. Here, we investigated the role of  $\text{Ca}_v\beta$ , type 4b, and actin cytoskeleton on synaptic transmission in hippocampal neurons from newborn

(postnatal day P0-P3) mice using electrophysiological methods in autaptic and continental cultures. We found that exogenously expressed  $\text{Ca}_v\beta_{4b}$  enhances spontaneous and evoked postsynaptic currents without altering synaptogenesis. Moreover,  $\text{Ca}_v\beta_{4b}$  increases the readily releasable pool (RRP) size of synaptic vesicles at rest conditions, assessed by hypertonic sucrose application, and accelerates their recovery time after depletion. Either inserting a double mutation in the  $\text{Ca}_v\beta_{4b}$  that inhibits its association with  $\text{Ca}_v\alpha_1$  or disrupting pharmacologically the actin cytoskeleton abolishes the potentiation of synaptic strength induced by  $\text{Ca}_v\beta_{4b}$ . Thus,  $\text{Ca}_v\beta_{4b}$  relies on an intact actin cytoskeleton to facilitate the mobilization of synaptic vesicles to a fusion competent state.

However, a  $\text{Ca}_v\beta_{4b}$  mutant with impaired binding to  $\text{Ca}_v\alpha_1$  does not alter the time course of the RRP recovery but preserves the ability to increase the RRP size suggesting an alternative path to mobilize synaptic vesicles to the RRP at rest conditions from a pool loosely coupled to the actin cytoskeleton. Altogether our results demonstrate that an interplay between  $\text{Ca}_v\beta$  and F-actin support synaptic transmission and uncover a new function of the  $\text{Ca}_v\beta$  that is independent of its association state with  $\text{Ca}_v\alpha_1$ .

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

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PCB254

### **Re-Organisation Of Frequency Discrimination In The Cochlear Tonotopy.**

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Frequency selectivity is a functional feature of the inner ear and this feature has not been fully resolved since the beginning of hearing studies (Manley and van Dijk 2016). Frequency selectivity is defined as a combination of passive cochlear mechanics due to travelling wave motion proposed by von Békésy (Békésy, 1960) and active processes evaluated by outer hair cells proposed by Dallos (Dallos, 1996). Psychoacoustic frequency selectivity is that resolution is sharper than mechanically travelling wave motion along the cochlea (Evans, 1975). In our study, reorganization of the frequency distribution in cochlear tonotopic which an important product/output of frequency selectivity was evaluated at electrophysiological, ultrastructural and molecular level. Following electrophysiological tests, 30 guinea pigs (60 ears) with auro-palpebral reflexes were randomly assigned to three groups, one control group and two study groups. All measurements were performed with the guinea pigs under general anesthesia achieved by an intramuscular injection of ketamine (50 mg/kg) and xylazine (10 mg/kg). At 1 and 8 kHz frequencies, an acoustic trauma was generated by giving a sound stimulus of 110 dB HL for 10 minutes. Frequency specific hearing loss was assessed by Distortion Product Otoacoustic Emission (DPOAE) and Auditory Brainstem Response (ABR) tests. For ultrastructural evaluation, temporal bones (Figure 1) were dissected and stereocilia morphology of outer hair cells (DTH) was evaluated. In the dissected temporal bones, the cochlea was homogenized and the gene expression

of Glu2/3 receptors was examined as a segment. As a result of electrophysiological tests pure tone acoustic trauma was performed at frequencies of 1 and 8 kHz. In ultrastructural evaluations and molecular analyzes, a heterogeneous distribution was observed in which OHC degeneration in certain segmental regions was not observed after acoustic trauma Glu2/3 receptors did not increase, and DTH degeneration and Glu2/3 receptors increased in different segmental regions due to wave propagation along the tonotopic axis of the cochlea. In our study, we suggest that basilar membrane movements play secondary role whereas outer hair cell electromotility provides primary mechanism in the cochlear analysis of sound. Thus, tonotopic map of cochlea should be reorganized in the light of these new findings.



The otic capsule in the tympanic cavity (Magnification 15×1.5×11.2)

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PCB255

### Mechanism of calcium-dependent activator protein for secretion 1 and 2 differential subcellular localization in dorsal root ganglion neuron

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The calcium-dependent activator protein for secretion (CAPS) is an established priming factor for large dense core vesicles (LDCVs) and has been reported to prime synaptic vesicles (SVs) as well [1, 2]. It is a multi-domain protein consisting of two family members CAPS1 and CAPS2. Recently, we showed that in dorsal root ganglion (DRG) neurons CAPS1 exclusively

mediates SV exocytosis, while CAPS2 only promote neuropeptide release from LDCVs. We further showed that their differential function is due to distinct localization. In fact, CAPS1 is highly enriched at synapses while CAPS2 has a more diffuse mainly somatic localization. To establish and maintain this differential localization, the subcellular transport mechanism of both CAPS paralogs must differ and be tightly controlled. In the peripheral nervous system, cell soma and synaptic contacts are separated by a great distance, which poses a challenge for synaptic targeting of cytoplasmic proteins. Active zone proteins overcome this problem by their association with so-called piccolo/bassoon transport vesicles. We propose that CAPS1 but not CAPS2 is able to bind to these vesicles to be directed to synapses. We investigated this question by targeted mutagenesis of CAPS2. We verified that our mutant CAPS2/CAPS1 was correctly expressed and functional. Using immunocytochemistry together with confocal microscopy we could show that the CAPS2/CAPS1 chimera protein was re-localized from the soma to synapses (Fig. 1). With this approach we identified a specific domain responsible for CAPS1 synaptic localization.

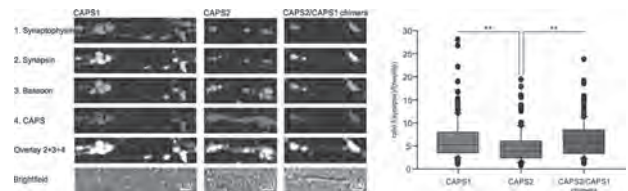


Figure 1: Differential CAPS2/CAPS1 localization to synapses. A: CAPS1, CAPS2 and CAPS2/CAPS1 chimera were attached to HaloTag and expressed via lentivirus. In addition, DRG neurons were infected with a lentivirus encoding for Synaptophysin-pHluorin to enable the recognition of DRG neurites. On DIV2 spinal cord neurons were added to the culture. Halo ligand chloralkane-atto590 labeling was performed at DIV6. On DIV9 cells were fixed and immuno-labeled against bassoon and synapsin. B: CAPS signal at synapses was quantified and normalized to the signal in adjacent areas of the neurites to account for variability in transfection efficiency. CAPS2 fluorescence intensity at synapses was significantly lower in comparison to CAPS1 and CAPS2-CAPS1 chimera ( $n = 182, 148$  and  $173$  for CAPS1, CAPS2 and CAPS2/CAPS1 chimera,  $p < 0.01$  ANOVA on rank with Dunn's post-test,  $N = 3$ )

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PCB256

### Spray-dried animal plasma supplementation attenuates aging neuroinflammation in SAMP8 mice

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Dietary supplementation with spray-dried animal plasma (SDP) has anti-inflammatory effects in several models of inflammation. In SAMP8 mice, a strain prone to senescence, SDP reduces brain neuroinflammation and oxidative stress and prevents the aging effects on short- and long-term memory (Miró et al, 2018). We have now studied if SDP can also improve neuronal integrity in senescent mice by reducing astrogliosis and microgliosis, both involved in the development of cognitive pathologies. Experiments were performed on male mice of the SAMP8 strain. The groups were: 2 months-old (young, 2M; 25 g, n=12) and 6 months-old (senescent) mice (32-34 g) fed either a Control diet (6M-CTL; n=12) or a diet containing 8% SDP (6M-SDP; n=12) for 4 months. Both diets were isoenergetic and isonitrogenous. Mice were anaesthetized by an i.p. injection of ketamine/xylazine (100/10 mg/kg). Brain tissue was perfused with 4% paraformaldehyde and stored at -80°C. Histological studies were carried out on 20 µm sections. Markers of astrogliosis (GFAP) and microgliosis (IBA and CD11b) were quantified by immunostaining. Digital fluorescence images were acquired by a SP1 confocal scanning laser microscope. The abundance of presynaptic marker synaptophysin (SYN), postsynaptic marker PSD95, and p-NF-kB were determined by Western blot. Endothelial adhesion molecules (*Vcam-1* and *Icam-1*) and *Tnf-α* expression were quantified by Real Time-PCR. Data were analyzed by one-way ANOVA. Senescence increased the expression (fold change) of neuroinflammation markers such as *Tnf-α* (2M: 1.0±0.3; 6M-CTL: 5.3±0.8, p=0.002, n=6) and p-NF-kB (2M: 1.0±0.1; 6M-CTL: 1.6±0.3, p=0.057, n=5) and adhesion molecules *Vcam-1* (2M: 1.0±0.2; 6M-CTL: 3.3±0.7) and *Icam-1* (2M: 1.0±0.2; 6M-CTL: 4.2±1.0), both p=0.015, n=5. The SDP-supplemented diet prevented in part neuroinflammation since the value of *Tnf-α* was 2.7±0.8 (p=0.026) and that of p-NF-kB of 0.9±0.1 (p=0.018). Aging decreased the abundance of SYN, from 1.0±0.1 (2M) to 0.7±0.1 (6M-CTL; p=0.037), indicating neural loss and this effect was prevented by SDP (1.1±0.1, p=0.012, n=6). Senescence increased the percentage of GFAP+ cells from 11.4±0.8% (2M) to 18±1.5% (6M-CTL, p=0.008 n=4), IBA1+ cells, from 3.7±0.3% (2M) to 4.9±0.3% (6M-CTL, p=0.033, n=4), and the mean fluorescence intensity of CD11b cells augmented 32% in senescent mice (2M: 99.1; 6M-CTL: 131.7±6.1, p=0.017, n=4). These effects of aging were also prevented by SDP (GFAP+, 11.3±1.5%, p=0.007; IBA+ cells, 3.6±0.3%, p=0.028; CD11b, 93.7±8.4, p=0.011). These results suggest that oral SDP supplementation can maintain neuronal integrity by reducing aging-associated neuroinflammation, astrogliosis and microgliosis.

Miró L, Garcia-Just A, Pérez-Bosque A, Polo J, Griñán-Ferré C, Pallàs M, Amat C, Moretó M (2018). Neuroprotective effects of dietary supplementation with spray-dried animal plasma in senescent mice (SAMP8), 11th FENS Forum of Neuroscience Meeting, Berlin, Germany (Ref. F18-3498)

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB257

### L-Arginine supplementation with Carbamazepine improves cognition in male Sprague-Dawley rats

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Oxidative stress has been linked to the development of cognitive impairment in various experimental models. Carbamazepine has been associated with cognitive impairment and oxidative stress. This study investigated the effects of add-on L-Arginine; an antioxidant on cognition and oxidative stress in male non-epileptic rats.

Adult male Sprague-Dawley rats from the colony in the animal facilities of the College of Medicine, University of Lagos, Idi-Araba campus were housed in groups of six per cage and were divided into 4 groups; (1) Control treated with distilled water., (2) L-Arginine treated with L-Arginine (100mg/kg BW) in distilled water., (3) Carbamazepine treated with carbamazepine (25mg/kg BW twice daily) in distilled water., (4) L-Arginine + Carbamazepine treated with L-Arginine and Carbamazepine as above for two weeks to assess the acute changes in cognition and oxidative stress markers. Carbamazepine (Tegretol) was obtained from Novartis Pharma AG, Basle, Switzerland while L-Arginine was obtained from Now Foods, Bloomingdale, USA.

All animals had free access to normal rat chow throughout the experiment. At two weeks of treatment, cognition was assessed using the Y maze. The rats were anaesthetized after cognition assessment with tribromoethanol (2, 2, 2-tribromoethanol, Aldrich), humanely sacrificed with the hippocampus and frontal lobes isolated from the whole brain and subsequently homogenized for assessment of oxidative stress markers (Catalase, superoxide dismutase (SOD), malondialdehyde (MDA), and reduced Glutathione (GSH)).

The number of arm entry and correct alternation were significantly higher (p < 0.05) in the L-Arginine and L-Arginine + Carbamazepine groups. In the frontal lobe, L-Arginine significantly increased (p < 0.05) catalase and GSH levels and no significant difference in SOD and MDA levels. Carbamazepine significantly increased (p < 0.05) SOD in the frontal lobe with no significant change in catalase and GSH levels. L-arginine + carbamazepine significantly (p < 0.05) increased glutathione levels and reduced SOD levels however there was no significant difference in the levels of catalase and MDA. In the hippocampus, L-Arginine significantly (p < 0.05) reduced MDA with no change in other parameters. Carbamazepine significantly reduced (p < 0.05) glutathione (GSH) levels but no significant change in catalase, SOD and MDA levels. SOD and MDA level were significantly lower (p < 0.05) in the L-Arginine + Carbamazepine group compared to other groups, GSH was also significantly higher (p < 0.05) in this group and catalase level was not significantly different in all the groups. Oral L-Arginine supplementation (100mg/kg BW) with carbamazepine improved performance on Y maze and also enhanced the cognitive process in the frontal lobe and hippocampus of male rats.



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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

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PCB258

**The peripheral nervous system *in vitro* - Characterization of a co-culture system with human derived neurones and Schwann cells**

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Peripheral neuropathy can be induced by inherited or acquired dysfunctions of the peripheral nervous system. The disease causing factors are largely unknown. Different cell types and cell-cell interactions, such as the axon-Schwann cell (SC) interaction, can be affected. In order to study these interactions in detail a reliable model is needed, which includes both neurones and SCs.

Progress in the differentiation of human induced pluripotent stem cells (hiPSCs) into different cell types has led to efficient protocols generating *human* sensory neurones and SCs (Chambers et al., 2012; Kim et al., 2017). Previous approaches to establish a co-culture were achieved in a heterologous system of rodent and human cells (Clark et al., 2017) and with long-term protocols using human embryonic stem cells derived sensory neurones and SCs (Ziegler et al. 2011).

By using a recently published protocol (Kim et al., 2017) we generate a homogeneous culture of hiPSCs derived SCs for co-culturing with hiPSCs derived sensory neurones, which we produce using a small molecule approach (Eberhardt et al., 2015, based on Chambers et al., 2012). The expression of typical SC markers, including S100, GFAP, p75 and Sox10, is approved by immunostaining and mRNA expression. We co-culture human SCs with iPSCs derived sensory neurones similar as described in Clark et al. 2017 for rodent SCs. Within an hour after adding SCs to iPSCs derived neurones, alignments of spindle-shaped SC-like cells to axonal structures are observed. To analyse the effect of SCs on sensory neurones in a co-culture system we study immunocytochemical characteristics and canonical markers in quantitative RT-PCR like MBP, Caspr and ion channel expression. In addition, we aim to study the electrophysiological aspects of nociceptors matured in the presence of SCs by using current clamp to measure action potential generation. This will help to explore whether SCs promote and accelerate the process of maturation of neurones.

By providing a human disease model for neuropathy which more faithfully follows physiology, we aim to explore underlying disease mechanisms and potentially to support the development of new drugs.

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## PCB260

### Foundations of neocortical high-fidelity synaptic transmission

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Highly reliable synaptic transmission between layer 5 pyramidal neurons (L5PNs) is thought to be crucial for neocortical feedforward excitation and ensemble formation (Frick et al., 2008; Markram et al., 1997). However, the synaptic foundations of this high reliability are largely unclear.

We analyzed these synapses in 3-4 weeks old C57Bl6 mice that were decapitated under deep Isoflurane inhalation anesthesia before excising the brain and cutting coronar slices of neocortical S1 region. In paired recordings from connected L5PNs we found strong depression of EPSC amplitudes (A) at 50 Hz (median A2/A1 = 0.78, 0.69 - 0.90 IQR, n = 86 pairs) and an exceptionally low initial synaptic failure rate ( $F_{\text{syn}} = 0, 0 - 0.04$ ) that does not significantly increase after 5 stimulations ( $F_{\text{syn}} = 0.07, 0.05 - 0.14$ , n = 24, P = 0.061, ANOVA on ranks). Using mean-variance analysis of EPSC amplitudes at different extracellular  $\text{Ca}^{2+}$  concentrations ( $[\text{Ca}^{2+}]_e$ ) we show that L5PN synapses operate at high vesicular release probability of 0.66 (0.55 - 0.71, n = 5) and 8 (3-19) release sites. Dual-dye two-photon  $\text{Ca}^{2+}$  imaging with the low-affinity  $\text{Ca}^{2+}$  indicator Fluo-5F and the  $\text{Ca}^{2+}$ -insensitive Alexa-594 revealed that  $\text{Ca}^{2+}$  influx into individual presynaptic terminals was small (129 nM, 79 - 219 nM, n = 41 cells) but highly reliable with no failures in single AP-induced  $\text{Ca}^{2+}$  influx and low trial-to-trial signal variability. The majority of the  $\text{Ca}^{2+}$  influx occurred through  $\omega$ -agatoxin-IVA (AgTx)-sensitive P/Q-type  $\text{Ca}^{2+}$  channels (64%, 50 - 66%, n = 11). Transmitter release was almost exclusively mediated by P/Q-type channels, since AgTx blocked EPSC amplitudes by 85% (82 - 88%, n = 7 pairs). Numerical simulation of influx-release coupling predicts a coupling distance of 32 nm. Indeed, transmitter release was insensitive to 10 mM EGTA since median EPSC amplitudes remained stable at 94% (71 - 102%, n = 5) of the baseline amplitude (P = 0.418 vs. 100%, t-test) after 40 min of presynaptic buffer application. This finding corroborates that the  $\text{Ca}^{2+}$  channels are tightly coupled to the  $\text{Ca}^{2+}$  sensor. We further investigated sustained synaptic transmission and vesicle replenishment during trains of stimuli at different frequencies. Cumulative EPSC amplitudes were plotted to estimate frequency-dependent reloading rates (at 50 Hz: 0.74 pA/ms, 0.62 - 0.84, n = 9) and the initial size of the ready-releasable pool (92 pA, 69 - 131 pA, n = 9; Schneggenburger et al., 1999).

From our data we conclude that the high reliability of sustained synaptic transmission between L5PNs is ensured at several stages translating electrical activity into transmitter release, ranging from highly reliable AP-mediated  $\text{Ca}^{2+}$  influx and high  $p_r$  of tightly coupled synaptic vesicles to rapid vesicle replenishment. These features allow L5PNs to reliably generate and spread neocortical oscillations (Sanchez-Vives and McCormick, 2000; Silva et al., 1991).

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Markram, H., Lubke, J., Frotscher, M., Roth, A. and Sakmann, B. (1997) 'Physiology and anatomy of synaptic connections between thick tufted pyramidal neurones in the developing rat neocortex', *The Journal of Physiology*, vol. 500, Pt 2, pp. 409-440.

Sanchez-Vives, M. V. and McCormick, D. A. (2000) 'Cellular and network mechanisms of rhythmic recurrent activity in neocortex', *Nature Neuroscience*, vol. 3, no. 10, pp. 1027-1034.

Schneggenburger, R., Meyer, A. C. and Neher, E. (1999) 'Released fraction and total size of a pool of immediately available transmitter quanta at a calyx synapse', *Neuron*, vol. 23, no. 2, pp. 399-409.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB261

### GABAergic modulation of cells within the neurogenic niche of the postnatal spinal cord

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Modulation of GABA mediated inhibition is one of the most important approaches for the treatment of CNS diseases. Precise targeting of such treatments depends on identification and characterisation of the different subunit complexes that exist. In the CNS, there are pools of neural stem cells (NSC), which can differentiate to become neurones, astrocytes or oligodendrocytes while progenitor cells have limited lineage. In addition to brain regions, where NSCs are now known to exist, the spinal cord area also has a neurogenic potential in the form of ependymal cells (ECs)<sup>1</sup>. Ependymal cells (ECs) around the central canal of the spinal cord respond to GABA<sup>2</sup>. One approach to develop pharmacological treatments to modulate stem cell activity is to determine how endogenous proteins and receptors on NSCs can regulate their behaviour. Responses to GABA were determined and compounds known to modulate GABA receptors at selective sites were tested using both bath and puff applications. Prolonged application of FGIN-1-27; 1  $\mu\text{M}$  (peripheral benzodiazepine (BZD) receptor (TSPO) agonist) significantly enhanced ( $P < 0.05$ ) responses to GABA in ECs. This may be due to the neurosteroidogenic effect of TSPO activation. Bath applications of octadecaneuropeptide (ODN), the endogenous ligand of the central BZD receptor alone caused both depolarising and hyperpolarising effects on ECs but did not significantly affect responses to GABA. Local ODN application caused fast hyperpolarisations that were mimicked by local application of low (1.25-2.5  $\mu\text{M}$ ) concentrations of GABA. To further characterise the GABA

receptor mediated responses, agonists and antagonists were used to enhance and block the receptor accordingly. Since TPMPA antagonised responses to ODN ( $P < 0.05$ ) and reduced the responses to low concentrations of GABA, this suggests that GABA receptors contain subunits from both GABA<sub>A</sub> and GABA<sub>C</sub>. Baclofen; 1  $\mu\text{M}$  & 5  $\mu\text{M}$  (a selective GABA<sub>B</sub> receptor agonist) also hyperpolarised ECs, an effect antagonised by CGP 55845 (GABA<sub>B</sub> antagonist), indicating a further role for GABA<sub>B</sub> receptors. To examine whether baclofen could affect the level of proliferation in acute spinal cord slices, the thymidine analogue 5-Ethynyl-2'-deoxyuridine (EdU) was added to the slices in the presence or absence of baclofen. The numbers of EdU-positive cells were significantly lower in baclofen treated slices ( $P < 0.05$ ) compared with control EdU-positive cells in central canal area. If cells within the central canal area respond to GABA, it is possible that following an injury or onset of pathological condition, GABA could be released in this area to modulate proliferation and differentiation. These experiments will help to confirm the role of GABA receptors in spinal cord cell proliferation.

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Corns, L.F. et al., 2013. GABAergic responses of mammalian ependymal cells in the central canal neurogenic niche of the postnatal spinal cord. *Neuroscience letter*. 553, pp. 57-62

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB262

### Aberrant Changes in Oxygen Levels in the Brain during Seizures in Awake Freely Behaving Rodents

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**Introduction:** Temporal lobe epilepsy (TLE) is a form of acquired epilepsy characterized as an electro-clinical syndrome in which seizures emanate from the limbic system (1, 2). TLE is particularly disabling due to the unpredictable and recurrent nature of seizures and incidence of antiepileptic drug resistance (3) many times an indication for resection surgery (4). Seizures are paroxysmal events in which increased neuronal activity is accompanied by an increase in localized energetic demand, which is followed by a hemodynamic response which overwhelms the tissue with energetic substrates such as oxygen and glucose. Both monitoring of electrical activity and hemodynamic response have been explored extensively in the identification of seizure onset zones as well as in epilepsy diagnosis. This underlies the importance of understanding brain energetic and cerebrovascular response during seizures, which we here address by concurrent recording of local field potential and brain tissue  $p\text{O}_2$  using high-frequency amperometric oximetry.

**Methods:** we used multisite Pt microelectrode arrays (MEA) (5) to perform high-frequency amperometric recording of  $p\text{O}_2$  and local field potential (LFP)-related currents in the CNS

of chronically implanted freely-moving rats during pilocarpine-evoked *status epilepticus*. Fast Fourier transform (FFT) filtering of the raw electrochemical signal allowed extraction of the low frequency component ( $< 1$  Hz), corresponding to the electrochemical reduction of  $\text{O}_2$  and high ( $> 1$  Hz) frequency component, corresponding to the LFP currents.

**Results:** We found that resting levels of  $p\text{O}_2$  in the rodent brain varied between  $6.63 \pm 0.74 \mu\text{M}$  in the DG region of the hippocampus and  $22.1 \pm 4.91 \mu\text{M}$  in the cerebral cortex. Induction of *status epilepticus* by intrahippocampal injection of pilocarpine produced typical and graded behavior correlate of temporal lobe seizures, from oro-alimentary automatisms to forelimb clonus, clonic extension and wild running and ultimately generalized tonic-clonic seizure. Reading of LFP currents in the hippocampus confirmed temporal lobe seizure with initial hyper-synchronization at low frequency followed by increased power at high frequency. Regarding interstitial levels of  $p\text{O}_2$ , we observed a biphasic change in the hippocampus. The initial dip at seizure onset ( $\Delta\text{O}_2 = -4.5 \pm 0.7 \mu\text{M}$ ) was followed by a prolonged hyperoxygenation phase ( $\Delta\text{O}_2 = +10.4 \pm 2.9 \mu\text{M}$ ). More interesting was the observation that the initial dip appeared to precede a more pronounced alteration in LFP, suggestion that increased oxidative metabolism in response to initial increase in neuronal firing may be a valuable feature in seizure prediction.

**Conclusions:** This strategy revealed that a single sensor can simultaneously report chemical ( $p\text{O}_2$ ) and electrophysiological (LFP currents) information, allowing concurrent monitoring of electrical and neurovascular/neurometabolic activities.

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PCB263

### Permethrin in low dose reduces excitability in murine peripheral sensory neurons

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Pyrethroid insecticides are a family of molecules commonly used for the control of insect pests. The most studied mechanism of pyrethroid action is their binding to the voltage-gated sodium channel (Navs) and the consequent inhibition of inactivation. This leads to neurotoxicity due to disruption of the neuronal conductivity. This mechanism of action works not only in the insect neural system but also in mammals. Nevertheless, the safety of pyrethroid use is ensured by the much higher sensitivity of insect channels to these compounds. Unfortunately, cases of acute pyrethroid intoxication are reported in human and pets, especially cats. The symptoms



range from slight burning and stinging sensations to tremor, seizures, coma, and death.

Beside heterologous expression systems, so far, only rodent dorsal root ganglia (DRG) *in vitro* models have been used to study pyrethroid actions (Soderlund 2012), therefore it is not known if species differences in mammal channels, as already described for a variety of insects, could also account for a difference in pyrethroid sensitivity of the Navs. We, therefore, assessed the mechanism of action of permethrin, a type I pyrethroid, on mouse DRGs and plan to compare it with a human *in vitro* model using human induced pluripotent stem cell (iPSC)-derived nociceptive neurons from healthy individuals. We isolated mouse DRG cells and measured the effect of 1  $\mu$ M permethrin applied in the extracellular bath solution on the action potential (AP) shape and frequency in current clamp. The AP threshold was significantly depolarized (permethrin treated cells (n=15)  $-34.34 \pm 5.85$  mV, vehicle-treated control cells (n=14)  $-41.06 \pm 6.93$  mV). The time from pulse onset to AP peak was significantly delayed from  $33.10 \pm 13.56$  ms in control to  $89.08 \pm 58.37$  ms in permethrin-treated cells, and the treated cells display a significantly reduced after-hyperpolarization ( $-63.34 \pm 6.40$  mV in control cells;  $-41.52 \pm 13.45$  in permethrin-treated cells). Supporting these unexpected findings, 1  $\mu$ M Permethrin reduced the firing rate of mouse DRGs, adding additional evidence for a reduction of excitability induced by this pyrethroid in the peripheral nervous system. Our current clamp data of mouse DRG are not easily explained with a simple mechanism of impairment of Nav inactivation. Therefore we hypothesize the contribution of another permethrin action mechanism on DRG cells, either on alternative Nav gating or on other ion channels such as potassium channels, which we aim to follow up. Furthermore, the human iPSC-derived nociceptive neurons model will be used in the future to assess if the same effect can be measured in human cells and possibly reversed using specific channel blockers. The application of permethrin on the human model will for the first time assess possible species differences of pyrethroid mechanisms of action, that can not be verified with the current rodent *in vitro* models.

Soderlund, David M. (2012): Molecular mechanisms of pyrethroid insecticide neurotoxicity. Recent advances. In: *Archives of Toxicology* 86 (2), S. 165–181. DOI: 10.1007/s00204-011-0726-x.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB264

## Ischaemic preconditioning induced tolerance in PC12 cells

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Background: Neuroprotection has received significant attention over the years with aims of limiting neurological damage following ischaemic stroke<sup>1</sup>. One such promising strategy is ischaemic preconditioning (IPC), where exposure to sub-lethal ischaemia can activate brain's own evolutionary endogenous mechanisms resulting in tolerance against subsequent ischaemic events<sup>2</sup>. This study was conducted to imitate the IPC neuroprotection in an *in vitro* ischaemic stroke model and elucidate the underlying mechanism by which IPC leads to neuroprotection.

Methods: An *in-vitro* model of ischaemic stroke was developed in a neuron-like PC12 cell line derived from rat pheochromocytoma. Oxygen-glucose deprivation (OGD) (2h,4h,6h, 0.3%

O<sub>2</sub>, glucose free) followed by re-oxygenation (24h) was used to mimic IPC before a subsequent OGD (6h). Cytotoxicity (LDH assay), Cell viability (MTT assay) and ATP concentration were measured compared to the controls. Flow cytometry studies of cells labelled with Annexin-V and 7-AAD were used to determine apoptosis and necrosis. Quantitative real-time PCR was performed to study gene expression. Significance was determined using one-way ANOVA, followed by Tukey's post-hoc analysis.

Results: Cytotoxicity (LDH assay) was significantly reduced in cells subjected to OGD-preconditioning (OGD-PC) for 4h ( $29.34 \pm 2.44$ , n=120) and 6h ( $23.60 \pm 2.56$ , n=120) in comparison to non-IPC cells ( $39.09 \pm 1.84$ , n=120) ( $P < 0.05$ ). Cell viability (MTT assay) was significantly ( $P < 0.05$ ) increased from  $24.39 \pm 2.56$  (n=120) in non-IPC cells to  $43.23 \pm 2.33$  (n=120) and  $44.26 \pm 5.72$  (n=120) in cells exposed to 4h and 6h of OGD-PC respectively. Both 4h and 6h of OGD-PC increased ATP concentration significantly ( $P < 0.05$ ) [ $10.1 \pm 0.04 \times 10^6$  moles / well (n=88) and  $11.2 \pm 0.02 \times 10^6$  moles / well (n=88)] compared to non-PC OGD [ $4.11 \pm 0.04 \times 10^6$  moles / well (n=88)]. In cells subjected to 4h OGD-PC ( $16.65 \pm 3.24$ , n=3) and 6h OGDPC ( $11.55 \pm 3.06$ , n=3), significant reductions in early apoptosis were seen in comparison to non-PC OGD cells ( $27.76 \pm 5.27$ , n=3) ( $P < 0.05$ ). No significant difference was observed in the necrotic cell percentage. Gene expression studies revealed significant ( $P < 0.05$ ) fold change in BNIP3 ( $1.56 \pm 0.02$ , n=3), Bcl-2 ( $18.71 \pm 0.06$ , n=3), VEGF ( $9.35 \pm 0.01$ , n=3), PHD-2 ( $2.98 \pm 0.06$ , n=3) and GLUT-1 ( $20.94 \pm 0.06$ , n=3) genes in cells exposed to 6h OGD.

Conclusion: This study has shown that *in-vitro* PC-12 cell model is a useful tool to study molecular mechanisms underlying IPC neuroprotection, reflected by increasing in cell viability and ATP production, as well as decreasing in cytotoxicity and suppressing apoptosis. This study indicates that IPC induced tolerance is associated with the increasing expression of a panel of hypoxic genes, such as VEGF (promoting angiogenesis)<sup>3</sup>, GLUT-1 (glucose transporter)<sup>4</sup> and BNIP3 (an inducer for apoptosis and protective autophagy)<sup>5</sup>.

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PCB265

### A classic updated - the sodium hypothesis of action potential generation

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I wholeheartedly agree with the view of Stephen Kuffler that students should consult original sources (Kuffler, 1972), since by the time such experimental information has reached textbooks it has inevitably been diluted, interpreted and edited to the extent that the original intentions of the researchers may have been obscured or lost (e.g. Purves *et al.*, 2008; Bear *et al.*, 2015). For students of membrane excitability interested in the classic Hodgkin and Huxley era of the 1940s and 1950s, there is a rich history of such information, a considerable amount of which is freely available via The Physiological Society website. However there is one major impediment that stands in the way of students' appreciation of these papers: the conventions and the associated terminology differ from those used today, and can be confusing even for the experienced researcher. This stems from the fact that the accepted norm in physics at the time was that an inward movement of +ve charge was depicted as an upward current deflection, with the resulting change in voltage depicted as a downward deflection (Patton, 1982). Using the classic Hodgkin and Katz 1949 paper to illustrate such difficulties we find that the resting membrane potential was regarded as a positive quantity with the action potential (AP) a negative deflection, thus the AP in this, and the subsequent classic papers by Hodgkin & Huxley, was displayed as an upward deflection with the y-axis defined as  $-V$ . Modern practice deviates from this convention and shows inward current as a downward deflection and membrane potential as "positive-up, negative-down". The modern convention depicts an electrode inserted into a cell submerged in a bath, with the amplifier connected to ground which is 0 mV, and a resting membrane potential ( $E_m$ ) of about -70 mV. The convention used by Hodgkin & Katz is based upon a reference system where changes in  $E_m$  were considered relative to rest, with increases or decreases, respectively, being equivalent to hyperpolarization or depolarization, respectively. This was due to technical considerations, since the junction potentials created by switching from seawater to the test solutions in the recording cell made accurate measurements of the potential difference across the axon membrane unreliable. Another source of confusion are their references to deviations of  $E_m$  from rest being variously described as 'depression, decrease, diminution, raising, larger, reduce, lowered', each description being context dependent.

Using the Hodgkin and Katz paper as a reference I describe how such papers can be updated to the modern convention without altering their meaning or content. This process involves not only substituting the appropriate modern terminology but in some instances redrawing figures and altering axes.

Kuffler SW. (1972). Introduction. In *Cellular Neurophysiology*, ed. Cooke I & Likin M, pp. 1-3. Holt, Reinhart & Winston, Inc., New York.

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Hodgkin AL & Katz B. (1949). The effect of sodium ions on the electrical activity of the giant axon of the squid. *J Physiol* 108, 37-77.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB266

### Investigation of NMDA receptor modulation by adenosine A2A and D2 dopamine receptor activation in rat substantia nigra dopaminergic neurones.

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Substantia nigra (SN) dopaminergic neurons degenerate in Parkinson's Disease (PD). Ropinirole, a D2 receptor agonist is currently used in PD therapy and D2 receptor activation has been shown to decrease NMDA responses in hippocampus and striatum. SN dopaminergic neurons express D2 autoreceptors and somatodendritically released dopamine mediates a negative feedback via D2 receptors on dopaminergic neuron activity. Via cAMP signalling,  $G_{as}$ -coupled adenosine A2A receptors (A2ARs) have the potential to counteract modulation by  $G_{ai}$ -coupled D2Rs. Because cAMP regulates protein kinase A, an enzyme that phosphorylates NMDARs and increases NMDAR responses, these mechanisms have the potential to regulate NMDAR signaling. Here we tested the effects of D2R activation with ropinirole, and A2AR activation with CGS-21680.

Whole-cell patch clamp recordings were made from DAergic neurons in the SNc of acute midbrain slices from neonatal (P7, P21 and P28) rats. Dopaminergic neurons were identified by the presence of a prominent hyperpolarisation-activated inward current (In P7 rats, amplitude,  $173 \pm 34$  pA; activation time constant,  $797 \pm 113$  ms; mean  $\pm$  S.E.M.,  $n = 11$ ) in response to a voltage step from -60 to -120 mV. In each cell, two successive responses to 20 mM NMDA with 10 mM glycine were recorded. In P7 rats, control experiments gave NMDA responses of  $919 \pm 89$  pA (1<sup>st</sup> response) and  $881 \pm 113$  pA,  $n = 11$  (2<sup>nd</sup> response) while in P28 rats the control NMDA responses were  $790 \pm 110$  pA (1<sup>st</sup> response) and  $800 \pm 93$  pA,  $n = 10$  respectively. In P7 or P28 rats, upon application of 200 nM ropinirole, the steady state NMDA current was not significantly changed suggesting D2R activation may not modulate NMDARs in neonatal rat SNc. The A2AR agonist, CGS-21680 (1 or 10 mM) was applied in absence or presence of ropinirole to P28 dopaminergic neurons to determine the effect of activating this  $G_{as}$ -coupled receptor which could be in a D2R-A2AR heteromeric complex. 1 mM CGS-21680 gave a  $46 \pm 16\%$  increase in NMDA response ( $P = 0.05$ , paired t-test;  $n = 12$ ) while in the combined presence of 10 mM CGS-21680 and 200 nM ropinirole, the NMDA current increased by  $59 \pm 15\%$  ( $P = 0.002$ , paired t-test;  $n = 16$ ). These data suggest D2Rs do not modulate dopaminergic neuron NMDA receptors, at least under the conditions of these experiments. It is possible a D2 effect might be observed in older rats. Furthermore, limitations involved in the whole-cell patch-clamp configuration,

such as dilution of cellular components could affect the result. In contrast, activation of A2A receptors enhanced the NMDA response.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCB267

### Metabolically induced changes of nerve conduction velocity of spinal fibers *in vivo* in mice

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The conduction velocity of a nerve fiber is not constant but may be modified. We now investigated the influence of acute metabolic changes on nerve conduction velocity (NCV) of spinal fibers in mice *in vivo*. We developed an electrophysiological set-up for *in vivo* recording of CNS fibers in the fasciculus gracilis of the spinal dorsal column in anesthetized mice (initially injection of pentobarbital 80 mg/kg intraperitoneal, continuance intravenously with methohexital 40-60 mg/kg/h; artificially ventilated; ECG, core body temperature and blood O<sub>2</sub> saturation permanently monitored for control of the anesthetic state; cf. P. Dibaj and E. D. Schomburg 2017<sup>1</sup>) and measured NCV under different metabolic conditions: a) under low and high anesthetic state indicated by relatively high and low heart rate, respectively; b) under normoxia and hypoxia. Increasing the anesthetic state as well as decreasing the oxygen saturation in the blood led to a decrease of NCV indicating an immediate influence of acute metabolic changes on the speed of signal propagation in spinal nerve fibers. The decrease of NCV under the above mentioned different systemic metabolic challenges was rescued by local supply of lactate which was already shown to be secreted by glial cells and metabolized by axons for ATP generation. Metabolic support of axons by glial cells is impaired in ALS, which considerably contribute to the pathophysiological progress of axonal damage during the course of the disease. In the ALS mouse model SOD1G93A, we observed an improvement of the decreased NCV in clinically affected mice after lactate supply. Thus, local energy supply with lactate leads to a compensation of metabolically induced changes of the NCV of spinal fibers *in vivo*, not only under acute systemic impairment like high anesthesia or hypoxia but also in the chronic paradigm of ALS. It will be discussed if the supplied lactate increases the availability of ATP in axons. The increased ATP would then improve the activity of the ion channels of the metabolically impaired axons leading to an accelerated depolarization and thus to an increased NCV.

<sup>1</sup> P. Dibaj and E. D. Schomburg; *In vivo* recording of nerve conduction velocity of spinal CNS fibers in the mouse. *Physiol Res.* 2017; 66:545-548.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCB268

### Comparison of high frequency monopolar and bipolar direct cortical stimulation protocols for motor mapping during neurosurgeries.

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Direct Cortical Stimulation (DCS) is considered the gold standard technique for the intraoperative functional mapping of motor areas. The stimulation protocols of DCS used world-wide vary in terms frequency of stimulation (low vs high) and monopolar vs bipolar (Kombos et al, 1999). The present study was done to compare the efficacy of monopolar and bipolar high frequency stimulation protocols (Kovac et al, 2014).

41 Patients (Age: 35.13±12.69 yrs; 25 Males, 16 Females) undergoing neurosurgery with lesion at/near the motor area (34 glioma, 1 meningioma, 2 glioblastoma, 4 intractable epilepsy) were recruited for the study. Pre-operative motor power assessment was done according to Medical Research Council (MRC) scale one day prior to surgery. Intraoperative motor mapping was performed with monopolar and bipolar stimulation probes successively delivering trains of 8 square wave 100µs duration biphasic pulses at the rate of 275p/s. The stimulation strength was started at 5 mA with incremental steps of 1 mA till a response was recorded or a maximum of 30 mA was reached. The motor evoked responses were recorded with paired needle electrodes placed in muscles on the side contralateral to cortical lesion. Post-operative assessment of motor power was done on the day of the surgery and again after 4 weeks. Statistical Analysis was done on the stimulation and recording data using GraphPad Prism v5 (GraphPad Software, Inc, California, USA).

Motor cortex was identified in 28 patients and could not be identified in exposed area in 13 patients even at 30 mA. In all 28 cases, the monopolar stimulation was successful while only in 14 cases mapping could be done with bipolar stimulation along with monopolar. The threshold current intensity with monopolar stimulation was 10 mA (7.75-18.00) and with bipolar stimulation was 18.50 mA (10.00-28.50), p<0.05. Data is expressed as Median (with IQR).

High frequency protocol with monopolar stimulation requires less stimulation current. (Szelényi et al, 2011). Monopolar high frequency stimulation should be preferred over bipolar high frequency stimulation for identification of motor cortex.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*



## PCB269

**The role of interstitial  $K^+$  in stimulating lactate release from adult mouse optic nerve**A.M. Brown<sup>1</sup>, L.R. Rich<sup>1</sup> and B.R. Ransom<sup>2</sup><sup>1</sup>*School of Life Sciences, University of Nottingham, Nottingham, UK* and <sup>2</sup>*Neurology, University of Washington, Seattle, WA, USA*

It is accepted that glucose is the primary energy support of the brain, but what is contentious is the form of glucose that neural elements use: glucose or glucose-derived metabolites? Neurones contribute 80% of brain metabolic rate, but astrocytes consume up to 60% of brain glucose. This dichotomy is resolved if we accept that glucose is converted to a conduit, lactate, within astrocytes that is released into the interstitial space for neuronal metabolism. A broad conclusion is that astrocytes are primarily glycolytic whereas neurones are primarily oxidative. We demonstrate that lactate is released from the mouse optic nerve (MON), and that stimulus evoked activity leads to elevations in  $[K^+]_o$  and  $[lac^-]_o$ . The logarithmic relationship between  $[K^+]_o$  and  $[lac^-]_o$  and the stimulatory effects of barium in the absence of elevated  $[K^+]$  supports the notion that it is the astrocyte membrane potential that dictates lactate release.

All procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986 under appropriate authority of project and personal licenses. Adult male CD-1 mice were killed by cervical dislocation and decapitated. Optic nerves were dissected, placed in a perfusion chamber, superfused with aerated aCSF containing 10 mM glucose. The stimulus evoked compound action potential was evoked with supra-maximal stimuli. Lactate biosensors (sarissa.co.uk) recorded  $[lac^-]_o$  at the glial pia boundary and  $K^+$  sensitive microelectrodes measure interstitial  $[K^+]$ .

We sought to establish the relationship between  $[lac^-]_o$  and  $[K^+]_o$  by altering aCSF  $[K^+]$ . aCSF containing increasing  $[K^+]$  resulted in elevations in  $[lac^-]_o$  compared to aCSF containing 3 mM. The relationship between aCSF  $[K^+]$  and  $[lac^-]_o$  was logarithmic. We used the  $K^+$  channel blocker barium to depolarize astrocytes, causing a sustained  $[lac^-]_o$  elevation. We quantified the relationship between stimulus intensity and  $[lac^-]_o$  by imposing 2-minute periods of stimulus ranging in intensity from 2 to 60 Hz, which produced stimulus frequency dependent increases in  $[lac^-]_o$  and  $[K^+]_o$ , which peaked at about 50 Hz. Stimulating the nerve with high intensity bursts of action potentials evoked increases in  $[lac^-]_o$  that attenuated with subsequent identical stimuli.

Our data provides convincing evidence of an intimate link between interstitial  $[K^+]$  and lactate release from astrocytes in MON. The relationship provides a means by which neural elements can signal to astrocytes their metabolic requirements. All neural elements release  $K^+$  in response to increased firing in a frequency dependent manner, and astrocytes are exquisitely sensitive to interstitial  $[K^+]$ , their membrane potential responding in a Nernstian manner to fluctuations in  $[K^+]_o$ . Thus astrocytes are uniquely equipped to respond rapidly to activity induced fluctuations of  $[K^+]_o$  and release lactate for use by neural elements as substrate.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## PCB270

**Long-term treatment of live HEK293 cells expressing  $\delta$ -opioid receptor with therapeutic concentration of lithium results in decrease of receptor level and attenuation of its function**L. Roubalova<sup>1</sup>, M. Vošahlíková<sup>1</sup>, H. Ujčíková<sup>1</sup>, J. Brejchová<sup>1</sup>, M. Alda<sup>2,3</sup> and P. Svoboda<sup>1</sup><sup>1</sup>*Department of Biomathematics, Institute of Physiology of the Czech Academy of Sciences, Prague 4, Czechia*, <sup>2</sup>*Dalhousie University, Halifax, NS, Canada* and <sup>3</sup>*National Institute of Mental Health, Klecany, Czechia*

Part of the biochemical research in the area of lithium (Li) therapies of mood disorders was focused on interference with signaling initiated by GPCR. Although opioids were traditionally associated with pain and opioid dependence, an increasing evidence links the opioid receptors (OR) and OR-mediated activation of G proteins to the psychiatric disorders. The functional state of  $\delta$ -OR signaling in live cells exposed to a therapeutic concentration of Li for a prolonged period of time (weeks) was tested in this work together with a biophysical state of plasma membrane (PM). HEK293 cells stably expressing  $\delta$ -OR were cultivated in the presence or absence of 1 mM LiCl for 7 (L7 Li-treated, C7 control cells) or 21 days (L21 and C21) and three independent PM preparations were isolated from each of four types of cells. Level of  $\delta$ -OR in PM was determined by specific radioligand [<sup>3</sup>H]DADLE binding and immunoblot assays; the functional coupling between  $\delta$ -OR and G proteins was determined as DADLE-stimulated high-affinity [<sup>35</sup>S]GTP $\gamma$ S binding. Li interaction with PM was characterized by fluorescent probes DPH, TMA-DPH and Laurdan. Values are means $\pm$ SEM, compared by unpaired Student's t-test (triplicate for each preparation). Li-treated cells exhibited the decreased amount of  $\delta$ -OR. This was evidenced by both [<sup>3</sup>H]DADLE binding (L7 83 $\pm$ 3%,  $p < 0.05$ ; L21 83 $\pm$ 3%,  $p < 0.05$ ; appropriate controls 100%) and immunoblot assays (L7 69 $\pm$ 6%,  $p < 0.01$ ; L21 60 $\pm$ 3%,  $p < 0.01$ ). Relatively high stimulation of the basal level of [<sup>35</sup>S]GTP $\gamma$ S binding by 10<sup>-4</sup>M DADLE in C7 ( $p < 0.05$ ) was diminished in L7 ( $p > 0.05$ ). The same result was found when C21 ( $p < 0.05$ ) were compared with L21 ( $p > 0.05$ ). The steady-state anisotropy of DPH and TMA-DPH fluorescence determined in PM isolated from Li-treated cells was not significantly different when compared with controls ( $p > 0.05$ ). FLIM studies in live cells indicated that Li treatment did not significantly alter the lifetime of TMA-DPH fluorescence ( $p > 0.05$ ). Analysis of the biophysical state of PM by Laurdan generalized polarization (GPex) indicated significantly higher values of this parameter in PM prepared from Li cells when compared with controls. The average value of GPex at 25°C in PM prepared from L7 cells (0.216 $\pm$ 0.001,  $p < 0.01$ ) was significantly higher than in C7 (0.185 $\pm$ 0.001). The same result was obtained in PM prepared from L21 (0.175 $\pm$ 0.001,  $p < 0.01$ ) and C21 (0.145 $\pm$ 0.002) and confirmed at 40°C. We conclude that prolonged exposure of  $\delta$ -OR-HEK293 cells to 1 mM Li results in down-regulation of  $\delta$ -OR level and attenuation of  $\delta$ -OR-G protein coupling. This change proceeds in parallel with alteration of the polar head-group region of PM; the hydrophobic core of membrane is unchanged.

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PCB271

### Expression of calcium sensing receptors increases in differentiated human odontoblasts at stable extracellular calcium

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The calcium-sensing receptor (CaSR) is a G protein-coupled receptor responsible for calcium ( $\text{Ca}^{2+}$ ) homeostasis and translating changes of extracellular  $\text{Ca}^{2+}$  into intracellular signalling pathways facilitating differentiation and mineralization of osteoblasts in the bone [1]. Odontoblasts are specialised cells in the dental pulp, that produce and regenerate dentin [2]. Increased expression of CaSR in odontoblasts, which was upregulated by elevated extracellular  $\text{Ca}^{2+}$ , was reported [3], however, whether expression of CaSR changes during differentiation of dental pulp cells when extracellular  $\text{Ca}^{2+}$  concentration is constant and how this differentiation influences electrophysiological properties of differentiated odontoblasts remains unclear.

We have used immunocytochemistry (ICC) to assess CaSR expression and electrophysiology to measure transmembrane currents, membrane potential and evaluate the cell capacitance in human telomerase reverse transcriptase dental pulp (hTERT DP) cells that were non-differentiated and differentiated *in vitro* into odontoblasts. hTERT DP-derived odontoblasts demonstrated prominent changes in the cell morphology upon differentiation, so that every single hTERT DP-derived odontoblast occupied larger area than non-differentiated hTERT DP cell without changes of the cell capacitance. Semiquantitative analysis showed that normalized fluorescence intensity of CaSR immunostaining was doubled in differentiated hTERT DP-derived odontoblasts when compared to non-differentiated hTERT DP cells. In addition, increased CaSR expression was evident in cellular processes and on the edges of differentiated odontoblasts.

Voltage-clamp experiments showed significantly higher outward transmembrane current densities in differentiated hTERT DP-derived odontoblasts vs. non-differentiated hTERT DP cells at 0.5 mM of extracellular  $\text{Ca}^{2+}$ . Outward transmembrane current density increased dramatically in the presence of 5 mM  $\text{Ca}^{2+}$  in differentiated hTERT DP-derived odontoblasts but not in non-differentiated cells. These data match with current-clamp experiments showing hyperpolarisation of differentiated hTERT DP-derived odontoblasts in response to application of 5 mM extracellular  $\text{Ca}^{2+}$  vs. non-significant depolarisation of non-differentiated hTERT DP cells.

To conclude, the expression levels and cellular localisation of the CaSR changes during *in vitro* odontoblast differentiation in stable extracellular  $\text{Ca}^{2+}$ . Differentiation of hTERT DP into odontoblasts significantly changed cell morphology as well as localization of CaSR expression within the cell. Also, hTERT DP-derived odontoblasts displayed increased sensitivity to extracellular  $\text{Ca}^{2+}$  which suggests of its pivotal role during dentinogenesis.

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PCB272

### The implication of Interstitial cells in understanding Hirschsprung's Disease Pathology

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Aim of the study:

Improved understanding of the pathophysiology of Hirschsprung Disease (HD) may allow development of surgical and non-surgical approaches. Whilst abnormal motility in HD is associated with Enteric Nervous System defects, non-neuronal Interstitial Cells (ICs) identified as positive for cKit or platelet derived growth factor receptor alpha (PDGFRα) are also implicated. However, the contribution of these IC types to HD pathology is unclear, typically due to different methods in tissue processing between studies. Having optimised our methodology, this project aims to allow clarification of the contribution of IC subtypes to HD pathology, and by comparing IC in the presence and absence of ganglia, to allow further clarification of IC biology.

Methods and results:

Resected bowel specimens from 7 patients with HD were compared to tissue matched samples from stoma closures (n=10) using immunofluorescence. Ten minute immersion fixation in acetic ethanol allowed rapid tissue processing and robust immunostaining revealed by staining intensity and number of visible cells in comparison to formalin and zinc fixation.

ICs were reliably labelled and differentiated with antibodies to the ion channels Ano-1 (c-kit) and SK3 (PDGFRα) Imaris and Image J software based quantification revealed IC to be significantly reduced, expressed as mean ± standard-deviation cells/mm<sup>3</sup>, in the distal section of the colon (4.7±1) in comparison to the proximal region (85.9±3). Reduction in protein was mirrored by significant reductions in mRNA for cKit (P1.56±0.16)(D0.45±0.092), ANO-1(P1.77±0.14)(D0.32±0.03), PDGFRα(P 1.33±0.14)(D 0.48± 0.4) and SK3 (P 1.86± 0.4) (D 0.33±0.35) in distal portion of the HD samples (p<0.05).

Conclusion:

This robust approach to study IC can be applied to compare IC distribution at the proximal and distal resection margins from HD, Initial steps have been made to examine mRNA expression in HD colon. Extension of these approaches will enhance our understanding of HD pathology.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB273

**Assessment of hearing status of Rickshaw drivers by Pure tone audiometry test in Karachi, Pakistan.**M.A. Kanpurwala<sup>1,2,3</sup> and F. Mirza<sup>2</sup>

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Workplace health is a key concern of the working person. Excessive exposure to occupational noise results in loss of hearing and is now a recognized professional hazard and is regarded as a global problem. For the maintenance of social and economic development, a disease-free environment and healthy worker is very significant. The aim of our study is to assess the hearing status of the rickshaw drivers of Karachi using pure tone audiometry. In this cross-sectional survey, a total of 128 rickshaw drivers with minimum experience of one year were selected and Pure Tone Audiometry was performed on selected subjects to assess hearing status. Data gathered through structured questionnaire after verbal and written consent. History was taken regarding hearing followed by local examination of ears in each subject to rule out the presence of wax or any abnormality like perforation in tympanic membrane. The average age of the participants was 46.25±15.20 years. Out of 128 rickshaw drivers, hearing impairment was observed in 87.5% (112/128) cases while 12.5% (16/128) had no hearing impairment. Hearing impairment was significantly associated with age groups, experience, awareness of the ill effects of noise on health, use of protective device. From our results it is concluded that public transport drivers are exposed to excess noise on roads in Pakistan and most of them are suffering from noise induced hearing loss. This hearing impairment among the rickshaw drivers may jeopardize their safety as they may not be able to hear warning signals or horns from other vehicles on the roads thus leading to road traffic accident. It is suggested that government and regulatory bodies should take necessary actions in reducing noise pollution as it may decrease the probability of road traffic accidents secondary to impaired hearing among drivers.

Comparison of Hearing status among Auto Rickshaw drivers with different duration of exposure

| Experience of driving an Auto Rickshaw in years | Right Ear     |                |            |       | Left Ear      |                |            |       | P value |
|-------------------------------------------------|---------------|----------------|------------|-------|---------------|----------------|------------|-------|---------|
|                                                 | 2 to 10 years | 11 to 20 years | > 20 years | Total | 2 to 10 years | 11 to 20 years | > 20 years | Total |         |
| No impairment                                   | 16            | 3              | 3          | 22    | 13            | 3              | 0          | 16    |         |
| Slight Impairment                               | 14            | 7              | 0          | 21    | 17            | 6              | 0          | 23    |         |
| Moderate                                        | 6             | 29             | 3          | 38    | 6             | 26             | 7          | 39    |         |
| Severe                                          | 3             | 19             | 13         | 35    | 3             | 16             | 3          | 22    |         |
| Profound Including deafness                     | 3             | 0              | 9          | 12    | 3             | 7              | 18         | 28    |         |
| Total                                           | 42            | 58             | 28         | 128   | 42            | 58             | 28         | 128   | < 0.01* |

\* p-value significant < 0.05

Frequency of drivers suffering from hearing problems

| Hearing problems                                                                      | Frequency | Percentage |
|---------------------------------------------------------------------------------------|-----------|------------|
| Ringing in the ears                                                                   | 10        | 7.8        |
| Difficulty in hearing on the phone                                                    | 16        | 12.5       |
| Difficulty in hearing other person in one to one conversation                         | 41        | 32         |
| Difficulty in understanding spoken communication in the presence of surrounding noise | 16        | 12.5       |
| None                                                                                  | 45        | 35.2       |
| Total                                                                                 | 128       | 100        |

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## PCB274

**P3a amplitude reduction in antipsychotic-naïve first-episode psychosis**R. Solís-Vivanco<sup>2</sup>, A. Mondragón-Maya<sup>1</sup> and C. de la Fuente-Sandoval<sup>3</sup>

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**INTRODUCTION:**

Schizophrenia is a chronic and disabling psychotic disorder associated with cognitive impairment which affects the patient's functional outcome. P3a is an event-related potential which seems to be involved in automatic orienting and involuntary attention processes. Reduced amplitude of p3a has been consistently observed in schizophrenia patients. However, most studies have reported p3a deficits using passive oddball paradigms in medicated patients (Atkinson, 2012; Jahshan, 2012; Kaur, 2011; Nagai, 2013; Takahashi, 2013). To our knowledge, no reports of p3a elicited using an active task in unmedicated patients have been performed. Thus, the aim of the study was to analyze the p3a component in antipsychotic-naïve first-episode psychosis patients (FEP) and healthy controls (HC), using an active oddball paradigm.

**METHODS:**

15 FEP patients and 13 HC were recruited for p3a recording at the National Institute of Neurology and Neurosurgery in Mexico City. All participants signed an informed consent letter. Subjects performed an active auditory involuntary attention task including frequent (1000 Hz, 90%) and deviant (900 and 1100 Hz, 10%) tones during an EEG recording. They were instructed to classify the tones according to their duration (i.e., long vs. short) regardless the pitch.

**RESULTS:**

Mixed ANOVA analyses showed that p3a amplitude was significantly reduced in antipsychotic-naïve FEP patients when compared to HC. The main effect was observed at frontocentral electrodes ( $p < .01$ ). No differences between groups were found regarding p3a latency.

**CONCLUSIONS:**

Decreased p3a amplitudes are evident in antipsychotic-naïve FEP patients. Such deficits indicate that involuntary attention processes may be affected at early stages of the disease. Moreover, such impairments are independent from medication. Thus, p3a amplitude reduction may be a core feature of schizophrenia and should be considered as a psychophysiological trait marker of the disease.



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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB275

### **High incidental physical activity is associated with faster speed of processing in a reading task. An Event-Related Potentials study**

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**Background.** High levels of physical activity seem to positively influence health and cognition across the lifespan. Several studies have found that aerobic exercise enhances cognition and may prevent cognitive decline in the elderly. Nevertheless, the association of incidental physical activity (IPA) with health and cognition in elderly people has not been studied. Thus, the aim of this study was to evaluate the association between IPA levels and performance on a reading task during an event-related potentials (ERP) recording in elderly individuals. **Methods.** Spanish speaking participants (mean age = 66.7 years, SD = 4.3) with normal scores on psychometric and neuropsychological tests and normal values in blood analyses were included. Two groups were identified using a cluster analysis based on the scores of the Yale Physical Activity Scale (YPAS): Actives (n = 34), with high levels of IPA, and Passives (n = 34), with low levels of IPA. Actives showed significant higher Leisure activity (kcal/week; p = 0.002), and Vigorous (p < 0.0001) and Moving (p = 0.006) index scores of YPAS than Passives. ERPs were recorded while participants performed a reading task in which they had to identify grammatical errors. The sentences were manipulated with regard to (1) the gender agreement of the noun and adjective (i.e., the adjective's gender either agreed or disagreed with the noun), and (2) the working memory (WM) load (i.e., the number of words between the noun and adjective in the sentence; high and low). **Results.** Actives showed earlier latencies of the left anterior negativity (LAN) than Passives in the low WM load condition. Moreover, their peak latencies were less variable

than Passives'. Both groups showed an earlier latency of LAN in the low than high WM load condition, but this difference was greater in Actives. These latency patterns were also observed in the P600 ERP component. **Conclusion/implication.** Actives showed to be faster and less variable in their brain responses than Passives during morphosyntactic processing for sentence comprehension. Longer ERP latencies could be associated with speed of processing decline in aging. Our findings imply that a high level of IPA in older people could be a neuroprotective factor against cognitive decline associated with aging.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB276

### **Plasma levels of the high mobility group box 1 protein are associated with severity of gastrointestinal dysfunction in individuals with autism spectrum disorder**

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**Introduction.** Autism spectrum disorder (ASD) is a life-long neurodevelopmental condition characterized by impairments in communication, social interaction, restricted interests and repetitive behavior. The aetiology of ASD is poorly understood, evidence indicates that inflammation may play a key role. In individuals with ASD, high prevalence of gastrointestinal (GI) disturbances is reported, that are linked to a low-grade chronic inflammation of the intestinal mucosa. The high mobility group box-1 protein (HMGB1) is an intracellular protein with potent proinflammatory cytokine activity that has been implicated in ASD, as well as in other neurological disorders. The objective of this study was to determine plasma levels of HMGB1 in individuals with ASD and to analyze their association with GI symptoms.

**Methods.** The study involved 31 subjects with low functioning ASD and 16 healthy controls aged 2-22 years. Subjects had a diagnosis of ASD based on the ICD -10 (International Classification of Diseases, 10<sup>th</sup> edition) criteria. History of GI disorders was collected by a questionnaire, and the score of gastrointestinal dysfunction based of frequency of symptoms was calculated. Plasma levels of HMGB1 were measured by a sandwich enzyme-linked immunosorbent assay (ELISA). Statistical analysis included the Chi-square test and Mann Whitney test. The study was approved by the Ethics Committee of the Faculty of Medicine, Comenius University and University Hospital in Bratislava, Slovak Republic. An informed consent from all the subjects or their caregivers was obtained. The study conformed to the code of ethics stated in the Declaration of Helsinki.

**Results.** Plasma HMGB1 levels were significantly higher in individuals with ASD than in controls (13.8 ± 11.7 ng/ml vs. 7.90 ± 4.0 ng/ml, p < 0.02). GI problems were significantly more prevalent in subjects with ASD (96.8 %, vs 66.6% of controls). In the subgroup of ASD cases with higher HMGB1 levels (11 ng/ml) a significantly higher percent of subjects (83.3 %) suffered by more severe forms of GI problems, whereas in subgroup with lower levels of HMGB1 (<11 ng/ml) mild forms of GI symptoms prevailed (c<sup>2</sup>=6.42, df=1, p < 0.02)

**Conclusions.** Results of the study support the involvement of the low-grade inflammation in the pathomechanisms of autism, and that inflammatory process may underlie the GI symptoms observed in some subgroups of subjects with ASD. More research is needed in order to specify the role of HMGB1 in the pathomechanisms of the autistic disorders.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCB277

### **The Preventive Effects of Oxytocin and Liraglutide to Vincristine-Induced Neuropathy: An Experimental Rat Model**

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**Introduction:** Vincristine is an anti-neoplastic drug that is a kind of a microtubule poison. The most common side effect is peripheral sensory and motor neuropathy. The aim of this study is to assess the effects of oxytocin and GLP-1 analogue (Liraglutide) on neuropathy.

**Methods:** Twenty-four Sprague-Dawley adult male rats were included in the study. Six rats were included in the study as normal group. No action was taken to this group. Eighteen rats were injected with Vincristine intraperitoneally (i.p.) at the dose of 4 mg / kg / week (2 injections per week) for 5 weeks to induce neuropathy development. The vincristine-administered rats were divided into 3 groups. The first group (n=6) were injected with 100 µg / kg / day Oxytocin i.p., the second group (n=6) were injected with 10 mg / kg / day Liraglutide i.p., whereas the third group (n=6) received 1 mL/kg %0.9 NaCl (saline) i.p. simultaneously with vincristine injections for 5 weeks. At the end of these 5 weeks, Electromyography (EMG) studies were recorded from the rats, rats were tested by inclined plane test and histopathological nerve and nerve sheath examination was performed by excising the sciatic nerve to evaluate neuropathy.

**Results:** In Vincristine + saline group, nerve conduction velocity and electrical potentials obtained from the muscle in EMG was found to be decreased when compared to the normal group (P<0,05). Additionally, In the inclined plane test, it was observed that the climbing angle was decreased in Vincristine + saline group when compared to the normal group (P<0,05). These findings were interpreted in the direction of neuropathy. In groups receiving Oxytocin and Liraglutide, Improvement in electrical potentials in EMG and inclined plane test were observed in groups receiving Oxytocin and Liraglutide when compared to the Vincristine + saline group (P<0,05). Histopathologically, myelin sheath was decreased in all groups. There was an increase in NGF (neuronal growth factor) expression, although there was no significant change in the treatment groups when compared to the Vincristine + saline group.

**Conclusion:** In conclusion, this is the first study which demonstrates that Oxytocin and Liraglutide have protective effects in Vincristine-induced neuropathy. Previously, positive effects of oxytocin and GLP-1 in neural recovery processes (in neurosurgical damage model, cisplatin neuropathy model, diabetic autonomic neuropathy model) have been demonstrated and published by our team. In this study it was observed that Oxytocin and Liraglutide showed Schwann protective effects in Vincristine neuropathy model. Based on these findings, Oxytocin and Liraglutide may be considered as potential agents that can be used for the prevention of Vincristine-induced neuropathy. However, new experimental and clinical studies are required for the clinical application of these treatments modality for preventing the neuropathy in cancer patients.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCB278

### **Evaluation of toxicity of gadolinium based contrast agents on neuronal cells**

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**Introduction:** Gadolinium based contrast agents (GBCAs) are widely used in magnetic resonance imaging (MRI). Recently, increased signal intensity has been reported in specific brain areas such as dentate nucleus, globus pallidus and cerebellum after repeated administrations of GBCAs. The aim of the present study was to investigate the toxic effects of GBCAs on neuronal cells by using SH-SY5Y neuroblastoma cell cultures. **Methods:** For toxicity assays, SH-SY5Y cells were seeded in 96-well dishes (5000 cells/well) and incubated with different doses (0-1000 µM) of several macrocyclic (gadoteric acid and gadobutrol) and linear GBCAs (gadoversetamide, gadopentetic acid, gadodiamide and gadoxetic acid) for 72 hours. Cell viability and mitochondrial metabolic activity were evaluated by using MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay, colony forming assay and Hoechst staining. The results were expressed as mean ± SEM. The data was analyzed using student t-test. A p value <0.05 was accepted as statistically significant.

**Results:** Both macrocyclic and linear GBCAs significantly and dose-dependently reduced cell viability in neuronal cells compared to untreated cells. Cell viability was measured between 89.49 ± 4.09 % and 60.99 ± 0.77 % in GBCAs-treated groups. In addition, neurotoxicity was more prominent in linear GBCAs-treated cultures (p<0.0005).

**Conclusion:** The results of the present study indicate that exposure to specific GBCAs, even at low micro-molar concentrations, may have detrimental effects on neuronal survival. Further investigations are required to clarify the molecular mechanism underlying GBCAs-induced cell death.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## PCB279

**Antiepileptic activity of ellagic acid in pentylenetetrazole-kindled rat and role in motor function**

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Epilepsy is a neurologic disorder affecting more than 50 million people. Motor function impairment is among the disorders caused by epileptic seizures. This study assessed the effect of ellagic acid in pentylenetetrazole (PTZ)-kindled rats and its role in motor functions. Thirty male wistar rats weighing 200 – 300g, with 6 groups of 5 rats each were used. Groups 1 – 5 received 35mg/kg PTZ, while group 6 received distilled water (s.c) on alternate days. One hour before PTZ administration, group 2, 3 and 4 received 15, 30 and 60 mg/kg (p.o) ellagic acid dissolved in 10% dimethylsulphoxide (DMSO) respectively while group 5 received 30mg/kg phenobarbital (i.p) and were observed for seizure activity for 30 minutes after PTZ injections. When kindling was achieved, the rats were subjected to beam walk assay for motor coordination and inverted screen test for grip strength. Rats were anaesthetized with Ketamine 10mg/kg and diazepam 5mg/kg before being sacrificed. The brain tissues were harvested, homogenized and the supernatant used for assaying neurochemicals such as total glutamate concentration, gamma amino butyric acid (GABA), CFOS, oxidative stress biomarker, malondialdehyde (MDA) and antioxidant enzymes, superoxide dismutase (SOD) and glutathione peroxidase (GPx). Results showed significant ( $P < 0.05$ ) decrease in transfer latencies of all the ellagic acid treated groups, 2, 3, and 4: ( $18.20 \pm 1.77$ ;  $19.60 \pm 5.80$ ;  $11.00 \pm 0.54$ ) respectively when compared to group 1 ( $54.00 \pm 4.00$ ). There was also a significant decrease ( $P < 0.05$ ) in foot slip for groups 2 ( $0.40 \pm 0.35$ ) and 3 ( $0.40 \pm 0.35$ ) when compared to the control ( $1.60 \pm 0.31$ ). Significant increase ( $P < 0.05$ ) in grip strength occurred in groups 3, 4, 5 and 6 compared to group 1 rats. For glutamate assay, group 1 ( $8.50 \pm 0.28$ ) is statistical significance when compared to group 2 ( $5.58 \pm 0.34$ ); 3 ( $5.37 \pm 0.23$ ); 4 ( $5.72 \pm 0.38$ ); 5 ( $3.59 \pm 0.21$ ) and 6 ( $4.13 \pm 0.42$ ) respectively and for GABA, decrease significantly in group 1 ( $135.16 \pm 1.23$ ) when compared to group 4 ( $218.51 \pm 6.93$ ) and 6 ( $104.92 \pm 3.26$ ). CFOS levels decreased significantly in all groups 2, 3, 4, 5 and 6 ( $4.31 \pm 0.22$ ;  $4.19 \pm 0.14$ ;  $4.58 \pm 0.43$ ;  $3.64 \pm 0.21$ ;  $3.50 \pm 0.15$ ) nMol/L respectively as compared to group 1 control ( $6.61 \pm 0.22$ ) nMol/L. Significant decrease ( $P < 0.05$ ) in MDA concentration in all the treated groups, 2, 3, 4 and 5 ( $1.08 \pm 0.08$ ;  $1.10 \pm 0.09$ ;  $1.16 \pm 0.08$ ;  $1.06 \pm 0.11$ )  $\mu$ Mol/L respectively when compared to control group 1 ( $1.84 \pm 0.07$ )  $\mu$ Mol/L. SOD increased significantly ( $P < 0.05$ ) in all the groups ( $1.90 \pm 0.08$ ;  $2.02 \pm 0.17$ ;  $1.94 \pm 0.07$ ;  $1.92 \pm 0.07$ ;  $1.88 \pm 0.04$ ) IU/L when compared to control group 1 ( $1.14 \pm 0.05$ ) IU/L while for GPx significant increase ( $P < 0.05$ ) was recorded only in group 4 ( $41.20 \pm 1.02$ ) compared to control ( $37.20 \pm 0.74$ ) IU/L. Findings indicate oxidative stress is involved in kindling which occur as a result of imbalance in Glutamate-GABA concentration. And ellagic acid has potent antiepileptic potentials with no associated side effects.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB280

**The structural basis for the interaction of TRPM3 channel proteins with G protein beta-gamma subunits**M. Behrendt<sup>1</sup>, F. Gruss<sup>2</sup>, M. Nys<sup>2</sup>, R. Enzeroth<sup>1</sup>, F. Mohr<sup>1</sup>, S. Philipp<sup>3</sup>, C. Ulens<sup>2</sup> and J. Oberwinkler<sup>1</sup>

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TRPM3 proteins form  $\text{Ca}^{2+}$ -permeable, non-selective cation channels that can be activated by diverse chemical compounds, notably the steroid pregnenolone sulfate, and by some physical stimuli, principally by heat. Accordingly, TRPM3 channels have been shown to play important roles in heat-induced nociception and in inflammatory thermal hyperalgesia (1, 2). Recently, we and others have shown that these channels are tightly regulated by  $\text{G}\alpha_i$ -coupled receptors (3 – 5). Notably, in peripheral nociceptor neurons  $\mu$ -opioid receptors have a strong inhibitory influence on TRPM3 channels. Hence, the peripheral activation of  $\mu$ -opioid receptors reduces TRPM3-dependent pain in vivo. The signaling pathway from  $\mu$ -opioid receptors to TRPM3 channels was shown to be dependent on  $\text{G}\beta\gamma$  subunits (3 – 5). However, it is not clear, whether TRPM3 channels and  $\text{G}\beta\gamma$  subunits interact directly, and if so, where on the respective proteins the interaction takes place. By screening naturally occurring isoforms of TRPM3 arising from alternative splicing, we discovered that a region of 10 amino acids, encoded by exon 17, is required for the inhibitory action of  $\text{G}\beta\gamma$  subunits on TRPM3. Mutational analysis indicated that 5 of these 10 residues are particularly important for this functional interaction. Biochemically, we showed that  $\text{G}\beta$  proteins that co-immunoprecipitated with TRPM3 were much reduced for a TRPM3 isoform that lacks exon 17-encoded amino acids. Conversely, short peptides containing parts or all of the sequence encoded by exon 17 preferentially displayed binding to  $\text{G}\beta\gamma$  proteins in vitro. Finally, X-ray analysis of  $\text{G}\beta\gamma$  co-crystallized with a short peptide encoded by exon 17 showed that this peptide adopts an  $\alpha$ -helical structure to interact with  $\text{G}\beta$ . The molecular interactions observed in



the crystal structure involved mainly the same amino acids previously identified through mutagenesis as important for the functional  $G\beta\gamma$ -mediated inhibition of TRPM3. Conversely, structure-guided mutations of  $G\beta$  proteins demonstrated that residues of  $G\beta$ , which interacted with the exon 17-encoded peptide in the crystal structure, are also important for the functional inhibitory effects of  $G\beta\gamma$  on TRPM3 in cellular assays. Together, these data provide evidence of considerable strength that  $G\beta$  directly interacts with TRPM3 proteins through amino acids of TRPM3 encoded by exon 17 and that this direct interaction is crucial for the inhibitory action of  $G\beta\gamma$  on TRPM3. The interaction surface between  $G\beta$  and TRPM3 that we have identified might be druggable and thus presents a good opportunity to develop selective TRPM3 inhibitors for the treatment of TRPM3-dependent pain.

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Vandewauw et al., 2018, *Nature*, 555: 662-666

Badheka et al., 2017, *eLife* 6: e26147

Dembla et al., 2017, *eLife* 6: e26280

Quallo et al., 2017, *eLife* 6: e26138

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## PCB281

### Low nanomolar concentrations of isradipine selectively reduce in vivo burst firing of dopamine neurons in the lateral substantia nigra

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Isradipine, a clinically approved L-type calcium channel inhibitor, is currently used in a clinical trial in order to slow the progression of Parkinson disease (PD-Steady III). Although there is in vitro evidence that isradipine reduces activity-dependent calcium loading of dopamine (DA) substantia nigra (SN) neurons, it is however unknown whether isradipine affects the in vivo activity of DA SN neurons in therapeutically relevant concentrations. We have previously presented evidence for a selective role of isradipine-sensitive L-type calcium channels in boosting in vitro burst excitability in lateral but not in medial DA SN neurons of adult C57Bl6N mice (Shin et al., Sfn 2017). To now probe for a role of isradipine in the therapeutically relevant, low nanomolar concentration range in controlling in vivo bursting of DA SN neurons, we carried out single unit recordings of DA SN neurons combined with juxtacellular labeling in anesthetized mice. Systemic applications of 3 mg/kg isradipine (i.p.) resulted in a selective reduction of in vivo bursting of lateral DA SN neurons (spikes fired in bursts: control,  $41.84 \pm 26.48\%$ , isradipine,  $25.69 \pm 23.03\%$ ,  $n=18$ ,  $p=0.0006$ ), while firing properties of medial DA SN neurons remained unaffected (spikes fired in bursts: control,  $14.63 \pm 18.67\%$ , isradipine,  $14.65 \pm 16.08\%$ ,  $n=17$ ,  $p=0.94$ ). The maximal in vivo effect on bursting was observed at about 15 minutes after systemic drug application, which corresponded to a free plasma concentration of ca. 3 nM isradipine ( $22.53 \pm 3.26$  ng/ml at 15 min. post-injection, assuming 95% plasma protein binding,  $n=5$ ). In vitro bath application of 3 nM

isradipine was sufficient to induce a partial inhibition of burst excitability in lateral DA SN neurons (gain: control,  $758.3 \pm 120.3$  Hz $\cdot$ nS,  $n=24$ , 300 nM isradipine,  $425.9 \pm 106.5$  Hz $\cdot$ nS,  $n=17$ , 3 nM isradipine,  $641 \pm 181.8$  Hz $\cdot$ nS,  $n=14$ ) consistent with our in vivo results.

We are currently probing for behavioral effects of low nanomolar isradipine locally infused into the lateral SN of freely moving mice to identify behavioral consequences of acutely suppressed burst activity in lateral SN DA neurons. In summary, our data identify a surprisingly selective effect of low nanomolar isradipine on burst firing of lateral SN DA neurons, which are the most vulnerable DA subpopulation in Parkinson disease.

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## PCB282

### Loss of pain due to a gain-of-function mutation: iPSC-derived human nociceptors as a disease model of a Nav1.9-linked pain syndrome

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Congenital insensitivity to pain (CIP) can be induced by the p.L811P *de novo* mutation in the gene coding for the voltage-gated sodium channel Nav1.9. The patients are devoid of any pain perception, resulting in severe self-inflicted wounds. Experiments on dorsal root ganglia (DRGs) from knock-in mice and a rat neuroblastoma cell line showed that the loss of pain phenotype is linked to a gain-of-function mutation of Nav1.9 (Leipold et al. 2013). Although a hyperpolarizing shift in channel activation and slowed deactivation was seen in DRGs and rat neuroblastoma cells, a hyperpolarizing shift in the channel's inactivation was solely observed in the murine DRGs. Patient-specific induced pluripotent stem cell (iPSC) derived nociceptors offer the possibility to study human sensory neurons of this patient, promising to clarify in part conflicting data and could provide insight into the physiological function of the voltage-gated sodium channel Nav1.9.

Therefore we reprogrammed patient fibroblasts carrying the p.L811P mutation in the Nav1.9 gene *scn11a* by induced expression of the Yamanaka factors. We subsequently differentiated the acquired induced pluripotent stem cells (iPSCs) of healthy controls and the patient into nociceptors using a small molecule approach. We are currently testing various differentiation protocols to enhance the expression of Nav1.9 in these human nociceptors. We perform a detailed analysis of nociceptor excitability by whole-cell patch clamp electrophysiology and non-invasive multi electrode array recordings. We focus on resting membrane potential, action potential characteristics as well as spontaneous and evoked excitability at different temperatures. These experiments are currently ongoing and the results will be presented at the meeting.

Nav1.9 is known to be an important contributor to pain signaling and has not only been found to lead to congenital insensitivity to pain but is also connected to hereditary pain syndromes such as familial episodic pain, painful peripheral neuropathy and erythromelalgia. This study will help to elucidate the role of Nav1.9 in action potential generation and

modulation in nociceptors and potentially help to develop new pain treatments with fewer side effects.

Leipold, E et al. (2013). "A de Novo Gain-of-Function Mutation in SCN11A Causes Loss of Pain Perception." *Nature genetics* 45(11): 1399–1404.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB283

### Establishing a neurotoxicological assay using human induced pluripotent stem cell (hiPSC)-derived sensory neurons

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During daily life we are exposed to multiple commercially used chemicals. Although there is a large number of compounds with the potential for neurotoxicological effects on humans, there is a lack of information about the impact of these compounds on human neural development and function. Currently, the majority of neurotoxicity data reported in scientific studies is based either on *in vivo* animal-based models or on *in vitro* models with immortalized cell lines. Oftentimes, the interpretation of experimental data obtained from animal-based studies poses difficulties due to the differences between species in both neuronal anatomy and physiology. In this case, immortalized human neuronal cell lines offer a better option for *in vitro* screening. However, these tumor-derived cells may not display the same phenotype as primary cultures of healthy human neurons. There is an increasing need for alternative species-specific toxicology screening assays, which should have a higher reliability and effectiveness to identify and characterize the neurotoxic potential of compounds on humans. The use of human induced pluripotent stem cells (hiPSCs) and their neural derivatives for assessing neurotoxicity offers many advantages, such as the potential of unlimited expansion, as well as the ability to portray not only structural, but also functional characteristics of neurons of both the CNS and PNS.

In this sense, sensory neurons (SNs) derived from hiPSCs were used to establish a screening platform, which is able to analyze the effects of multiple compounds on structure and function of human SNs. For this aim, two common pyrethroid-based insecticides (deltamethrin and bioallethrin) were selected for this proof-of-concept. These insecticides are known to disrupt the kinetics of voltage-gated sodium channels in both insect and mammalian neurons by prolonging the time the channel is open. This causes neuronal hyperexcitability and is the basis for their insecticidal and toxicological mode-of-action.

To distinguish between general cytotoxicity and specific neurotoxicity, the no observed effect concentrations (NOECs) of deltamethrin and bioallethrin are identified using the neutral red uptake assay. The selected test concentrations are applied on mature SNs. After exposure, both neurite morphology and spontaneous activity are assessed. Using immunostaining, neurites and cell bodies of neurons are quantified with an imaging reader. Considering the fact that many disruptions to neuronal excitability occur in the absence of morphological changes to the neurons, we proceed to assess changes in neuronal activity using the Multielectrode array (MEA) technology. Thus, we here established a human-based

method, which embraces both structural and functional effects of potential neurotoxins.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB284

### Pharmacological activation of Nav1.9 – a model for Nav1.9 channelopathies linked to chronic pain or congenital insensitivity to pain

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Chronic pain disorders, such as inherited erythromelalgia, paroxysmal extreme pain disorder or small fiber neuropathy are debilitating diseases. Gain of function mutations in the voltage-gated sodium channel subtypes Nav1.7, Nav1.8 and Nav1.9 have been shown to cause these disorders. However, Nav1.9 is set apart from other sodium channels as gain of function mutations in this channel do not only cause chronic pain disorders, but are also linked to congenital insensitivity to pain. The mechanism underlying this discrepancy is not yet completely understood.

Here, we aim to find a way to activate Nav1.9 pharmacologically which will allow better investigation of its role in sensory neuron excitability. However, electrophysiological studies on Nav1.9 are challenging, as this channel has been notoriously difficult to express heterologously. Here, we use the Nav1.9\_C4 chimera in which the C-terminus of Nav1.9 is exchanged for that of Nav1.4 (Goral et al., 2015). This chimera was reported to express relatively well in neuroblastoma cell lines. Nevertheless, when patch-clamping ND7/23 cells transfected with this chimera, we recorded only small currents.

In a first step, we therefore optimized the transfection protocol. Following transfection and overnight incubation at 37 °C, we incubated the cells at 28 °C, 33 °C, or 37 °C for periods of 48 h to 120 h. The best results for the transient transfection of the Nav1.9\_C4-chimera were achieved, when the cells were incubated at 37 °C and patched 72 h to 96 h post transfection. At 28 °C and 33 °C, all cells had Nav1.9\_C4 currents below 1 nA. Incubation for less than 72 h resulted in lower currents, whereas incubation for more than 96 h resulted in fewer cells with a current.

In a second step, we aim to activate the chimera using compounds known to activate voltage gated sodium channels, such as deltamethrin and other toxins. At a concentration of 1 µM, the pyrethroid deltamethrin was shown to induce large persistent currents in other sodium channels such as Nav1.5. In our experiments, this concentration did not cause larger effects on Nav1.9 gating. We will present a detailed analysis of deltamethrin effects on gating of Nav1.9\_C4 at the conference.

Our study will help to better understand the pharmacology of Nav1.9 which has a prominent role in human pain perception.

Goral, R. O. et al. (2015) 'Heterologous expression of Nav1.9 chimeras in various cell systems', *Pflügers Archiv European Journal of Physiology*, 467(12), pp. 2423–2435. doi: 10.1007/s00424-015-1709-1.

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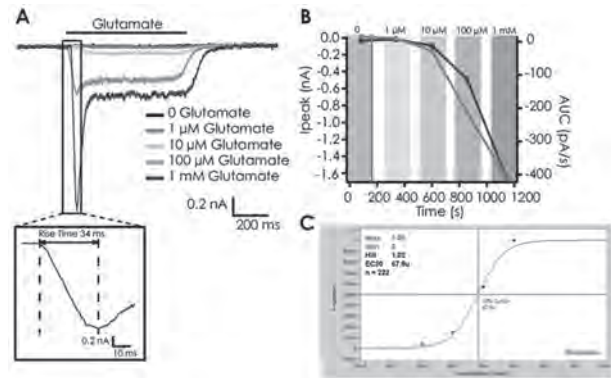
## PCB285

**Expression and pharmacology of GluA2-containing AMPA receptors in cell lines and stem cell-derived neurons**

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The vast majority of excitatory neurotransmission is mediated by AMPA ( $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors. The functional receptor exists as a tetramer, either homomers or heteromers, from a repertoire of 4 different subunits, GluA1 – GluA4. It is well known that glutamate is a neurotoxin and it is proposed that overactivation of ionotropic glutamate receptors may underlie many neurodegenerative disorders such as ischemic stroke, epilepsy, Parkinson's and dementia, amongst others. Enhancement of AMPA receptor activation by, for example, BDNF, has been proposed to have beneficial effects of learning and memory and has potential therapeutic value in the treatment of depression, Huntington's and Parkinson's diseases. We have used GluA2 receptors expressed in HEK cells on 3 different automated patch clamp systems recording from either 1, 8 or 384 wells simultaneously. We have compared the glutamate  $EC_{50}$  obtained on the different platforms and found that they are similar, ranging from approximately 30 – 80  $\mu$ M. The speed of glutamate application greatly affected activation and inactivation kinetics of GluA2. Responses to glutamate were reproducible using a concentration of 30 or 100  $\mu$ M, GluA2 could be repetitively activated at least 4 or more times, making the assay suitable for pharmacological characterization. GluA2 was also recorded at physiological temperature as well as room temperature. Using pharmacological agents we could either inhibit or enhance the glutamate elicited responses. The inhibitor, CNQX, blocked glutamate responses mediated by GluA2 with an  $IC_{50}$  of 600 nM using a glutamate concentration of 100  $\mu$ M and the concentration response curve for CNQX was dependent on the glutamate concentration. The potentiator, LY404187, was pre-incubated prior to co-application with 100  $\mu$ M glutamate giving an  $EC_{50}$  of around 500 nM. In addition to GluA2 expressed in HEK cells, we also recorded glutamatergic-enriched cortical neurons derived from induced pluripotent stem cells on an automated patch clamp platform. In these neurons we recorded NaV currents which could be blocked by low concentrations of TTX with an  $IC_{50}$  of 12 nM. In addition, glutamate responses were recorded which were potentiated by LY404187.



The AMPA receptor (GluA2) was activated by increasing concentrations of glutamate on the SyncroPatch 384PE. L-glutamate was applied for approximately 500 ms in increasing concentrations (A) and a cumulative concentration response curve for glutamate was constructed for 222 wells (C). The online analysis values peak amplitude and area under the curve (AUC) are shown versus time in Panel B. The fast activation of GluA2 could be captured at higher concentrations (inset; 1 mM).

We thank SB Drug Discovery for providing the HEK expressing GluA2 cell line and Cellular Dynamic International for providing the glutamatergic-enriched cortical neurons.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB286

**Modulation of Transient Receptor Potential Melastatin 8 Channel by tyrosine phosphorylation**

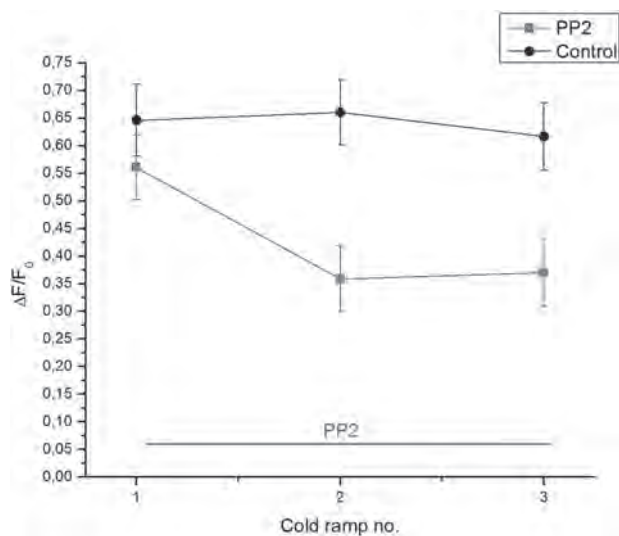
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TRPM8 is a polymodal cation channel found in sensory neurons and other tissues. Its main agonists are low temperatures ( $<26^{\circ}\text{C}$ ) and natural compounds like menthol. TRPM8 is also modulated by many signaling pathways which can change its activity. Thus, we studied tyrosine phosphorylation of TRPM8, an important process in cell signaling that had not been investigated for this particular receptor-channel. HEK293T cells were co-transfected with human TRPM8 which had a VSV-G tag (VSV-G-TRPM8) and Src kinase (SrcY527F). After 48h, cells were treated with PP2 (10  $\mu$ M), a selective Src kinase inhibitor. VSV-G-TRPM8 was immunoprecipitated from cell lysates with VSV-G antibody and its phosphorylation was analyzed using the Western blot assay. VSV-G-TRPM8 is phosphorylated in cells expressing SrcY527F and PP2 treatment prevented TRPM8 phosphorylation (3 replicates). HEK293T cells stably transfected with human TRPM8 (HEK293T-hTRPM8) were transfected with siRNA to reduce endogenous Src expression (Src\_siRNA) and a scrambled RNA was used as control. A Western blot assay was performed to verify Src expression. Src kinase expression was reduced after 96h in Src\_siRNA-transfected cells, compared to scrambled RNA transfection (2 replicates). Calcium microfluorimetry was used to monitor the acute effect of PP2 on cold-induced calcium transients in HEK293T-hTRPM8 cells. Cold ramps (from  $\sim 32$  to  $\sim 25^{\circ}\text{C}$ ) were applied seven times at 5 min interval, in the presence and in the absence of PP2 (10  $\mu$ M). Values represent mean  $\pm$  S.E.M. compared by Student's *t*-test. Tachyphylaxis of



cold-induced calcium transients occurred in the presence of PP2 in HEK293T-hTRPM8 cells transfected with both Src\_siRNA and scrambled RNA but the effect was significantly more pronounced in scrambled RNA-transfected cells. The ratio between the amplitudes of the 7<sup>th</sup> and the 1<sup>st</sup> cold-induced calcium transients was  $0.73 \pm 0.01$  in scrambled RNA-transfected cells ( $n=336$ ) and  $0.90 \pm 0.01$  in Src\_siRNA-transfected cells ( $n=335$ ),  $p < 0.01$ . Adult Wistar rats (male,  $n=3$ ) were used. Rats were killed by inhalation of 100% CO<sub>2</sub> followed by decapitation. Dorsal root ganglia (DRG) were removed and prepared for the primary culture. Acute PP2 treatment (20 min) also partly inhibited cold-induced calcium transients in menthol-sensitive DRG neurons, from  $0.56 \pm 0.06$  to  $0.37 \pm 0.06$ ,  $n=27$ ,  $p < 0.05$  (Fig.1). In the absence of PP2 there was no significant change in the amplitude of cold-induced calcium transients ( $0.64 \pm 0.07$  during the first cold ramp, compared to  $0.61 \pm 0.06$  during the 3<sup>rd</sup> cold ramp,  $n=24$ ). These results indicate that TRPM8 is tyrosine phosphorylated by Src kinase, as PP2 treatment prevents TRPM8 phosphorylation. The calcium microfluorimetry data suggest a positive modulation of the Src protein kinase on the TRPM8 channel, the phosphorylation of TRPM8 potentiating its response to moderate cooling. It is important that TRPM8 activity is reduced by PP2 treatment in neurons, where both TRPM8 and Src are expressed natively.



Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB287

### The thermosensitivity of two-pore potassium channels depends on their phosphorylation status

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Thermosensitivity of ion channels is a prerequisite for the perception of internal and external temperatures. In mammals, it is critical for the homeostasis of the body temperature, the avoidance of extreme temperatures and survival. In accordance, previous studies revealed an expression of the thermosensitive members of the two-pore K<sup>+</sup> channel (K2P) family in tissues important for temperature sensation (e.g. DRG neurons, hypothalamus). Here, we investigated the thermosensitivity of different members of the K2P family (TREK-1, TREK-2, TRAAK, TALK-2; TRESK). While TREK-1 and TREK-2 were strongly activated by elevating

temperatures all other K2P channels showed only modest sensitivity with Q10 values around 2. For the TREK channels we observed a clear temperature-optimum. However, this optimum was depending on the heating speed. Slow heating (<0.1°C/s) of the cells resulted in maximal currents at 37°C while a faster heating (>0.2°C/s) resulted in maximal currents at higher temperatures. This observation suggests that the temperature-sensitivity is not an intrinsic feature of the ion channels themselves but rather depends on another regulatory factor. TRAAK channels were previously described as being also temperature-activated. However, in our investigations, these channels were not temperature-activated. Furthermore, chimeras of TREK-1 channels with the C-terminus of TRAAK were not temperature-activated anymore. Vice-versa TRAAK channels with the C-terminus of TREK-1 became thermosensitive. By gradually truncating the C-terminus of TREK-1 channels we identified an 18 amino acid stretch as being essential for the thermosensitivity. Interestingly, the previously identified PKA phosphorylation site (S348) is located within this sequence. The activation of PKA with forskolin/IBMX led to a strong reduction of the basal ion current and further to a complete loss of thermosensitivity. As expected, mutating the S348 to A (mimicking a dephosphorylated state) yielded channels with high basal activity and mutating the serine to D (mimicking the phosphorylated state) reduced the basal activity significantly. However, while S348A still was temperature-activated, S348D was not. This observation stays in agreement to the current postulation that TREK-1 channels are opened by the elevation of their C-terminus to the plasma membrane. Consequently, the introduction of the negative charge in S348D provokes channel closure due to a disrupted contact to the plasma membrane. However, the observed temperature-sensitivity of S348A points to a more indirect involvement of the PKA site in temperature perception, as S348A could neither be phosphorylated nor dephosphorylated. Together with the observation that TREK-1 channel activation by temperature depends on the heating speed this may indicate that an additional factor binds to the dephosphorylated S348 to determine the thermosensitivity.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB288

### Heterodimerization across K2P Channel subfamilies: A systematic BiFC screen reveals interaction between members of different subfamilies

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Two-pore Domain Potassium (K2P) Channels are the most recently identified potassium channel class. They are widely expressed in the body - in the central nervous system as well as the periphery, e.g. lung, heart and endocrine cells - with strongly overlapping expression patterns of the 15 K2P members. Their large basal activity hyperpolarizes the resting membrane potential and dampens the excitability of neurons and other cells.

K2P channels are modulated by a variety of mechanical and chemical stimuli like stretch, heat, acidification, lipids or anaesthetics, denoting their role in many physiological and pathophysiological processes including sleep, pain or epilepsy. Unlike other K<sup>+</sup> channels, they show a particular topology of four transmembrane helices with two pore domains. Therefore, only two subunits assemble as dimers with the typical

tetrameric symmetry of the pore. The 15 K2P members are classified into six subfamilies by sequence similarity and most channels form functional homodimers in heterologous expression systems. Only a few heterodimers within the subfamilies have been reported, e.g. THIK-1/THIK-2, TREK-1/TREK-2, TASK-1/TASK-3. Nearly nothing is known about the formation of heterodimeric channels across the K2P subfamilies. We used bimolecular fluorescence complementation (BiFC) to systematically screen for all possible heterodimers. All K2P subunits were cloned into the pBiFC-VN173 and pBiFC-VC155 vectors, producing fusion proteins with half of a split fluorescent protein each. All possible combinations were coexpressed, and fluorescence by recombination of split proteins revealed the formation of several heterodimeric channels also across different subfamilies. We particularly examined the TWIK/TALK combination because of a strong fluorescence in the BiFC assay, and estimated the homodimeric and heterodimeric fractions of the coexpressed BiFC constructs. Co-immunoprecipitation experiments confirmed their existence and they were characterized in electrophysiological measurements. Moreover, fluorescence microscopy showed a change in TWIK-1 localization compared to the wildtype homodimer. Interestingly, TWIK-1 mRNA is ubiquitously expressed, but the homodimeric channel protein is reported to be nonfunctional because of intracellular sequestration. Hence, heterodimerization may be a requirement for functional plasma membrane expression of TWIK-1, and TWIK family members could function as regulator subunits for many K2P channels from other subfamilies, modulating trafficking, plasma membrane expression levels and gating characteristics. The cross-subfamily combination of K2P channel monomers could confer intermediate or even completely new properties to these mixed channels and contribute to the diversity of K<sup>+</sup> channels adapted to their specific cellular environment *in vivo*.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB289

### Studying ligand binding and gating of retinal CNGA1 channels by FRET

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Ligand-gated ion channels are multimeric membrane proteins with key roles within various signal transduction pathways of different cell types. A detailed knowledge of the ligand binding and activation mechanism of these channels is essential for understanding basic cellular functions and pathological processes. Information regarding the gating mechanism, single-channel conductance and selective ion permeability of ligand-gated ion channels can be gathered by means of the patch-clamp technique. The confocal patch-clamp fluorometry (cPCF) technique can additionally measure ligand binding and the triggered conformational changes within the channel protein. Unfortunately, the ligand concentration which can be used in combination with cPCF is restricted to the lower micromolar range. Herein, we propose a new approach to measure binding and gating by combining cPCF with Förster resonance energy transfer (FRET) measurements. As model system we choose retinal homotetrameric cyclic

nucleotide-gated (CNGA1) channels. Binding of a cyclic nucleotide to the intracellularly-located binding domains (CNBDs) promotes an allosteric conformational change that leads to channel opening. Due to the concentration range in which this channel operates, ligand binding studies with fluorescently labelled ligands were not possible until now.

To this aim, CNGA1 channels were expressed in *Xenopus* oocytes and their function was tested using inside-out patches. As FRET pair, the CNGA1 channel labelled at the C-terminus and a novel fluorescently-labelled cGMP derivative, 8-[DY547-P1]-AET-cGMP (P1-cGMP) were used. When the labelled ligand binds to the channel's binding site, energy from the donor fluorophore (eGFP) is transferred to the acceptor fluorophore (DY547-P1), whose absorption spectrum overlaps with the emission spectrum of the donor. FRET efficiency was determined by measuring the decrease of donor fluorescence, allowing in this way the quantification of ligand binding. The efficiency of P1-cGMP to activate the CNGA1-eGFP channel was close to that of the physiological ligand, cGMP,  $90 \pm 3\%$  ( $\pm$  SEM) in the presence of saturating [P1-cGMP]. The potency of the P1-cGMP to activate the CNGA1-eGFP channels was factor 10 higher in comparison with that of cGMP. The eGFP-labelling procedure had only a minor influence on the channel's functional characteristics ( $EC_{50}=27.9 \mu\text{M}$  P1-cGMP,  $H=2.1$ ,  $n=5-8$  vs.  $EC_{50}=35.3 \mu\text{M}$  cGMP,  $H=2.6$ ,  $n=5-8$ ). The high FRET efficiency observed in combination with cPCF allowed us to study simultaneous ligand binding and channel activation. Our results showed that at concentrations lower than  $\sim 10 \mu\text{M}$  P1-cGMP, binding exceeds gating and at higher concentrations gating exceeds binding. In conclusion, the method proposed herein represents an elegant approach to measure ligand binding over a wider concentration range and to monitor binding to individually labelled subunits selectively.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB290

### Functional Evidence for P2X7 Receptor-Mediated Ionic Currents and Macromolecule Uptake in Cochlear Glial Cells

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The auditory nerve transmits acoustic signals from cochlear hair cells to the brain. The spiral ganglion neurons (SGNs) that form the primary afferent pathway are reliant on glial cells for their function and long-term survival, but neuro-glial interactions remain largely uncharacterized. Purinergic signaling is recognized as a regulator of auditory nerve physiology, following the detection of various P2X and P2Y receptors, and demonstration of neuronal ionotropic and metabotropic responses (Housley et al, 2002). Here, we examined P2X7 receptors (P2X7Rs) as potential mediators of neuro-glial communication in the cochlea.

In rat cochlear vibratome sections, P2X7R immunofluorescence was first detected at postnatal day 6 (P6) in Schwann cells wrapping SGN peripheral neurites and in satellite cells surrounding the SGN cell bodies, but not in oligodendrocytes in the central portion of the auditory nerve. This expression pattern was conserved through to adulthood. Elsewhere in the peripheral nervous system P2X7Rs are non-selectively permeable to small cations when stimulated transiently, but during prolonged agonist exposure there is an increased permeability to larger cationic molecules up to 1kDa (e.g. fluorescent dyes).

To confirm functional expression and investigate the properties of P2X7Rs in cochlear glia, we prepared dissociated spiral ganglion cultures from hearing rats (P13-P14). In electrophysiology and dye uptake experiments P2X7Rs were stimulated using the P2X agonist BzATP (10  $\mu$ M) and blocked by the specific P2X7R antagonist A-740003 (100 nM). Patch clamp recordings demonstrated BzATP-activated currents that increased in magnitude with prolonged exposure time, and that were inhibited by A-740003. BzATP activated rapid YOPRO-1 uptake into glia during time series confocal imaging, and the YOPRO-1 uptake was prevented by incubation with A-740003. Where an SGN and its satellite cell remained paired, BzATP-mediated YOPRO-1 uptake could be detected only within the satellite cell.

The present study demonstrates functional expression of P2X7Rs in glial cells within the peripheral portion of the auditory nerve, raising the possibility that these enigmatic ion channels play roles in cochlear neuro-glial communication. We suggest that P2XRs may act as sensors of tissue stress during periods of damaging noise or ischemia.

Housley, G. D. *et al.* (2002) 'Purineric regulation of sound transduction and auditory neurotransmission', *Audiology and Neuro-Otology*, 7(1), pp. 55–61. doi: 10.1159/000046865.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCB291

##### Effects of dynamic arm cycling on motor cortical excitability in humans

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Isometric contractions of upper limb muscles have been shown to increase corticospinal excitability of trunk muscles, known as crossed-corticospinal facilitation. Neural mechanisms underlying this effect are likely to be cortical in origin as work has demonstrated a reduction in intracortical inhibition but no change in spinal excitability during the contractions. Further, we have previously shown that excitability of corticospinal pathways controlling contralateral erector spinae (ES) muscles was facilitated by unilateral arm cycling, a dynamic contraction of arm muscles, in healthy subjects. However, the neural mechanisms underlying the increased corticospinal excitability of the trunk muscles during dynamic arm contractions, a rhythmic movement, remains unclear. Hence, the aim of this study was to investigate activity of intracortical neural circuits within the motor cortex during static and dynamic arm muscle contractions in healthy subjects using paired-pulse transcranial magnetic stimulation (TMS) paradigms. EMG activity of 9 subjects were recorded at the contralateral erector spinae (ES) muscle to the cycling arm (T12 vertebral level), and at the biceps brachii (BB) of the cycling arm. Three tasks were conducted; a control task (arms by the side), dynamic task (arm cycling at 60 revolutions per minute), and a static task (exerting a level of EMG matched to the dynamic task). TMS was applied to the hotspot for ES over the primary motor cortex and short-interval intracortical inhibition (SICI) and short-interval intracortical facilitation

(SICF) were examined at the pedal position corresponding to maximal BB EMG (6 o'clock position). Prestimulus EMG in ES was matched across the tasks. Our results showed that ES MEPs were greater during the dynamic and static tasks than the control task (dynamic: 172.348 [7.94]% of MEP during the control task; static: 143.53 [29.86]%). SICI was reduced and SICF was increased during the dynamic (SICI: 106.85 [18.77]%; SICF: 228.17 [93.32]%) and static tasks (SICI: 109.41 [23.57] test MEP; SICF: 212.93 [38.09]%) compared to the control task (SICI: 68.76 [15.38]%; SICF: 152.59 [73.17%]) when the test stimulus intensity was adjusted for the dynamic and static tasks. Our findings show that both dynamic and static contractions of upper limb muscles can increase corticospinal excitability of the contralateral trunk muscles. We further demonstrate that the increased corticospinal excitability of the trunk muscles likely involves cortical mechanisms. These findings improve our understanding to cortical control of trunk muscles during voluntary contractions of upper limb muscles and could have implications for development of rehabilitation programmes in subjects with impaired function of the trunk, such as those with spinal cord injury and back pain.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

#### PCB292

##### Pathological changes in muscle signaling mechanisms in muscle contractures of children with cerebral palsy

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Background: Cerebral palsy (CP) is a non-progressive motor disorder that affects the posture and gait of the patients. One major complication in CP is the development of muscle contractures. The mechanisms behind contractures are still poorly understood. Aim: The aim of the present study was to elaborate whether specific signaling pathways in the muscle are correlated to increased passive stiffness and reflex activity in CP when compared with typical developing children (TD), and further to investigate sarcomere length and possible mitochondrial mutations in both groups. Methods: Next Generation Sequencing of 92 candidate targets was performed in muscle biopsies from the m. Gastrocnemius muscle in n=10 children with CP and n=10 TD children. In addition, stretch reflexes and passive stiffness was measured in the lower legs. The sarcomere length was measured using electron microscopy and the mitochondria were also sequenced in all samples. All participants gave informed consent and the protocol was approved by the Regional Ethics Committee for Copenhagen (H-4-2014-047), and the protocol was in compliance with the Helsinki Declaration. Results: The sequencing results from all candidate genes are shown in Figure 1. The red colour indicates a downregulation of the target in children with CP while the green colour indicates an upregulation in CP when compared to TD children. Passive stiffness was significantly correlated to mRNA expressions of *HSPG2* ( $p=0.02$ ;  $R^2=0.67$ ), *PRELP* ( $p=0.002$ ;  $R^2=0.84$ ), *RYR3* ( $p=0.04$ ;  $R^2=0.66$ ), *COL5A3* ( $p=0.0007$ ;  $R^2=0.88$ ), *ASPH* ( $p=0.002$ ;  $R^2=0.82$ ) and *COL4A6* ( $p=0.03$ ;  $R^2=0.97$ ). In addition the reflex activity



was significantly correlated to mRNA expressions of *HSPG2* ( $p=0.02$ ;  $R^2=0.67$ ), *LAMC1* ( $p=0.04$ ;  $R^2=0.69$ ) and *COL4A6* ( $p=0.05$ ;  $R^2=0.96$ ). Furthermore, the sarcomere length was significantly increased in children with CP when compared with TD ( $p=0.04$ ). Subsequently children with CP showed more pathogenic variants in the mitochondria compared to TD children (variants shown as variants in total), especially in *COII* (CP=11; TD=4), *COIII* (CP=11; TD=6) and *ATPase6* (CP=10; TD=5). Conclusion: The present study shows that some of the investigated targets potentially might be associated to the severity of passive stiffness and reflex activity in cerebral palsy. Perspectives: The cause and effect between the expression of certain genes and muscle stiffness still has to be proven in order to create a better treatment for muscle contractures. Funding

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| Collagen |                 |              | Cytoskeleton |                 |              | Mitochondria |                 |              | Muscle growth |                 |              |
|----------|-----------------|--------------|--------------|-----------------|--------------|--------------|-----------------|--------------|---------------|-----------------|--------------|
| Target   | fold difference | Significance | Target       | fold difference | Significance | Target       | fold difference | Significance | Target        | fold difference | Significance |
| COL3A1   | 1.1             | ns           | ACTA1        | 1.2             | ns           | ND1          | 1.1             | ns           | MYH2          | 1.1             | ns           |
| COL4A1   | 1.1             | ns           | SOX1         | 1.2             | ns           | ND2          | 1.1             | ns           | MYH7          | 1.1             | ns           |
| COL4A2   | 1.1             | ns           | SOX2         | 1.2             | ns           | ND3          | 1.1             | ns           | MYH8          | 1.1             | ns           |
| COL4A3   | 1.1             | ns           | SOX3         | 1.2             | ns           | ND4          | 1.1             | ns           | MYH9          | 1.1             | ns           |
| COL4A4   | 1.1             | ns           | SOX4         | 1.2             | ns           | ND5          | 1.1             | ns           | MYH10         | 1.1             | ns           |
| COL4A5   | 1.1             | ns           | SOX5         | 1.2             | ns           | ND6          | 1.1             | ns           | MYH11         | 1.1             | ns           |
| COL4A6   | 1.1             | ns           | SOX6         | 1.2             | ns           | ND7          | 1.1             | ns           | MYH12         | 1.1             | ns           |
| COL4A7   | 1.1             | ns           | SOX7         | 1.2             | ns           | ND8          | 1.1             | ns           | MYH13         | 1.1             | ns           |
| COL4A8   | 1.1             | ns           | SOX8         | 1.2             | ns           | ND9          | 1.1             | ns           | MYH14         | 1.1             | ns           |
| COL4A9   | 1.1             | ns           | SOX9         | 1.2             | ns           | ND10         | 1.1             | ns           | MYH15         | 1.1             | ns           |
| COL4A10  | 1.1             | ns           | SOX10        | 1.2             | ns           | ND11         | 1.1             | ns           | MYH16         | 1.1             | ns           |
| COL4A11  | 1.1             | ns           | SOX11        | 1.2             | ns           | ND12         | 1.1             | ns           | MYH17         | 1.1             | ns           |
| COL4A12  | 1.1             | ns           | SOX12        | 1.2             | ns           | ND13         | 1.1             | ns           | MYH18         | 1.1             | ns           |
| COL4A13  | 1.1             | ns           | SOX13        | 1.2             | ns           | ND14         | 1.1             | ns           | MYH19         | 1.1             | ns           |
| COL4A14  | 1.1             | ns           | SOX14        | 1.2             | ns           | ND15         | 1.1             | ns           | MYH20         | 1.1             | ns           |
| COL4A15  | 1.1             | ns           | SOX15        | 1.2             | ns           | ND16         | 1.1             | ns           | MYH21         | 1.1             | ns           |
| COL4A16  | 1.1             | ns           | SOX16        | 1.2             | ns           | ND17         | 1.1             | ns           | MYH22         | 1.1             | ns           |
| COL4A17  | 1.1             | ns           | SOX17        | 1.2             | ns           | ND18         | 1.1             | ns           | MYH23         | 1.1             | ns           |
| COL4A18  | 1.1             | ns           | SOX18        | 1.2             | ns           | ND19         | 1.1             | ns           | MYH24         | 1.1             | ns           |
| COL4A19  | 1.1             | ns           | SOX19        | 1.2             | ns           | ND20         | 1.1             | ns           | MYH25         | 1.1             | ns           |
| COL4A20  | 1.1             | ns           | SOX20        | 1.2             | ns           | ND21         | 1.1             | ns           | MYH26         | 1.1             | ns           |
| COL4A21  | 1.1             | ns           | SOX21        | 1.2             | ns           | ND22         | 1.1             | ns           | MYH27         | 1.1             | ns           |
| COL4A22  | 1.1             | ns           | SOX22        | 1.2             | ns           | ND23         | 1.1             | ns           | MYH28         | 1.1             | ns           |
| COL4A23  | 1.1             | ns           | SOX23        | 1.2             | ns           | ND24         | 1.1             | ns           | MYH29         | 1.1             | ns           |
| COL4A24  | 1.1             | ns           | SOX24        | 1.2             | ns           | ND25         | 1.1             | ns           | MYH30         | 1.1             | ns           |
| COL4A25  | 1.1             | ns           | SOX25        | 1.2             | ns           | ND26         | 1.1             | ns           | MYH31         | 1.1             | ns           |
| COL4A26  | 1.1             | ns           | SOX26        | 1.2             | ns           | ND27         | 1.1             | ns           | MYH32         | 1.1             | ns           |
| COL4A27  | 1.1             | ns           | SOX27        | 1.2             | ns           | ND28         | 1.1             | ns           | MYH33         | 1.1             | ns           |
| COL4A28  | 1.1             | ns           | SOX28        | 1.2             | ns           | ND29         | 1.1             | ns           | MYH34         | 1.1             | ns           |
| COL4A29  | 1.1             | ns           | SOX29        | 1.2             | ns           | ND30         | 1.1             | ns           | MYH35         | 1.1             | ns           |
| COL4A30  | 1.1             | ns           | SOX30        | 1.2             | ns           | ND31         | 1.1             | ns           | MYH36         | 1.1             | ns           |
| COL4A31  | 1.1             | ns           | SOX31        | 1.2             | ns           | ND32         | 1.1             | ns           | MYH37         | 1.1             | ns           |
| COL4A32  | 1.1             | ns           | SOX32        | 1.2             | ns           | ND33         | 1.1             | ns           | MYH38         | 1.1             | ns           |
| COL4A33  | 1.1             | ns           | SOX33        | 1.2             | ns           | ND34         | 1.1             | ns           | MYH39         | 1.1             | ns           |
| COL4A34  | 1.1             | ns           | SOX34        | 1.2             | ns           | ND35         | 1.1             | ns           | MYH40         | 1.1             | ns           |
| COL4A35  | 1.1             | ns           | SOX35        | 1.2             | ns           | ND36         | 1.1             | ns           | MYH41         | 1.1             | ns           |
| COL4A36  | 1.1             | ns           | SOX36        | 1.2             | ns           | ND37         | 1.1             | ns           | MYH42         | 1.1             | ns           |
| COL4A37  | 1.1             | ns           | SOX37        | 1.2             | ns           | ND38         | 1.1             | ns           | MYH43         | 1.1             | ns           |
| COL4A38  | 1.1             | ns           | SOX38        | 1.2             | ns           | ND39         | 1.1             | ns           | MYH44         | 1.1             | ns           |
| COL4A39  | 1.1             | ns           | SOX39        | 1.2             | ns           | ND40         | 1.1             | ns           | MYH45         | 1.1             | ns           |
| COL4A40  | 1.1             | ns           | SOX40        | 1.2             | ns           | ND41         | 1.1             | ns           | MYH46         | 1.1             | ns           |
| COL4A41  | 1.1             | ns           | SOX41        | 1.2             | ns           | ND42         | 1.1             | ns           | MYH47         | 1.1             | ns           |
| COL4A42  | 1.1             | ns           | SOX42        | 1.2             | ns           | ND43         | 1.1             | ns           | MYH48         | 1.1             | ns           |
| COL4A43  | 1.1             | ns           | SOX43        | 1.2             | ns           | ND44         | 1.1             | ns           | MYH49         | 1.1             | ns           |
| COL4A44  | 1.1             | ns           | SOX44        | 1.2             | ns           | ND45         | 1.1             | ns           | MYH50         | 1.1             | ns           |
| COL4A45  | 1.1             | ns           | SOX45        | 1.2             | ns           | ND46         | 1.1             | ns           | MYH51         | 1.1             | ns           |
| COL4A46  | 1.1             | ns           | SOX46        | 1.2             | ns           | ND47         | 1.1             | ns           | MYH52         | 1.1             | ns           |
| COL4A47  | 1.1             | ns           | SOX47        | 1.2             | ns           | ND48         | 1.1             | ns           | MYH53         | 1.1             | ns           |
| COL4A48  | 1.1             | ns           | SOX48        | 1.2             | ns           | ND49         | 1.1             | ns           | MYH54         | 1.1             | ns           |
| COL4A49  | 1.1             | ns           | SOX49        | 1.2             | ns           | ND50         | 1.1             | ns           | MYH55         | 1.1             | ns           |
| COL4A50  | 1.1             | ns           | SOX50        | 1.2             | ns           | ND51         | 1.1             | ns           | MYH56         | 1.1             | ns           |
| COL4A51  | 1.1             | ns           | SOX51        | 1.2             | ns           | ND52         | 1.1             | ns           | MYH57         | 1.1             | ns           |
| COL4A52  | 1.1             | ns           | SOX52        | 1.2             | ns           | ND53         | 1.1             | ns           | MYH58         | 1.1             | ns           |
| COL4A53  | 1.1             | ns           | SOX53        | 1.2             | ns           | ND54         | 1.1             | ns           | MYH59         | 1.1             | ns           |
| COL4A54  | 1.1             | ns           | SOX54        | 1.2             | ns           | ND55         | 1.1             | ns           | MYH60         | 1.1             | ns           |
| COL4A55  | 1.1             | ns           | SOX55        | 1.2             | ns           | ND56         | 1.1             | ns           | MYH61         | 1.1             | ns           |
| COL4A56  | 1.1             | ns           | SOX56        | 1.2             | ns           | ND57         | 1.1             | ns           | MYH62         | 1.1             | ns           |
| COL4A57  | 1.1             | ns           | SOX57        | 1.2             | ns           | ND58         | 1.1             | ns           | MYH63         | 1.1             | ns           |
| COL4A58  | 1.1             | ns           | SOX58        | 1.2             | ns           | ND59         | 1.1             | ns           | MYH64         | 1.1             | ns           |
| COL4A59  | 1.1             | ns           | SOX59        | 1.2             | ns           | ND60         | 1.1             | ns           | MYH65         | 1.1             | ns           |
| COL4A60  | 1.1             | ns           | SOX60        | 1.2             | ns           | ND61         | 1.1             | ns           | MYH66         | 1.1             | ns           |
| COL4A61  | 1.1             | ns           | SOX61        | 1.2             | ns           | ND62         | 1.1             | ns           | MYH67         | 1.1             | ns           |
| COL4A62  | 1.1             | ns           | SOX62        | 1.2             | ns           | ND63         | 1.1             | ns           | MYH68         | 1.1             | ns           |
| COL4A63  | 1.1             | ns           | SOX63        | 1.2             | ns           | ND64         | 1.1             | ns           | MYH69         | 1.1             | ns           |
| COL4A64  | 1.1             | ns           | SOX64        | 1.2             | ns           | ND65         | 1.1             | ns           | MYH70         | 1.1             | ns           |
| COL4A65  | 1.1             | ns           | SOX65        | 1.2             | ns           | ND66         | 1.1             | ns           | MYH71         | 1.1             | ns           |
| COL4A66  | 1.1             | ns           | SOX66        | 1.2             | ns           | ND67         | 1.1             | ns           | MYH72         | 1.1             | ns           |
| COL4A67  | 1.1             | ns           | SOX67        | 1.2             | ns           | ND68         | 1.1             | ns           | MYH73         | 1.1             | ns           |
| COL4A68  | 1.1             | ns           | SOX68        | 1.2             | ns           | ND69         | 1.1             | ns           | MYH74         | 1.1             | ns           |
| COL4A69  | 1.1             | ns           | SOX69        | 1.2             | ns           | ND70         | 1.1             | ns           | MYH75         | 1.1             | ns           |
| COL4A70  | 1.1             | ns           | SOX70        | 1.2             | ns           | ND71         | 1.1             | ns           | MYH76         | 1.1             | ns           |
| COL4A71  | 1.1             | ns           | SOX71        | 1.2             | ns           | ND72         | 1.1             | ns           | MYH77         | 1.1             | ns           |
| COL4A72  | 1.1             | ns           | SOX72        | 1.2             | ns           | ND73         | 1.1             | ns           | MYH78         | 1.1             | ns           |
| COL4A73  | 1.1             | ns           | SOX73        | 1.2             | ns           | ND74         | 1.1             | ns           | MYH79         | 1.1             | ns           |
| COL4A74  | 1.1             | ns           | SOX74        | 1.2             | ns           | ND75         | 1.1             | ns           | MYH80         | 1.1             | ns           |
| COL4A75  | 1.1             | ns           | SOX75        | 1.2             | ns           | ND76         | 1.1             | ns           | MYH81         | 1.1             | ns           |
| COL4A76  | 1.1             | ns           | SOX76        | 1.2             | ns           | ND77         | 1.1             | ns           | MYH82         | 1.1             | ns           |
| COL4A77  | 1.1             | ns           | SOX77        | 1.2             | ns           | ND78         | 1.1             | ns           | MYH83         | 1.1             | ns           |
| COL4A78  | 1.1             | ns           | SOX78        | 1.2             | ns           | ND79         | 1.1             | ns           | MYH84         | 1.1             | ns           |
| COL4A79  | 1.1             | ns           | SOX79        | 1.2             | ns           | ND80         | 1.1             | ns           | MYH85         | 1.1             | ns           |
| COL4A80  | 1.1             | ns           | SOX80        | 1.2             | ns           | ND81         | 1.1             | ns           | MYH86         | 1.1             | ns           |
| COL4A81  | 1.1             | ns           | SOX81        | 1.2             | ns           | ND82         | 1.1             | ns           | MYH87         | 1.1             | ns           |
| COL4A82  | 1.1             | ns           | SOX82        | 1.2             | ns           | ND83         | 1.1             | ns           | MYH88         | 1.1             | ns           |
| COL4A83  | 1.1             | ns           | SOX83        | 1.2             | ns           | ND84         | 1.1             | ns           | MYH89         | 1.1             | ns           |
| COL4A84  | 1.1             | ns           | SOX84        | 1.2             | ns           | ND85         | 1.1             | ns           | MYH90         | 1.1             | ns           |
| COL4A85  | 1.1             | ns           | SOX85        | 1.2             | ns           | ND86         | 1.1             | ns           | MYH91         | 1.1             | ns           |
| COL4A86  | 1.1             | ns           | SOX86        | 1.2             | ns           | ND87         | 1.1             | ns           | MYH92         | 1.1             | ns           |
| COL4A87  | 1.1             | ns           | SOX87        | 1.2             | ns           | ND88         | 1.1             | ns           | MYH93         | 1.1             | ns           |
| COL4A88  | 1.1             | ns           | SOX88        | 1.2             | ns           | ND89         | 1.1             | ns           | MYH94         | 1.1             | ns           |
| COL4A89  | 1.1             | ns           | SOX89        | 1.2             | ns           | ND90         | 1.1             | ns           | MYH95         | 1.1             | ns           |
| COL4A90  | 1.1             | ns           | SOX90        | 1.2             | ns           | ND91         | 1.1             | ns           | MYH96         | 1.1             | ns           |
| COL4A91  | 1.1             | ns           | SOX91        | 1.2             | ns           | ND92         | 1.1             | ns           | MYH97         | 1.1             | ns           |
| COL4A92  | 1.1             | ns           | SOX92        | 1.2             | ns           | ND93         | 1.1             | ns           | MYH98         | 1.1             | ns           |
| COL4A93  | 1.1             | ns           | SOX93        | 1.2             | ns           | ND94         | 1.1             | ns           | MYH99         | 1.1             | ns           |
| COL4A94  | 1.1             | ns           | SOX94        | 1.2             | ns           | ND95         | 1.1             | ns           | MYH100        | 1.1             | ns           |
| COL4A95  | 1.1             | ns           | SOX95        | 1.2             | ns           | ND96         | 1.1             | ns           | MYH101        | 1.1             | ns           |
| COL4A96  | 1.1             | ns           | SOX96        | 1.2             | ns           | ND97         | 1.1             | ns           | MYH102        | 1.1             | ns           |
| COL4A97  | 1.1             | ns           | SOX97        | 1.2             | ns           | ND98         | 1.1             | ns           | MYH103        | 1.1             | ns           |
| COL4A98  | 1.1             | ns           | SOX98        | 1.2             | ns           | ND99         | 1.1             | ns           | MYH104        | 1.1             | ns           |
| COL4A99  | 1.1             | ns           | SOX99        | 1.2             | ns           | ND100        | 1.1             | ns           | MYH105        | 1.1             | ns           |
| COL4A100 | 1.1             | ns           | SOX100       | 1.2             | ns           | ND101        | 1.1             | ns           | MYH106        | 1.1             | ns           |
| COL4A101 | 1.1             | ns           | SOX101       | 1.2             | ns           | ND102        | 1.1             | ns           | MYH107        | 1.1             | ns           |
| COL4A102 | 1.1             | ns           | SOX102       | 1.2             | ns           | ND103        | 1.1             | ns           | MYH108        | 1.1             | ns           |
| COL4A103 | 1.1             | ns           | SOX103       | 1.2             | ns           | ND104        | 1.1             | ns           | MYH109        | 1.1             | ns           |
| COL4A104 | 1.1             | ns           | SOX104       | 1.2             | ns           | ND105        | 1.1             | ns           | MYH110        | 1.1             | ns           |
| COL4A105 | 1.1             | ns           | SOX105       | 1.2             | ns           | ND106        | 1.1             | ns           | MYH111        | 1.1             | ns           |
| COL4A106 | 1.1             | ns           | SOX106       | 1.2             | ns           | ND107        | 1.1             | ns           | MYH112        | 1.1             | ns           |
| COL4A107 | 1.1             | ns           | SOX107       | 1.2             | ns           | ND108        | 1.1             | ns           | MYH113        | 1.1             | ns           |
| COL4A108 | 1.1             | ns           | SOX108       | 1.2             | ns           | ND109        | 1.1             | ns           | MYH114        | 1.1             | ns           |
| COL4A109 | 1.1             | ns           | SOX109       | 1.2             | ns           | ND110        | 1.1             | ns           | MYH115        | 1.1             | ns           |
| COL4A110 | 1.1             | ns           | SOX110       | 1.2             | ns           | ND111        | 1.1             | ns           | MYH116        | 1.1             | ns           |
| COL4A111 | 1.1             | ns           | SOX111       | 1.2             | ns           | ND112        | 1.1             | ns           | MYH117        | 1.1             | ns           |
| COL4A112 | 1.1             | ns           | SOX112       | 1.2             | ns           | ND113        | 1.1             | ns           | MYH118        | 1.1             | ns           |
| COL4A113 | 1.1             | ns           | SOX113       | 1.2             | ns           | ND114        | 1.1             | ns           | MYH119        | 1.1             | ns           |
| COL4A114 | 1.1             | ns           | SOX114       | 1.2             | ns           | ND115        | 1.1             | ns           | MYH120        | 1.1             | ns           |
| COL4A115 | 1.1             | ns           | SOX115       | 1.2             | ns           | ND116        | 1.1             | ns           | MYH121        | 1.1             | ns           |
| COL4A116 | 1.1             | ns           | SOX116       | 1.2             | ns           | ND117        | 1.1             | ns           | MYH122        | 1.1             | ns           |
| COL4A117 | 1.1             | ns           | SOX117       | 1.2             | ns           | ND118        | 1.1             | ns           | MYH123        | 1.1             | ns           |
| COL4A118 | 1.1             | ns           | SOX118       | 1.2             | ns           | ND119        | 1.1             | ns           | MYH124        | 1.1             | ns           |

In the dorsal region of the dentate gyrus, Tegaserod group had significantly fewer proliferating cells, ( $32.6 \pm 1.0$ ,  $p < 0.05$ ) compared to the control group ( $40.3 \pm 2$ ,  $p < 0.05$ ). Furthermore there was a reduction in the percentage colocalisation of EdU and the neuronal marker HuC/D in treatment group ( $8.8\% \pm 0.8$  vs  $13.7\% \pm 1.2$ ,  $p < 0.05$ ). EdU+ve cells were counted in central canal, grey matter and white matter of the spinal cord. Tegaserod-treated mice had lower numbers of proliferating cells in specific regions of spinal cord compared to control. Overall in cervical regions there were fewer proliferating cells in Tegaserod-treated mice compared with control ( $79 \pm 8.8$  vs  $102.5 \pm 8.2$ ,  $p < 0.05$ ). Furthermore the central canal had fewer proliferating cells compared to control group ( $4.4 \pm 1.4$  vs  $7.8 \pm 1.3$ ,  $p < 0.05$ ). However there were no significant differences in the numbers of EdU+ve cells between Tegaserod-treated and control mice in either gray matter or white matter. In sacral spinal cord, Edu+ve cells were significantly fewer in all regions including white matter ( $19.6 \pm 1.4$  vs  $30.5 \pm 3.6$ ,  $p < 0.05$ ), gray matter ( $28.3 \pm 2.0$  vs  $47.4 \pm 4.5$ ,  $p < 0.05$ ) and central canal ( $5.5 \pm 0.9$  vs  $9.8 \pm 1.2$ ,  $p < 0.05$ ) in treatment group compared to control. The percentages of differentiation markers were determined and there were no significant difference between groups for all markers. In conclusion, activation of 5-HT<sub>4</sub> reduced the extent of cell proliferation generally and the differentiation into neurones specifically in hippocampus. It might suggest this activation can help in reducing the rate of cell proliferation in cancerous conditions.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB295

### Association of serotonin levels in urine with the autism severity.

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Autism-spectrum disorder (ASD) is a neurodevelopmental disorder characterized by deficits in social communication and repetitive patterns of behavior. However, most of the children with autism have gastrointestinal disorders (GI) and there is a correlation between the severity of autism manifestations and GI symptoms. Although the association between GI dysfunction and ASD behavior is not completely understood, research over the past years suggests that the gut-brain axis plays a critical role. Bidirectional communication between gut microbiome and the brain might be the responsible for at least some specific behavioral symptoms in people with ASD and

serotonin is the possible signal molecule of this pathway. Serotonin pleiotropic effect across multiple brain systems is well known, it is a critical modulator of enteric and central nervous systems development and function. It has been shown on animal models that serotonin is associated with behavioral abnormalities. Recently a correlation between a quantitative measure of GI symptoms and whole blood serotonin levels was observed in children and adolescents with ASD.

In order to assess the possible use of serotonin detection for diagnosis of autism or for establishing autism severity, we used the morning urine of 34 children with autism (age  $6.39 \pm 0.38$  years) and 31 neurotypical children (age  $6.88 \pm 0.39$  years). All children in autistic group were boys, 28 children in the control group were boys and three girls. Children with autism were recruited from the Autism Centre based at the Institute of Physiology, Comenius University in Bratislava, Faculty of Medicine. The protocol was approved by the Ethics Committee of the University. The study conformed to the code of ethics stated in the Declaration of Helsinki. Psychological evaluation of the children with autism was performed using ADIR and ADOS-2 scales. LC-MS/MS was used for serotonin detection.

The results of the in our study revealed that children with autism had higher level of urine serotonin ( $0.1058 \pm 0.0039$  mmol serotonin/mmol creatinine) than children without ( $0.1014 \pm 0.0058$  mmol serotonin/mmol creatinine), although the difference was not significant ( $p = 0.26$ ). Interestingly within the group of children with autism a significant association between the autism severity and serotonin levels was found. Children with more than 50% of the symptoms registered by ADOS-2 had significantly higher ( $0.1139 \pm 0.0063$  mmol serotonin/mmol creatinine) levels of serotonin, than children with less than 50% of the symptoms ( $0.09864 \pm 0.0042$  mmol serotonin/mmol creatinine)  $p = 0.024$ . Age difference in the groups was not significant  $5.969 \pm 0.61$  vs  $6.811 \pm 0.49$  years,  $p = 0.15$ .

The results of our study support the hypothesis of association of serotonin levels with the autism manifestations, but only more complex study will reveal the mechanism of serotonin modulation of the gut-brain axis.

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### Effect of High Fructose Corn Syrup Consumption and Stress on TRPM2 Immunoreactivity in Rat Brain

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Metastin-like Transient Receptor Potential Channel 2 (TRPM2) is a nonselective cation channel that is activated under oxidative stress conditions and leads to increased intracellular free calcium concentration and cell apoptosis. The protein channels are widely expressed in central nervous system and brain, which are quite vulnerable to oxidative stress. In this study it has been researched consumption of high fructose corn syrup

(HFCS), which is quite widespread use in the food industry in recent years, and stress on the TRPM2 channels immunoreactivity in rat brain.

In the study, 28 Wistar-Albino male rats were divided randomly into 4 groups and with 7 animals in each group: Group I (Control), Group II (applied a 2 weeks 20 % (v/v) HFCS solution), Group III (applied a 2 weeks 40% (v/v) HFCS solution) and Group IV (rats were exposed to a chronic restraint stress procedure: 3h/day in first week, 6h/day in second week). At the end of the study, blood samples were taken from the animals. Total oxidant (TOS) and total antioxidant levels (TAS) were measured by ELISA method (Enzyme-Linked Immunosorbent Analysis) and the oxidative stress index (OSI) was calculated. Immunohistochemistry was performed in some stress-related brain regions (prefrontal cortex, amygdala, nucleus accumbens, hippocampus). Brain samples were removed and fixed 10% formaldehyde solution. Paraffin blocks were prepared and 5 µm brain section stained with Hematoxylin-Eosin and Periodic Acid Schiff (PAS) method to analyses TRPM2 ion channel immunoreactivity.

Immunohistochemical study showed that TRPM2 immunoreactivity were increased in all brain regions in HFCS (20%), HFCS (40%) and stress groups compared to control group. TOS levels and OSI values, which are reflecting oxidative stress status of body, were found to be higher in HFCS (20%, 40%) and stress groups compared to control. There was no significant difference in TAS levels, reflecting body antioxidant systems, between groups.

As a result, it was seen that consumption of high fructose corn syrup may lead to oxidative stress and increased TRPM2 immunoreactivity in the brain similar to stress.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

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### Circadian rhythms in a rat model of depression.

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The circadian system plays an important role in normal physiology and its abnormal regulation can result in cardiovascular disease, metabolic syndrome, obesity, immune dysfunction, increased risk for cancer, etc. In depressed patients, specific abnormalities in circadian system are individual and might be expressed as a phase advance or phase delay of rhythms and/or increase or decrease in the amplitude (McClung 2007). To analyse the involvement of circadian system in the development of depression, we used Wistar rats exposed for 3.5 weeks to chronic mild stress paradigm. The development of anhedonic-like behaviour was controlled weekly by sucrose consumption test and animals with more than 30% reduced sucrose intake within 3 weeks were used for further analysis. All methods and procedures are described in detail in Christiansen *et al.*, 2016a and Christiansen *et al.*, 2016b. We report that 3-week chronic exposure to mild stressors induces anhedonic-like behavior in some Wistar rats. Anhedonic-like rats demonstrate hypothermia ( $\Delta=0.83^{\circ}\text{C}$  in core body temperature at zeitgeber time (ZT) 22), hypercortisolemia (presence

of the second peak at ZT2), and hypermelatoninemia (at peak ZT18  $21.4 \pm 1.6$  vs.  $15.5 \pm 1.4$  mmol/l ( $p<0.05$ ) and at ZT22  $12.2 \pm 2.1$  vs.  $5.2 \pm 1.9$  mmol/l ( $p<0.05$ )). The *in situ* hybridization analysis of clock genes (Per1, Per2, and Bmal1) in brain slices of anhedonic-like and control rats revealed shift in phase in the expression of these genes in different brain areas (table 1). In suprachiasmatic nucleus (master clock), the expression of Per1 is sustainable in maintaining the circadian rhythm, while Per2 and Bmal1 demonstrate sensitivity to chronic stress and involvement into the development of the depression-like condition. In pineal gland, both Per1 and Bmal1 demonstrated their involvement in the development of the depression-like condition, while the expression level of Per2 was undetectable. In hippocampal areas, we were able to detect the Per1 only, which was phase-advanced in anhedonic-like rats. Thus, the normalization of the expression patterns of clock genes is likely to be essential for the recovery from the pathological depression state, induced by chronic stress. Despite the fact that mechanisms, underlying the development of depression are still under investigation, the involvement of circadian system in this process is clear and requires now a molecular biological description of the causal relationship between circadian rhythm and mood disorders. This is necessary for screening for the possible targets in order to achieve better results in the treatment of worldwide distributing pathology.

|                                           | Control Wistar Rats |      |       | Anhedonic-like Wistar Rats |      |       |
|-------------------------------------------|---------------------|------|-------|----------------------------|------|-------|
|                                           | Per1                | Per2 | Bmal1 | Per1                       | Per2 | Bmal1 |
| Pineal gland                              |                     |      |       |                            |      |       |
| Peak                                      | 18                  | -    | 2     | 14                         | -    | 22    |
| Nadir                                     | 10                  | -    | 14    | 6                          | -    | 10    |
| Suprachiasmatic nucleus                   |                     |      |       |                            |      |       |
| Peak                                      | 6                   | 10   | 14    | 6                          | 6    | 18    |
| Nadir                                     | 18                  | 18   | 6     | 18                         | 2    | 6     |
| Hippocampus (CA1, CA3, and dentate gyrus) |                     |      |       |                            |      |       |
| Peak                                      | 22                  |      |       | 18                         |      |       |
| Nadir                                     | 14                  |      |       | 10                         |      |       |

Table 1. Zeitgeber time (ZT=0 at 6:00 am) of peak and nadir expression level of clock genes in brain areas.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

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### Investigating the neuronal networks responsible for insulin sensing in the dorsal vagal complex

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Obesity is an epidemic that is spreading worldwide, by 2020 8/10 men and 7/10 women will be overweight or obese. Obesity leads to negative metabolic consequences including insulin resistance and diabetes. Obesity-related diseases e.g. diabetes will likely cost the NHS an extra £2.5 billion per year by 2035. New anti-obesity treatments are essential and recent research has begun to consider how targeting the CNS may be the answer.

The dorsal vagal complex (DVC) of the brain can sense changes in insulin levels and regulate glucose metabolism and feeding behaviour. However, the neuronal circuitry in



the DVC that senses insulin to regulate metabolic function are still unknown. Direct insulin infusion to the DVC lowers food intake and glucose levels in healthy rats but fails to do so following 3 days of high fat diet (HFD), indicating DVC insulin resistance. The exact mechanism for these changes following HFD is unknown, however changes in mitochondria, through alteration of mitochondrial dynamics, may be involved. HFD increases mitochondria fission in the DVC via activation of Dynamin Related Protein 1 (Drp1) which causes an increase in ER stress that leads to insulin resistance. It is still unclear how HFD-dependent activation of Drp1 causes ER stress and insulin resistance. A potential mediator of this process is iNOS as DVC iNOS levels increase following HFD. It is crucial to understand this event to prevent the loss of insulin sensitivity in the brain. We aim to characterise the neural cell types responsible for insulin sensing in the DVC and understand how changes in mitochondrial fission affect iNOS levels in these different cell types.

Adenoviruses expressing a constitutively active form of Drp1 (S637A), a dominant negative form of Drp1 (K38A) and a GFP-expressing control were injected into the NTS of the DVC. IHC of DVC slices taken from GFP-expressing control rats showed that 24-26% of GFP-expressing cells were NeuN<sup>+</sup>, while 38-40% were GFAP<sup>+</sup> glial cells. Close appositions between GFP<sup>+</sup> cells and V-GAT<sup>+</sup> or 5HT<sup>+</sup> projections were also observed. On-going work will characterise Drp1-S637A<sup>+</sup> and Drp1-K38A<sup>+</sup> cells in the same way. The majority of GFP<sup>+</sup> cells were glial cells, suggesting that glial-neuronal crosstalk may be a central aspect of NTS-dependent regulation of glucose levels and feeding behaviour. Current work aims to use Drp1 adenoviral constructs with a GFAP-promotor for glial-targeted expression of Drp1-S637A/K38A to further analyse this possibility.

Fluorescence intensity of iNOS and the ER stress marker p-PERK are significantly greater in the NTS of HFD fed rats. iNOS and p-PERK IHC have also been carried out in NTS of Drp1-S637A- and Drp1-K38A-expressing DVC to determine whether these animals exhibit increased ER stress following changes in mitochondrial dynamics. Such results link HFD, ER stress, and insulin resistance, and experiments are currently underway to further examine this link by NTS-specific lentiviral-mediated KO of iNOS.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

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PCB299

#### **Differential selectivity and distributed pattern of S1 neurons for multiple features of touch and pain mechanosensations**

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The primary somatosensory (S1) cortex plays an important role in the perception and discrimination of touch and pain mechanosensations. Traditionally, neurons in the somatosensory system including S1 cortex have been classified into low/high threshold (non-nociceptive/nociceptive) and wide dynamic range (convergent) neurons by their electrophysiological

responses to innocuous and noxious stimuli, such as brush-stroke and forceps-pinch. Besides this 'modality' (innocuous/noxious) feature, each stimulus also includes other stimulus features: 'texture' (brush hairs/forceps steel arms), 'dynamics' (dynamic stroke/static press) and 'intensity' (weak/strong). However, it remains unknown how S1 neurons comprehensively encode such diverse features of cutaneous mechanical stimuli at single cell and population levels. Using *in vivo* two-photon Ca<sup>2+</sup> imaging in lightly anesthetized (1% isoflurane) mice expressing GCaMP6s in the layer 2/3 neurons of S1 cortex, we identified clearly separated response patterns of S1 neural population with distinct tuning properties of individual cells to texture, dynamics and modality features of the cutaneous stimuli. Among cells other than broadly tuned neurons, the majority showed highly selective response to the difference in texture, but low selectivity to the difference in dynamics or modality with slightly more specificity to dynamics while they could be decoded using the response patterns of neural populations. In addition, more neurons are recruited and stronger Ca<sup>2+</sup> responses are evoked as the intensity of forceps-pinch is gradually increased (<2g, 100g, 200g, 300g pressure), showing a non-linear positive relationship with steep and gentle slopes. Our results suggest that S1 neurons inclusively encode multiple features of cutaneous mechanosensations through differential selectivity and distributed pattern responses, and raise a caution about ignoring other aspects of the sensory stimuli when classifying neurons by a single stimulus feature.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

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PCB300

#### **Dopamine and glutamate dysbalance in the dorsal striatum of parkinsonian mice: glial cells induced inflammatory environment**

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L-DOPA-induced dyskinesia (LID) is associated to a pro-inflammatory environment with the contribution of glial cells (astrocytes and microglia) in the dopamine-denervated striatum. This setting results from a combination of dopamine (DA) accumulation following L-DOPA reposition and abnormal glutamate (GLU) release from motor cortex. Our aim was to determine *in vivo* in the lesioned striatum and *in vitro* purified cultured glial cells whether the response to increased DA and GLU in composition with activation of glial cells would influence LID and the production of inflammatory cytokines. C57BL/6 6-hydroxydopamine-lesioned mice received L-DOPA (25 mg/kg + Benserazide 10 mg/kg i.p.) for twenty-one days and had their striatum analyzed by immunohistochemistry. Primary glial cells culture preparation was performed based on an established protocol and stimulated with L-DOPA, DA or GLU. In the DA-denervated dorsal striatum of dyskinetic

mice there was an augmentation of the cytokines TNF- $\alpha$ , IL-1 $\beta$  and IL-6, astrocytic activation and an increase of reactive microglia. Cultured primary astrocytes stimulated by L-DOPA and DA (3 and 10  $\mu$ M) presented reduced TNF- $\alpha$  and IL-6 levels; GLU (50 and 500  $\mu$ M) increased GFAP expression and TNF- $\alpha$  production. In cultured microglial cells, DA diminished Iba-1 expression, whereas GLU induced Iba-1 increase. The combination of anti-inflammatory compounds capsazepine/cannabidiol (5 mg/kg + 30 mg/kg i.p.) attenuated LID. This effect was, at least in part, via inhibition of the pro-inflammatory cytokine TNF- $\alpha$  production. We concluded that glial activation accompanies cytokine production in striatum of dyskinetic mice, and an imbalance of GLU and DA might contribute to this inflammatory environment.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB301

## A New Human-Machine Interface: Using Hall Effect Sensor to Control Prosthetic Fingers.

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Despite the fact that wooden hand prostheses date back hundreds of years, mechatronic hand prosthetics have only

been possible in the last few decades. Mechatronic prostheses have many problems though. Moving the prostheses on demand, synchronously and physiologically is an important problem. Several methods have been proposed for human-machine interfaces designed for this purpose and some of them are actively used in prostheses sold in the market. Electroencephalography (EEG) signals and prosthesis control are still in the research stage because of their many challenges. Control with electromyography (EMG) signals is an approach used by the majority of commercially available advanced prostheses. With this approach, the signals from the residual muscles are processed and transmitted to mechatronic prosthetic. The difficulties in retrieving and manipulating EMG signals have led to the need to develop new methods. There have been intensive researches on controlling prosthetic hand. Myoelectric sensors were surgically implanted into the muscular segments in order to receive signals from the muscle sections with different functions. The energy required to operate these sensors is provided wirelessly from the outside. However, this method is very expensive and difficult to apply. In addition, prolonged use is not suitable.

We propose a new method that has not been tried before to control prosthetic fingers. Actually we plan to implement our method in three consequent parts as mockup, animal and human models. We have just finished the mockup model while we are still working for the animal and human models. In this work we present the concept of the using Hall effect sensor to control prosthetic hand with its design features, and a proof of concept of a prototype. In this model we implanted neodymium magnets (3x2 mm round) into the residual muscle tendons in the mockup hand as shown in Figure 1B. The Hall effect sensors located on the prosthetic fingers outside the body just above magnets measure the magnetic field as shown in Figure 1A. The signals coming from Hall effect sensors are processed by a tiny microcontroller (Atmega32u4), and then transferred to servo motors which moves the mockup fingers.

The proposed method has several advantages. First of all, since we used passive magnets there is no need of battery. Second since the design is simple, there is no need of high computing power, we replaced the computer by a microcontroller. Our design is simple, and the power of our design comes from its simplicity. It is small, portable, and highly accurate. As much as we know, the study we propose will be the first study in the literature.

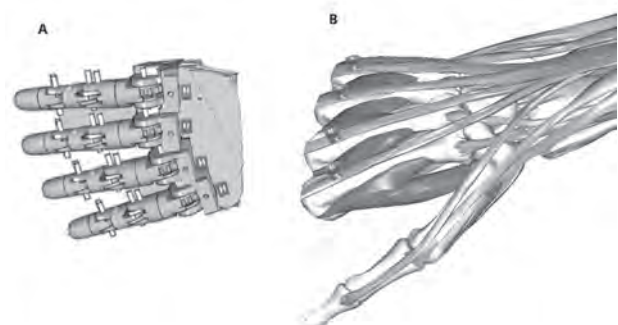


Figure 1. The concept of the using Hall effect sensor to control prosthetic hand: (A) Mechatronic hand prosthesis. "H" represents Hall effect sensors. (B) Mockup hand. "NS" represents neodymium magnets.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB302

### Feedforward and internal feedback motor circuit dysfunction impairs reaching movements in EphA4 knockout mice

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Skilled reaching movements are thought to be initiated by feedforward motor commands conveyed by supraspinal motor pathways<sup>1</sup>, and modified by peripheral feedback as well as internal feedback systems that transmit copies of motor commands to cerebellar circuits for rapid online updating<sup>2,3</sup>. Mutation of the axon guidance molecule EphA4 has been shown to produce axonal misrouting of the corticospinal tract<sup>4</sup> and spinal interneurons<sup>5</sup>. Mice lacking EphA4 receptors display a specific motor deficit, a rabbit-like hopping gait in hind limbs<sup>4</sup>. However, it remains unknown whether the loss of EphA4 signaling disrupts the function of forelimb motor circuits and the execution of dexterous movements. In this study, we use behavioral and *in vivo* electrophysiological techniques to investigate how loss of EphA4 function affects feedforward command and internal feedback neural circuits that influence forelimb motor neurons (MNs) and the execution of skilled forelimb movements. We show that EphA4 knockout mice display an impairment in goal-directed reaching. We demonstrate that cortico-reticulospinal excitation of forelimb MNs is increased, whereas direct reticulospinal excitation of MNs is decreased in EphA4 knockout mice. We further identify reduced direct propriospinal neurons excitation of forelimb MNs and dysfunction in the lateral reticular nucleus - cerebellar pathway in EphA4 knockout mice. Together, our findings reveal that loss of EphA4 produces abnormal transmission in both descending feedforward command pathways as well as ascending internal feedback pathways, resulting in impaired reaching movements.

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PCB303

### Dicarbonyls from the diabetic metabolism activate nociceptors

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In about one third of diabetes patients neuropathy affects primary sensory neurons and causes loss of cutaneous sensitivity and/or pain. There is growing evidence that certain metabolites arising from the glycolytic metabolism are

responsible for neuronal hyperexcitability or damage. These reactive dicarbonyls such as methylglyoxal (MG), glyoxal and 3-deoxyglucosone (3-DG) accumulate and lead to non-enzymatic glycation of ion channels that are responsible for transduction of sensory stimuli, generation and finally conduction of action potentials. It has already been shown that MG directly activates cutaneous nociceptors through TRPA1 receptors (Eberhardt et al. 2012, Andersson et al. 2013) and causes pain-related behaviour when applied systemically in mice (Bierhaus et al. 2012). Here we investigate the impact of different dicarbonyls on the excitability of cutaneous nociceptors.

Using the isolated skin-saphenous nerve preparation the effects of the metabolites on characterized C and A-delta fibers are investigated. Response properties as well as mechanical and thermal thresholds before and after stimulation with the dicarbonyls are compared as well as their potencies. TRPA1 involvement is tested using TRPA1 knockout animals or the TRPA1 antagonist A967079. These experiments are complemented by measurement of stimulated CGRP release from isolated mouse hind paw skin flaps *in vitro* using an enzyme-immuno-assay (Bertin Pharma France). The *ex vivo* animal research plan was approved by the Government of Central Franconia in Würzburg.

Recordings from cutaneous nociceptors from C57Bl/6J control mice showed that 86% of classical polymodal nociceptors (n=15) respond to glyoxal (10mM) superfusion of their receptive fields with an ongoing low frequency discharge that comprises about  $128 \pm 25$  S.E.M. spikes/10min (n=13). 30% of low threshold, rapidly adapting A-delta fibers (n=23) were activated by MG (10mM) and, in contrast to C fibers, exhibited a high frequency and long lasting discharge pattern with about  $1095 \pm 409$  S.E.M. spikes/10min (n=7). Regarding the C fibers a clear tendency of threshold changes could not be detected whereas 43% of the responding A-delta fibers (n=3) showed an increase of mechanical thresholds by more than 362mN. We could also show that 77% of the C-fiber responses were TRPA1 dependent. MG (1, 3, 5mM), glyoxal (1, 3, 5, 10mM) and 3-DG (1, 3, 5mM) induced a TRPA1- and concentration-dependent increase of CGRP release (n=6-10 each). While the MG-induced release was reversible, a clearly prolonged CGRP release was found when the skin was stimulated with glyoxal or 3-DG.

These data show that beside MG also glyoxal and 3-DG activate nociceptors in a differential manner. The dicarbonyls accumulate with different time courses and reach different concentrations. Our future work will focus on a possible cooperation of these metabolites. Supported by the DFG (SA-2126/2-1).

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.



PCB304

### Effect of Agomelatine on Transient Receptor Potential Vanilloid-1 Expression in Mice

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The transient receptor potential vanilloid type-1 (TRPV1) is a nonselective cation channel that is considered to be an important activator of various pain stimuli such as exogenous and endogenous physical and chemical stimuli. TRPV1 is high levels expressed in the central nervous system and play an important role in pain mediated by central sensitization. This work presents new data concerning the effect of agomelatine, a new antidepressant, on expression of TRPV1 in periaqueductal gray (PAG), raphe nucleus, amygdala and nucleus accumbens (NAC) of healthy and diabetic mice.

Agomelatine was administered for acute and chronic (15 consecutive days (twice per day)) in healthy and diabetic mice (n=7 for each group). Diabetes was induced in mice by intraperitoneal (i.p.) injection of streptozotocin (STZ). Agomelatine (10mg/kg) treatment was performed six weeks after STZ injection in diabetic groups.

Expression of TRPV1 in PAG, raphe nucleus, amygdala and NAC were detected by real time-PCR. Agomelatine administration caused significant changes in TRPV1 mRNA levels in amygdala, PAG and raphe nucleus in healthy and diabetic mice ( $p < 0.05$ ,  $p < 0.01$ ).

The findings suggest that agomelatine change TRPV1 expression in diabetic mice. Agomelatine may have exerts its beneficial effects on pain transmission and affective disorders via TRPV1.

Key words: Agomelatine;

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PCB305

### Exploration of contributions from retinal pigment epithelium and retinal ganglion cells to the "Light Adaptation Effect" of the human photopic electroretinogram

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The electroretinogram (ERG) represents the electrical response of the retina to light, and can be recorded non-invasively *in vivo*. The "Light Adaptation Effect" (LAE) refers to the growth in amplitude of the photopic electroretinogram elicited by light flashes delivered over several minutes after onset of a photopic, rod-saturating light background. The processes underlying this effect are not understood. An increase in potential is known to occur over the retinal pigment epithelium (RPE) over a similar time course. Also, intrinsically photosensitive retinal ganglion cells have been shown, in animal models, to affect the light-adapted ERG. We compared the

time course of the LAE in three healthy subjects with that recorded in two patients: one patient had a genetic condition (Best disease) that abolished the increase in potential over the RPE; the other patient had loss of ganglion cells in one eye. The study had research ethics committee approval. In each experiment, the subject dark adapted for 20 minutes and was then exposed to a white background (30 photopic  $\text{cd m}^{-2}$ ). White flashes (3 photopic  $\text{cd m}^{-2} \text{ s}$ ) were delivered (in groups of 36 flashes, repeated every 2 minutes) for the next 20 minutes. Pupils were pharmacologically dilated. ERG responses were recorded using a conductive fibre electrode placed in the lower conjunctival fornix. ERG b-wave amplitudes were measured and plotted as a function of time following onset of the white background. In the healthy subjects, the b-wave amplitude rose with time after background onset, reaching a plateau at approximately 10 minutes. The ratio of the amplitude at 20 minutes to the initial amplitude ranged from 1.8 to 2.0. The time course of the increase in b-wave was similar in the patients. The ratio was 2.0 in the patient with Best disease. In the patient with unilateral loss of ganglion cells, the ratio was 1.9 in the affected eye and 2.0 in the healthy eye. We found that the LAE was present in both patients, and followed a similar time course to that seen in healthy subjects. Thus, the processes underlying the LAE appear not to depend on the increase in potential over the RPE despite the two having similar time courses. Also, loss of ganglion cells does not seem to affect the LAE, suggesting that the effect does not depend on ganglion cell function.

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PCB306

### Investigating the effect of scotopically or photopically matched pre-adapting backgrounds on the "Light Adaptation Effect" of the human electroretinogram

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The retina adapts over a large range of light intensities. The summed electrical response of the retina to light can be recorded non-invasively, and is termed the electroretinogram (ERG). If a light background is switched on after a period of dark adaptation, the ERG response to flashes increases in amplitude over several minutes. This has been termed the "Light Adaptation Effect" (LAE). It is unclear whether this is driven by rod or cone stimulation. We explored relative contributions from rod and cone systems by evaluating the effect of pre-adapting backgrounds that were either matched scotopically (for rod stimulation) or photopically (for cone stimulation). In three healthy subjects (ages 21, 24, 39), the LAE was measured as follows. Subjects dark-adapted for 20 minutes, and then were exposed to a standard white background (30 photopic  $\text{cd m}^{-2}$ ). White flashes (3  $\text{cd m}^{-2} \text{ s}$ ) were delivered over 20 minutes (36 flashes at 0.5 s intervals, repeated every 2 min). Flash and background intensities were chosen to correspond with international clinical standards. Pupils were pharmacologically

dilated. ERGs were recorded from both eyes with conductive fibre electrodes placed in the lower conjunctival fornices. The study had research ethics committee approval. B-wave amplitudes were plotted against time following background onset. In subsequent experiments, following dark adaptation, prior to the white background, the subject viewed a "pre-adapting" background (red or blue) for 10 minutes. Background intensities were chosen to yield pairs that were photopically or scotopically matched. Scotopically matched backgrounds were 2.27 scotopic cd m<sup>-2</sup> (photopic intensities were 30 and 0.15 cd m<sup>-2</sup>); photopically matched backgrounds were 5 photopic cd m<sup>-2</sup> (scotopic intensities were 0.38 and 146 cd m<sup>-2</sup>). With no pre-adapting background, the b-wave amplitude was initially 55-57 microvolts, increasing over several minutes, reaching a plateau by c.10 minutes, after which increase in amplitude was minimal. Amplitudes at 20 min ranged from 101 to 112 microvolts. Initial amplitudes were 50-54% of the final amplitude; amplitudes at 10 minutes were 94-97% of the final amplitude. Pre-adapting backgrounds reduced the LAE to varying degrees, such that the initial amplitude was closer to the final amplitude. However, photopically matched backgrounds did not produce identical effects on the LAE, and the same was found for scotopically matched backgrounds. Our findings suggest that both rod and cone input may play a role in the LAE. Also, the observing of a plateau at 10 min (with no pre-adapting background) suggests arrival at a steady state, supporting the clinical recommendation of a minimum 10 min light adaptation prior to assessment of cone system function.

Wellcome Trust, Fight for Sight

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB307

### Pruritogen-induced scratching behaviour in mice is a heritable trait

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Pruritus is an agonizing medical condition with limited treatment options. Major challenges in the treatment of pruritus are individual but also ethnical differences in the experience of pruritus. Epidemiological studies have shown that ethnic group and sex influence the prevalence of itch (1,2), indicating an important role for genetic factors that alter the individual susceptibility to itch (3). To increase our understanding of the influence of genetics on pruritus, the heritable sensitivity to acute itch stimuli was assessed in 20 murine inbred strains (N=18-28). The scratch response towards 10 intradermally injected acute pruritic stimuli (e.g. histamine, chloroquine, endothelin) was objectively determined for 30 min using a magnet-based recording technology. For that, small magnets were subcutaneously implanted during isoflurane anaesthesia (2-3%) into both hind paws of mice. After a regeneration time of one week, scratch measurements were performed in individual cages surrounded by coils inducing electrical currents upon movement of the mice. Scratching was distinguished from other movements according to its frequency (10-20 Hz), intensity (threshold 300 mV) and repetition (min. 4 beats). Our results show significant differences in scratch behavior among the various inbred strains for all pruritogens

(repeated measures two-way ANOVA,  $p < 0.05$ ). For instance, endothelin caused outstandingly high scratching in 4 strains (>150 scratch events/30min) while 3 strains barely responded to this acute itch stimulus (<50 scratch events/30min). These results confirm the hypothesis of heritable differences in scratching behavior. When looking at the relative sensitivity of the individual inbred strains over all pruritogens, it was noticeable that most strains exhibited coherent itch sensitivity independent of the stimuli. Thus, strains that showed high or medium response rates compared to the total average of the population remained high or medium responders throughout most measured pruritogens. To further assess the characteristics of the strain-dependent variability, we compared the course of scratching between a low responding strain (NZB/BINJ) and a high responding strain (KK/HIJ). Noticeably, NZB/BINJ showed no onset of scratching throughout our recordings for 9 out of 10 pruritogens measured, suggesting the presence of genetic factors preventing itch induction. In contrast, the high responding KK/HIJ strain exhibited an intensified rather than an elongated course of scratching. Furthermore, there were mild sex differences detected. Collectively, our results demonstrate heritable differences in scratch behavior between murine strains. Besides confirming the impact of genetic factors on acute itch, the scope of the data acquired allows the identification of promising gene candidates using haplotype mapping which may pave the way for future development of novel anti-itch therapies.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB308

### Effects of 5-HT<sub>1</sub> and 5-HT<sub>2</sub> receptor agonists on electromagnetic field induced analgesia in rats

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Numerous evidence demonstrates the antinociceptive effect of the magnetic field. However, the analgesic action mechanism of the electromagnetic field (EMF) is not exactly understood. The aim of present study was to investigate the effects of 5-HT<sub>1</sub> and 5-HT<sub>2</sub> receptor agonists (serotonin HCl and DOI hydrochloride) on EMF induced analgesia. In total, 66 adult male Wistar albino rats with an average weight of 225±13 g were used in this study. The animals were subjected to repeated exposures of alternating 50 Hz and 5 milli Tesla (mT) EMF for 15 days. Prior to analgesia tests, serotonin HCl (5-HT<sub>1</sub> agonist) 4 mg/kg, WAY 100635 (5-HT<sub>1</sub> antagonist) 0.04 mg/kg, DOI hydrochloride (5-HT<sub>2</sub> receptor agonist) 4 mg/kg and SB 204741 (5-HT<sub>2</sub> antagonist) 0.5 mg/kg doses were injected into rats. For statistical analysis of the data, analysis of variance (ANOVA) was used and the multiple comparisons determined by Tukey test. Administration of serotonin HCl magnetic field

(5 mT) exposed rats produce a significant increase in % MPE as compared to EMF group ( $p < 0.05$ ). On the contrary, injection of WAY 100635 to magnetic field exposed rats produce a significant decrease in analgesic activity ( $p < 0.05$ ). Similarly, administration DOI hydrochloride significantly increases % MPE values as compared to EMF group while SB 204741 reduces it ( $p < 0.05$ ). In conclusion, our results suggested that serotonin 5-HT<sub>1</sub> and 5HT<sub>2</sub> receptors play an important role in EMF induced analgesia. However, further research studies are necessary for the mechanism of action of the magnetic field.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCB309

### **Epilepsy and Neuropathic Pain: Investigation of Pain Sensitivity in Schiatic Nerve Chronic Constriction Injury-Induced Neuropathic Pain Model in Absence Epileptic WAG/Rij Rats**

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By using Wistar albino Glaxo rats of Rijswijk (WAG/Rij) rats, we have previously documented that there may be increased sensitivity for acute pain in epilepsy (1) but it is not clear whether the case is also valid for chronic pain. The aim of this study was to investigate chronic neuropathic pain sensitivity in WAG/Rij rats, in comparison with normal Wistar rats.

The WAG/Rij groups consisted of asymptomatic (2 months old) and symptomatic (5 months old) males and sex-matched healthy Wistars (5 months old) served as control. Neuropathic pain was induced by schiatic nerve chronic ligation under general anaesthesia. Pain sensitivity was assessed, one week after schiatic nerve injury, by using mechanical (von Frey test) and heat-induced (plantar test) behavioral pain tests, which was performed in interictal period (in symptomatic group). Measurements were performed as animals were awake and freely moving. Post-operative pain sensitivity was measured in contralateral hind-paw of sciatic nerve constricted leg. Statistical analysis of pain threshold values were performed by use of paired sample t test and Dunnett's test as follow-up to two-sample ANOVA.  $P < 0.05$  was accepted as statistically significant. All protocols were approved by the Ethical Committee on Animal Experimentation of Karadeniz Technical University.

Schiatic nerve injury caused a significant increase in mechanical pain sensitivity in Wistar ( $74.5 \pm 16.3$  sec vs.  $57.3 \pm 15.1$  sec,  $P < 0.02$ ,  $n = 8$ , pre-op vs. post-op, respectively). Similarly non-symptomatic WAG/Rij rats showed increase in pain sensitivity, although not statistically significant, following schiatic nerve injury ( $43.4 \pm 4.5$  sec vs.  $35.1 \pm 8.1$  sec,  $P > 0.20$ ,  $n = 8$ , pre-op vs. post-op, respectively). But in symptomatic WAG/Rij rats an opposite effect was observed following chronic nerve ligation ( $37.3 \pm 2.5$  sec vs.  $69.8 \pm 2.5$  sec,  $P < 0.001$ ,  $n = 8$ , pre-op vs. post-op, respectively). Similar pain sensitivity change profiles were observed in heat-induced pain responses of symptomatic epileptic WAG/Rij rats compared to control Wistar and asymptomatic WAG/Rij rats.

Results from this study indicates that epileptic WAG/Rij rats develop different response to chronic neuropathic pain, this is despite they having higher nociceptive pain sensitivity compared to healthy Wistars under basal conditions, following

chronic constriction injury of the schiatic nerve. The possible mechanism underlying this response is not clear and deserve further investigation.

Velioglu SK, Gedikli O, Yildirim M, Ayar A. Epilepsy may cause increased pain sensitivity: Evidence from absence epileptic WAG/Rij rats. *Epilepsy Behav.*, 2017, 75:146-150.

The study was supported by a scientific grant from TUBITAK (The Science and Technological Research Council of Turkey, Project No 214S206).

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCB310

### **Association between iron depletion and cognitive development in Santal children of Purulia District, West Bengal, India**

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The impairment of children's cognitive development has been found to be closely associated with the iron deficiency. Recently, poor cognitive development, poor nutritional status and a positive association between these two have been evaluated in Santal children. Deficiency of iron in their diet was also reported in recent past. The association between cognitive development and iron status has not been identified in Santal tribal population. It is pertinent to investigate this association as it may provide a valuable contribution in the field of cognitive research. Therefore, the purpose of the present study was to characterize a relationship between iron status and cognitive development in Santal children of Purulia district of India. The present study was conducted on 170 (90 boys and 80 girls) school children aged 5-12 years from Balarampur and Bagmundi areas of the Purulia district. Some biochemical parameters such as hemoglobin concentration, serum iron, serum ferritin, total iron binding capacity (TIBC), serum transferrin, and transferrin saturation were measured. Stages of iron depletion have also been measured in surveyed children. Raven's Coloured Progressive Matrices (RCPM) was applied for measuring the general intelligence. The protocol and procedures employed were in accordance with the human ethical guidelines of the Institutional Human Ethical Committee, Department of Physiology, University of Calcutta (Ref. No. IHEC/TKG/P07/11 dated 13.07.2011). The Hb concentration ( $p < 0.05$ ), serum iron ( $p < 0.01$ ), serum ferritin ( $p < 0.05$ ), transferrin saturation ( $p < 0.05$ ) levels of both boys and girls having RCPM scores below 5<sup>th</sup> percentile value were significantly lower than that of RCPM scores above 5<sup>th</sup> percentile value of standard. The Hb concentration, serum iron, serum ferritin and transferrin saturation levels of 'intellectually deficit' group (for both boys and girls) and 'below average' group (only in girls) were significantly lower ( $p < 0.05$ ) than that of 'above average' group of IQ level. Pearson's correlation study indicated that all the iron status parameters were found to be significantly correlated ( $p < 0.05$ ) to RCPM scores. The present study suggests that the iron status of surveyed children is linked with their cognitive development. Vulnerable iron status may be one of the causes of the impairment of the cognitive functions in Santal children. Cognitive development was found to be affected more in girls than boys.

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Hulthen L (2003). Iron deficiency and cognition. *Scandinavian Journal of Nutrition*. 47(3): 152-156.

Dallman PR (1986). Biochemical basis for the manifestations of iron deficiency. *Annu Rev Nutr* 6, 13–40.

We would like to acknowledge the authorities of all primary and secondary schools providing permission to carry out the work. We are greatly indebted to Mr. Abani Mohon Sandil for his constant help in the field work and Dr. Suman Chakraborti for his help in statistical analyses.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

### PCB311

#### The role of chloride ions in contraction of rat saphenous artery changes during postnatal development partly due to influence of sympathetic innervation

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Special feature of smooth muscle cells (SMC) is that opening of  $\text{Cl}^-$  channels leads to  $\text{Cl}^-$  efflux and depolarization of the cell which enhances contraction. The role of  $\text{Cl}^-$  currents may change during postnatal development: in antenatal and early postnatal neurons activation of GABA(A) receptors results in exit of  $\text{Cl}^-$  and depolarization, while in mature neurons it leads to entrance of  $\text{Cl}^-$  and hyperpolarization. It is still unknown if the role of chloride changes in arteries during postnatal development and what factors may lead to this change. Some changes of cardiovascular system during maturation are attributed to sympathetic nervous system (Puzdrova et al. 2014). In this study, we tested the hypothesis that the role of  $\text{Cl}^-$  in the regulation of arterial contractile responses differs in young and adult rats due to the trophic action of sympathetic innervation.

We used the young (10-14 days old) and adult (2-4 months old) male Wistar rats. In order to reveal trophic action of sympathetic nervous system we used 2 months old rats that were subjected to chronic neonatal sympathectomy with the use of guanethidine (Johnson, O'Brien, and Werbitt 1976) and compared them to control animals that got 0.9% NaCl injections. We registered contractile responses of endothelium-denuded saphenous artery in isometric myograph (DMT A/S) that were evoked by the cumulative addition of the  $\alpha 1$ -adrenoreceptor agonist methoxamine (MX,  $10^{-8}$ - $10^{-4}$  M). The role of  $\text{Cl}^-$  was studied by  $\text{Cl}^-$  removal from the solution (equimolar substitution of NaCl and KCl by respective aspartate salts). Comparison of NKCC1, AE2, and  $\text{Cl}^-$  channels (TMEM16A and Bestrophin 3) mRNA expression levels in the saphenous arteries without endothelium were studied by RT-PCR (GAPDH, 18S and RPLP0 as reference genes).

Removal of  $\text{Cl}^-$  decreased effects of MX stronger in arteries of young rats (66% decline of force response,  $n=9$ ) compared to adults (32% decline of force response,  $n=13$ ) (t-test,  $p=0.001$ ) and in sympathectomized rats (33% decline of force response,  $n=8$ ) compared to control rats (18% decline of force response,  $n=8$ ) (t-test,  $p=0.002$ ). mRNA expression of NKCC1

(Mann-Whitney test,  $p=0.003$ ), TMEM16A (t-test,  $p=0.03$ ) and Bestrophin 3 (Mann-Whitney test,  $p<0.0001$ ) were higher in young ( $n=9$ ) compared with adult ( $n=8$ ), AE2 did not differ between adult and young animals. The mRNA expression level of these genes did not differ between sympathectomized and control rats.

Hence, the impact of  $\text{Cl}^-$  on contractile responses of rat saphenous artery decreases during early postnatal development at least in part due to trophic action of sympathetic innervation. Johnson, Eugene M., Ferdinand O'Brien, and Rand Werbitt. 1976. "Modification and Characterization of the Permanent Sympathectomy Produced by the Administration of Guanethidine to Newborn Rats." *European Journal of Pharmacology* 37: 45–54.

Puzdrova, V. A., T. V. Kudryashova, D. K. Gaynullina, S. V. Mochalov, C. Aalkjaer, H. Nilsson, A. V. Vorotnikov, R. Schubert, and O. S. Tarasova. 2014. "Trophic Action of Sympathetic Nerves Reduces Arterial Smooth Muscle  $\text{Ca}^{2+}$  Sensitivity during Early Post-Natal Development in Rats." *Acta Physiologica* 212 (2): 128–41.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

### PCB312

#### Perivascular nerve-mediated vasodilation is predominantly $\beta_1$ -AR mediated in vivo in rat mesenteric small arteries.

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Mesenteric arteries are densely innervated by perivascular nerves, the density of the innervation increasing towards the resistance arteries. Perivascular nerves are important regulators of vascular tone and consequently blood pressure. Sympathetic nerve activity has been shown to be increased in patients with hypertension and perivascular sensory nerves function is increased in some animal models of hypertension and decreased in others. Perivascular sensory nerves have been shown to possess potent vasodilating properties in vitro. However, little is known about the function of the perivascular sensory nerves *in vivo*.

Male Wistar rats (10-12 weeks old;  $n=72$ ) were anesthetized with isoflurane (3%) before subcutaneous injection of ketamine ( $3 \text{ mg kg}^{-1}$ ) and xylazine ( $0.75 \text{ mg kg}^{-1}$ ) or intraperitoneal injection of pentobarbital ( $60 \text{ mg kg}^{-1}$ ). After a laparotomy, the small intestine was exteriorized and a 2nd order mesenteric artery segments isolated in an organ bath and the vessel visualized after minimal removal of perivascular adipose tissue. The diameter and flow changes to perivascular nerve stimulation (PNS) or drugs, were recorded with intravital microscopy and laser speckle imaging (LSI) respectively. PNS induced vasodilation was induced in arteries incubated with endothelial cell blockers ( $100 \mu\text{M}$  L-NAME,  $3 \mu\text{M}$  indomethacin,  $1 \mu\text{M}$  TRAM-34 and  $50 \text{ nM}$  apamin) and precontracted with  $1 \mu\text{M}$  U46619 in the presence of  $140 \mu\text{M}$  suramin and  $1 \mu\text{M}$  prazosin. The vasodilation was sensitive to:  $1 \mu\text{M}$  tetrodotoxin,  $1 \mu\text{M}$  atenolol and  $5 \mu\text{M}$  guanethidine. The NPY Y1 antagonist BIBP3226 ( $0.3 \mu\text{M}$ ) increased the vasodilation; the CGRP antagonist BIBN4096bs ( $1 \mu\text{M}$ ) caused a small decrease in vasodilation while atenolol inhibited a large part particularly at the high frequencies (fig. 1). Increasing the extracellular potassium concentration to  $20 \text{ mM}$  caused vasodilation which was sensitive to  $1 \mu\text{M}$  BIBN4096 and the contraction to  $30 \text{ mM}$

potassium was potentiated by BIBN4096bs. Atenolol - but not BIBN4096bs - increased contraction to PNS. PNS induced nerve-mediated vasodilation was predominantly  $\beta_1$ -adrenoceptor dependent since it was TTX, guanethidine, and atenolol sensitive. A minor neurogenic CGRPergic vasodilation was observed, but only after blocking NPY-Y1 or after increasing extracellular potassium.

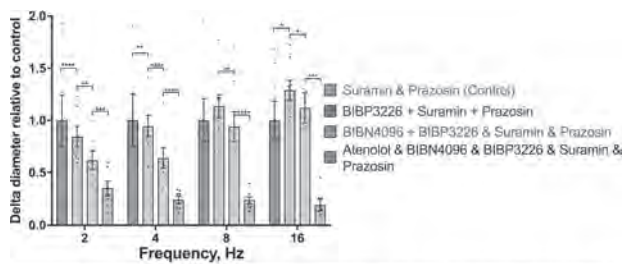


Figure 1. PNS induced nerve-mediated vasodilation, showing the effect of NPY Y1, CGRP1 and  $\beta_1$ -adrenoceptors. Data normalised to suramin and prazosin for each frequency. 1  $\mu$ M BIBN4096bs inhibits vasodilation compared with 1  $\mu$ M BIBP3226 and atenolol further inhibits vasodilation. 2-way with Sidak multiple comparison test. 0.0332(\*), 0.0021(\*\*), 0.0002(\*\*\*) <0.0001(\*\*\*\*), n=7.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB313

### Knockout of Tumor Necrosis Factor in myeloid cells does not protect against development of Abdominal Aortic Aneurysms in Mice

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Abdominal aortic aneurysm (AAA) is an inflammatory, irreversible dilation of the abdominal aorta, which affects elderly males and is associated with high mortality. In developing AAA macrophages accumulate in the aortic wall either as the pro-inflammatory M1 macrophage releasing cytokines like tumor necrosis factor (TNF), or the tissue repairing M2 macrophage. Besides macrophages, TNF is synthesized in vascular smooth muscle cells and endothelial cells. It exists as a membrane bound (tmTNF) and a soluble form (solTNF) cleaved by the TNF converting enzyme (TACE). The pro-inflammatory solTNF acts primarily via activating of TNF receptor (TNFR) 1 and induces apoptosis, while the tissue repairing effect is mediated by tmTNF acting via TNFR2. TNF is elevated in aneurysmal tissue and inhibition of TNF limits AAA expansion in experimental models. However, the cellular source of the elevated TNF levels measured in AAA and its contribution to AAA progression is not fully understood.

We hypothesize that a major source of TNF in the aneurysm wall is macrophages and that mice with conditional knockout of TNF in cells of myeloid origin will have smaller AAA compared to controls due to diminished inflammation. Nine weeks old male mice, either lacking *tnf* in myeloid cells (LysMcrexTNF<sup>fl/fl</sup>) or their floxed littermate controls (TNF<sup>fl/fl</sup>) were anaesthetized i.p. with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg). AAA was induced by intraluminal elastase infusion for 5 min in the infrarenal region. AAA was allowed to develop within a period of 14 days.

AAA size was based on a difference in outer diameter of the abdominal aorta at baseline and day 14 after elastase infusion. Quantitative RT-PCR (qPCR) was performed on RNA isolated from the abdominal aorta 14 days after elastase treatment. Both TNF<sup>fl/fl</sup> and LysMcrexTNF<sup>fl/fl</sup> developed AAA, defined by a 50 % increase in aortic diameter, but no difference was found between genotypes (98.3±19.5%, n=8 vs. 121.0±27.3%, n=8, p=0.51). In the aneurysmal tissue no differences between LysMcrexTNF<sup>fl/fl</sup> and TNF<sup>fl/fl</sup> in relative TNF mRNA levels (7.60±2.78, n=8 vs. 6.20±1.75, n=8, p=0.68), or TACE mRNA levels (2.01±0.20, n=8 vs. 1.69±0.23, n=8, p=0.32) was observed. Also, no difference between genotypes were detected in the level of the macrophage marker F4/80 (1.18±0.17, n=8 vs. 0.79±0.20, n=8, p=0.16) nor in the M2 macrophage marker CD206 (0.19±0.03, n=8 vs. 0.20±0.030, n=8, p=0.75) or the levels of TNFR1 (5.49±0.80, n=7 vs. 4.93±0.79, n=8, p=0.63), or TNFR2 (8.11±1.03, n=7 vs. 6.94±1.38, n=8, p=0.52).

In conclusion, conditional knock out of TNF in myeloid cells does not have an impact on AAA size suggesting that myeloid-derived cells are not the major contributors of aneurysmal TNF at mRNA levels.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB314

### Selective inhibition of tumour necrosis factor signalling diminishes the formation of abdominal aortic aneurysms

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The pro-inflammatory cytokine tumour necrosis factor (TNF) is elevated in abdominal aortic aneurysms (AAA). TNF inhibition dampens AAA expansion in experimental AAA. TNF exists as a membrane-bound (tmTNF) and a soluble form (solTNF). SolTNF primarily activates TNFR1 and induces inflammation and apoptosis, while TNFR2 mainly signals via tmTNF and signals tissue preservation and immune regulation. Non-selective TNF inhibition has successfully treated chronic inflammatory disorders, but has been associated with many adverse effects believed to be caused by blocking the tmTNF-TNFR2 signaling pathway.

We hypothesize that selective inhibition of solTNF/TNF-R1 signalling, by XPro1595, will inhibit AAA expansion, but preserve tissue repairing mechanisms and immune regulating functions induced by tmTNF-TNFR2 signalling and thus limit the side effects seen by non-selective anti-TNF therapy, by etanercept (ETN).

Nine week old C57Bl/6 male mice were anesthetized i.p. with xylazine (10 mg/kg) and ketamine (100 mg/kg) and AAA was induced by intraluminal elastase infusion in the infrarenal region of the aorta. Mice were treated i.p. with XPro1595 (20mg/kg), ETN (20 mg/kg) or vehicle twice a week for 14 days. Aneurysm size was measured *in vivo* as maximal outer abdominal aortic diameter (AD). At termination, AAA tissue was isolated and proteins extracted. Aneurysmal cytokine profile and TNFR1 and TNFR2 levels were determined using multiplex ELISA.

Fourteen days after elastase infusion all mice had developed AAA, defined as an increase in AD greater than 50%.

In XPro1595 treated mice, AD was significantly lower when compared to vehicle treated mice (117.419.0, vs. 205.5 23.9%, n=8, p=0.01). A similar trend was observed in the ETN treated group, though not significant (130.819.7%, n=9). Both TNF and Interferon (IFN) $\gamma$  aneurysmal protein levels showed a trend to be elevated in the ETN treated mice when compared to vehicle, this increase were significantly lowered in the XPro1595 treated mice (TNF: 18.1 $\pm$ 2.1 vs. 26.78.0, vs 11.61.3 pg/mg, n=5-8, p=0.04); (IFN $\gamma$ : 2.5 $\pm$ 0.4 vs. 3.50.9 vs. 1.30.2 pg/mg, n=7-8, p<0.05). The mean values of aneurysmal interleukin 6 levels displayed a drug dependent decrease, though it did not reach significance (891.8202.7 vs. 681.4 $\pm$ 226.8 vs. 318.289.0 pg/mg, n=5-7, p=0.2). Lastly, the aneurysmal protein levels of TNFR1 was reduced by 50% in both ETN and XPro1595- compared to vehicle-treated mice, though only ETN was significantly downregulated (737.9 vs. 323.181.12 vs. 345.9  $\pm$  49.1 pg/mg, n= 6-8, p=0.03). No differences in aneurysmal TNFR2 protein levels were detected; however there were a trend towards elevated TNFR2 in the XPro1595 treated group when compared to ETN (801.3 $\pm$ 253.4 vs. 1632 $\pm$ 175.6 pg/mg, n=5, p=0.03 by unpaired t-test). In conclusion, selective inhibition of solTNF, by XPro1595, limits AAA expansion.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB315

### Repeated episodes of forearm ischemia and reperfusion and its remote effects on mechanical properties of the arterial pressure waveforms on the contralateral arm in healthy volunteers.

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Repeated episodes of ischemia and reperfusion (I&R) applied to one organ may trigger protection against ischemia in other organs - this phenomenon is called as the remote ischemic preconditioning (RIPC).

We aimed to study the effects of repeated episodes of forearm I&R on one site on different features of arterial pressure waveforms on the contralateral arm in healthy people.

86 healthy volunteers (22 $\pm$ 1.9 years) were resting in a sitting position. Finger arterial pressure waveform was recorded continuously and non-invasively (Portapres 2, FMS, The Netherlands) during 100-second rest, then 100-second forearm ischemia of the contralateral forearm (caused by inflation of the brachial arm with the pressure 60 mmHg over personal systolic blood pressure), and 100-second reperfusion. The recordings were then repeated twice, each time the ischemia was started 10-minutes after the onset of the preceding reperfusion. With the pulse contour analysis, we measured mean blood pressure (MBP), arterial compliance (Compl), distensibility (Dist), and total peripheral resistance (TPR). Two-way ANOVA was applied for testing the effects of I&R as one factor and preconditioning (3 repetitions of I&R) as another factor. Results are presented as mean  $\pm$  standard deviation.

There were no significant effects of I&R on the studied features of arterial pressure waveforms (the first factor). However, the repetitions of I&R (i.e., the preconditioning as the second

factor) induced significant reductions in MBP (p<0.001) and TPR (p=0.001), and increases in Compl (p=0.007) and Dist (p=0.001). Pairwise comparisons showed that the significant differences were present as early as during the second I&R and further improved after the third I&R.

RIPC induces significant vascular changes in healthy individuals. RIPC reduces blood pressure and vascular resistance and improves arterial compliance and distensibility on the contralateral arm.

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D. J. Hausenloy et al. *Remote ischaemic preconditioning: underlying mechanisms and clinical application Cardiovascular Research*, Volume 79, Issue 3, 1 August 2008, Pages 377 – 386

G. Heusch et al. *Molecular basis of cardioprotection: signal transduction in ischemic pre-, post- and remote conditioning*. *Circ Res*. 2015 ; 116 : 674 – 99

P. Kleinbongard et al. *Cardioprotection by remote ischemic conditioning and its signal transduction*, *Eur J Physiol* (2017) 469 : 159.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB316

### Applying Bayesian data analysis for automated image segmentation – overcoming a gap between computer scientists and biomedical researches

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Time lapse microscopy of living cells is essential in the assessment of their physiological function. Due to the technical progress image data sets become bigger and exceed the possibility of manual segmentation. Many potent imaging algorithms already exist, however it is a task on its own to adjust them to specific data. While life science researchers often have specific ideas about the distinct features of interest, the same researches do often not know how to convert their thoughts into machine readable form. We address a gap between computer scientists and biomedical researchers by an algorithm, which is trained solely by the expertise of a life science researcher.

Thus, it is the aim of this study to construct a trainable image segmentation algorithm enabling life science researchers to convert their ideas to algorithms without coding. We apply our approach to the example of a wound healing assay (HUVEC cells were observed over 24 h with 6 frames per hour resulting in 145 images) to assess collective cell motility quantitatively. To assess the so-called cell covered area over time a three step procedure was established. First the experimenter used a paintbrush like tool to specify cell covered areas within a single frame of the image series. In a second step Bayesian parameter estimation based on the nested sampling algorithm was performed to automatically adjust all parameters of a corresponding image processing pipeline, which segments the cell covered areas. The imaging pipeline consisted of individual parameter dependent imaging functions such as edge detection, blurring or intensity as well as particle size thresholding. The Bayesian parameter estimation is based on a distance metric allowing quantifying the difference of the



manually segmented image and the result of the pipeline. Thus, the trained parameter set of the optimized pipeline was obtained by the algorithm as well as their uncertainties. In the final step the imaging pipeline was applied to all frames of the data set reducing the time expenditure for user input from 145 to a single frame. We applied the algorithm to many different images recorded using bright-field, phase contrast or differential interference contrast microscopy to check its stability. In all cases, even for bad image qualities, we obtained reliable results. In addition we could show that different prior knowledge of the experimenter is considered by the algorithm: The input of four persons with differences in accuracy of the manual segmentation caused uncertainties of the border of the cells between  $6.8 \pm 0.1$  pixels and  $37.9 \pm 0.5$  pixels.

In conclusion, we present a new robust algorithm allowing automated segmentation of bio-medical image sequences. Our approach is highly modular, since user input, image processing pipelines and metrics are interchangeable if necessary for other problems. We believe that this tool will help to overcome the increasing gap between computer scientists and biological researches.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCB317

#### **Efficacy and Safety of Botulinum Toxin Type A (Botox®) Injections to Rat aorta**

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**AIM:** Botulinum toxin A is an acetylcholine release inhibitor and a neuromuscular blocking agent indicated in adult patients for the temporary improvement in the muscle. The aim of the present study is to investigate the effect of botulinum toxin A on rat aorta contraction.

**MATERIAL AND METHODS:** Descending thoracic aorta was isolated after cervical dislocation from wistar albino rats (n=12). Aorta tissues were cleaned and sectioned into 3-4 mm long rings. Rings were placed in organ baths containing Krebs solution, thermoregulated at 37°C and aerated (95 % O<sub>2</sub> and 5% CO<sub>2</sub>). Changes in isometric tension of aorta rings were recorded using a four channel force displacement transducer. Phenylephrine (PE 10<sup>-6</sup>M) was applied and contractions were recorded in both groups. 100 units vacuum-dried powder Botulinum Toxin Type A (Botox) was diluted by 5ml 0.9% NaCl solution. Botox doses of 0.1 mL (4 units), 0.2 mL (8 units) and 0.3 mL (12 units) were added in organ baths and contractions were recorded.

**RESULTS AND CONCLUSIONS:** After 0.3 mL of botox was given, significant inhibition of spontaneous contractions was noticed (p<0.05). Vasodilatation of aorta suggests that botox may have beneficial effects on treatment of peripheral arterial diseases and hypertensive disorders. Further studies are needed to clarify the mechanism of Botulinum Toxin Type A.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCB318

#### **The effect of short term high salt diet on cerebrovascular reactivity in response to orthostatic position change in young healthy subjects**

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**Introduction:** Brain tissue is protected from variations in blood pressure by cerebral autoregulation (CA), maintaining stable cerebral perfusion and brain tissue oxygenation in spite of these variations (1). It is well accepted that increased dietary salt intake is positively correlated with incidence of stroke (2). High salt (HS) diet, even short termed, impairs endothelium-dependent vasodilator responses in many vascular beds, including cerebral microcirculation (3). The present study aimed to evaluate the effect of short term HS diet on middle cerebral artery (MCA) reactivity to orthostatic position change in healthy human subjects.

**Methods:** 25 persons were included (men:6, women:19) in this study. The study was designed as prospective, "cross-over" study with repeated measurements. Participants took low salt (LS) diet for 1 week (<2,3g kitchen salt/day) followed by 1 week of HS diet (>11,2 g kitchen salt/day). 24h urine samples were collected and analyzed for sodium excretion after diet protocols. Measurements of MCA reactivity during sitting in a relaxed state and in response to orthostatic position change (standing up and sitting down) were performed after LS and HS diet protocols by Transcranial Doppler (TCD) Flowmetry. Blood pressure (BP), heart rate (HR), R-R interval, mean cerebral blood flow (CBF), systolic CBF (sCBF), diastolic CBF (dCBF), velocity time integral (VTI), cerebral vasomotor reactivity (CVR) and cerebral pulsatility index (CPI) were simultaneously recorded during TCD measurements. This study was approved by the Ethical Committee of the Faculty of Medicine University of Osijek

**Results:** Changes in 24h urinary sodium excretion confirmed that subjects conformed to the diet protocols. CBF, sCBF and dCBF decrease during sitting down was significantly more pronounced after HS diet compared to measurements taken after LS diet (relative CBF change -0.0518 vs. -0.00109, P=0.011; sCBF -0.0286 vs. -0.0021, P=0.019; dCBF -0.0662 vs. -0.00412, P=0.015). VTI was significantly decreased during sitting down after HS diet, whereas in the period after LS diet there was significant increase during sitting down from the measurement conducted in the relaxed sitting state (relative VTI change -0.0344 vs. 0.0309, P=0.036). sCBF decrease after standing up was also significantly more prominent after HS diet compared to the decrease during standing up measured after LS diet (-0.0536 vs. -0.0222, P=0.050). Changes of HR, R-R interval, CVR and CPI during orthostatic position changes were not significantly affected by the HS diet compared to LS diet. Furthermore, HS diet did not significantly affect any of the arterial BP values.

**Conclusion:** One week of HS diet can impair the cerebrovascular reactivity in humans, even without affecting BP values.

**Support:** University of Osijek research grant, INGI-2015-16  
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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## PCB319

### Effect of exercise on venoarterial response in young healthy subjects

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An increase in the transmural venous pressure, triggered by the acute venous congestion of a limb below the heart level, leads to localized vasoconstriction called veno-arteriolar reflex (VAR) [1]. This response exists in all tissues of the limbs, including subcutaneous adipose tissue, muscles and skin and is absent in diabetes mellitus. It could be associated with the activation of sympathetic vasoconstrictor or vasodilator nerves [2] but the complete mechanism is not clear yet. The aim of our study was to compare the VAR in the skin spots with different vascular anatomy and sympathetic innervation: in glabrous (presence of arteriovenous anastomoses, sympathetic adrenergic innervation) and nonglabrous skin (no arteriovenous anastomoses, cholinergic sympathetic innervation) and at the different levels of sympathetic tone, provoked by whole body exercise. The transmural venous pressure in the calf (TMVPC) was changed by the use compression calf sleeves (CCS). In nine young healthy volunteers laser-Doppler skin blood flow (LDF) was measured in the finger pulp of the toe (glabrous skin) and on the dorsum of the foot (non-glabrous skin) at different transmural venous pressures in the calf: in the sitting position, wearing the compression calf sleeves or not. Arterial blood pressure was assessed simultaneously. Measurements were conducted at rest and three times in the recovery after submaximal cycling: in the 1<sup>st</sup>, 15<sup>th</sup> and 25<sup>th</sup> minute after exercise. Cutaneous vascular conductance (CVC) was calculated. At rest, statistically significant differences were found in LDF and CVC with respect to TMVPC in glabrous (LDF: 56,51±12,48PU without and 87,06±15,93PU with CCS; P=0,04) and in nonglabrous skin (LDF: 6,76±1,15PU without and 9,41±1,54PU with CCS; P=0,04). After exercise, there were no statistically significant changes in LDF and CVC of both skin types with respect to TMVPC at 1<sup>st</sup>, 15<sup>th</sup> and 25<sup>th</sup> minute. VAR is present in glabrous as well as in nonglabrous skin at rest. On the contrary, after physical exertion, VAR disappeared in both skin spots indicating that blood flow through arteriovenous anastomoses, present in glabrous skin after exercise due to thermoregulation is not involved in VAR. Sympathetic tone, declining during recovery, does not affect VAR in vasodilator nerves of nonglabrous skin. Further investigation should be conducted to elucidate the mechanism of this reflex.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## PCB320

### Efficacy and Safety of Botulinum Toxin Type A Injections on Rat aorta

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**AIM:** Botulinum toxin A is an acetylcholine release inhibitor and a neuromuscular blocking agent indicated in adult patients for the temporary improvement in the muscle. The aim of the present study is to investigate the effect of botulinum toxin A on rat aorta contraction.

**MATERIAL AND METHODS:** Descending thoracic aorta was isolated after cervical dislocation from wistar albino rats (n=12). Aorta tissues were cleaned and sectioned into 3-4 mm long rings. Rings were placed in organ baths containing Krebs solution, thermoregulated at 37°C and aerated (95 % O<sub>2</sub> and 5% CO<sub>2</sub>). Changes in isometric tension of aorta rings were recorded using a four channel force displacement transducer. Phenylephrine (PE 10<sup>-6</sup>M) was applied and contractions were recorded in both groups. 100 units vacuum-dried powder Botulinum Toxin Type A (Botox) was diluted by 5ml 0.9% NaCl solution. Botox doses of 0.1mL (4 units), 0.2 mL (8 units) and 0.3 mL (12 units) were added in organ baths and contractions were recorded.

**RESULTS AND CONCLUSIONS:** After 0.3 mL of botox was given, significant inhibition of spontaneous contractions was noticed (p<0.05). Vasodilatation of aorta suggests that botox may have beneficial effects on treatment of peripheral arterial diseases and hypertensive disorders. Further studies are needed to clarify the mechanism of Botulinum Toxin Type A.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## PCB321

### Efficacy of Botulinum Toxin Type A Injection on Human Atrium

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**AIM:** Botulinum toxin type A (botox) is an acetylcholine release inhibitor and a neuromuscular blocking agent indicated in adult patients for the temporary improvement in the muscle. There isn't sufficient number of studies with botulinum toxin type A about cardiac tissues. We aimed to investigate the effects of botulinum toxin A on human atrium contraction.

**METHODS:** Human atrium tissues (n=12) were taken from patients who underwent coronary bypass surgery. The patients' ages were between 50 to 75. All tissues were placed in organ baths containing Krebs solution, thermoregulated at 37 °C and aerated (95 % O<sub>2</sub> and 5% CO<sub>2</sub>). Changes in isometric

tensions were recorded using a four channel force displacement transducer. All tissues washed for 3 hours in order to diminish the effects of anaesthetic agents. Adrenaline ( $10^{-1}$  M) was administered in tissue cabs to induce isometric contractions. 100 units vacuum-dried powder Botox was diluted by 5ml 0.9% NaCl solution. Botox doses of 0.1 mL (4 units), 0.2 mL (8 units), 0.3 mL (12 units) and 5mL (20 units) were added in organ baths and contractions were recorded accordingly. Contraction with measurements were used as contraction parameters. Friedman and Kruskal Wallis tests were used for statistical evaluation.

**RESULTS AND CONCLUSIONS:** After last dose of botulinum toxin type A (0.5mL, 20 units) was given, significant inhibition of spontaneous contractions was noticed ( $p < 0.05$ ). Vasodilatation of atrium suggests that contraction of atrium and ejection fraction decrease. Further studies are needed to clarify the mechanism of Botulinum Toxin Type A.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCB322

### Humanized Sickle Cell Disease Mice Exhibit a Blunted Pressor Response to Acute Behavioral Stress

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Sickle cell disease (SCD) patients experience an elevated burden of psychosocial stressors. This study was designed to determine the impact of SCD on the hemodynamic response to acute behavioral stress. We utilized humanized sickle cell disease (HbSS) and hemoglobin A control mice (HbAA). Blood pressure, heart rate, and locomotor activity were measured by telemetry. Baseline 24-hour blood pressure was significantly lower in HbSS compared to HbAA ( $p < 0.05$ ) mice similar to humans with SCD. Mice were exposed to cage switch stress (CSS), a model of acute psychosocial stress. CSS rapidly induced a pressor response in all groups. HbSS mice exhibit a blunted rise in systolic blood pressure ( $46.6 \pm 4.8$  vs.  $24.3 \pm 3.5$  mmHg-hours,  $p = 0.036$ ), and diastolic blood pressure ( $47.2 \pm 4.6$  vs.  $21.0 \pm 3.2$  mmHg-hours,  $p = 0.021$ ) compared to HbAA mice. CSS induced a similar rise in heart rate and activity in HbSS and HbAA, suggesting that both genotypes experienced a similar level of acute stress. These results suggest that SCD dampens the normal physiological pressor response to acute stress. We then hypothesized that SCD mice would display enhanced sensitivity to  $\alpha 1$ -adrenoreceptor-mediated vasoconstriction but this enhancement would be absent in resistance arteries. Vascular reactivity of isolated aortic and resistance mesenteric artery (100-150 $\mu$ m diameter) rings to phenylephrine (PE) and potassium chloride (KCl) were examined using wire myography. Aortae of HbSS mice demonstrated enhanced sensitivity to  $\alpha 1$ -adrenoreceptor-mediated vasoconstriction as evidenced by significantly lower EC<sub>50</sub> ( $-6.22 \pm 0.14$  vs.  $-6.90 \pm 0.04$  log[PE, M],  $p = 0.002$ ) and elevated E<sub>max</sub> ( $118.7 \pm 3.2$  vs.  $155.9 \pm 5.2$  %KCl,  $p < 0.001$ ) compared to HbAA. In contrast, resistance mesenteric arteries from HbSS and HbAA mice displayed similar sensitivity to  $\alpha 1$ -adrenoreceptor-mediated vasoconstriction as indicated by comparable EC<sub>50</sub> and E<sub>max</sub> ( $p > 0.05$ ). Graded concentration responses to KCl were similar between genotypes. Aorta of HbSS mice exhibited increased  $\alpha 1A$ -adrenoreceptor mRNA expression compared to HbAA mice ( $1.00 \pm 0.18$  vs.  $2.25 \pm 0.40$ ,

$p = 0.016$ ), while resistance mesenteric arteries of HbSS mice displayed similar  $\alpha 1A$ -adrenoreceptor expression compared to HbAA mice ( $p > 0.05$ ).  $\alpha 1B$ -adrenoreceptor expression was similar in aorta of HbSS mice and HbAA mice ( $p > 0.05$ ) and was not detected in resistance mesenteric arteries. Additionally,  $\alpha 1D$ -adrenoreceptor expression was similar in aorta ( $p > 0.05$ ) and resistance mesenteric arteries ( $p > 0.05$ ) of HbSS mice and HbAA mice. These findings suggest that enhancement of arterial sensitivity to  $\alpha 1$ -adrenoreceptor-mediated vasoconstriction in HbSS mice is specific to conduit arteries and results from increased  $\alpha 1A$ -adrenoreceptor expression. Similar sensitivity to  $\alpha 1$ -adrenoreceptor-mediated vasoconstriction in resistance arteries suggests that increased vasodilator mechanisms may be responsible for the blunted cardiovascular responses to stress in HbSS mice.

U01 HL117684 to DMP, JSP, and F30 DK107194 to BMF.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCB323

### Perinatal exposure of dams to a high salt diet Impaired vascular function and elevated biomarkers of inflammation in the offspring

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Hypertension is an important risk factor for cardiovascular diseases and excess of dietary salt is the most common environmental factor that contributes to the development of hypertension (Meneton *et al.*, 2005). However, *in utero* factors has been implicated in the pathogenesis of hypertension (Alexander, 2006). To assess the effect of perinatal high salt diet (HSD) on the offspring, pregnant Sprague-Dawley rats were exposed to perinatal normal (0.3%) or high (8%) salt diet from day 1 of pregnancy till term. The following experiments were conducted in the offspring at 12 weeks of age: Blood pressure (BP) was measured via arterial cannulation under urethane and  $\alpha$ -chloralose anesthesia (5ml/kg body weight i.p) using ADInstrument powerlab. After BP measurement and blood collection through the cannulated artery, rats were killed by cervical dislocation. Isolated abdominal aorta reactivities to noradrenaline (NA) and acetylcholine (ACh) in the presence or absence of endothelial nitric oxide synthase (eNOS) inhibition by L- nitro arginine (L-NA) were determined and serum concentrations of C-reactive proteins (CRP), TNF- $\alpha$  and IL-6 were measured using commercially available kits. Data were expressed as mean $\pm$ S.E.M and analysed using one way analysis of variance with Student Newman Keuls posHoc test for difference between means. Confidence interval was placed at 95%. BP was elevated ( $p < 0.05$ ) in the offspring of dams exposed to perinatal HSD ( $116 \pm 3$  vs.  $103 \pm 2$  mmHg). Maximum contractile response to noradrenaline was similar in both groups of offspring ( $72.22 \pm 5.57\%$  vs.  $79.45 \pm 5.76\%$ ). However, maximum relaxation response to ACh in the presence of L-NA was significantly reduced ( $p < 0.05$ ) when compared with that in the absence of L-NA ( $84.23 \pm 4.92$  vs.  $54.23 \pm 3.92\%$ ) in the offspring of dams that were exposed to perinatal normal salt diet. In the offspring of dams that were exposed to a perinatal high salt diet, maximum relaxation responses to ACh in the presence or absence of L-NA were similar ( $70.46 \pm 4.76$  vs.  $66.15 \pm 3.76\%$ ). Serum concentrations of CRP ( $500 \pm 6.83$



vs.  $606.7 \pm 19.6$  ng/ml), IL-6 ( $112.5 \pm 2.7$  vs.  $147.7 \pm 2.5$  pg/ml) and TNF - alpha ( $2000 \pm 85.63$  vs.  $3783 \pm 97.18$  pg/ml) were significantly elevated ( $p < 0.05$ ) in the offspring of dams that were exposed to perinatal high salt diet when compared with offsprings from dams that were fed a normal salt diet. Findings from this study suggest that perinatal exposure to a high salt diet increases BP in the offspring by impairing vascular relaxation and increasing systemic and vascular inflammation. Meneton, P. et al., (2005). *Physiol Rev* 85: 679–715.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB324

### Long non-coding RNA MANTIS is induced by statins and limits monocyte adhesion to human endothelial cells

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Long non-coding RNA MANTIS is an important regulator of the endothelial angiogenic function. Here, we sought to determine the mechanisms of MANTIS gene regulation for endothelial function during vascular disease development.

Exposure of human umbilical vein endothelial cells to laminar flow or to HMG Co-A reductase inhibitors (statins) induced the expression of lncRNA MANTIS. MANTIS upregulation was caused by the transcription factors KLF2 and KLF4. Mutation of specific KLF binding motifs on the MANTIS promoter abolished the induction. RNA-Sequencing after laminar shear stress demonstrated that MANTIS repressed ICAM-1 gene expression. Consequently, MANTIS was required for endothelial flow alignment and its knockdown increased monocyte adhesion to endothelial cells, which was blocked by ICAM-1 antibodies. CRISPR activation and RNAi confirmed the findings. Mechanistically, the observed regulation was an effect of MANTIS Alu element, blocking the binding of the chromatin remodelling factor BRG1 to the ICAM-1 promoter. These effects were also seen in a putative mouse homologue of MANTIS in murine endothelial cells and murine retina. In the human situation, MANTIS was downregulated in atherosclerotic carotid arteries from patients with >85% stenosis; an effect that was reversed in patients under statin therapy. Moreover, MANTIS expression positively correlated to KLF2 and KLF4, and negatively to ICAM-1 in the human cohort.

Expression of MANTIS is tightly regulated by KLF2 and KLF4 in atherosclerotic plaques, in response to shear stress and statin treatment. MANTIS upregulation limits ICAM-1 expression, which affects monocyte adhesion to endothelial cells. MANTIS might regulate the initiation step of atherogenesis in humans.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB325

### The epidermal growth factor receptor in vascular smooth muscle cells and endothelial cells is needed for an appropriate vasoactive response.

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Epidermal growth factor receptor (EGFR) is activated by its canonical ligands and transactivated by various vasoactive substances, e.g. angiotensin II. Vascular EGFR has been proposed to be involved in vascular tissue homeostasis and remodeling. Thus, most studies focused on its role during long term vascular changes whereas the relevance for acute regulation of vascular function *in vivo* and *ex vivo* is insufficiently understood.

To investigate the role of the EGFR in the vasculature, we generated mouse models with cell-specific deletion of the EGFR either in smooth muscle cells (VSMC) or endothelial cells (EC). The impact of the EGFR on vasoconstriction and vasodilation was analyzed by Mulvany myography and pressure myography. Aortic rings or mesenteric arteries were utilized respectively. In aortic rings dose response curves for angiotensin II (Ang II, 1-1000 nmol/l) were performed and vasoconstriction upon the application of a single dose U46619 (30 nM), a thromboxan analogue was tested. After precontraction with U46619 vasorelaxation was analyzed by applying cumulative doses of carbachol ( $10^{-7}$ - $10^{-3}$  M) or S-Nitroso-N-acetylpenicillamine (SNAP,  $10^{-9}$ - $10^{-5}$  M). Precontracted mesenteric arteries (U46619) were treated as described for aortic rings. Performing dose response curves to ang II the generation of force was significantly weaker by aortic rings from mice with deletion of the EGFR in VSMC. While the maximum response was about 3.5 mN for wildtype aortic rings, only 1 mN additional force could be generated by knockout aortic rings by application of 100 nM ang II. We hypothesize that the EGFR in VSMC inhibits the homologous desensitization of the AT1-receptor as repetitive application of ang II reduced the force generation in knockout aortic rings faster. Upon application of U46619 there was a similar increase in force generation by aortic rings with or without (w/o) deletion of the EGFR in VSMC. No difference in the response to carbachol or SNAP could be observed between aortic rings and mesenteric arteries from mice w/o deletion of the EGFR in VSMC.

Force generation of aortic rings from mice w/o deletion of the EGFR in EC was the same in response to ang II and U46619. Additionally, the relaxation upon SNAP application was the same by aortic rings from wildtype and knockout animals, in contrast, relaxation towards carbachol was reduced in aortic rings as well as in mesenteric arteries from mice with deletion of the EGFR in EC. This indicates that the EGFR in EC inhibits the development of endothelial dysfunction.

In conclusion: the EGFR in EC is needed for appropriate endothelial function but not the acute ang II vasoconstrictive response, while EGFR in vascular smooth muscle cells increases ang II response but is not necessary for endothelial function.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB326

### Stimulation of Epac activates PI3K/Akt and MEK/ERK signalling in endothelial cells: Role in endothelial barrier stabilisation and survival

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**Background and Aims:** Activation of the cAMP/Epac signalling stabilises endothelial barrier function. cAMP/Epac also activates PI3K/Akt and MEK/ERK signalling in diverse cell types, but the impact of this activation on endothelial barrier function is largely unknown. Here the role of PI3K/Akt and MEK/ERK signalling in cAMP/Epac-mediated endothelial barrier stabilisation was analysed.

**Methods:** Endothelial barrier function was analysed in cultured human umbilical vein endothelial cells (HUVECs) by measuring albumin flux. A modified cAMP analogue 8-pCPT-2'-O-Me-cAMP was used to specifically activate cAMP/Epac signalling. Activation of PI3K, Akt, MEK, and ERK was measured by Western blotting. Caspase activity was determined by Caspase-Glo kit. qPCR was used to quantify the expression level of PI3K isoforms. Cytoskeletal proteins and cell-cell junctions were visualised by immunohistochemistry using phalloidin TRITC and antibody against VE-cadherin, respectively. Endothelial cell proliferation was determined by expression of Ki67.

**Results:** The Epac agonist reduced basal and significantly attenuated thrombin-induced endothelial hyperpermeability ( $n=5$ ;  $p<0.05$  for all further parameters); this was accompanied by an activation of PI3K/Akt and MEK/ERK signalling. Quantitative (q)PCR demonstrated that HUVECs express PI3K $\alpha$ , PI3K $\beta$ , and PI3K $\gamma$  but not PI3K $\delta$  isoforms. Western blotting confirmed that the Epac agonist preferentially activates PI3K $\alpha$  and PI3K $\beta$  isoforms ( $n=3$ ). Inhibition of the MEK/ERK using U0126 but not the PI3K/Akt pathway by Akt inhibitor VIII, potentiated the endothelial barrier-protective effects of cAMP/Epac signalling. Inhibition of MEK/ERK signalling in the presence of the Epac agonist induced reorganisation of the actin cytoskeleton to the cell periphery, reduced stress fibre formation, and enhanced VE-cadherin localisation at cell-cell junctions. Moreover, the Epac agonist promoted endothelial cell (EC) survival but not proliferation via reduction in activities of pro-apoptotic caspases (caspase 3 and 7) in a PI3K/Akt and MEK/ERK signalling-dependent manner as determined by respective selective pharmacological inhibitors.

**Conclusion:** Our data demonstrate that stimulation of the cAMP/Epac axis simultaneously activates PI3K/Akt and MEK/ERK pathways, which govern the pro-survival effects of Epac signalling on ECs. Inhibition of MEK/ERK but not PI3K/Akt signalling enhances barrier-stabilising and barrier-protective effects of cAMP/Epac activation.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB327

### Estrogen determines the sex-differences in adrenergic vessel tone regulation

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Vessels of female rats react with stronger vasorelaxation and weaker vasoconstriction to adrenergic stimulation than vessels of males. Although we have previously shown that these sex-specific differences rely on endothelial  $\beta$ -adrenoceptors, the role of sex-hormones in endothelial  $\beta$ -adrenoceptor expression and related vessel tone regulation is unknown. Here we investigated the role of female and male sex-hormones on  $\beta$ -adrenoceptor expression and adrenergic vessel tone regulation.

Five weeks old female and male Wistar rats were ovariectomized and orchietomized, respectively, under anesthesia with 3% isoflurane and analgesia with 1 mg/kg Carprofen at the age of 5-weeks. As controls, a sham-operated, a hormone substituted (2 mg/kg, twice a week) and a vehicle group of rats were examined. At the age of 12-weeks, the rats were sacrificed with a Ketamine overdose (90 mg/kg plus 10 mg/kg Xylazine). Vasoconstriction (norepinephrine) and vasorelaxation (isoprenaline and  $\beta_3$ -agonist BRL) of isolated aortas were assessed with Mulvany myograph. Additionally, a qRT-PCR for quantification of  $\beta$ -adrenoceptor mRNA levels in aorta was performed.

The sex-specific differences in vasoconstriction and relaxation in rat aorta was eliminated after ovariectomy in female rats. Compared to sham operated females, ovariectomy increased aortic vasoconstriction in response to norepinephrine more than 2-fold. Vasorelaxation by isoprenaline and a  $\beta_3$ -agonist was significantly ( $P<0.01$ ) reduced after ovariectomy. Compared to vehicle, estrogen substitution largely ( $P<0.05$ ) restored sex-specific differences in vasoconstriction and vasorelaxation in ovariectomized rats. Differences in vasoconstriction and vasorelaxation between sexes were profoundly diminished in presence of selective  $\beta_1$ - and  $\beta_3$ -adrenoceptor antagonists and L-NMMA which blocks eNOS. mRNA levels of  $\beta_1$ - and  $\beta_3$ , but not  $\beta_2$ -adrenoreceptors were significantly ( $P<0.05$ ) higher in aortas of sham operated females than in aortas of sham operated males. Ovariectomy abolished this difference by decreasing  $\beta_1$ - and  $\beta_3$ -adrenoreceptor expression in female rats, without affecting the expression of  $\beta_2$ -adrenoreceptors. Consistently, estrogen substitution in ovariectomized female rats almost completely ( $P<0.05$ ) restored  $\beta_1$ - and  $\beta_3$ -adrenoreceptor expression. Orchiectomy or testosterone treatment did not affect aortic vasoconstriction and vasorelaxation nor  $\beta$ -adrenoceptor expression in aortas of male rats.

In conclusion, the sex-specific differences in vasoconstriction and vasorelaxation in rat aorta are estrogen, but not testosterone-dependent. Estrogen determines these differences via regulation of vascular endothelial  $\beta_1$ - and  $\beta_3$ -adrenoreceptor expression.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB328

**Investigation Effects of Metformin in rats ileum contractility**S. Sahinturk<sup>1</sup>, A. YARDIMCI<sup>2</sup>, N. Ulker<sup>2</sup>, I. Serhatlioglu<sup>3</sup> and E. Kacar<sup>2</sup><sup>1</sup>Department of Physiology, Uludag University, Bursa, Turkey,<sup>2</sup>Department of Physiology, Firat University, Elazig, Turkey and<sup>3</sup>Department of Biophysics, Firat University, ELAZIG, TurkeyS. Sahinturk<sup>1</sup>, A. Yardimci<sup>2</sup>, N. Ulker<sup>2</sup>, I. Serhatlioglu<sup>3</sup>, E. Kacar<sup>2</sup>  
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Type 2 diabetes mellitus (T2DM) is among the most common disorders at world and affects peoples daily-life adversely. Metformin is one of the most effective agent for treatment of T2DM around worldwide. It is an antihyperglycemic medication, which improves glucose tolerance in patients with T2DM, decreasing both basal and postprandial plasma glucose levels. Metformin has some adverse effects, most common is gastrointestinal symptoms, including vomiting, nausea, diarrhea, stomachache, loss of appetite and indigestion. One of the most important these effects is diarrhea, because it can lead to disruption of patient adaptation to the treatment. Because of metformin cause to decrease intestinal glucose absorbtion, so it may result in diarrhea. However, effects of metformin on rat intestinal contractility is almost unknown and these effects may contribute to the diarrhea. In this study, we investigated metformin effects on rat ileum contractility in isolated tissue bath. Adult female Sprague-Dawley rats (n=8) were used in this study. In order to investigate of metformin effects on ileum conractility, ileum (nearly 10 mm in length) was quickly removed from 5 cm proximal to the ileocecal valve, placed in Krebs solution and then mounted in (tension 1 g) isolated tissue baths (5, 10 and 20 mL) bubbled with 95 % O<sub>2</sub> and 5 % CO<sub>2</sub> at 37°C. The tissues were equilibrated for one hour with rinsing every 15 min under a resting tension of 1 g before starting experiment. The tension formed by the tissues was measured using transducers MP150 instrument (Biopac Systems, Inc., U.S.A.). After equilibration, acetylcholine (10<sup>-4</sup> M) was given to the all baths. After one hour rinsing, metformin (2 mM) was given and incubated 15 min. After 15 min, again acetylcholine was given same dose without rinsing and then these part was repeated with metformin 20 mM dose. Control group values were accepted as 100 %, and change calculated as percent (%) for all treatment groups for peak to peak amplitude and area values. Data were given as means ± SE and statistical analysis of the results was implemented by one way ANOVA. In all conditions, differences were considered significant when p<0.05. Area values compared to control group, there was no significant changes at any doses. However, peak to peak amplitude values compared to control group, there was significant decrease in 2 mM (73.68±10.21, change %, p<0.01) and 20 mM (76.83±5.48, change %, p<0.05). As a result, it was concluded that metformin may reduce acetylcholine-induced contractile activity in rat ileum.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB329

**Cardio-Ankle Vascular Index in obese adolescents**B. Czippelova<sup>1,2</sup>, Z. Turianiková<sup>1,2</sup>, J. Krohova<sup>2</sup>, Z. Lazarova<sup>2</sup>, K. Pozorciakova<sup>3</sup>, M. Ciljakova<sup>3</sup> and M. Javorka<sup>1,2</sup><sup>1</sup>Biomedical Center Martin, Comenius University in Bratislava, Jessenius Faculty of Medicine in Martin (JFM CU), Martin, Slovakia,<sup>2</sup>Department of Physiology, Comenius University in Bratislava, Jessenius Faculty of Medicine in Martin (JFM CU), Martin, Slovakiaand <sup>3</sup>Clinic of Children and Adolescents, Comenius University in Bratislava, Jessenius Faculty of Medicine in Martin and University Hospital Martin, Martin, Slovakia

**Introduction.** Obesity is regarded as a risk factor contributing to the development of atherosclerosis. Cardio-Ankle Vascular Index (CAVI) quantifies arterial stiffness from the aortic arch to the distal arteries of the lower extremities potentially increased by atherosclerotic process and is therefore regarded as a measure of early atherosclerotic development. Surprisingly, most previous studies show a negative correlation between CAVI and the BMI (Body Mass Index). However, CAVI involves not only the structural changes in the vessel wall related to the ATS process, but also the functional stiffness – arterial vasomotor tone controlled by the sympathetic branch of autonomic nervous system. Vasodilation – a lower peripheral vascular resistance – observed in previous hemodynamic studies in obese subjects could explain the reduced CAVI in this patient population. The aim of our study was to compare CAVI values, peripheral vascular resistance (PVR) and sympathetic indices in obese adolescents and lean controls and to explain paradoxically decreased CAVI in obesity.

**Methods.** We examined 30 obese (15f, age: 16.27 ± 2.8y, BMI: 33.03 ± 4.4kg.m<sup>-2</sup>) and 30 non-obese (15f, age: 16.31 ± 2.8y, BMI: 20.93 ± 2.3kg.m<sup>-2</sup>) gender and age matched adolescents. CAVI was measured using VaSera VS-1500 (Fukuda Denshi, Japan). At supine rest, we recorded continuously and non-invasively (300 beats long recordings) blood pressure by volume-clamp plethysmography (Finometer Pro, FMS, Netherlands) and impedance cardiography (ICG) measures (CardioScreen 2000, Medis GmbH, Germany). PVR was calculated as follows: PVR = 80 \* (median MBP/median CO); MBP - mean blood pressure from Finometer, CO – cardiac output from ICG. Sympathetic nervous system activity related indices included Velocity Index (VI) and Heather index (HI) from ICG reflecting myocardial contractility. The statistical analysis was performed using SYSTAT 13. Values are expressed as means ± SD and Kruskal-wallis test and Spearman correlation were used for the analysis.

**Results.** As expected, significantly lower CAVI was found in obese group compared to control group (4.57 ± 0.87 vs. 5.19 ± 0.62, p = 0.001). PVR (850 ± 145 vs. 1112 ± 235 dyn.s/cm<sup>5</sup>, p < 0.001) and sympathetic nervous system activity indices VI (61.8 ± 17.5 vs. 88.7 ± 16.1 1/1000 s, p < 0.001) and HI (16.1 ± 5.2 vs. 21.4 6.5 ± Ω/s<sup>2</sup>, p = 0.002) were all significantly lower in obese group. In addition, we found positive correlation between CAVI and PVR (ρ = 0.386, p = 0.038) in the group of obese subjects.

**Conclusion.** In accordance with recent studies, we observed lower CAVI in obese adolescents. This result could be at least partially attributed to the lower PVR observed in obese group related to lower sympathetic activity. Thus, lower CAVI values in obese patients may not mean the absence of atherosclerotic damage of the vessels and could lead to underestimation of the complications development in this high-risk patients.



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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB330

**Effect of omega-3 on endothelial dysfunction in rat model adjuvant arthritis: Role of vascular cell adhesion molecule** Eman A. Allam, Eman M. Omar Medical Physiology Department, Faculty of Medicine, Alexandria University, Egypt

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**INTRODUCTION:** Rheumatoid arthritis (RA) is the most common systemic autoimmune disease resulting in excessive cardiovascular complication and mortality secondary to endothelial dysfunction. Recently many clinical trials have been carried on the role of omega-3 fatty acid supplementation in regression of symptoms of diseases. The exact mechanisms underlying such association are still not fully elucidated.

**AIM OF THE WORK:** To evaluate the effect of omega-3 on endothelial dysfunction as well as assess the level of vascular cell adhesion molecule (VCAM) and malonaldehyde (MDA) in rats with adjuvant arthritis.

**MATERIALS AND METHODS:** After approval of the Ethics Committee of Faculty of Medicine, Alexandria University, 30 male albino rats (125-150 grams) were used in this study. Rats were kept under standard laboratory conditions. The rats were divided into 3 groups 10 rats each. Group I: control group, Group II: RA induced group and Group III: RA induced group + omega3 supplementation. Induction of RA in 20 rats was done by intradermal injection at base of tail (120 µL of 1 mg of heat-killed *Mycobacterium butyricum* suspended in 0.1 ml of mineral oil). Induction of RA was confirmed by clinical sign of arthritis on day 11 of induction. The study duration was 30 days from the day of induction. In group III Omega 3 was given orally once daily by an intra-gastric tube, at a dose of 300 mg/kg (equivalent to 0.02 ml fish oil/rat, the omega-3 started on the day 11 after the induction of RA (appearance of clinical arthritis) continued to end of the study. At the end of study, clinical scoring of arthritis was repeated. Then all rats anaesthetized by ether inhalation to be sacrificed by decapitation, blood samples were collected for VCAM and MDA assessment in serum and section of Aorta was taken to test vascular reactivity to acetylcholine and noradrenaline using power lab AD instrument.

**RESULTS:** Values are expressed as means  $\pm$  SD, compared by ANOVA. Both VCAM and MDA were increased significantly after RA induction in group II ( $10 \pm 1.3$  ng/ ml &  $8.9 \pm 1.6$  nmom/ml respectively) in comparison to group I ( $5 \pm 0.45$  ng/ ml &  $3.5 \pm 0.7$  nmom/ml respectively) with  $p < 0.05$  for both of them. Treatment with omega-3 significantly decreased VCAM ( $7.2 \pm 1.42$  ng/ ml) & MDA ( $5.2 \pm 1.2$  nmom/ml) in group III in comparison to group II with  $p < 0.05$  for both of them. Plasma VCAM is negatively correlated with Ach-induce relaxation of thoracic Aorta. ( $r = -0.723$  and  $P = 0.000$ )

**CONCLUSION:** The present study highlights the importance of omega-3 in RA to slow down the progression of endothelial dysfunction by its effect on vascular reactivity and oxidative stress. The decreased VCAM levels seen with omega-3 may contribute to this improvement.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB331

**Neurovascular coupling is blunted during acute poikilocapnic hypoxia**

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Neurovascular coupling (NVC) is responsible for the close temporal and regional linkage of cerebral blood supply to local alterations in cerebral metabolism during neuronal activation and remains an important determinant of cognitive performance. Despite its clinical importance, the mechanisms underpinning hypoxia-induced cognitive impairment remain elusive, but may relate to blunted NVC given the potential of hypoxia to impair cerebrovascular function. To examine this, we measured NVC in twelve healthy participants (11 male and 1 female) aged  $28 \pm 8$  years (mean  $\pm$  standard deviation) during 30 min of passive exposure to isocapnic normoxia ( $100 \pm 4$  mmHg  $P_{ET}O_2$  and  $43 \pm 3$  mmHg  $P_{ET}CO_2$ ) and poikilocapnic hypoxia equivalent to a simulated altitude of  $\sim 4400$ m ( $50 \pm 3$  mmHg  $P_{ET}O_2$  and  $27 \pm 1$  mmHg  $P_{ET}CO_2$ ), achieved through dynamic end-tidal forcing. Posterior cerebral artery blood velocity (PCAv) was assessed using transcranial Doppler ultrasound and beat-by-beat blood pressure for the determination of mean arterial pressure (MAP) via finger photoplethysmography. Following a 60 s supine baseline, all participants completed five consecutive trials of 30 s eyes open with standardised visual stimulation (flashing checkerboard), followed by 30 s of eyes closed. The NVC response was characterised as the percent peak and average increase (relative to the baseline control) in PCAv during 25 s of visual stimulation, averaged across the five trials. Distribution normality was confirmed by Shapiro Wilks W tests and data analysed using paired samples *t*-tests. Poikilocapnic hypoxia reduced baseline PCAv from  $42 \pm 6$  cm.s<sup>-1</sup> to  $33 \pm 6$  cm.s<sup>-1</sup> ( $P < 0.01$ ) and attenuated the peak ( $10 \pm 4\%$  vs.  $16 \pm 6\%$ ,  $P = 0.04$ ) and average ( $3 \pm 3\%$  vs.  $7 \pm 4\%$ ,  $P < 0.01$ ) percent increases in PCAv, across the visual stimulation period. MAP remained unchanged ( $3 \pm 3\%$  vs.  $3 \pm 3\%$ ,  $P = 0.81$ ). These are the first data to demonstrate that NVC is blunted following acute poikilocapnic hypoxia in humans. This may potentially be related to hypocapnia-induced vasoconstriction (1) and/or oxidative inactivation of nitric oxide-induced vasodilation (2) that out-competes the normal NVC dilatory mechanisms. A functional attenuation in NVC may contribute to the cognitive deficits observed in patients characterised by arterial hypoxemia and individuals sojourn to terrestrial high altitude.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB332

## A novel physiological role for dynein motor proteins in arterial smooth muscle

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**Background** - Microtubules are key components of the cell cytoskeleton involved in many biological processes, including cell division and intracellular transport. Although little is known about the role of microtubules in smooth muscle, recent work has shown that the microtubule network can regulate arterial tone by controlling Kv7.4 channel membrane expression in smooth muscle cells. However, the precise mechanism underlying microtubule transport of Kv7.4 channels is yet to be determined.

Dyneins are small molecular motor proteins that bind to microtubules and walk toward the minus ends. They are essential for the transport and distribution of certain membrane proteins. The role of dynein in arterial smooth muscle cells is unknown; therefore the aim of this study was to investigate whether dynein was involved in the microtubule-dependent regulation of Kv7.4 in vascular smooth muscle.

**Methods and results** - Experiments were performed on 15- to 18-week-old male Wistar rats euthanized by cervical dislocation. Isometric tension recordings were performed on third-order mesenteric artery segments (~2 mm) in a wire myograph. Mesenteric artery segments were incubated with the dynein inhibitor ciliobrevin, after which either the  $\beta$ -adrenoceptor agonist, isoprenaline, or Kv7.2-7.5 activator, S-1, were applied at increasing concentrations to precontracted artery segments. Treatment with ciliobrevin enhanced isoprenaline- and S-1-mediated relaxations compared to non-treated controls. Morpholino-induced knockdown of Kv7.4 channels prevented ciliobrevin from enhancing S-1-mediated relaxations and partially attenuated the enhanced isoprenaline relaxations. Structured Illuminescence Microscopy on myocytes isolated from arteries showed that dynein and Kv7.4 can locate in close proximity to each other. Given that Kv7.4 channel knockdown only partially attenuated ciliobrevin-enhanced isoprenaline-mediated relaxations, we investigated if there was also a vasorelaxant component unrelated to Kv7.4 channels that was enhanced by dynein inhibition. Isoprenaline stimulation in presence of bisoprolol ( $\beta$ 1-adrenergic antagonist) inhibited isoprenaline relaxations in control artery segments. However, when artery segments were incubated with ciliobrevin, bisoprolol had no effect on the relaxations.

**Conclusion** - This study is the first to show regulatory effects of dynein on  $\beta$ -adrenoceptor-mediated relaxations of rat mesenteric arteries. We show that dynein inhibition enhanced Kv7.4 channel function, which contributed to increased  $\beta$ -adrenoceptor mediated relaxations. In addition, dynein inhibition may prevent agonist-induced internalisation of the  $\beta$ 2-adrenoceptor, thus increasing isoprenaline-mediated relaxations through mechanisms independent of Kv7 channel recruitment.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB333

## Remodeling of vascular smooth muscle ion channels contributes to the UTP-induced vasoconstriction in essential hypertension.

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Vascular smooth muscle cells (VSMCs) from hypertensive mice (BPH mice) have a depolarized membrane potential and an increased vascular tone when compared with myocytes from their normotensive controls (BPN mice). We have previously shown an increased functional expression of a member of the DAG-activated TRPC family of non-selective cation channels, namely TRPC3, in mesenteric VSMC of hypertensive mice which could contribute to the increased vascular tone by favoring depolarization (Alvarez-Miguel *et al.* 2017). However, changes in the functional expression of some G-protein coupled receptors (GPCRs) could also contribute to the altered vascular tone by modulating the TRPC3 channels or  $\text{Ca}^{2+}$  activated  $\text{Cl}^-$  currents (CaCl). Therefore, here we explored if changes in the molecular constituents of the purinergic signaling cascades could contribute to the hypertensive phenotype of the BPN/BPH model.

BPN and BPH mice were anesthetized by isoflurane inhalation (5%  $\text{O}_2$  at 2.5  $\text{Lmin}^{-1}$ ) and sacrificed by cervical dislocation, according to the European Community guiding principles with respect to the care and use of animals (Directive 2010/63/UE). Endothelial-denuded mesenteric arteries and isolated VSMC from BPN and BPH mice were used to analyze the expression and association pattern of GPCRs and TRPC and CaCl channels by qPCR and PLA assays. Their functional contribution was explored using electrophysiology and myography studies.

Pressurized mesenteric arteries showed larger, dose-dependent, vasoconstrictor effects to UTP and UDP in BPH compared to BPN. Both the pharmacological profile of these responses and qPCR data indicated an increased expression of P2Y6 receptors in BPH. Patch-clamp studies showed a significantly larger UTP-induced depolarization in BPH VSMCs which could be abolished by niflumic acid and by Tmem16A inhibitor. Moreover, these blockers induced a significantly higher hyperpolarization in BPH VSMC, pointing to a relevant contribution of CaCl channels to determine VSMC resting Vm. These effects agreed with the higher mRNA expression of  $\text{Cl}^-$  channels obtained in BPH cells by qPCR. Surprisingly, voltage-clamp studies showed no differences in UTP-activated whole-cell currents between BPH and BPN VSMC even though a fraction of these currents could be blocked by niflumic acid, Tmem16A inhibitor or Pyr10 (a TRPC blocker). In this context, we propose that differences between BPN and BPH UTP-mediated responses are the result of a combination of changes in the expression of P2Y6 receptors, TRPC and CaCl channels, together with some variation in their functional coupling. Altogether, these findings could be relevant to explain the increased vascular tone in the hypertensive phenotype.

Álvarez-Miguel, I. *et al.* (2016) 'Differences in TRPC3 and TRPC6 channels assembly in mesenteric vascular smooth muscle cells in essential hypertension.', *The Journal of physiology*, 0, pp. 1-17. doi: 10.1113/JP273327.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB334

### Kv1.3 channel blockers as therapeutical agents against intimal hyperplasia: New evidences in human arteries from diabetic patients.

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Vascular occlusive disease is a leading cause of death and disability worldwide. The development over the last decades of vascular interventions has represented a huge therapeutical improvement. A limitation of these procedures is restenosis of the target vessel because of the arterial repair mechanisms. Mechanical or chemical injury promotes dedifferentiation of the vascular smooth muscle cells (VSMCs) through a process known as phenotypic modulation. VSMCs develop a proliferative, migratory and secretory phenotype directed to intimal hyperplasia (IH). Restenosis after vascular surgery is accelerated in pathological vessels, as the underlying disease will determine prognosis. Among these, metabolic syndrome and type II diabetes mellitus (DM), represent the dark side of restenosis, showing increased IH and worse outcomes.

We have recently found that phenotypic modulation associates with an increased functional expression of the voltage-dependent potassium channel Kv1.3, and that selective blockade of this channel decreases human VSMC proliferation *in vitro* and neointima formation *in vivo*.

Here, we compare the effect of PAP-1 (a Kv1.3 selective blocker) with everolimus (the gold standard in clinical practice) on the remodeling of human mammary artery (hMA) in organ culture and on proliferation and migration of VSMC in culture. These studies were carried out in non-diabetic (NDM) and diabetic patients (DM). hMAs were obtained from donors after approval by the Hospital Ethics Committee according to the standards of the Helsinki Declaration. IH in organ culture was induced after two weeks incubation using fetal bovine serum. Intimal/media ratio (I/M) shows that IH is increased in DM vessels compared to NDM. PAP-1 treatment inhibited IH in both groups, being more effective in DM vessels (I/M ratio of  $0.46 \pm 0.13$  in DM versus  $0.73 \pm 0.04$  in NDM,  $p < 0.05$ , t-test). However, everolimus was only effective in DM vessels. Surprisingly, no inhibitory effect was observed when both drugs were applied together. Additionally, PAP-1 inhibited proliferation and decreased the amount of elastin in organ culture. In primary VSMCs cultures from hMAs, PAP-1 and everolimus inhibited proliferation to a similar extent, and their effects were additive only in NDM VSMCs. Migration assays showed that PAP-1 had an inhibitory effect in NDM and DM cultures but everolimus only inhibited DM samples. Altogether, our data highlight Kv1.3 channels as a promising target against IH in human vessels and suggest that diabetes induces long-term changes in the IH signaling pathways that could be relevant when possible therapeutical approaches are considered.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB335

### Human myometrial artery function is critically affected by maternal BMI

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The incidence of obesity in the pregnant population is growing and is a well-recognised risk factor for adverse pregnancy outcome. We have studied human myometrial artery function in order to better understand the (patho)physiology of myometrial vessels and how obesity might affect this. We have therefore examined whether maternal BMI affects the ability of the human myometrial arteries to contract and relax *in vitro*.

Human myometrial biopsies were collected during elective c-section from women with uncomplicated singleton term pregnancies, after informed consent, at Liverpool Women's Hospital. Small arteries were dissected (2mm sections), mounted in a wire myograph containing oxygenated physiological salt solution at 34°C and set to their normalized diameter ( $0.9 \times IC_{100}$ ). Vessels were separated into 2 groups: BMI < 25 (normal weight) and BMI ≥ 25 (overweight/obese). There was no significant difference in vessel size between groups. Vessel function was assessed using contractile (AVP, U-46619) and relaxatory (bradykinin (BK), CCh, SNAP) agonists. In addition, vessels (BMI ≥ 30) were treated with 2% methyl cyclodextrin (MCD), to sequester cell membrane cholesterol, for 20min prior to assessing function. Data are expressed as mean ± sem, where n=vessels, N=biopsies.

Contractile responses were altered in the BMI ≥ 25 group. U-46619 was significantly less potent ( $pEC_{50} = 6.83 \pm 0.13$ ,  $n = 21$  /  $N = 11$  v  $7.14 \pm 0.15$ ,  $n = 22$  /  $N = 11$ , t-test  $p = 0.018$ ) compared to BMI < 25. AVP showed a similar trend ( $pEC_{50} = 8.94 \pm 0.09$ ,  $n = 17$  /  $N = 9$  v  $9.20 \pm 0.09$ ,  $n = 21$  /  $N = 13$ ,  $p = 0.054$ ). When pre-contracted with AVP, all vessels relaxed equally well to BK (BMI < 25:  $pEC_{50} = 7.71 \pm 0.14$ ,  $n = 12$  /  $N = 9$  v BMI ≥ 25:  $pEC_{50} = 7.80 \pm 0.06$ ,  $n = 20$  /  $N = 12$ ). In contrast, only a subset of vessels relaxed when challenged with CCh (14 out of 44). The BMI of women whose vessels responded to CCh ( $23.64 \pm 0.72$ ,  $N = 14$ ) was significantly lower than those not responding ( $29.22 \pm 1.11$ ,  $N = 30$ ). Overall, 15% of BMI ≥ 25 vessels (4/27) and 59% of BMI < 25 vessels (10/17) responded to CCh. In fact, no vessels from obese women (BMI > 30) responded to CCh, although they all relaxed to BK. The CCh  $pEC_{50}$  in responding vessels was  $7.41 \pm 0.25$  ( $n = 9$  /  $N = 6$ ). No significant difference in response to SNAP was observed. Exposure to MCD failed to restore a response to CCh in vessels from obese women.

Vasoconstriction and vasodilation are impaired in myometrial arteries from overweight/obese women, although responses are agonist-specific: the CCh response is ablated and U-46619 is less potent. Full relaxation was observed with SNAP, suggesting no deficit in arterial smooth muscle responses with increased BMI. If hyperlipidemia caused the loss of the CCh response, then treatment with MCD could potentially restore



sensitivity to CCh, however this was not the case. Impaired vessel function may contribute to the significant adverse pregnancy outcomes experienced by overweight/obese women.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB336

### Dietary Salt Induces Vascular Dysfunction in C57BL/6J Mice

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Average individual salt intake is ~10g/day worldwide, exceeding the 5g/day recommended by the World Health Organisation as the upper limit tolerable to health[1]. This habitually high salt intake is associated with cardiovascular and renal disease, with the effect upon blood pressure (BP) as a pivotal factor in the development of these global health problems. The underlying mechanisms linking high salt intake to increased BP remain controversial. Impaired renal sodium (Na) excretion[2] and vasodysfunction[3] are proposed as key players in salt-induced BP rises. Here, we hypothesised that dietary salt induces endothelial dysfunction, which would be reflected in an increase in BP. We examined this in adult male C57BL/6J mice fed diets containing either 0.3% Na (n=10-15), 1.3% Na (n=7-8) or 3.0% Na (n=10-11) for 1-2 weeks. Mice were culled by decapitation and blood was collected to measure plasma aldosterone levels. Vascular reactivity to acetylcholine (ACh) and sodium nitroprusside (SNP) was determined in dissected mesenteric arteries using wire myography. Data are mean±SEM with groups compared by one-way ANOVA with post-hoc Bonferroni testing, unless specified otherwise. Plasma aldosterone (pg/mL) was significantly suppressed by dietary salt (0.3% 68.5±5.2 vs 1.3% 30.4±5.9, P=0.0003; or vs 3% 30.9±6.4, P=0.0002), confirming normal regulation of the renin-angiotensin system here. In mesenteric arteries, sensitivity to ACh was diminished by high salt intake (Fig.1A) expressed as logIC<sub>50</sub> (0.3% 7.189±0.195 vs 1.3% 6.054±0.120, P=0.0023), which was particularly evident in response to 3% Na (Fig.1C; 3% 6.057±0.120, P=0.0005). This reduced sensitivity was not observed with direct nitric oxide (NO) provision by SNP (Fig.1B 0.3% 6.624±0.180 vs 1.3% 6.845±0.314, P=0.936; or vs Fig.1D 3% 6.824±0.139, P=0.910). In a separate cohort (n=7) of conscious, unrestrained C57BL/6J mice, BP measurement was carried out using radiotelemetry as previously described[4]. A modest but statistically significant rise in mean BP (MBP) was noted when dietary salt content was increased from 0.3% Na (103.3±2.3mmHg) to 3% Na (114.4±2.8mmHg, P=0.0006; paired t-test). To conclude, elevating salt intake decreases mesenteric artery reactivity to ACh but not to SNP, indicating that dietary salt impairs endothelial-dependent NO production and would be likely to increase vascular tone *in vivo*. We also found that high salt intake increased MBP in these mice, which is supportive of the role of vasodysfunction thus far. The next steps involve evaluating renal function following salt intake and its possible contribution to the salt-induced increases in BP seen.

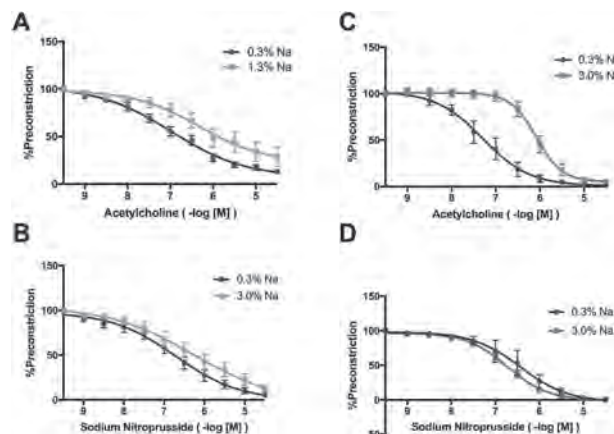


Figure 1: Cumulative concentration-response curves to acetylcholine (A, C) and sodium nitroprusside (B, D) following a 0.3% Na (blue) vs 1.3% Na (orange)(A, B) or vs 3% Na (red) diet (C, D). Points represent mean±SEM.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB337

### Novel insights into the role of oxidant-activation of Protein Kinase G within the vascular endothelium

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A healthy endothelium is vital for cardiovascular health. Within the endothelium of small resistance arteries, vasodilatory pathways are predominantly dependent on localised calcium (Ca<sup>2+</sup>) signalling. These endothelial cells (ECs) Ca<sup>2+</sup> signals control potassium (K<sup>+</sup>) channel activity, and this regulates arterial membrane potential. The principal of endothelial vasodilatory Ca<sup>2+</sup> mechanisms is controlled by inositol trisphosphate-induced Ca<sup>2+</sup> release from the endoplasmic reticulum<sup>1</sup> and extracellular Ca<sup>2+</sup> entry through TRPV4 channels<sup>2</sup>. In the vascular smooth muscle cells, Protein Kinase G (PKG) plays an important role in K<sup>+</sup> dilation mechanism. Our previous study has shown that oxidants generated in response to the intraluminal pressure are able to activate PKG<sup>3</sup>. However, the crosstalk between PKG and Ca<sup>2+</sup> pathways in the endothelium remains unclear. Effects of oxidant-activation of PKG on Ca<sup>2+</sup> signalling pathways were assessed via the use of a genetically modified mouse model whereby an amino acid switch (Cysteine to Serine) renders PKG insensitive to oxidant activation (PKG[C42S]<sup>KI</sup>). EC Ca<sup>2+</sup> signals were

visualised using high-speed (50Hz) spinning disc confocal microscopy of third order mesenteric arteries which were slit open and imaged in an 'en-face' configuration. Results were correlated with diameter changes via the same  $\text{Ca}^{2+}$  induced pathways using pressure myography.  $10\mu\text{M}$  Acetylcholine (ACh) increased  $\text{IP}_3$  (Pulsar frequency  $\text{WT}=6$ ;  $15.37\pm 3.64$  vs  $\text{KI}=n$ ;  $7.99\pm 1.55$ ) and TRPV4 (Sparklets activity  $34.92\pm 7.35$  vs  $22.43\pm 2.37$ )  $\text{Ca}^{2+}$  signalling pathways with intact vascular endothelium compared to  $\text{PKG}[\text{C42S}]^{\text{KI}}$  arteries. Similarly, ACh dilated WT arteries but not in the oxidant resistant model ( $0.75\pm 0.15\%$  vs  $0.16\pm 0.08\%$ ). Vasodilation to TRPV4 agonists ( $10\text{nM}$  GSK1016790A) was also impaired in the  $\text{PKG}[\text{C42S}]^{\text{KI}}$  arteries. In contrast, direct  $\text{K}^+$  channel vasodilation using  $0.3\mu\text{M}$  NS309 was preserved ( $0.89\pm 0.18\%$  vs  $0.91\pm 0.27\%$ ). The principal vasodilatory pathways of the resistance artery endothelium are critically dependent on oxidant generation which activate PKG. The oxidant-activation of PKG controls  $\text{Ca}^{2+}$  signal generation within the EC rather than the vasodilatory K channel function.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB338

### Angiotensin II effect on dynamics of leukocyte's subpopulations in rats on short-term high salt diet

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AIM: In previous studies, we have demonstrated that short-term high-salt diet affects microcirculation and impairs flow-induced dilation in cerebral vessels and skin microcirculation via increased oxidative stress, in animal models (1) and in humans (2). Present study aimed to determine the effect of subpressor doses of angiotensin II administration via miniosmotic pumps on activated monocytes', granulocytes' and T-lymphocytes' expression levels in rats fed high salt diet (4% NaCl).

MATERIALS AND METHODS: Healthy male Sprague-Dawley rats (age of 10-12 weeks), were divided in three groups ( $n=6$ /per group; 7-day protocol): 1) LS group – control group fed low salt diet (0,4% NaCl); 2) HS group – fed high salt diet (4% NaCl); 3) HS+ANG II group – fed high salt diet (4% NaCl), on the 5th day of protocol  $100\text{ ng}$  of angiotensin II/  $\text{kg}$ /  $\text{min}$  was administered by Alzet miniosmotic pumps for 3 consecutive days, till the end of protocol. Rats were anaesthetized (IP) with ketamine ( $75\text{ mg/kg}$ ) and midazolam ( $2.5\text{ mg/kg}$ ). Arterial blood was collected on the 8th day and used for flow cytometry measurements of cell adhesion molecules CD11a/ LFA-1 and CD11b/Mac-1 as the ligands for ICAM-1, and CD49d/ VLA-4 as the ligand for VCAM-1 on subpopulations of leukocytes (BD FACS Canto II flow cytometer; Flowlogic v7.2.1 software). Statistical analyses were performed with Sigma Plot v12.0 and GraphPad Prism v6.01 software. To test differences

among groups One-way ANOVA was used;  $p<0.05$  was considered significant. All experimental procedures conformed to the European Guidelines for the Care and Use of Laboratory Animals (directive 86/609) and were approved by the local and national Ethical Committee (Class: UP/I-322-01/14-01/36, No. 525-10/0255-15-6).

RESULTS: Expression of CD49d on monocytes was significantly higher in HS group compared to HS+ANG II and LS groups ( $p=0.04$  and  $p=0.016$ , respectively). CD49d expression on granulocytes was significantly lower in HS group compared to LS group ( $p=0.041$ ). CD49d expression on CD8 lymphocytes was lower in HS+ANG II group compared to HS and LS groups ( $p=0.0204$ ). Granulocytes CD11b expression was significantly higher in HS+ANG II group compared to LS group ( $p=0.0161$ ). CD11a expression of CD8 lymphocytes was significantly higher in HS group compared to LS and HS+ANG II groups ( $p=0.027$  and  $p=0.005$  respectively).

CONCLUSION: Decrease in angiotensin II levels caused by high salt dietary intake may be related to altered immunoreactivity of rats on HS diet. HS leads to a significant increase of CD49d expression on monocytes and CD11a expression on CD8 lymphocytes, potentially increasing their ability to adhere to endothelium. Angiotensin II infusion reversed their expression levels to the values comparable to the LS group, restoring normal immunophenotype.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB339

### Exploring the effect of an oral glucose load on the cutaneous microvascular dynamics with the wavelet transform

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Chronic hyperglycemia leads to microvascular dysfunction associated with several cardiovascular and metabolic complications. It has also been established that acute hyperglycemia impacts endothelial function by increasing the level of oxidative stress and reducing NO bioavailability, as well as interfering with the activity of the sympathetic nervous system. On the other hand, hyperinsulinemia has been shown to increase the endothelium-dependent vasodilation. How these mechanisms integrate at the level of microcirculation is less well investigated and contradictory results have been found in *in vivo* studies where both physiological stimuli are present. Our objective was to explore the effect of a standard oral glucose load (OGL) on the reactivity of the cutaneous microcirculation. 16 healthy subjects ( $21.4\pm 1.3\text{ yr.}$ ; 11 males,

5 females) participated in the study after informed consent was obtained. Preliminary experiment where plasma glucose dynamics was traced spectrophotometrically (HemoCue, Glucose201+) for 2 h by a 5 min blood sampling revealed that the highest plasma conc. was achieved after 35 min ( $8.9 \pm 1.2$  mmol/L). After fasting for 12 h, microcirculation was assessed on the finger pulp and on the volar surface of the forearm by using laser Doppler fluxmetry (LDF), quantified in arbitrary units (AU). To test microvascular reactivity, we induced post-occlusive reactive hyperemia (PORH: 5 min baseline tracing, 3 min occlusion of the brachial artery, 7 min post-occlusion) before (pre-load) and after OGL (75 g/250 mL). As a control experiment, subjects performed the same protocol after receiving 250 mL of bottled water. The main spectral components of the LDF signal (cardiac, respiratory, myogenic, sympathetic and endothelial NO-dependent) were obtained after decomposition with the wavelet transform, and their contribution to the overall signal was expressed as percent ratio (%). Nonparametric tests were applied and a  $p < 0.05$  adopted. The post-pre PORH perfusion difference was significantly lower with glucose than with water (glucose:  $-2.0 \pm 4.9$  AU; water:  $5.4 \pm 7.9$  AU;  $p = 0.001$ ) for the forearm. The glucose load significantly affected the sympathetic activity of the forearm region. On the post-load PORH, this activity was significantly lower than control during the reactive hyperemia phase (glucose:  $20.3 \pm 8.4$  %; water:  $26.0 \pm 4.1$  %;  $p = 0.006$ ), but significantly higher during the recovery phase (glucose:  $20.4 \pm 6.7$  %; water:  $18.4 \pm 7.8$  %;  $p = 0.039$ ). These changes were not noted on the pre-load PORH and in neither of the control experiments. No changes in the PORH response profile were detected for the finger. These results suggest that, in healthy subjects, an oral glucose load might affect the local cutaneous microvascular response to a PORH test through modulation of the sympathetic activity, rather than through endothelium-dependent vasodilation.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCB340

#### **Extra virgin olive oil phenol, hydroxytyrosol, vasodilates uterine arteries in an estrogen receptor- and nitric oxide-dependent manner**

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Extra Virgin Olive Oil (EVOO), a major ingredient of the Mediterranean diet, has been shown to confer benefits to human health by protecting against cardiovascular disease. Special attention has been on phenols (a component of EVOO) which have been shown to produce vasodilation in some type of vessels, although the underlying mechanisms remain unknown. Phenols have not been tested on uterine arteries, which must vasodilate and grow during pregnancy to increase uteroplacental blood flow to meet fetal demands. These processes are compromised in some gestational diseases such as preeclampsia and fetal growth restriction.

In this study, we tested the effects of EVOO phenols on isolated, pressurized rat uterine (UA) and mesenteric (MA) resistance arteries. The contribution of the two major EVOO phenols - Hydroxytyrosol (HT) and Oleuropein (Ole) was examined, and the underlying mechanisms probed by using L-NAME and ICI182,780 to inhibit nitric oxide synthase (NOS) and estrogen receptors (ERs) respectively. Both HT and Ole

induced vasodilation of UA in a concentration-dependent manner. At the highest concentration ( $3 \times 10^{-5}$  M), the vasodilation was  $85.7 \pm 8\%$  by HT and of  $26.3 \pm 6\%$  by Ole. Neither compound had any effect on MAs. UA vasodilation to HT was both ER- and NO-dependent; conversely, inhibition of ERs or NOS did not affect UA vasodilation to Ole, indicating that other, as yet unrecognized mechanisms are responsible.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCB341

#### **Omega-3 enriched eggs consumption reduces oxidative stress in granulocytes in men**

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AIM: Previously, we have demonstrated that 3 weeks of intake of omega-3 enriched eggs reduced the level of oxidative stress in young healthy men (1) and had a positive effect on reducing systemic oxidative stress in young healthy women due to increased blood antioxidative capacity (2). Present study aimed to investigate the effects of n-3 polyunsaturated fatty acids (n-3 PUFA) enriched eggs consumption on the levels of oxidative stress in human leukocyte's subsets.

MATERIALS AND METHODS: 33 healthy participants (18 men and 15 women, age: 20-26) included in the protocol were divided into 2 groups: OE group consumed n-3 PUFA enriched chicken eggs (n=18; 2 eggs/day, n-3 PUFA=403.10 mg/day) while RE group consumed regular chicken eggs (n=15; 2 eggs/day, n-3 PUFA=74.63 mg/day) for 3 weeks. Peripheral leukocytes' oxidative stress was measured by flow cytometry (BD FACS Canto II flow cytometer; Flowlogic v7.2.1 software) with DCF-DA (2',7' - dichlorofluorescein diacetate) assay at the beginning and at the end of the study. Levels of intracellular ROS were assessed prior and 30 minutes after PMA (phorbol-12-myristate-13-acetate) (1:1000; 50µl) mediated induction of ROS production. Statistical analyses were performed using Sigma Plot v12.0 and GraphPad Prism v6.01 softwares. T-test was used for differences among groups and intra-group differences were tested by paired t-test;  $p < 0.05$  was considered significant. The study protocol and procedures conformed to the standards of the Declaration of Helsinki and were approved by the Ethical Committee of the Faculty of Medicine, University of Osijek.

RESULTS: Leukocytes' baseline and PMA stimulated ROS production did not differ between the study groups at the beginning of the study. At the end of the study, OE group exhibited significantly reduced levels of ROS upon PMA stimulation in male granulocytes ( $p = 0.0442$ ), in contrast to significantly increased ROS production upon PMA stimulation in male and the female monocytes from OE subjects ( $p = 0.0006$  and  $p = 0.0227$ , respectively). At the end of the study, OE men had significantly lower level of oxidative stress in granulocytes prior and upon PMA stimulation ( $p = 0.0366$  and  $p = 0.0049$ , respectively) compared to RE men. At the end of study OE men had significantly lower levels of ROS in monocytes and lymphocytes compared to OE women ( $p < 0.0001$  and  $p = 0.0153$ , respectively).



**CONCLUSION:** Omega-3 consumption reduced levels of oxidative stress upon PMA stimulation in granulocytes of men, while it had an opposite effect on the monocytes of both sexes. Results suggest that the omega-3 supplementation during 3 weeks reduces oxidative stress in granulocytes probably by increasing the cellular antioxidative capacity, and this effect seems to be restricted to male subjects.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB342

### Assessment of the reliability of subdural intracranial pressure measurement method in preclinical ischaemic stroke models

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Monitoring intracranial pressure (ICP) is critical in patients at risk of raised ICP. Intraventricular pressure (IVP) monitoring is still the gold standard in the clinical setting due to its reliability and accuracy over epidural (ED) or subdural (SD) ICP monitoring. However, ED and SD monitoring can be beneficial in reducing the risk of infection and bleeding associated with IVP monitoring. Therefore, we evaluated the reliability of SD ICP monitoring in preclinical stroke models by comparing it against IVP and ED ICP monitoring methods at different ICP levels over time. Male Wistar rats (250-350g) were anaesthetised using isoflurane (5% induction, 1.5-2% maintenance) in 50:50 N<sub>2</sub>:O<sub>2</sub>. The rats were subjected to either middle cerebral artery occlusion or photothrombotic stroke, and ICP was measured from 18-24hrs post-stroke. Briefly, a burr hole (2mm posterior, 2mm lateral to Bregma) was drilled in the right parietal bone and the dura was gently pierced using an atraumatic Sprotte-type needle. A fibre optic pressure sensor probe (Opsens, Canada) was inserted through the burr hole to access the epidural space. Another burr hole in the left parietal bone (0.8mm posterior, 1.8mm lateral to Bregma) was made, and a second ICP probe was either inserted into the left lateral ventricle for IVP monitoring or into the epidural space (dura intact) for ED ICP monitoring. The probes were sealed and either IVP (n=99) or ED ICP (n=107) was measured simultaneously with SD ICP in real-time. Values are mean ± SD. The Pearson correlation coefficients of the mean intraventricular-subdural and epidural-subdural ICP values were  $r = 0.91$  ( $p < 0.0001$ ) and  $r = 0.95$  ( $p < 0.0001$ ), respectively. Using the Bland–Altman analysis, intraventricular-subdural and epidural-subdural methods showed a mean difference of  $0.15 \pm 2.1$  mmHg and  $0.16 \pm 1.7$  mmHg respectively.

ICP measured using the modified SD method was reliable and reproducible, and was comparable to data obtained from both the 'gold-standard' IVP method and the ED ICP method. The results suggest that SD method is a valid alternative to the more invasive IVP monitoring, as well as the ED ICP method.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB343

### Structural remodeling in cerebral and mesenteric arteries from obese Göttingen minipigs with or without diabetes

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Obesity and diabetes are associated with structural remodeling and hypercontractility of small arteries. The degree and type of remodeling depends on the vascular bed and severity of the obese/diabetic state. In this study, remodeling was investigated using pressure myography of small pial arteries (PA) and terminal mesenteric arteries (TMA) from male castrated Göttingen minipigs (79.5 ± 0.3 weeks; N=21) after high-fat feeding (HFD; N=7), HFD with streptozotocin-induced diabetes (HFD/STZ; N=7), or chow feeding (lean control; LC; N=7). *In vivo* plasma parameters (implanted IV-catheter; anaesthesia w. tiletamin and zolazepam *i.m.*, both 0.81 mg/kg) included total cholesterol (TC), triglyceride (TG), glucose (GLU), fructosamine (FRA), and insulin (INS). We quantitated, by Q-PCR, vessel expression levels of putative structural remodeling genes (ROCK1; MMP2; TIMP1; TGM2), and several Ca<sup>2+</sup> (Cacna1C/G/H, TRPV4) and K<sup>+</sup> channel genes (Kcnj2, Kcnn3, Kcnn4) involved in tone regulation. Using linear regression analysis, we correlated structural parameters, plasma parameters, and gene expression levels. Hypotheses: 1) Remodeling in HFD and HFD/STZ is correlated with distinct changes in plasma parameters and gene expression. 2) Hyperglycemia leads to altered ion channel expression, which potentially is involved in contractility changes.

In TMAs from HFD/STZ pigs the passive lumen diameter (PLD) and wall cross-sectional area (CSA) were increased vs. LC, with little or no change in wall:lumen ratio and elastic modulus. In TMAs from HFD pigs, PLD was in-between that observed in LC and HFD/STZ, and CSA was not different from that observed in HFD/STZ. We observed qualitatively similar, albeit non-significant, structural changes in PA from HFD and HFD/STZ vs. LC. HFD pigs had increased BW, % body fat, TC, TG, and INS. HFD/STZ pigs had increased TC, TG, FRA, and GLU. As BW was not correlated with PLD, outward remodeling was not an effect of body size. Two genes (Cacna1G, MMP2) were down-regulated in HFD vs. LC. Ca<sup>2+</sup> channels (Cacna1C, Cacna1G, Cacna1H), K<sup>+</sup> channels (Kcnj2, Kcnn3, Kcnn4), and remodeling genes (Rock1, MMP2, and TGM2) were upregulated in HFD/STZ vs. LC or HFD. Structural parameters (PLD, CSA) were positively correlated with plasma lipids (TC, TG), and with ROCK1 and TGM2 expression. ROCK1 and TGM2 expression was positively correlated with GLU and FRA. In general, ion channel

expression was positively correlated with GLU and FRA, and negatively correlated with INS.

Obesity with diabetes causes outward hypertrophic remodeling of small arteries in Göttingen minipigs. Remodeling is positively correlated with plasma lipid levels and expression of ROCK1 and TGM2 genes, which are upregulated in hyperglycemia. Outward structural remodeling in small arteries is driven by several factors present in obesity/diabetes, and this translational model allows for investigation of the molecular mechanisms involved.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCB344

### **Role of the catalytic beta-subunit of calcineurin (PPP3CB) in angiotensin II-induced vascular changes**

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Angiotensin II (angII), a key effector of the renin-angiotensin-aldosterone system (RAAS), is an important mediator of pathological cardiac and vascular changes by contributing to cardiac hypertrophy and altered vascular tone, inflammatory processes and structural remodeling with fibrosis. Calcineurin (PPP3), a serine/threonine protein phosphatase consisting of an enzymatic and a catalytic subunit, is known for its role in inflammatory processes and cellular hypertrophy. One of its ubiquitously expressed enzymatic subunits, PPP3CB, has been shown to influence T cell development and also angII-induced cardiac hypertrophy [1, 2]. To investigate the role of PPP3CB for vascular changes stimulated by angII, we analysed the effect of a global PPP3CB knockout (KO) on the murine aorta. Under basal conditions, no upregulation of the PPP3CA subunit due to PPP3CB KO was detectable excluding a compensatory effect in adult male mice. Additionally, KO animals had no obvious phenotype under basal conditions compared to wild-type (WT) littermates.

Contractility of aortic rings from PPP3CB WT and KO mice was measured via mulvany wire myography and was not altered after acute angII-receptor activation, indicating normal functionality under these conditions. Additionally, endothelium-dependent relaxation mediated by carbachol and SNAP showed no significant difference excluding an endothelial dysfunction.

Subsequently, angII was applied chronically for 28 days (500ng/kg/day) to mice by subcutaneous implantation of osmotic minipumps (surgery under isoflurane anesthesia (2%, 1 L/min O<sub>2</sub>)) and systolic blood pressure was measured via tailcuff. In PPP3CB KO mice, angII-induced systolic blood pressure increase was smaller than in WT mice. In histological stainings, KO mice showed less aortic wall thickening after angII treatment and an increase in lumen area compared to WT mice was detectable. Aortic gene expression, assessed by Next Generation Sequencing, was significantly altered in angII-treated WT mice with an upregulation of fibrosis related genes (COL1a1, CTGF) inflammation markers (PAI-1) and adhesion related genes (ACAN, FN-1) compared to KO animals. A slight difference in collagen I and fibronectin-1 secretion upon angII-stimulation, analysed with ELISA in freshly isolated aortic vascular smooth muscle cells (aVSMCs) of WT and KO animals, revealed a possible involvement of aVSMCs in the observed angII-induced vascular changes.

Overall, our results suggest that the pathological effects of angII in the vasculature are partially mediated by PPP3CB. While the acute effects of angII on vascular tone are not affected by PPP3CB, chronic vascular remodeling and blood pressure is reduced in PPP3CB KO mice compared to WT controls.

[1] Bueno et al. Impaired cardiac hypertrophic response in calcineurin A beta -deficient mice. *Proc Natl Acad Sci USA*. 2002 Apr 2;99(7):4586-91.

[2] Bueno et al. Defective T cell development and function in calcineurin A beta -deficient mice. *Proc Natl Acad Sci USA*. 2002 Jul 9;99(14):9398-403.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCB345

### **Evidence that altered redox status results in KCa3.1 channel reduced endothelial cell surface expression**

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Endothelial derived hyperpolarization (EDH) is an important path to vasodilatation especially in small arteries, and is more important in women and the elderly. Our previous work has shown that this becomes impaired in mice that had experienced a high fat diet during gestation up to weaning, with concomitant reduction in endothelial surface KCa3.1 expression (Stead et al. 2016). We have tested the idea that altered redox status affects the location of KCa3.1 in human dermal microvascular endothelial cells (HDMECs).

HDMECs were obtained from PromoCell (Heidelberg, Germany) and cultured in MV2 growth medium containing 1% penicillin-streptomycin in 5% O<sub>2</sub>, 5% CO<sub>2</sub> at 37°C to passage 6 & 7 in the SCI-tive Physiological Oxygen Workstation from Baker Ruskinn (Maine, USA) for at least 5 days before experiments. The cells were plated on fibronectin (10µg/ml) coated 8 well micro-slides (Ibidi, Martinsried, Germany) and incubated for 48h, treated with PBS or H<sub>2</sub>O<sub>2</sub> 100µM in PBS for 10min., and fixed with 4% formalin. Immunofluorescence was performed by incubating with anti-KCa3.1 mouse monoclonal antibody (AL-051 Alomone, Israel) at 4°C overnight, with half of the wells being treated with 2% Triton-X to permeabilize the cells. The Alexa 488 labelled anti-mouse antibody for 1h and Texas Red Lycopersicon Esculentum Lectin (to stain the cell surface) for 20 min, were applied to the cells at room temperature and finally the Dapi nuclear stain was applied for 3 min. The cells were examined using a x60 1.3 NA oil-immersion objective and images captured via a Hamamatsu RG3 camera into a computer. Using ImageHopper software and computer-driven focussing device (Prior Instruments, UK) we took 10 serial images of KCa3.1 at 1.0 µm intervals, which were integrated using Image J. These images were analysed for the fractional area of an endothelial cell occupied by KCa3.1, defined as bright spots between 1 to 4 µm diameter in the relevant fluorescence channel.

The density of channels on the surface, defined as the integrated image of non-permeabilized KCa3.1 channels, was  $9.5 \pm 0.68 \mu\text{m}^{-2} \times 10^3$  (mean  $\pm$  sem), while in the permeabilized cells the density within the whole cell area was  $13.9 \pm 1.89 \mu\text{m}^{-2} \times 10^3$  ( $p < 0.05$ , 't' test, 4 experiments with 20 cells per experimental group) indicating that about 70% of the channels

normally reside on the cell surface. Treatment with  $H_2O_2$  had little effect on the total cell density (reduced to  $12.3 \pm 1.56$ ), but reduced the surface density to  $5.25 \pm 0.47$  ( $p < 0.01$ ) i.e. reduced to 43%.

Thus even a brief exposure to oxygen stress will result in the diminution of KCa3.1 on endothelial surface, and this may have implications for the ability of arterioles to dilate.

Stead R et al. (2016) J Hypertens. 34:452-63

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB346

### Double-edged vasomotor regulation of healthy resistance arteries by COX: Role of isoforms

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The cyclooxygenase (COX) enzyme in the vascular endothelium has the unique ability to produce both vasodilators and vasoconstrictors (Davidge, 2001). This makes it an important determinant of endothelial function and dysfunction and therefore a double-edged regulator of vascular tone. However, it is not clear, particularly in healthy resistance arteries whether these opposing roles are determined by the relative expression and activity of its isoforms COX-1 and COX-2. Thus we investigated whether the double-edged role of the enzyme in healthy resistance arteries is isoform-specific.

Skeletal muscle (SKM) and mesenteric vasa recta (MVR) resistance arteries were isolated from samples obtained from healthy male sheep euthanized in a government-controlled slaughter house in Doha and therefore did not require additional ethics approval. Endothelium-dependent relaxation to Acetylcholine (Ach,  $10^{-10}$ - $10^{-4.5}$  M) was studied by wire myography in the absence and presence of 10  $\mu$ M indomethacin (non-selective COX inhibitor), 1  $\mu$ M FR122047 (COX-1 selective inhibitor) or 4  $\mu$ M Celecoxib (COX-2 selective inhibitor). Experiments were also carried out in the absence and presence of 100  $\mu$ M N( $\omega$ )-nitro-L-arginine methyl ester (L-NAME, nitric oxide synthase inhibitor). Messenger RNA for both isoforms of the enzyme were also measured by RT-PCR. Values are means  $\pm$  SEM., and comparisons were made with ANOVA or students t-test as appropriate.

Both arteries were relaxed by Ach in a dose-dependent manner. Greater maximum relaxation was recorded in SKM arteries ( $I_{max}$   $90.6 \pm 1.7$  %,  $n=21$ ) compared with MVR arteries ( $85.5 \pm 4.1$  %,  $n=19$ ,  $p < 0.01$ ). The SKM arteries were also more sensitive to Ach compared with MVR arteries (log IC50  $-7.6 \pm 0.1$  vs.  $-6.9 \pm 0.2$  M for SKM vs. MVR,  $p < 0.001$ ). Indomethacin had opposite effects on the relaxation of the two arteries: It attenuated SKM relaxation but enhanced the relaxation of MVR arteries. FR122047 attenuated relaxation of both arteries with significant rightward shift in the curve for SKM arteries ( $n=8$ ,  $p < 0.001$ ). On the other hand, Celecoxib significantly enhanced the relaxation of MVR arteries ( $n=5$ ,  $P < 0.01$ ,) but had no noticeable effect on the SKM arteriolar relaxation. In the presence of L-NAME, the curves for both arteries were shifted to the right ( $n=7-8$ ,  $p < 0.01$ ). COX-1 mRNA was more abundant in SKM arteries compared with MVR arteries ( $n=6-8$ ,  $p < 0.05$ ). On the other hand, COX-2 mRNA was slightly more abundant in the MVR compared with SKM arteries, although

the difference was not statistically significant. These results suggest that the opposing roles of COX in these apparently healthy ovine resistance arteries are isoform-specific, with COX-1 driving positive regulation in SKM arteries, and COX-2 responsible for negative vasomotor regulation of MVR arteries.

Davidge ST (2001). Circ Res. 89(8):650-60.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB347

### Optical coherence tomography as a novel method to measure endothelial dysfunction in mice *in vivo*

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#### Objective

Endothelial dysfunction is an early process in the pathogenesis of atherosclerosis. Transgenic mouse models are widely used to study the development of endothelial dysfunction and atherosclerosis. However, a non-invasive *in vivo* measurement to analyse vascular function in mice is missing.

#### Methods and Results

Transluminal optical coherence tomography (OCT) imaging provides high resolution images of the saphenous artery in their natural surroundings of the vascular bed.

To determine endothelial vessel function, we set a vessel clamp and analysed the changes in vessel diameter after release of the occlusion. In C57BL/6J mice, a significant increase in flow-mediated dilation was detectable. We did choose LDL receptor knockout mice as an atherosclerosis-prone model to study flow-mediated dilation by OCT. The saphenous artery of these mice showed a significant reduced flow-mediated dilation compared to wild-type mice, verifying an endothelial dysfunction. Furthermore, we confirmed our findings by *ex vivo* analysis in the Aorta thoracalis by classical Mulvany Myograph analysis. The reproducibility of results in both different assays was tested in a Bland-Altman plot.

#### Conclusion

Changes in vessel diameter could be detected by optical coherence tomography. We provide a novel method for the visualization and quantification of endothelial function in a mouse model *in vivo*. Our new approach allows analysing progression and reversibility of endothelial dysfunction in the same animal for a prolonged period of time *in vivo*.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.



PCB348

**Intracellular  $\text{Ca}^{2+}$  dynamics govern Weibel-Palade Body trafficking**

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Endothelial-specific Weibel Palade bodies (WPBs) and their constituents play major roles in vascular haemostasis, angiogenesis and inflammation. Disruption or improper exocytosis of WPBs contributes to the progression of inflammatory and cardiovascular diseases (Wang *et al.*, 2010). Several functionally distinct endothelial agonists induce WPB trafficking and exocytosis by raising intracellular  $\text{Ca}^{2+}$  (Lowenstein *et al.*, 2005). We hypothesize that agonists evoke distinct  $\text{Ca}^{2+}$  signatures that give rise to differential WPB trafficking and thereby modulate endothelial function. The aim of the project is to examine the role of  $\text{Ca}^{2+}$  signalling on WPB trafficking using high-resolution cellular imaging alongside intracellular  $\text{Ca}^{2+}$  measurements and biochemical techniques.

Here we show that histamine and thrombin induce dose-dependent  $\text{Ca}^{2+}$  responses in HUVECs with peak  $\text{Ca}^{2+}$  release from intracellular stores observed within 10 secs thereafter the  $\text{Ca}^{2+}$  slowly declined and was sustained at 300 secs. To relate the effects of  $\text{Ca}^{2+}$  signalling to WPB trafficking we imaged HUVECs immunostained for vWF over a similar time frame. Surprisingly, after 5 mins we observed histamine, but not thrombin evoked trafficking of a subpopulation of WPBs to a perinuclear region. This subpopulation was P-selectin negative but interestingly contained angiopoietin-2. RT-PCR analysis identified  $\text{H}_1\text{R}$  as the predominant histamine receptor expressed in HUVECs. Treatment of HUVECs with an  $\text{H}_1\text{R}$  specific agonist-evoked a dose-dependent  $\text{Ca}^{2+}$  response and induced perinuclear trafficking of WPBs. These findings suggest that in order to elicit an agonist appropriate response, endothelial cells control the release of WPB subpopulations by distinguishing different agonist-evoked  $\text{Ca}^{2+}$  signatures.

Lowenstein CJ *et al.* (2005). *Trends Cardiovasc Med* 8, 302-308.Wang JW *et al.* (2010). *Hamostaseologie* 3, 150-155.

British Heart Foundation

Medical Research Council

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCB349

**Platelet releasate upregulates mRNA expression of various angiogenesis-related genes in cultured endothelial cells: a new approach to study intercellular cross-talk in humans**

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The human vasculature is extremely plastic. This is clearly demonstrated by the broad-ranged adaptations seen in the vasculature in response to an exercise training period. However, the molecular mechanisms by which the vasculature communicates are largely unknown. Recent studies have demonstrated that substances are released into the circulation during exercise training. In addition, it has been

shown that platelets carry and release Vascular Endothelial Growth Factor (VEGF) and Thrombospondin-1 (TSP-1) which are important for vascular plasticity, and it is further speculated that platelets are the main origin of extracellular vesicles in the circulation. Thus, we aimed to elucidate the angiogenic potential of human platelets and to study if platelets activate molecular pathways associated with angiogenesis in cultured endothelial cells.

In this pilot study, platelets were isolated from four healthy male subjects at baseline and following an acute bout of exercise training on a bicycle ergometer (45 minutes at 80%  $\text{HR}_{\text{max}}$ ). In brief, blood was drawn using Sodium Citrate as anticoagulant and centrifuged to obtain platelet-rich-plasma. Highly purified platelets were then isolated from the platelet-rich-plasma and platelet-free-plasma was collected. To study the effect on endothelial cells in culture, platelet releasate was prepared by activation of isolated platelets using Thrombin Receptor Activator Peptide 6 (TRAP-6) under stirring conditions. The releasate was collected and added to Human Umbilical Vein Endothelial Cells (HUVEC) in culture for 3- and 6 hours.

Preliminary data demonstrate that platelets carry the pro- and antiangiogenic proteins VEGF and TSP-1, and exposure of isolated platelets to TRAP-6 leads to a release of these angiogenic proteins. VEGF levels in platelet releasate are ranging from 32 – 116 pg/mL. Furthermore, stimulation of cultured endothelial cells with platelet releasate upregulates mRNA expression of various angiogenesis-related genes up to 4-fold compared to control situation.

Our preliminary data suggest that platelets mediate inter-cellular cross-talk in the vasculature by releasing angiogenic proteins consequently leading to an upregulation of molecular pathways related to angiogenesis in endothelial cells. Further studies are being conducted to verify the mechanism and analysis is ongoing.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCB350

**Mitochondria in skeletal muscle endothelial cells – A new method designed for investigating the development of cardiovascular disease**

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**Introduction:** It is hypothesized, that increased production of reactive oxygen species (ROS) in endothelial cells may contribute to the development of endothelial dysfunction in cardiovascular disease. In order to test this, we have developed a novel method to determine mitochondrial respiration and ROS production in a limited number of endothelial cells isolated from skeletal muscle samples of healthy humans and rats and, for comparison, human umbilical vein endothelial cells (HUVEC).

**Method:** Muscle endothelial cells were isolated from muscle samples obtained from the thigh of healthy young subjects and the lower limb of rats by use of antibody coated magnetic beads. The  $\text{H}_2\text{O}_2$  emission (ROS) was measured fluorometric in intact endothelial cells with an Oxygraph (Oxygraph-2K, Oroboros Instruments). The method allows for measuring

real time oxygen consumption using a respirometer, and ROS production is measured fluorometric with an add-on module to the Oxygraph for the simultaneous measurement of respiration and  $H_2O_2$  production of approximately 100,000 cells in the chamber. High (11mM) compared to normal physiological (5mM) glucose levels were acutely incubated with measurement in the Oxygraph in HUVEC to simulate the effect of high glucose levels in the bloodstream.

**Results:** The isolation procedure of cells resulted in over 1 million cells per preparation from the rat tissue and the human muscle biopsies.

The oxygen consumption tended to be higher in endothelial cells from the microvascular system in rat muscle compared to HUVEC at baseline level ( $88 \pm 16$  vs.  $24 \pm 4$  pmol  $O_2/S \times 10^6$  cells;  $p=0.060$ ;  $n=4$ ). Oxygen consumption in human microvascular endothelial cells was 75% ( $p<0.001$ ;  $n=4$ ) lower than in microvascular endothelial cells from rat. No difference in oxygen consumption found between human microvascular endothelial cells and HUVEC. However, the ROS production was not different between the cell types.

The oxygen consumption was 12% lower ( $9 \pm 2$  vs.  $10 \pm 2$  pmol  $O_2/S \times 10^6$  cells;  $n=2$ ) in acutely incubated high glucose compared to normal levels of glucose in HUVEC. The ROS production was 7% higher ( $0.37 \pm 0.03$  vs.  $0.35 \pm 0.01$  pmol  $H_2O_2/S \times 10^6$  cells;  $n=2$ ) in high compared to normal levels of glucose. Despite the oxygen consumption was lowered in the cells incubated in high glucose, the ROS production seems to be higher from the preliminary data. These preliminary data is very important for understanding, how the environment affects the endothelial mitochondria. Furthermore, understanding how mitochondrial function may influence cell function will provide important novel information regarding cardiovascular disease.

**Conclusion:** Our data show, that it is possible to isolate sufficient number of endothelial cells from skeletal muscle biopsy samples for analysis of mitochondrial respiration and ROS formation. The procedure should provide a useful in vitro model for studying various aspects of microvascular biology and pathology.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCB351

### **Are endothelium-dependent dilator responses blunted in young South Asian women relative to young White European women?**

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The prevalence of cardiovascular disease (CVD) is greater in South Asians (SAs) than White Europeans (WEs). Oestrogen is generally considered to protect against CVD; it facilitates the dilator influences of nitric oxide (NO) and cyclooxygenase (COX) products (prostaglandins: PGs). However, there is evidence the prevalence of hypertension is greater in SA than WE women<sup>1</sup> and that SA women have greater risk of coronary artery disease, present at an earlier age and have higher risk of mortality<sup>2</sup>. Whether markers of CVD can be recognised in young SA women is unknown. Thus, in 12 SA and 12 WE women (18-26 years old), we compared endothelium-dependent vasodilator responses evoked in forearm cutaneous circulation by iontophoresis of acetylcholine (ACh; 7 pulses at 0.1mA followed by 1 pulse at 0.2mA for

30s each at 1 min intervals) and following release of arterial occlusion for 2 min (the reactive hyperaemia), by using laser Doppler fluximetry to continuously record cutaneous red cell flux in perfusion units (pu). All women were normotensive (SP/DP < 140/90 mmHg) and non-diabetic. All were tested in the early follicular phase of the menstrual cycle when oestrogen is low. Under control conditions, both responses were blunted in SA relative to WE women: average ACh responses were  $+230 \pm 25$  vs  $134 \pm 20^*$  (\*:  $P<0.05$  2-way ANOVA WE vs SA), while peak reactive hyperaemia was  $+98 \pm 11$  vs  $57 \pm 10^*$  pu. After topical application of the NO synthase inhibitor L-NAME (100nM for 30min), ACh-evoked dilatation and reactive hyperaemia were attenuated in WE women ( $161 \pm 16^{\delta}$ pu and  $65 \pm 7^{\delta}$ pu;  $\delta$ :  $P<0.01$ , paired t-test vs control), but not in SA women ( $104 \pm 11$  pu and  $48 \pm 8$  pu;  $P>0.05$ ). Similarly, 30 min after the COX inhibitor aspirin (600 mg p.o.), ACh-evoked dilatation was attenuated in WE women ( $155 \pm 18^{\delta}$ pu) while peak reactive hyperaemia was  $73 \pm 14$ pu;  $p=0.06$  vs control), whereas neither ACh-evoked dilatation, nor reactive hyperaemia were significantly altered in SA women ( $123 \pm 20$  and  $63 \pm 10$  pu respectively). Finally, with dual NO synthase and COX inhibition, ACh-evoked dilatation and reactive hyperaemia were similar to control responses in WE women ( $208 \pm 19$  and  $74 \pm 11$ pu respectively) and in SA women, but they were blunted in SA relative to WE women ( $117 \pm 19^*$  and  $50 \pm 8^*$  pu respectively).

These results suggest that in young adult SA women with no overt CVD, endothelium-dependent dilatation is blunted relative to that of young WE women and this can be attributed to attenuated contributions of NO and vasodilator PGs in SA women. Further, assuming responses evoked in the presence of COX and NOS inhibition reflect the influences of endothelium-derived hyperpolarising factor (EDHF), it seems these influences are substantial in both WE and SA women, but are also blunted in young SA women. We propose the protective influences of oestrogen on endothelium-dependent dilatation may be deficient in young SA women and explain their increased risk of CVD.

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Ahmed A & El-Menyar A (2015). South Asian ethnicity and cardiovascular risk: the known, the unknown and the paradox. *Angiology* 66, 405–415.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCB352

### **Enhancing endothelial cell signaling in resistance arteries to reverse vasospasm.**

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Endothelial cell dysfunction is a ubiquitous feature of cardiovascular disease, associated with enhanced vasoreactivity, or vasospasm. This increased vasoreactivity is due to loss of ability to generate nitric oxide (NO) and endothelial cell hyperpolarization (EDH). The possibility that altering endothelial function may prevent the development of vasospasm was studied here.

The vasoreactivity of rat small mesenteric resistance arteries was assessed with wire myography and analyzed with a bespoke MATLAB script, to quantify vasoconstriction/

vasodilation using Fourier transform with power spectra analysis. Arterial vasomotion, reflecting normal vasoreactivity, was evoked by vasoconstriction to the  $\alpha_1$ -adrenoceptor agonist phenylephrine. Vasomotion presented as symmetrical cycles of vasoconstriction/vasorelaxation (0.2Hz, signal to noise (S/N) ratio=0.87, n=18). Rat coronary arteries were also utilized, to study spontaneous myogenic tone development. Vasomotion was disrupted by the NO synthase inhibitor, 100 $\mu$ M L-NAME (0.07Hz/0.55, n=13), the sGC inhibitor 10 $\mu$ M ODQ (0.08Hz/0.50, n=11) or by endothelial cell removal (0.02Hz/0.37, n=5). Loss of vasomotion with L-NAME could be reversed by the NO donor, 2 $\mu$ M SNOG (0.2Hz/0.80, n=11) or the direct sGC stimulator, 1 $\mu$ M BAY 41-2272 (0.2Hz/0.84, n=5). SNOG failed to re-establish vasomotion in the presence of ODQ/after endothelium removal. Spontaneous myogenic tone in coronary arteries (22%  $\pm$  3 of maximum constriction) was augmented upon addition of L-NAME (78%  $\pm$  6 of maximum constriction). This increase was reversed with NNC 55-0396, a T-type  $Ca^{2+}$  channel inhibitor, to 23%  $\pm$  4 of maximum constriction. Further addition of nifedipine fully relaxed the arteries.

These data indicate endothelium-derived NO facilitates vasomotion via cGMP-dependent mechanisms. Waveform analysis highlighted differences between chemical block of NO synthesis and removal of the endothelium. The former was associated with vasospasm interspersed by vasodilation, probably due to intercellular feedback via endothelial potassium channels and EDH1. Sustained vasospasm was observed in denuded arteries, which lack this signaling circuit. These data show a physiological profile of vasoreactivity can be re-established by enhancing cGMP-signaling. Data collected from myogenically active vessels suggests that T-type channels are responsible for enhanced vasoreactivity but not myogenic tone. Defining the cellular pathways responsible for the loss of vasomotion and increased vasoreactivity may suggest potential targets to address the vasospasm associated with endothelial dysfunction.

Garland CJ, Bagher P, Powell C, Lemmey HAL, Borysova L, Dora KA. Sci. Signal. 10, eaal3806 (2017).

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

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PCB353

### Insulin therapy does not reverse the gestational diabetes mellitus-associated human umbilical vein endothelial dysfunction

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Gestational diabetes mellitus (GDM) associates with maternal hyperglycaemia, foetoplacental endothelial dysfunction, and impaired insulin signaling (1). Women with GDM treated with diet (GDMd) show normal glycaemia at delivery (2). However, some patients do not reach the recommended glycaemia with diet and they are passed to insulin therapy (GDMi). Human umbilical vein endothelial cells (HUVECs) from GDMi show

increased L-arginine transport via the human cationic amino acid transporters 1 (hCAT-1), endothelial nitric oxide synthase (eNOS), and insulin receptor A (IR-A) (3). GDMi alterations were reversed by treating these cells in vitro with insulin (i.e., exogenous insulin). However, the cell signaling triggered by exogenous insulin in HUVECs from GDMi is unknown. We studied whether insulin signaling via the 44 and 42 kDa mitogen-activated protein kinases (p44/42<sup>mapk</sup>) and protein kinase B/Akt (Akt) is altered in HUVECs from GDMi. HUVECs were isolated from full term normal (n = 13), GDMd (n = 14), or GDMi (n = 18) pregnancies collected at the Clinical Hospital UC-CHRISTUS and Hospital San Juan de Dios (HSJD) (Chile) after informed consent approved by the UC-CHRISTUS and HSJD ethics committees. The investigation conforms to the principles outlined in the Declaration of Helsinki. IR-A, insulin receptor B (IR-B), hCAT-1, and eNOS mRNA expression were evaluated by qPCR. Phosphorylation of p44/42<sup>mapk</sup> and Akt were evaluated by Western blot. L-Arginine transport kinetics was measured in Krebs solution and the NO level was evaluated in 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM)-loaded cells in the absence or presence of N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME, 100  $\mu$ mol/L, NOS inhibitor). Experiments were performed in the absence or presence of exogenous insulin (1 nmol/L, 8 h), Akt inhibitor IV (1  $\mu$ mol/L, Akt inhibitor, Inh IV), and PD-98059 (10  $\mu$ mol/L, mitogen-activated protein kinase kinase 1/2 inhibitor). Values are means  $\pm$  S.E.M., compared by ANOVA, P<0.04. IR-A but not IR-B mRNA expression was higher (2.6  $\pm$  0.5 fold) and phosphorylated p44/42<sup>mapk</sup> protein abundance was higher (1.7  $\pm$  0.1 fold) but phosphorylated Akt was unaltered in GDMi compared with normal pregnancies but similar to cells from GDMd pregnancies. In the absence of exogenous insulin, PD-98059 but not Inh IV partially blocked (33  $\pm$  0.32%) the GDMi-increased maximal L-arginine transport capacity ( $V_{max}/K_m$ ) but none of these inhibitors altered the GDMi- and GDMd-increased NO level, eNOS, and hCAT-1 mRNA expression. Exogenous insulin blocked the GDMi and GDMd effects on NO level, eNOS, and hCAT-1 mRNA expression, an effect inhibited by the Inh IV but not PD-98059. In conclusion, insulin beneficial effect on HUVECs from GDMi requires Akt activity. Westermeier F et al. (2015). FASEB J. 29, 37-49.

Guzmán-Gutiérrez E et al. (2016). Purinergic Signal. 12, 175-190.

Subiabre et al. (2017). Biochim Biophys Acta – Mol Basis Dis. 1863, 2987-2998.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

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PCB354

### Hyperglycaemia disrupts conducted vasodilation in the resistance vasculature of db/db mice

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Stimulation of proteinase-activated receptor 2 (PAR2) on endothelial cells (ECs) leads to an increase in intracellular  $Ca^{2+}$  stimulating endothelium-derived hyperpolarization (EDH), which spreads to adjacent vascular smooth muscle (VSM) causing vasodilation. In the resistance vasculature,



EDH can also rapidly spread to neighbouring ECs to trigger VSM relaxation along the length of the artery in a process termed conducted vasodilation. Previously, mouse models of diabetes mellitus have been reported to exhibit diminished EC  $\text{Ca}^{2+}$  signalling and EDH. However, the effect of chronic hyperglycaemia, the hallmark of diabetes mellitus, on conducted dilation has not been characterized.

Small mesenteric arteries were isolated from healthy and diabetic db/db mice, which were used as a model of chronic hyperglycaemia. Endothelium-dependent vasodilation via the  $\text{G}_{q/11}$ -coupled proteinase-activated receptor 2 (PAR2) was stimulated with the selective agonist SLIGRL. The  $\text{Ca}^{2+}$ -sensitive fluorescent indicator fluo-8 reported changes in endothelial cell (EC)  $[\text{Ca}^{2+}]_i$  and triple-cannulated bifurcating mesenteric arteries were used to study conducted vasodilation. Localized intraluminal perfusion of SLIGRL into the bifurcation of triple-cannulated arteries from WT mice induced conducted vasodilation 2.5mm away from the initial site of local vasodilation (Figure 1). This conducted response was reduced during acute exposure to a high glucose (40mM) or hyperosmolar (29mM mannitol) solution. Furthermore, arteries isolated from db/db mice also exhibited diminished conducted vasodilation. However, chronic hyperglycaemia did not affect either EC  $\text{Ca}^{2+}$  mobilization or EDH-dependent vasodilation stimulated by SLIGRL.

The current investigation demonstrates for the first time that the spread of a hyperpolarizing current along the endothelium of the resistance vasculature is attenuated in a mouse model of chronic hyperglycaemia. In addition, diminished conducted vasodilation was also observed during acute exposure to a hypertonic solution containing glucose or mannitol. Our findings reiterate the importance of studying the effects of hyperglycaemia in the vasculature, and provide the basis for further studies regarding the modulation of junctional proteins involved in cell to cell communication by diseases such as diabetes.

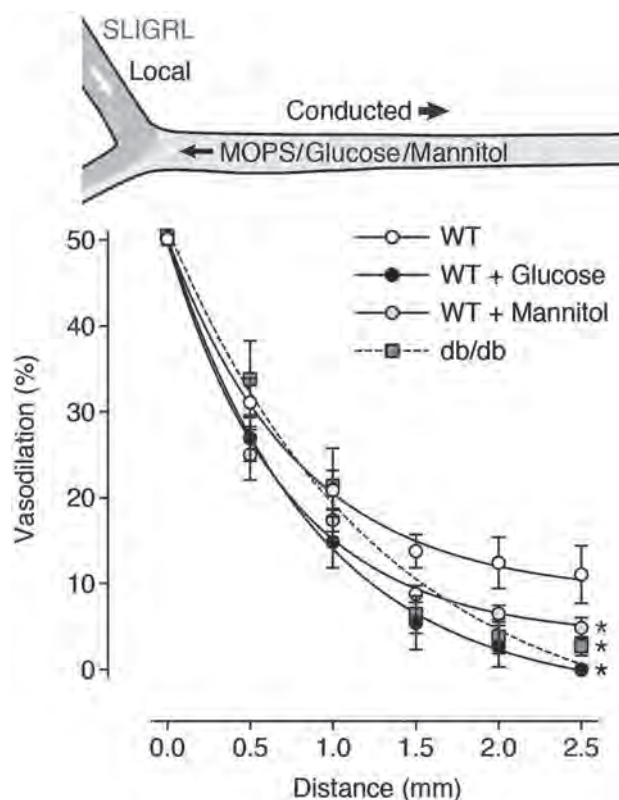


Figure 1. SLIGRL-mediated conducted vasodilation during acute and chronic hyperglycaemia.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB355

### Thyroxine direct vasodilating effect in skeletal muscle artery is mediated by integrin $\alpha\text{v}\beta 3$

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The thyroid hormones, triiodothyronine (T3) and thyroxine (T4), play essential roles in the regulation of almost all organ systems including the cardiovascular system. Traditionally their action was associated with activation of thyroid hormone receptors which alter the transcription of target genes (so-called genomic action). However, in the past two decades, the rapid nongenomic effects of thyroid hormones were discovered. Several authors had observed the direct relaxant action of thyroid hormones on small resistance arteries but the underlying mechanisms, in particular, the receptors mediating the arterial response, were not established. In some tissues, the nongenomic action of thyroid hormones is initiated by integrin  $\alpha\text{v}\beta 3$ . The aim of this study was to explore the role of integrin  $\alpha\text{v}\beta 3$  in vasodilation induced by thyroid hormones.

All experiments were performed on adult Wistar rats (body weight 250-350 g). Animals were killed by decapitation under  $\text{CO}_2$  anaesthesia, then sural arteries ( $d=260-380$  micron) with intact endothelium were isolated and studied in a wire myograph. Arterial responses were analyzed with 2-way ANOVA, the significance level was set below 0.05 and  $n$  was 5-12 for each experimental group.

T4 (0.02 to 10  $\mu\text{M}$ ) induced concentration-dependent vasorelaxation of the methoxamine-precontracted vessels, the difference from time-control appeared starting from the concentration of 2  $\mu\text{M}$ . T3 applied in the same concentration range did not induce vasorelaxation. The effect of T4 was not associated with its partial conversion to the T3 since the incubation with the deiodinase 2 inhibitor (iopanoic acid, 100  $\mu\text{M}$ ) did not alter the relaxation. In addition, the incubation with T4 (3  $\mu\text{M}$ ) diminished the maximum force and induced the leftward shift of methoxamine concentration-response curve (0.01 to 100  $\mu\text{M}$ ). The contractile responses of arteries incubated with T4 in the presence of tetrac (3  $\mu\text{M}$ ), a competitive inhibitor of integrin  $\alpha\text{v}\beta 3$ , were not different from the responses of arteries, incubated with tetrac alone. These results suggest that the vasodilatory effect of T4 was mediated by integrin  $\alpha\text{v}\beta 3$ .

To conclude, in skeletal muscle arteries T4 has direct nongenomic relaxant effect mediated by the integrin  $\alpha\text{v}\beta 3$ . Such data are relevant for improvement of our knowledge on T4 effects in the cardiovascular system. Moreover, the direct effects of T4 on the vasculature should be taken into account in hormone replacement therapy. Thyroid hormones could also have beneficial effects in the perioperative period of cardiac surgery because of their vasodilatory action on coronary arteries. The research was supported by the Russian Science Foundation (Grant N 14-15-00704).

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB356

**Somatostatin effects on endothelial barrier function and angiogenesis**M. Aslam<sup>1,2</sup>

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**Background and Aims:** Somatostatin is a peptide hormone that regulates the release of several other endocrine hormones and affects neurotransmission and cell proliferation by activation of one or more of five well-known G protein-coupled somatostatin receptors (SSTR1-5). Although, its endocrine regulatory and anti-tumour effects are well studied, little is known about its effect on vascular system. The aim of the present study was to analyse the effects of somatostatin on endothelial barrier function and angiogenesis in vitro.

**Methods:** The study was carried out on cultured human umbilical vein endothelial cells (HUVEC). EC barrier function was analysed by measuring flux of albumen through EC monolayers cultured on filter membranes. Angiogenesis was analysed by endothelial cell migration (wound assay), 3-D tubes and spheroid formation. Somatostatin-14 (10-100 pM) synthetic peptide was used to specifically activate somatostatin receptors.

**Results:** Quantitative PCR data suggested that HUVEC express somatostatin receptors 4 and 5. Treatment of EC monolayers with somatostatin (24h) sensitized endothelial monolayer to thrombin-induced hyperpermeability in a concentration-dependent (10pM-1nM) manner. The Western blot data demonstrated that somatostatin caused an activation of p42/44 MAPK and pharmacological inhibition of MAPK abrogated the barrier sensitizing effects of somatostatin. Similarly, treatment of ECs with somatostatin potentiated pro-proliferative and pro-angiogenic effects of VEGF which were also abrogated by pharmacological inhibition of MAPK.

**Conclusion:** The data of present study demonstrate that long-term treatment of ECs with somatostatin sensitizes endothelial monolayer towards thrombin-induced endothelial hyperpermeability and enhance the angiogenic effects of VEGF. The pro-angiogenic property of somatostatin can be exploited to enhance cardiac recovery in ischemic injury.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

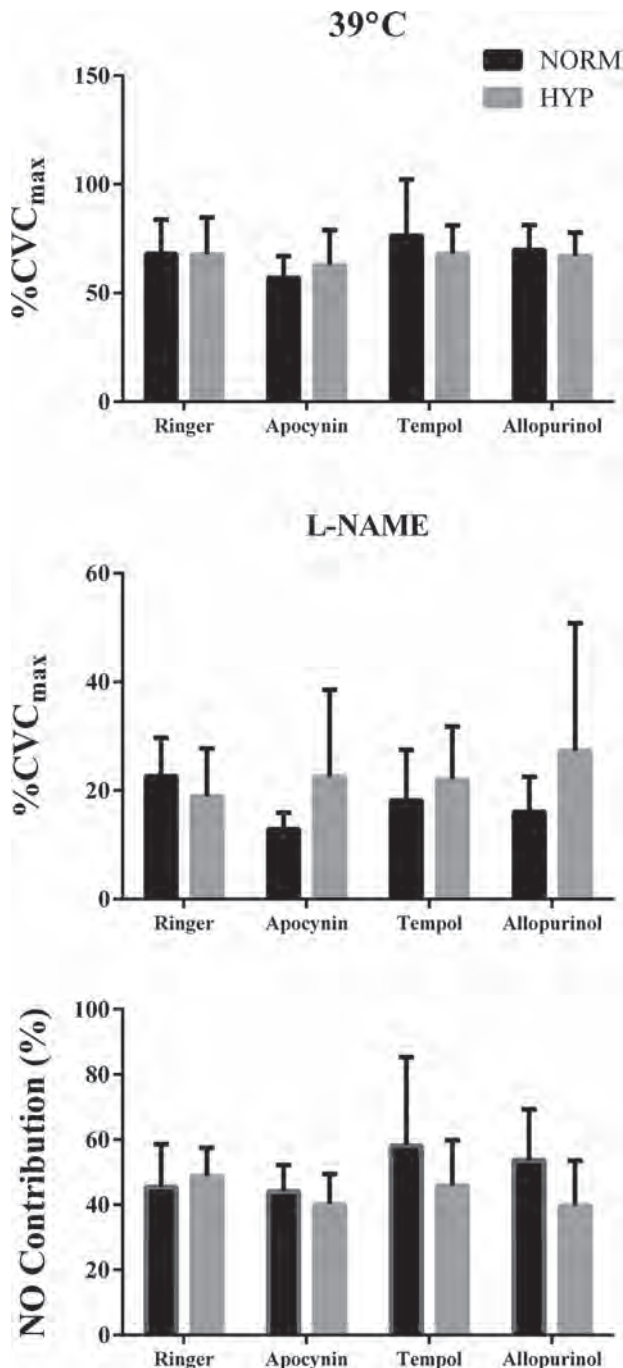
hypothesis that cutaneous vasodilation is impaired by reactive oxygen species from NADPH oxidase and xanthine oxidase pathways. The contribution of nitric oxide (NO) to vasodilation was also quantified. Four intradermal microdialysis membranes were inserted in the forearms of six male participants [age: 25 (3) years; BMI: 24.2 (2.2) kg/m<sup>2</sup>] on two separate days of either normobaric normoxia (NORM) or normobaric hypoxia (F<sub>I</sub>O<sub>2</sub>=0.13; HYP). The order of the days were counterbalanced between participants. The four sites were infused with 1) lactated Ringer solution (control), 2) 100 µM Apocynin (NADPH oxidase inhibitor), 3) 10 µM Tempol (superoxide scavenger), and 4) 10 µM Allopurinol (xanthine oxidase inhibitor). Skin blood flux was measured at each site with laser-Doppler flowmetry continually during the baseline, 39°C local heating, 20 mM L-NAME infusion, and 44°C local heating combined with 28 mM sodium nitroprusside infusion to achieve maximal vasodilation. Data are presented as cutaneous vascular conductance (CVC; flux/mean arterial pressure) as a percentage of maximal values (%CVCmax). During 39°C heating, there was no difference in vasodilation between conditions at the control site [NORM: 68 (16)% vs. HYP: 68 (17)%] and the contribution of NO-dependence was also similar in each condition [NORM: 45 (13)% vs. HYP: 49 (9)%]. There was no significant interaction (two-way ANOVA) between condition and any drug at 39°C heating (P=0.34) or during L-NAME infusion (P=0.38). Therefore, our preliminary data indicate that, at least in acute normobaric hypoxia, oxidative stress does not impair cutaneous vasodilation or its NO-mediated contribution.

PCB357

**Influence of oxidative stress on microvascular function in humans during acute hypoxia**G. Coombs<sup>1</sup>, J.C. Patik<sup>2</sup>, J.D. Akins<sup>2</sup>, P. Ainslie<sup>1</sup> and M. Brothers<sup>2</sup>

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Oxidative stress is associated with vascular dysfunction in several conditions. Acute exposure to hypoxia also results in reduced vascular function, which has been attributed to increases in hypoxemic-induced elevations in sympathetic nerve activity and oxidative stress. Although the former mechanism has been investigated, it has yet to be determined whether there is a direct link between hypoxia-induced oxidative stress and microvascular dysfunction. We tested the



Plateau responses of forearm cutaneous vascular conductance (%CVC<sub>max</sub>) at 39°C local heating (top) and during L-NAME infusion (middle). The nitric oxide (NO) contribution to vasodilation (bottom) is presented for normoxia (NORM) and hypoxia (HYP;  $F_iO_2=0.13$ ). Data are mean (SD).

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

### Oxidant activation of PKG controls vascular tone by regulating $Ca^{2+}$ spark frequency

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We have recently shown that oxidant activated Protein Kinase G (PKG) contributes to small artery contractility by regulation of the  $Ca^{2+}$  spark/Large conductance  $Ca^{2+}$ -activated  $K^+$  (BK) channel axis.

To explore characteristics of this signalling pathway in more detail, age-matched (12-week-old) male wild type C57BL/6J (WT) and PKG[Cys42Ser]<sup>KI</sup> (KI) mice were used, in which PKG is resistant to oxidant induced dimerization. Mice were euthanized by cervical dislocation.

Vascular function of mesenteric resistance arteries was examined in vitro with pressure myography and wire myography.  $Ca^{2+}$  sparks and caffeine-induced  $Ca^{2+}$  transients (indicative of sarcoplasmic reticulum (SR)  $Ca^{2+}$  load) were imaged with high-speed spinning-disc confocal microscopy. Western blot protocols examined ryanodine phosphorylation.

In WT arteries,  $Ca^{2+}$  spark frequency increased with intraluminal pressure but reached a ' $Ca^{2+}$  spark frequency-ceiling' at 80 mmHg ( $n=19$  arteries from 8 mice).  $Ca^{2+}$  spark frequency was significantly increased at 80 mmHg in WT (from  $8.3 \pm 1.3$  Hz to  $23.6 \pm 2$  Hz;  $n=9$  arteries from 4 mice;  $P=0.0045$ ) but not in the KI arteries (from  $5.76 \pm 1.6$  Hz to  $6.9 \pm 0.9$  Hz;  $n=9$  arteries from 3 mice). Similarly, exogenous  $H_2O_2$  increased  $Ca^{2+}$  spark frequency in WT arteries at low pressures ( $n=7$  arteries from 3 mice) but did not have this effect at higher intraluminal pressures ( $n=8$  arteries from 3 mice). Conversely, caffeine-induced  $Ca^{2+}$  transients showed very little relationship with intraluminal pressure, although at higher pressures SR  $Ca^{2+}$  content was reduced in WT ( $n=7$  arteries from 4 mice) compared with KI arteries ( $n=6$  arteries from 4 mice,  $P<0.001$  between WT and KI arteries at 110 mmHg). Both exogenous  $H_2O_2$  and the BK agonist NS11021 vasodilated pressure-constricted mesenteric arteries equivalently (WT:  $99 \pm 1.6\%$ ,  $n=7$  arteries from 5 mice; KI:  $97 \pm 4\%$ ,  $n=7$  arteries from 6 mice in the presence of  $30 \mu M H_2O_2$  and WT:  $95.3 \pm 2.4\%$  vasodilation  $n=3$  arteries from 3 mice; KI:  $93.9 \pm 1\%$  vasodilation  $n=3$  arteries from 2 mice at  $3 \mu M$  NS11021).  $30 \mu M H_2O_2$ -induced vasodilation of WT arteries mounted in a wire myograph ( $67.3 \pm 11.9\%$ ,  $n=8$  arteries from 7 mice) but not the PKG[C42S]KI ( $7.3 \pm 3\%$ ,  $n=5$  arteries from 4 mice).

$H_2O_2$  caused phosphorylation of the ryanodine receptor at S2808 from freshly dissected, pooled (unpressurized) mesenteric arteries with or without incubation with  $30 \mu M H_2O_2$  ( $P=0.0426$  between WT and WT+  $30 \mu M H_2O_2$ ,  $n=4$  arteries from 4 mice, respectively).

Oxidant activated PKG appears to target the ryanodine receptor to increase  $Ca^{2+}$  spark frequency and maintain BK function during pressure induced constriction. However, once activated, further increases of pressure or exogenous oxidants cannot additionally increase the activity of this vasodilatory pathway, despite only low-level activity of the BK channel.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.



PCB359

# **Role of adenosine kinase in gestational diabetes mellitus-associated fetoplacental vascular alterations.**

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Gestational diabetes mellitus (GDM) is associated with hyperglycaemia, endothelial inflammation, altered DNA methylation and mitochondrial (mt) gene expression in the fetoplacental vasculature. Adenosine regulates inflammation and DNA methylation, in which the DNA methyltransferase 1 (DNMT1) and S-adenosylhomocysteine hydrolase (SAHH) are involved [1]. Adenosine is regulated by adenosine kinase (AK) and human equilibrative nucleoside transporter 1 (hENT1), which are likely to be altered in the fetoplacental vasculature from GDM [2]. A cytoplasmic AK (c-AK) and nuclear AK (n-AK) isoforms of AK and at least three groups (D, C, and B) of hENT1 transcriptional variants, i.e., hENT1<sup>TV-D</sup>, hENT1<sup>TV-C</sup>, or hENT1<sup>TV-B</sup>, have been described [3]. AK inhibition leads to a decrease in TNF $\alpha$ -induced endothelial inflammation [4]. Whether this phenomenon is present in fetoplacental vasculature in GDM is unknown. This study aims to determine the expression of AK isoforms and hENT1 TVs and the role of AK in TNF $\alpha$ -induced endothelial inflammation, mt gene expression and DNA methylation in human umbilical vein endothelial cells (HUVECs) exposed to a high extracellular D-glucose. The investigation conforms to the principles outlined in the Declaration of Helsinki. HUVECs from normal pregnancies were provided by the Endothelial Cell Facility at UMCG. HUVECs were isolated (collagenase digestion) and cultured in RPMI medium (20% FCS, 5.5 mmol/L D-glucose (basal glucose, BG)). At ~70% confluency, cells were exposed to 25 mmol/L D-glucose (high glucose, HG) and incubated (24 h) with TNF $\alpha$  (2 ng/mL), ABT-702 (2  $\mu$ mol/L, AK inhibitor) or both. The mRNA expression was evaluated by RTq-PCR. GAPDH and  $\beta$ -actin were used as housekeeping. Values are mean  $\pm$  S.D., compared by non-parametric t-test or ANOVA, P<0.05. hENT1<sup>TV-D</sup> (relative expression (RE) = 0.012  $\pm$  0.0037), hENT1<sup>TV-C</sup> (RE = 0.0038  $\pm$  0.0013) and hENT1<sup>TV-B</sup> (RE = 0.00011  $\pm$  0.00004) mRNA were differentially expressed. The RE of n-AK was higher than c-AK (RE = 0.00026  $\pm$  0.0003 and 0.000007  $\pm$  0.000005, respectively). HG increased the expression total hENT1 and hENT1<sup>TV-C</sup>, DNMT1 and SAHH expression (1.1  $\pm$  0.1, 1.2  $\pm$  0.03, 1.1  $\pm$  0.1 and 1.14  $\pm$  0.08 fold, respectively), tends to increase mRNA expression of ICAM-1, IL-8 and E-selectin and tends to reduce cytochrome c oxidase I (COX1), with no changes in total AK, n-AK, and mt-ribosomal 12S and 16S. TNF $\alpha$  associated with increased n-AK and total AK mRNA expression and perhaps hENT1<sup>TV-D</sup> and with decreased expression of total hENT1, COX1, 12S, and 16S in BG and HG HUVECs. ABT-702 prevented (~50%) TNF $\alpha$ -increased ICAM-1, IL-8, and E-selectin and increased total AK, n-AK, total hENT1, DNMT1, and SAHH mRNA expression in BG and HG, and hENT1<sup>TV-D</sup> in HG treated cells. In conclusion, AK and differential expression of hENT1 transcriptional variants could be a new target to prevent GDM-associated pathophysiological and epigenetic alterations in the fetoplacental vasculature.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB360

# **In vitro effects of alarin on human saphenous vein contraction**

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AIM: Alarin is a novel endogenous vasoactive neuropeptide. Oriented information of vascular effects of alarin has been obtained from micro vascular dermis vessels. In this study we aimed to investigate the effects of alarin on human saphenous vein muscle contractility.

MATERIAL AND METHODS: Human saphenous vein (n=28) were taken from patients who underwent coronary bypass surgery. The patients' ages were between 47 to 72. All samples were cleaned and sectioned into 3-4 mm long rings. Rings were placed in organ baths containing Krebs solution, thermoregulated at 37°C and aerated (95 % O<sub>2</sub> and 5% CO<sub>2</sub>). Changes in isometric tension of rings were recorded by using a four channel force displacement transducer. All tissues washed for 3 hours in order to diminish the effects of anaesthetic agents. Phenylephrine (PE 10<sup>-6</sup> M) was administered in tissue chamber for producing isometric contractions. Amplitudes of contractions were determined and used for statistical analyses. Cumulative alarin (10<sup>-8</sup> to 10<sup>-5</sup>) doses were added in organ baths. The contractions were recorded accordingly. Friedman and Kruskal Wallis tests were used for statistical evaluation.

RESULTS AND CONCLUSIONS: Alarin treatments at 10<sup>-8</sup>, 10<sup>-7</sup>, 10<sup>-6</sup> doses had no significant changes on muscle contractility. But alarin inhibited the contraction at 10<sup>-5</sup> M concentration dose following PE administration (p<0.05). This effect of alarin occurred in a dose dependent manner. These findings have a potential to contribute to studies owing to effect of alarin on cardiovascular system. Further studies are needed to clarify the mechanism(s) of alarin.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB361

**Effects of sertraline treatment on saphenous vein contraction**A. Koc<sup>3</sup>, H. Solak<sup>1</sup>, Z. Solak Gormus<sup>1</sup>, R. Ozen Koca<sup>1</sup>, F. Taki<sup>1</sup>, S. Kutlu<sup>1</sup> and N. Gormus<sup>2</sup><sup>1</sup>Department of Physiology, Necmettin Erbakan University Meram Faculty of Medicine, Konya, Turkey, <sup>2</sup>Department of Cardiovascular Surgery, Necmettin Erbakan University, Meram Faculty of Medicine, Konya, Turkey and <sup>3</sup>Physiology, Hitit University Medicine Faculty, Çorum, Turkey

**AIM:** Selective serotonin reuptake inhibitor (SSRI) group of anti-depressant drugs are commonly used in patients with cardiovascular disorders in order to diminish anxiety or depression. However, their effects on saphenous veins are not investigated particularly in vitro. We aimed to investigate the effects of sertraline in human saphena.

**MATERIAL AND METHODS:** Human saphenous veins (n=12) were taken from patients who underwent coronary bypass surgery. The patients' ages were between 47 to 72. Saphena tissues were cleaned and sectioned into 3-4 mm long rings. Rings were placed in organ baths containing Krebs solution, thermoregulated at 37°C and aerated (95 % O<sub>2</sub> and 5% CO<sub>2</sub>). Changes in isometric tension of saphena were recorded using a four channel force displacement transducer. All tissues were washed for 3 hours in order to diminish the effects of anaesthetic agents. Phenylephrine (PE 10<sup>-6</sup>M) was administered in tissue baths for producing isometric contractions. Contraction with measurements were used as contraction parameters. Cumulative sertraline (10<sup>-9</sup> to 10<sup>-3</sup>) doses were added in organ baths. The contractions were recorded accordingly. Friedman and Kruskal Wallis tests were used for statistical evaluation. Amplitudes of contractions were determined and used for statistical analyses.

**RESULTS and CONCLUSIONS:** Sertraline treatments at 10<sup>-9</sup>, 10<sup>-8</sup>, 10<sup>-7</sup>, 10<sup>-6</sup>, 10<sup>-5</sup> doses had no significant changes on muscle contractility. But sertraline inhibited the contractions at 10<sup>-4</sup> and 10<sup>-3</sup> M dose following PE administration significantly (p<0.05). This effect of sertraline occurred in a dose dependent manner. This means we can use SSRI group of antidepressants in these doses confidently. These findings have a potential to contribute to studies owing to effect of sertraline on cardiovascular system.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCB362

**Effects Of Alarin In Healthy And Damaged Rat Aorta**H. Solak<sup>1</sup>, Z. Solak Gormus<sup>1</sup>, R. Ozen Koca<sup>1</sup>, A. Koc<sup>3</sup>, F. Taki<sup>1</sup>, N. Gormus<sup>2</sup> and S. Kutlu<sup>1</sup><sup>1</sup>Meram Faculty of Medicine Department of Physiology, Necmettin Erbakan University, Konya, Turkey, <sup>2</sup>Meram Faculty of Medicine Department of Cardiovascular Surgery, Necmettin Erbakan University, KONYA, Turkey and <sup>3</sup>Physiology, Hitit University Medicine Faculty, Çorum, Turkey

**AIM:** The new peptide alarin, a member of galanin neuropeptide family as well, probably has effects on different receptors except galanin receptors. So it can possibly reveal both similar effects like galanin neuropeptide family and exactly converse effects. Therefore determination of effects of endogenous

peptide alarin on vessels is important for contributing physiology and also pathophysiology of smooth muscle related cases like ever increasing hypertension, at the present time enables scientists to obtain new symptoms. Their effects on vessels are not investigated experimentally. We aimed to investigate the effects both on healthy and damaged rat aorta. **MATERIAL AND METHODS:** Wistar albino rats divided into 2 groups (n=24). Group 1 aorta-intact endothelium (n=12), group 2 aorta-damaged endothelium (n=12). Descending thoracic aorta was isolated after cervical dislocation. Aorta tissues were cleaned and sectioned into 3-4 mm long rings. Rings were placed in organ baths containing Krebs solution, thermoregulated at 37°C and aerated (95 % O<sub>2</sub> and 5% CO<sub>2</sub>). Changes in isometric tension of aorta rings were recorded using a four channel force displacement transducer. Phenylephrine (PE 10<sup>-6</sup>M) was applied and contractions were recorded in both groups. Then alarin was given cumulatively (10<sup>-8</sup>-10<sup>-5</sup>M) to group 1. In group 2 aorta damage was achieved by tearing endothelium with needle. After controlling endothelial damage by applying acetylcholine (ACh 10<sup>-6</sup>M), damaged strips were washed for one hour and second dose of PE was administered and then alarin was given cumulatively to group 2, contractions were recorded.

**RESULTS AND CONCLUSION:** After alarin was given cumulatively to group 1, significant inhibition of spontaneous contractions was noticed in alarin doses of 10<sup>-8</sup>-10<sup>-5</sup> (p<0.05). Alarin 10<sup>-8</sup>-10<sup>-5</sup> also made significant inhibition in contractions when compared to 10<sup>-7</sup>-10<sup>-6</sup>. Group 2 inhibition of second PE contractions continued after alarin doses, but it was less significant when compared with group 1 (p<0.05). These results suggest that alarin may have beneficial effects on treatment of hypertensive disorders. Further studies were needed to clarify the mechanism of alarin.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

PCB363

**Histamine promotes osteo-/chondrogenic trans-differentiation of vascular smooth muscle cells through the H1 histamine receptor**M. Estepa<sup>1</sup>, L. Henze<sup>1</sup>, B. Boehme<sup>1</sup>, T. Luong<sup>1</sup>, F. Lang<sup>2</sup>, B. Pieske<sup>1,3,4</sup>, J. Voelkl<sup>1</sup> and I. Alesutan<sup>1,3</sup><sup>1</sup>Center for Cardiovascular Research Cardiology CVK, Charité – Universitätsmedizin Berlin, Berlin, Germany, <sup>2</sup>Department of Physiology I, Eberhard-Karls University, Tübingen, Germany, <sup>3</sup>Berlin Institute of Health, Berlin, Germany and <sup>4</sup>Department of Internal Medicine and Cardiology, DHZB, Berlin, Germany

**Background:** Medial vascular calcification is a crucial risk factor for cardiovascular events and mortality in patients suffering from chronic kidney disease (CKD). This calcification develops as part of an active process mediated mainly by vascular smooth muscle cells (VSMCs), which are able to transdifferentiate into an osteo-/chondrogenic phenotype. The regulation of this transdifferentiation comprises complex intracellular signaling pathways and is related with inflammatory processes. Histamine levels are elevated in CKD and histamine may promote vascular inflammation. Thus, the present study explored the possible effects of histamine in vascular calcification.

**Methods:** For this purpose, primary human aortic smooth muscle cells (HAoSMCs) were treated with histamine in the presence or absence of fexofenadine, an H1 histamine

receptor antagonist. The mRNA expression was determined by quantitative RT-PCR and the protein abundance by Western blotting.

**Results:** Histamine induced in a dose-dependent manner osteo-/chondrogenic transdifferentiation of HAoSMCs, as shown by up-regulation of the mRNA expression of osteogenic transcription factors *MSX2* and *CBFA1* and of osteogenic enzyme alkaline phosphatase (*ALPL*). The H1 receptor was the main histamine receptor expressed in HAoSMCs whereas the expression of H2, H3 and H4 receptors was low. Additional treatment with the H1 receptor antagonist fexofenadine suppressed the histamine-induced *MSX2*, *CBFA1* and *ALPL* mRNA expression in HAoSMCs. These effects were paralleled by increased phosphorylation of p38 MAPK and SAPK/JNK MAPK in histamine treated HAoSMCs, effects again abolished by addition of fexofenadine. Histamine treatment did not significantly affect the phosphorylation of ERK1/2 MAPK. Moreover, *TNF $\alpha$*  and *IL6* mRNA expression was up-regulated by histamine in HAoSMCs, effects blunted in the presence of fexofenadine.

**Conclusions:** Histamine promotes osteo-/chondrogenic transdifferentiation of VSMCs, an effect mediated at least in large part through the H1 histamine receptor. These effects involve activation of the p38 and SAPK/JNK MAPK signaling pathways. Thus, histamine may participate in the progression of vascular calcification during CKD.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

#### PCB364

##### **Natriuretic peptides BNP and ANP and soluble guanylate cyclase agonists relax human intrarenal arteries**

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Plasma concentrations of natriuretic peptides ANP and BNP increase in states of volume expansion and after nephrectomy. The particulate guanylate cyclase (pGC) pathway, through ANP/BNP activation of natriuretic receptor type A (NPRA), as well as the soluble guanylate cyclase (sGC) pathway could cause renal vasorelaxation and improve GFR. We hypothesized that ANP and BNP cause concentration-dependent vasorelaxation in human intrarenal arteries comparable to soluble guanylate cyclase pathway-dependent vasodilatation. Human intrarenal arteries (n=31, 755.6  $\pm$  86.8  $\mu$ m 95% CI) were dissected from nephrectomy samples at 4 degrees celsius under a stereomicroscope and stored for up to 20 h at 4 degrees celsius in organ-buffer for drug washout. Segmental murine intrarenal arteries (n= 60, diameter 305.3  $\pm$  15.5  $\mu$ m 95% CI) were dissected from euthanized BL6J-mice. Development of isometric tension was recorded in a wire-myograph. Concentration-response curves for K<sup>+</sup> and  $\alpha_1$ -adrenoceptor agonist phenylephrine (PE) were established. Rings were precontracted with EC<sub>50</sub> of either PE or high K<sup>+</sup> and subjected to cumulatively increasing concentrations of BNP, ANP, the phosphodiesterase (PDE)-5-inhibitor sildenafil, or the sGC activator BAY 60-2770 or stimulator BAY 41-2272. EC<sub>50</sub>-values were 30 mmol/L K<sup>+</sup> for human arteries and 3x10<sup>-7</sup> M phenylephrine for murine arteries. BNP (IC<sub>50</sub> human/mouse = 10<sup>-7.1</sup>/10<sup>-7.3</sup>), ANP (IC<sub>50</sub> mouse = 10<sup>-8.3</sup>), sildenafil (IC<sub>50</sub> human/mouse = 10<sup>-4.9</sup>/10<sup>-6.3</sup>), BAY 60-2770 (IC<sub>50</sub> mouse = 10<sup>-7.5</sup>)

and BAY 41-2272 (IC<sub>50</sub> human/mouse = 10<sup>-5.1</sup>/10<sup>-6.6</sup>) caused concentration-dependent relaxation in human and murine intrarenal arteries. Pretreating vessels with sildenafil or BAY 41-2272 did not affect the BNP concentration response curve. PCR analysis showed consistent expression of PDE5, sGC and NPRA while NPRA was less consistently observed and NPRB was not present in human kidney vasculature.

In conclusion, natriuretic peptides and sGC relax human and murine intrarenal arteries, in a concentration dependent fashion, suggesting that the renal vascular bed contains functional NPR, sGC and PDE-5. In perspective, drugs that interact with the pGC/sGC pathways may have renoprotective effects.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

#### PCB365

##### **Perivascular P2X7-expressing cells in hypertensive renal vascular injury**

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Hypertension is associated with macrovascular and microvascular remodelling. Chronic high blood pressure impacts target organs including the kidney, resulting in proteinuria and impaired perfusion. We have reported that systemic infusion of a P2X7 antagonist reduces blood pressure and increases renal perfusion in a model of ANGII-dependent hypertension (Menzies et al, Kidney International, 2015). In the present study we developed a model of hypertensive vascular remodelling in C57BL/6 mice through a 4-week administration of ANGII (100ng/kg/min) and deoxycorticosterone (DOCA; 50mg), combined with high salt feeding (ANGII DOCA salt; n= 12) versus Sham controls (n=11). ANGII DOCA salt increased systolic blood pressure (ANGII DOCA salt = 136.5 $\pm$ 3.2mmHg vs. Sham = 113.3 $\pm$ 1.9mmHg; P<0.01) and urinary albumin excretion (ANGII DOCA salt: 357.3 $\pm$ 23.2 $\mu$ g/24hr vs. Sham: 22.0 $\pm$ 2.1 $\mu$ g/24hr; P<0.0001). Renal histology identified a perivascular fibrosis (ANGII DOCA salt: 54.9 $\pm$ 3.7pixels, Sham: 40.2 $\pm$ 3.2pixels normalised to vessel thickness; P<0.05). Interstitial fibrosis was not observed. Flow activated cell sorting identified that ANGII DOCA salt caused a 5-fold increase in renal perivascular PDGFRb<sup>+</sup> cells, but no change in endothelial, epithelial or macrophage cell types compared with Sham. Immunofluorescence of renal sections colocalised PDGFRb and P2X7 at focal points in the intermediate arterial architecture of the kidney, particularly renal interlobar arteries. These data suggest that perivascular P2X7-expressing cells of the intermediate arteries in the kidney are involved in hypertensive remodelling processes.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*



PCB366

### Serum- and glucocorticoid-inducible kinase 1 promotes vascular smooth muscle cell calcification via NF- $\kappa$ B

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**Introduction:** Medial vascular calcification, a risk factor for cardiovascular events and mortality in chronic kidney disease (CKD) patients, is a complex process tightly regulated by intracellular signaling pathways controlling osteo-/chondrogenic transdifferentiation of vascular smooth muscle cells (VSMCs). However, the intracellular signaling involved in vascular calcification remained ill defined. The present study explored the role of serum- and glucocorticoid-inducible kinase 1 (SGK1) in vascular calcification.

**Methods:** Experiments were performed in primary human and mouse aortic VSMCs, in human coronary arteries from CKD patients as well as in a mouse model of cholecalciferol overload and a mouse model of subtotal nephrectomy, induced surgically during isoflurane anaesthesia.

**Results:** SGK1 expression was up-regulated in VSMCs under calcifying conditions. In human coronary arteries from hemodialysis patients, SGK1 expression was increased and localized in the calcified arterial regions as compared to control patients. Overexpression of constitutively active SGK1<sup>S422D</sup>, but not inactive SGK1<sup>K127N</sup>, increased osteo-/chondrogenic transdifferentiation of primary human aortic VSMCs. SGK1<sup>S422D</sup> induced nuclear translocation of NF- $\kappa$ B p65 and increased NF- $\kappa$ B-dependent transcriptional activity, while blockage of NF- $\kappa$ B activation blunted the osteoinductive effects of SGK1. Genetic deficiency, silencing and pharmacological inhibition of SGK1 suppressed phosphate-induced calcification and osteo-/chondrogenic transdifferentiation of VSMCs. In addition, aortic calcification and osteo-/chondrogenic transdifferentiation as well as vascular stiffness in mice following cholecalciferol overload was suppressed in Sgk1-deficient mice or by pharmacological inhibition of Sgk1 with EMD638683. Similarly, vascular calcification induced in ApoE-deficient mice after subtotal nephrectomy was suppressed in Sgk1-deficient mice. In primary human aortic VSMCs, uremic serum-induced osteo-/chondrogenic transdifferentiation was ameliorated by SGK1 inhibitor EMD638683.

**Conclusions:** SGK1 is a key regulator of vascular calcification participating in the signaling that controls osteo-/chondrogenic transdifferentiation of VSMCs, effects involving NF- $\kappa$ B

activation. Thus, inhibition of SGK1 may reduce the progression of vascular calcification in CKD.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB367

### Investigation of the effects of tetrahydrobiopterin on renal nitric oxide synthase isoforms in Experimental Myoglobinuric Acute Kidney Injury

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The three nitric oxide synthase (NOS) isoforms; neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS), play an important role in pathological and physiological conditions in kidneys. Nitric oxide (NO) plays an important role in the pathophysiology of Myoglobinuric Acute Kidney Injury (MAKI). We aimed to investigate the effects of Tetrahydrobiopterin (BH4), L-arginine, L-NAME (NOS, inhibitor) and BH4+L-arginine treatment on renal NOS isoenzymes at different time periods in experimental MAKI model.

Sprague Dawley male rats were divided into 4 groups (A, B, C and D) and each group had 6 subgroups (n=8) as Control (saline, 8 ml/kg, im), MAKI (%50 glycerol, 8 ml/kg, im), MAKI+BH4 (20 mg/kg, ip), MAKI+L-Arginine (125 mg/kg, ip), MAKI+BH4+Arginine 20+125 mg/kg, ip) and MAKI+L-NAME (10 mg/kg, ip). To form a mild MAKI condition in group D, glycerol was applied at a dose of 5 ml/kg of body weight. Group A, B, C and D rats were received the ip injection of treatments for 1, 2, 4 and 4 times, respectively. The rats were euthanized after the glycerol injection; 24 hours for group A, 48 hours for group B, 96 hours for group C (8 ml/kg) and 96 hours for group D (5 ml/kg) after. Values are means $\pm$ SD, compared by Mann Whitney U Test.

While the MAKI group compared to the control group for the all sub-groups, kidney tissue nNOS, iNOS and eNOS gene expressions were significantly higher, but they were significantly lower in group D (p<0,05). In the group A and B, the treatment of BH4, L-Arginine and BH4+L-Arginine decreased the gene expression of nNOS (p<0,05). In the group A, while the gene expression of iNOS increased by the treatment in BH4 and BH4+L-Arginine groups, it decreased by BH4 treatment in group B. In the group A, the treatment of BH4, L-Arginine and BH4+L-Arginine decreased the gene expression of eNOS (p<0,05). In group B, treatment of BH4 decreased the eNOS gene expression (p<0,05).

While MAKI groups compared with the controls of all sub-groups, quantitative analysis of immunostaining for nNOS not changed, iNOS and 3-nitrotyrosine levels were significantly higher and eNOS was significantly lower (p<0,05). Similarly, in all groups, L-Arginine, BH4+L-Arginine treatment decreased the iNOS and 3-nitrotyrosine immunostaining. And again for the all groups, L-Arginine and BH4+L-Arginine treatment increased the eNOS immunostaining.

Our results support the hypothesis that administration of BH4, L-Arginine and BH4+L-Arginine were effective in attenuating nitrosative stress in MAKI. These results also suggest that BH4, L-Arginine and BH4+L-Arginine therapy may have

a clinical prevention against MAKI after rhabdomyolysis and may provide further evidence of future work in this regard.

This study is supported by TUBITAK (115S183).

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB368

### Uromodulin inhibits medial vascular calcification by interfering with pro-inflammatory cytokines

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**Background:** Serum levels of uromodulin (also known as Tamm-Horsfall protein) are reduced in patients with chronic kidney disease (CKD), but the physiological functions of serum uromodulin are still elusive. In CKD, medial vascular calcification is a common complication and associated with increased risk for cardiovascular events and mortality. The present study investigated the effects of uromodulin on medial vascular calcification.

**Methods:** Experiments were performed in serum samples from patients with CKD, in primary human (HAoSMCs) and mouse (MOVAS) aortic smooth muscle cells and in a cholecalciferol-overload mouse vascular calcification model following AAV-mediated uromodulin overexpression.

**Results:** As a result, in three independent cohorts of patients with CKD, serum uromodulin concentrations were inversely correlated with serum calcification propensity, a marker for increased risk for vascular calcification. Uromodulin supplementation in the cell culture medium suppressed phosphate-induced osteo/chondrogenic transdifferentiation and calcification of HAoSMCs. Similarly, uromodulin supplementation suppressed uremic serum-induced osteo/chondrogenic transdifferentiation of HAoSMCs. Furthermore, uromodulin blunted osteo/chondrogenic signaling and activation of the pro-inflammatory transcription factor NF- $\kappa$ B induced by the cytokines TNF $\alpha$  and IL-1 $\beta$ . Uromodulin supplementation also blunted phosphate-induced NF- $\kappa$ B-dependent transcriptional activity in HAoSMCs. In human serum, TNF $\alpha$  and IL-1 $\beta$  co-immunoprecipitated with uromodulin, suggesting that uromodulin is able to bind circulating pro-inflammatory

cytokines. In-vivo, AAV-mediated overexpression of uromodulin reduced vascular calcification and stiffness as well as aortic osteo/chondrogenic signaling in mice with cholecalciferol overload. In accordance, phosphate-induced osteo/chondrogenic transdifferentiation of murine MOVAS cells was suppressed following mouse uromodulin supplementation or overexpression.

**Conclusions:** Uromodulin inhibits osteo/chondrogenic transdifferentiation and calcification of vascular smooth muscle cells, at least partly, through inhibition of osteoinductive signaling induced by pro-inflammatory cytokines.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB369

### Genetic deletion of cyclooxygenase-2 impairs glomerular slit diaphragm formation during late stages of kidney development

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Renal cyclooxygenase-2 (COX-2) expression is necessary for normal glomerular development. Impaired COX-2 activity has been associated with decreased expression of VEGF, a vascular growth factor of significant importance for glomerular capillary development. Therefore experiments were designed to test the hypothesis that COX-2 expression supports glomerular development through stimulation of capillary loop formation. A developmental series of COX-2 knock-out (KO) mice and wild-type littermates (WT) was used. Animals were sacrificed by decapitation (P7, P14) or after anaesthesia using a mixture of ketamine (50 mg/kg) and xylazine (10 mg/kg) and kidney tissue snap frozen in liquid nitrogen or fixed in 4% paraformaldehyde. The number of glomeruli was counted by stereology, and glomerular morphology was examined by electron microscopy. mRNA expression of COX-2 and vascular growth factors were examined by quantitative PCR. Renal COX-2 expression showed developmental regulation with significantly higher COX-2 expression at postnatal (P) days 1 and P7 than at any later time points (P14, P21 and P40). Quantitative unbiased stereology at P28 showed significantly reduced glomerular number in COX-2 KO mice compared to WT ( $8188 \pm 781$  and  $12251 \pm 454$  glomeruli/kidney respectively,  $P=0.0001$ ). Subcapsular accumulation of small and immature glomeruli was seen in COX-2 KO mice whereas glomeruli deeper in the cortex towards the medullary junction appeared normal. Tissue abundance of VEGF, angiopoietin-1 and -2 mRNAs was significantly reduced in COX-2 KO mice compared to WT at P7. By electron microscopy at P28, immature subcapsular glomeruli showed normal appearance of fenestrated endothelial cells but severe podocyte foot process effacement and absence of mesangial cells. Normal morphology was confirmed in glomeruli deeper in the cortex. At P7, COX-2 KO mice showed reduced expression of slit diaphragm proteins nephrin and podocin but not synaptopodin compared to WT. In summary, deletion of COX-2 leads to decreased renal expression of vascular growth factors without changes to glomerular endothelial cell ultrastructure and severe podocyte foot process effacement and impaired expression of slit diaphragm proteins. In conclusion, COX-2 activity is necessary

for normal podocyte function and slit diaphragm formation during late stages of kidney development.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB370

### Plasminogen deficiency attenuates angiotensin II-induced hypertension in diabetic mice

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Diabetic nephropathy (DN) is associated with hypertension, urinary plasmin excretion and ability of urine to activate epithelial sodium channel (ENaC) current *in vitro*. It was hypothesized that deletion of plasminogen and amiloride treatment protects against hypertension in diabetes. Male plasminogen knockout (plg<sup>-/-</sup>) and wild-type mice were rendered diabetic by streptozotocin injections. After 4 weeks, urine was analyzed for albumin, plasmin(ogen) and the effect on current in collecting duct cells by patch clamp. Arterial blood pressure was recorded continuously by chronic indwelling catheters for 10 days (8 days ANGII-30-60 ng/kgxmin). Albuminuria was more pronounced in diabetic plg<sup>-/-</sup> compared to diabetic wildtype. Immunoblotting showed plasminogen in plasma from wildtype mice only and in urine from diabetic wildtype mice only. Baseline mean arterial blood pressure did not differ between groups and while ANGII induced hypertension in wildtype, diabetic wildtype and plg<sup>-/-</sup> control mice, this response was abolished in diabetic plg<sup>-/-</sup>. In ongoing studies, amiloride treatment for 3 days (n=4) in wildtype ANGII-hypertensive diabetic mice showed a tendency to counteract hypertension compared to ANGII infusion alone (n=2). Urine from diabetic and control wildtype mice evoked significantly larger amiloride-sensitive current than urine from diabetic and control plg<sup>-/-</sup> mice. Gamma ENaC displayed marked proteolysis in all 4 groups after ANGII infusion with no difference in abundance. No statistical significant difference between groups was found in kidney tissue protein level of prorenin receptor, TLR4 or TGFβ. Results indicated no change in infiltration of T<sub>H</sub>17 cells. In Plg<sup>-/-</sup> there was reduced infiltration of CD45-positive leukocytes. In summary, disruption of plasminogen in diabetic mice is associated with increased albuminuria, similar baseline blood pressure, but protection from ANGII-induced hypertension. In conclusion, plasmin promotes hypertension in diabetes with albuminuria compatible with an action through aberrant filtration.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

## PCB371

### Mechanisms involved in relaxations induced by electrical field stimulation in murine airways smooth muscle.

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**Purpose:** The mechanism underlying electrical field stimulation (EFS)-induced relaxations in airways smooth muscle (ASM) is unknown. This study tested the hypothesis that EFS relaxes ASM by neuronal-release of substance P (SP) that activates NK<sub>1</sub> receptors, causing release of inhibitory prostaglandin, PGE<sub>2</sub>. **Methods:** Isometric tension was measured in bronchial rings from mouse. Bronchi were pre-contracted with carbachol and EFS applied (0.3 ms pulse, 4 Hz frequency, amplitude 20 V, 10 sec).

**Results:** EFS relaxed precontracted rings. Tetrodotoxin (10 μM) failed to block the relaxations (control mean: 1.61 ± 0.41 mN, TTX mean: 2.40 ± 0.40 mN; P > 0.05, n=4). Tetrodotoxin plus PF-01247324 (1 μM), which selectively blocks tetrodotoxin-resistant Na<sub>v</sub>1.8 channels, also failed to block the responses (control mean: 1.61 ± 0.41 mN, TTX plus PF-01247324 mean: 2.60 ± 0.53 mN; P > 0.05, n = 4). However, when rings were contracted with 60 mM KCl, the mean EFS-induced relaxation amplitude was reduced from 0.50 ± 0.06 mN to 0.03 ± 0.03 mN (P < 0.05, n = 4), suggesting that membrane potential was involved, hence the responses might be nerve-mediated. SP mimicked EFS-induced relaxations. Both responses were blocked by indomethacin (P < 0.05), a blocker of cyclooxygenase, which catalyses the synthesis of prostaglandins from arachidonic acid. Mean relaxation amplitude caused by EFS was significantly inhibited by indomethacin (10 μM) from 1.12 ± 0.17 mN to 0.28 ± 0.12 mN (P < 0.05, n = 8). Indomethacin (10 μM) also significantly inhibited mean relaxation amplitude caused by SP from 1.69 ± 0.28 mN to 0.10 ± 0.06 mN (P < 0.05, n = 3). SR-140333 (100 nM), a NK<sub>1</sub> receptor blocker, abolished the SP response (from 0.74 ± 0.14 mN to 0.04 ± 0.02 mN, P < 0.05, n = 4) but had no effect on EFS-induced relaxations (control mean: 0.73 ± 0.16 mN, SR-140333 mean: 0.69 ± 0.13 mN; P > 0.05, n = 4). Mean relaxation amplitude caused by SP was significantly inhibited by SR-140333 from 0.74 ± 0.14 mN to 0.04 ± 0.02 mN (P < 0.05, n = 4). Addition of GR-159897 (100 nM), a NK<sub>2</sub> receptor blocker, also failed to block EFS relaxations (P > 0.05, n = 4). To test if PGE<sub>2</sub>, acting on EP<sub>2</sub> receptors was involved, PF-04418948 (100 nM), an EP<sub>2</sub> receptor blocker, was used. This caused variable reductions in amplitude (from 2.25 ± 0.66 mN to 1.52 ± 0.32 mN, P > 0.05, n = 8) but significantly shortened the durations of relaxations. The latter was assessed by measuring the half maximal recovery times, which decreased from 213 ± 34 sec to 115 ± 28 sec (P < 0.05, n = 8).

**Conclusion:** EFS-induced relaxations occur independently of SP release and NK<sub>1</sub>/NK<sub>2</sub> receptors activation, but they involve cyclooxygenase activity. Also, the relaxations appear to be partially mediated by EP<sub>2</sub> receptor activation, as evidenced by the shortening of their recovery time in PF-04418948. However, the early part of the response was less sensitive to blockade of EP<sub>2</sub> receptors, suggesting that more than one mechanism is involved.



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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB372

### The Epidermal growth factor receptor mediates cardiac hypertrophy and structural associated gene expression alteration in the aorta of diabetes mellitus type II mice.

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In the industrialized society incidence of diabetes mellitus type II (DMT2) increases, probably due to unbalanced lifestyle comprising an excess of fatty foods and insufficient physical exercise. DMT2 is associated with cardiovascular complications, yet the underlying mechanisms are incompletely understood. During the last years the importance of the epidermal growth factor receptor (EGFR) in the cardiovascular system has been recognized, besides its well-known role in embryo- and tumorigenesis. In vascular smooth muscle cells (VSMC) e.g. the EGFR is needed for an appropriate vasoconstrictive response upon angiotensin II stimulation. Additionally, increased EGFR phosphorylation and pressure-induced myogenic tone in mesenteric arteries was reported in DMT2 mice, which could be attenuated by EGFR inhibition.

To reveal the role of EGFR in cardiovascular pathophysiology associated with DMT2, our group established a mouse model with an inducible EGFR knock out (KO) in VSMCs and fed these, as well as wild type (WT) mice for 18 weeks either with a high fat diet (HFD, 5241.255kcal/kg) to induce DMT2 or standard chow (SD, 3188.487kcal/kg). Afterwards mice were sacrificed and morphology of organs was analyzed using formalin-fixed-paraffin embedded tissue sections of heart and kidney stained with Sirius Red (SR) and periodic acid Schiff (PAS). Next generation sequencing (NGS) and quantitative PCR of aortic tissue were performed to detect EGFR dependent gene expression.

During the observation period the HFD mice gained more weight (SD:  $\Delta 66\%$ , HFD:  $\Delta 107\%$ ) and blood glucose levels was increased (not fasted, SD: 7.9mmol/l, HFD: 9.7mmol/l) compared to SD mice. No difference between the genotypes could be observed, indicating that this model is a versatile tool to study the impact of EGFR on cardiovascular damages caused by DMT2. As expected an increased kidney weight in HFD mice compared to SD were detectable (SD: 362mg, HFD: 398mg), however lung weight was unaffected. Both genotypes displayed signs of HFD induced increased renal artery lumen (SD:  $846\mu\text{m}^2$ , HFD:  $1864\mu\text{m}^2$ ), and glomerular sclerosis (sclerotic score SD: 2.5, HFD: 2.8). Interestingly an EGFR dependent cardiac hypertrophy under HFD conditions was detectable. NGS followed by high stringency gene set enrichment analyses (GESA) revealed an EGFR dependent upregulation of genes involved in extracellular matrix formation in the aortas of DMT2 mice.

In conclusion, EGFR in VSMCs mediates DMT2 induced cardiac hypertrophy and gene expression in the aorta, this might lead to an altered functional response in blood vessels. Therefore inhibiting the EGFR might be an interesting approach in DMT2 treatment, although this opportunity needs to be investigated further.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB373

### The Gq protein inhibitor FR900359 induces strong vasodilation in pulmonary arteries

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Gq proteins are of high relevance in the pulmonary vasculature, since they determine tone regulation in health and disease. Previously, we have identified the depsipeptide FR900359 (FR) as a strong and specific pharmacological pan-Gq inhibitor. In the current study we have investigated the effect of FR on pulmonary arterial tone regulation in mouse *ex* and *in vivo*, as well as on human pulmonary artery smooth muscle cell (hPASMC) growth *in vitro*.

The effect of FR was examined in isometric force measurements of murine pulmonary arteries in a wire-myograph, in precision cut lung slices and in the isolated perfused lung model of mouse. Additionally, cell growth was investigated in hPASMCs with and without FR treatment. The effect of FR on pulmonary blood pressure *in vivo* was determined in healthy CD1 WT mice and animals after induction of hypoxia-induced PH by exposure to hypoxia (10 % O<sub>2</sub>) for 3 weeks. One hour after intratracheal application of FR (2.5  $\mu\text{g}$  per mouse, 5 % isoflurane) right ventricular systolic pressure was recorded under ketamine (50mg/kg)/xylazine (5 mg/kg) and 1.5 % isoflurane anesthesia.

Our results show that a single dose application of FR (1  $\mu\text{M}$ ) induced a pronounced pulmonary arterial vasorelaxation after pre-treatment with different Gq-dependent vasoconstrictors in the wire-myograph. After endothelin induced pre-constriction FR caused a dose-dependent (1 nM - 10  $\mu\text{M}$ ) pulmonary vasorelaxation reaching  $74 \pm 2.8\%$  (n=7) at the highest dose. In contrast, drugs currently used for PH in humans were less effective at 10  $\mu\text{M}$  (bosentan:  $8.8 \pm 2.9\%$ , n=5; iloprost:  $27.5 \pm 3.9\%$ , n=7; sildenafil  $34.6 \pm 3.5\%$  vasorelaxation, n=6,  $p < 0.001$  vs FR). Maximal vasorelaxation in these experiments could be strongly enhanced by the additional application of 1  $\mu\text{M}$  FR (bosentan + FR:  $70.4 \pm 2.3\%$ , n=5; iloprost + FR:  $68.2 \pm 4.4\%$ , n=7; sildenafil + FR  $73.4 \pm 3.9\%$ , n=6). FR (1  $\mu\text{M}$ ) also induced strong vasorelaxation after serotonin-mediated pre-constriction in lung slices (FR:  $93 \pm 1.2\%$ , n=7) as well as a pronounced decrease of the pulmonary arterial pressure in the isolated perfused lung model of mouse (FR:  $101.1 \pm 4.5\%$ , n=3). In a cell growth assay FR (1  $\mu\text{M}$ ) reduced hPASMC numbers to  $76.8 \pm 2.1\%$  (n=3,  $p < 0.01$ ) compared to vehicle controls. A similar reduction of hPASMC growth was found after bosentan and iloprost treatment, but not after exposure to sildenafil. Right ventricular catheter measurements showed that FR can reduce pulmonary blood pressure in hypoxia-induced PH mice (n=8) *in vivo*.

Thus, we show that the pharmacological Gq inhibitor FR is a strong pulmonary vasorelaxant under (patho)physiological conditions in mice *ex* and *in vivo*. Moreover, FR limits hPASMC growth *in vitro*. When comparing the action and efficacy of FR with currently applied drugs for the treatment of PH, FR appears superior, as it can reduce both vascular tone and hPASMC growth.

Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB374

### Platelet derived growth factor driven vascular smooth muscle cell remodelling potentiates abdominal aortic aneurysm

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Abdominal aortic aneurysm (AAA) is a focal dilation of the abdominal aorta which typically occurs in hypertensive male patients and progresses to rupture. Patients undergo surveillance for many years until the threshold for intervention is reached. Repair poses risk to life in addition to significant morbidity. A better understanding of the mechanisms of disease progression would aid the development of novel medical treatments.

The recent AAA Meta-GWAS identified potential enrichment of pathways linked to abnormal smooth muscle physiology in AAA. Platelet-derived growth factor (PDGF) is the major driver of vascular smooth muscle cell (VSMC) remodelling in physiology. We hypothesised that a transgenic mouse line with a tamoxifen inducible smooth muscle cell (SMC) specific constitutively active PDGFR $\beta$  (PDGF Receptor-  $\beta$ ) mutant (PDGFR $\beta^{D849V}$ ) would experience enhanced disease progression compared to wild-type littermates following elastase induced aneurysm induction.

We first validated the spatial specificity of tamoxifen inducible SMC specific Cre (SM-MHC.CreER<sup>T2</sup>) by crossing the mice with *Rosa26R.LacZ* reporter mice. Upon tamoxifen administration, LacZ expression was observed only in SMC layers of aorta and bowel and not in other cells/tissue types like heart (cardiac) and skeletal muscle. Then, we generated PDGFR $\beta^{D849V}$  knock-in mice and induced the transgene in 8 weeks old adult male mice by injecting with 2 mg of tamoxifen for 5 consecutive days. Prior to Cre recombination, no expression of the constitutively active PDGFR $\beta$  mutant isoform was observed. Upon tamoxifen administration, the Cre-mediated recombination results in expression of mutant PDGFR $\beta$  and increased PDGFR $\beta$  signalling. The supra and infra-renal aortic diameters were measured by Vevo 2100 ultrasound and then the vessels were harvested at 12 weeks. Apart from a subtle but statistically significant increase ( $0.53 \pm 0.02$  Vs  $0.68 \pm 0.07$ ;  $p=0.04$ ;  $n \geq 9$  for each group) in abdominal aortic diameter, we observed no difference in the basic phenotypic characteristics such as the body weights and organ weights between the PDGFR $\beta^{WT}$  and the PDGFR $\beta^{D849V}$  mice. The histological analysis of paraffin embedded thoracic and abdominal aortic sections showed no major architectural changes ( $n=6$  each). Next we induced AAA by peri-adventitial application of elastase to the infra renal aorta. The size of the aneurysm was monitored at 2 & 4 weeks using 3D ultrasound volumetric measurement. In wild-type mice, the AAA was stable between 2 and 4 weeks. In the transgenic littermates however, there was evidence of progression of AAA between weeks 2 and 4 ( $10.70 \pm 0.99$  Vs  $17.72 \pm 1.76$ ;  $p=0.017$ ;  $n \geq 3$  each). Thus our data show that PDGF driven VSMC proliferation may be important in the development and/or progression of AAA.

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Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.

PCB375

### Inverse relationship between the media-to-lumen ratio and smooth muscle contractile function in resistance arteries from patients with residual cardiovascular disease

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The media-to-lumen ratio (M:L) of resistance arteries (RA) is increased in hypertension, diabetes and obesity and is an independent risk factor for the development of life threatening cardiovascular events. We hypothesized that smooth muscle contractile function (CONTR) positively relates to M:L and that both outcomes are related to and influenced unidirectionally by patient characteristics.

Human pericardial RA from patients with residual cardiovascular diseases were obtained as described (1-4) after informed written consent. The Regional Committees on Health Research Ethics for Southern Denmark and the Danish Data Protection Agency approved the project. Procedures adhere to the guidelines of the World Medical Association's Declaration of Helsinki. RA ( $n = 503$ ) were isolated from 128 patients and maximal contractile response and structural properties were determined by combining wire myography and histomorphometry. Relations between CONTR (maximal force/tunica media volume; mean $\pm$ SD  $2.60 \pm 0.94$  MN/m<sup>3</sup>), M:L (median (IQR) 5.32 (4.43–6.17) %) and patient characteristics were analyzed by linear regression models using STATA after log<sub>10</sub> transformation of the outcomes.  $p < 0.05$  was considered significant.

Contrary to the hypothesis, there was a robust *negative* relationship between M:L and CONTR in univariate analysis (UVA), and after adjusting for patient age, gender, body mass index (BMI), mean arterial blood pressure (MAP) and diagnosis of diabetes prior to surgery (adj.  $p=0.029$ ,  $b=-0.283$  (95% CI: -0.535; -0.030)). Recent hyperglycemia (HbA1c, 38 (35–43) mmol/mol) had a small, significant increasing effect on M:L in both UVA and the model adjusted for age, gender, BMI, MAP (adj.  $p=0.035$   $b=-0.003$  (0.000; 0.007)), whereas CONTR was negatively affected by HbA1c in both models (adj.  $p=0.041$ ,  $b=-0.004$  (-0.008; -0.000)). Interestingly, there was a robust, persisting negative relationship between CONTR and plasma N-terminal fragment of proBNP (BNP) in all models applied, independent of the type of surgery (coronary artery bypass

grafting, valve replacement or both) both in UVA and adjusted models (age, gender, BMI, MAP, adj.  $p=0.964$ ), whereas plasma BNP was not related to M:L. Finally, M:L, but not CONTR, was strongly, negatively related to RA wall stiffness (Young's elastic modulus, 127 (91 – 159) kPa) in all models applied ( $p<0.0001$ ,  $b=-0.627$  (-0.870; -0.383)).

Smooth muscle contractile function is inversely related to M:L. Quality rather than quantity of tunica media is highly important for smooth muscle function. Circulating clinically relevant diagnostic biomarkers relate differently to structure and function of the arterial smooth muscle. Further analyses will determine relations and mixed effects of other patient characteristics and patients' pharmacotherapy on the outcomes.

Leurgans TM, Bloksgaard M, Irmukhamedov A, Riber LP, De Mey JGR. Relaxing Responses to Hydrogen Peroxide and Nitric Oxide in Human Pericardial Resistance Arteries Stimulated with Endothelin-1. *Basic Clin Pharmacol Toxicol*. 2018;122(1):74-81.

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*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

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PCB376

### **Disruption of the smooth muscle actin cytoskeleton decreases human resistance artery stiffness and abolishes smooth muscle contractile function**

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Resistance arteries (RA) are remodeled in hypertension, diabetes and obesity. We hypothesized that actin cytoskeleton polymerization, and thereby wall stiffness, is increased in resistance arteries from hypertensive human individuals compared to normotensive controls.

RA were isolated from human omental fat obtained from either bariatric or other abdominal surgeries at The University of Missouri Hospital after patients were informed and written consent secured. Collection and use of human specimens were approved by IRB #2003033 and all procedures adhere to the guidelines in the World Medical Association's Declaration of Helsinki. Resistance artery segments ( $n = 58$  patients, 37 females (11 / 26 non-bariatrics / bariatrics), 18 males (7 / 11 non-bariatrics / bariatrics), 3 unknown (excluded from analyses)) were isolated, subjected to pressurization from 5 to 120 mmHg and depressurization to 5 mmHg under passive conditions (calcium-free buffer with 100  $\mu$ M adenosine and 2 mM EGTA) before and after 1 hour incubation with 2  $\mu$ M mycalolide B at 5 mmHg to disrupt the actin cytoskeleton. Internal diameter and left and right wall thicknesses

were recorded continuously. Smooth muscle cell contractile ability was assessed at 40 mmHg as a decrease in internal diameter (constriction) after exposure to 80 mM K<sup>+</sup> at the beginning and end of the experimental protocol. The effect of mycalolide B, K<sup>+</sup> and the influence of surgery type hereon was evaluated by two-tailed, paired t-test and two-way, repeated measurement ANOVA, respectively. The level of significance was pre-set to 0.05.

Mean diameter, wall thickness and wall-to-lumen ratio at 100 mmHg were  $366 \pm 158 \mu\text{m}$ ,  $28 \pm 10 \mu\text{m}$  and  $16.6 \pm 0.06 \%$ , respectively. Mycalolide B did not affect the passive, internal diameter at 100 mmHg, but increased wall thickness slightly, yet significantly ( $3.5 \pm 6 \mu\text{m}$ ,  $p < 0.0001$ ). As expected, mycalolide B completely abolished smooth muscle contractile function (constriction in % upon K<sup>+</sup> exposure was  $27 \pm 20$  and  $0.4 \pm 5$ , before and after mycalolide B treatment, respectively). Stress-strain relationships were calculated and the Young's elastic modulus,  $E_{\text{inc}}$ , determined as described (1).  $E_{\text{inc}}$  was decreased significantly by Mycalolide B treatment ( $9 \pm 21 \text{ kPa}$ ,  $p = 0.0016$ ) and the ability of the RA wall to absorb energy (calculated as described (2)) decreased significantly ( $10 \pm 16 \%$ ,  $p < 0.0001$ ). There were no differences between RA from bariatric patients versus non bariatrics.

We conclude that the smooth muscle cytoskeleton has a small, but highly significant impact on the passive RA mechanics contributing to vascular wall stiffness and as expected, is essential for smooth muscle contractile function. Inclusion of additional patient demographic and clinical data and further analyses are required to confirm or reject the hypothesis.

Bloksgaard M, Leurgans TM, Spronck B, Heusinkveld MH, Thorsted B, Rosenstand K, et al. Imaging and modeling of acute pressure-induced changes of collagen and elastin microarchitectures in pig and human resistance arteries. *Am J Physiol Heart Circ Physiol*. 2017;ajpheart 00110 2017.

Kim J, Staiculescu MC, Cocciolone AJ, Yanagisawa H, Mecham RP, Wagenseil JE. Crosslinked elastic fibers are necessary for low energy loss in the ascending aorta. *J Biomech*. 2017;61:199-207.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

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PCB377

### **The Effect of Visfatin on Pregnant Mouse Myometrial Contractility In vitro**

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Obesity is a worldwide disorder influencing women's health and childbearing. There is a close relationship between obesity and pregnancy-related complications. Dyslipidemia and adipokine dysregulation are core environmental changes that may mechanistically link these complications with obesity in pregnant women. We have previously found that visfatin has a relaxant effect on the mouse, rat and human myometrial contractility. We hypothesised that visfatin inhibits mouse myometrial contractility through the NAD<sup>+</sup> pathway. This study was designed to examine the mechanism of action of visfatin on myometrial contractility. To examine the NAD<sup>+</sup> pathway, FK866 which is a potent inhibitor of NAD<sup>+</sup> biosynthesis was used. Methods: Myometrial strips from term pregnant mice were dissected, superfused with physiological saline and the effects of visfatin (10nM) on oxytocin-induced contractions (0.5nM) alone and after the infusion of FK866 (10uM) were studied. After regular contractions were



established, contractility was examined for control (100%) and test response at 37 °C for 10 min each. Results: FK866 was found to inhibit the effect of visfatin on myometrial contractility (the AUC increased from  $89 \pm 2\%$  of control,  $P=0.0009$  for visfatin alone to  $97 \pm 4\%$  of control,  $P>0.05$  for visfatin combined with FK866,  $n=8$ ). In conclusion; NAD<sup>+</sup> pathway appears to be involved in the mechanism of action of visfatin on mouse myometrium. This could have a role in making new targets to prevent obesity-related complications.

Zhang J, Bricker L, Wray S, Quenby S. Poor uterine contractility in obese women. *Br J Obstet Gynaecol* 2007;114: 343-348.

Mumtaz S, AlSaif S, Wray S, Noble K. Inhibitory effect of visfatin and leptin on human and rat myometrial contractility. *Life sciences*. 2015;125:57-62.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

TA were similar ( $1174 \pm 71$  vs.  $1250 \pm 66$  mm<sup>2</sup>) and ( $1501 \pm 93$  vs.  $1267 \pm 51$  mm<sup>2</sup>), respectively. These data suggest that muscle fibre composition and capillarity of the two strains are different, and indicate that fast muscles of Brown rats are more aerobic than those of Wistar rats, potentially increasing exercise tolerance.

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

## PCB378

### **Influence of muscle mass on capillarity and fibre composition in fast muscles of inbred and wild type rats**

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*University of Leeds, Leeds, UK*

Skeletal muscle composition is usually heterogeneous containing different fibre types with specific contractile and metabolic properties. Typically, Type IIa and IIb fibres are used for phasic activity and Type I to maintain posture. Genetic background may contribute to variability in composition of individuals, e.g. aerobic capacity is thought to be higher in inbred compared to wild strains. This study compared fibre type composition and capillarity of fast extensor digitorum longus (EDL) and tibialis anterior (TA) muscles between inbred (Wistar,  $n=6$ , body mass  $207 \pm 3$ g) and wild type (Brown,  $n=6$ , body mass  $209 \pm 2$ g) strains. Rats were sacrificed and cryosections ( $10 \mu\text{m}$  at  $-20^\circ\text{C}$ ) stained to identify fibre type (monoclonal anti-MHC antibodies; BA-D5 for Type I fibre and SC-71 for Type IIa fibre, DSHB, University of Iowa) and capillaries (*Griffonia simplicifolia* lectin I, Vector) and visualised (20x and 16x magnification) under fluorescent illumination (Nikon E600), with systematic-random sampling. Capillary to fibre ratio (C:F), capillary density (CD, mm<sup>2</sup>), and mean fibre area (MFA,  $\mu\text{m}^2$ ) were determined using in-house morphometric analysis programmes. Both EDL and TA muscle mass were higher in Brown rat ( $0.1021$  vs.  $0.0851$ g,  $P<0.05$  and  $0.400$  vs.  $0.355$ g,  $P<0.05$ ) despite similar body masses. In EDL, global capillarity (C:F) and MFA were greater in Brown than Wistar rats ( $1.29 \pm 0.04$  vs.  $1.14 \pm 0.03$  and  $1388 \pm 16$  vs.  $1108 \pm 93$   $\mu\text{m}^2$ , respectively, both  $P<0.05$ ) with similar CD ( $984 \pm 35$  and  $1001 \pm 45$  mm<sup>2</sup>, n.s.). In the TA, C:F was not significantly different in either core or cortex but CD of Wistar rat was greater in cortex ( $556$  vs.  $445$  mm<sup>2</sup>,  $P=0.022$ ) while MFA was slightly higher in Brown rat (n.s.). Both EDL and TA of Brown rat showed higher Type I numerical density ( $N_N$ ) than Wistar ( $0.058 \pm 0.003$  vs.  $0.038 \pm 0.003$ ,  $P<0.05$ ) and ( $0.060 \pm 0.005$  vs.  $0.038 \pm 0.004$ ,  $P<0.05$ ), respectively. Type IIb  $N_N$  in EDL was lower ( $0.67 \pm 0.01$  vs.  $0.75 \pm 0.02$ ,  $P<0.05$ ) than Wistar rat but was similar in TA. Areal density ( $A_A$ ) of TI fibres of EDL and TA were higher in Brown than Wistar rat ( $0.038 \pm 0.001$  vs.  $0.023 \pm 0.003$ ,  $P<0.01$ ) and ( $0.050 \pm 0.01$  vs.  $0.028 \pm 0.004$ ,  $P<0.01$ ), respectively. Local capillary to fibre ratio (LCFR) of Brown EDL was higher than Wistar rat ( $1.55 \pm 0.05$  vs.  $1.08 \pm 0.03$ ) while it was similar in TA. Accounting for differences in fibre size, local capillary density (LCD) of EDL and

# A

|                             |                                                         |
|-----------------------------|---------------------------------------------------------|
| Aakhus, S. ....             | SA038                                                   |
| Aalkjaer, C. ....           | SA066, PCA327, PCB312                                   |
| Aalto-Setälä, K. ....       | C001, PCB036                                            |
| Aasum, E. ....              | PCB031                                                  |
| ABA ALKHAYL, F. ....        | PCB159*                                                 |
| Abd-El hamid, M.S. ....     | PCB224                                                  |
| Abdala, A.P. ....           | PCA004                                                  |
| Abdelrahman, M. ....        | PCA302                                                  |
| AbdelWahed, D.M. ....       | PCB224                                                  |
| Abdulla, M. ....            | PCA071, PCB002                                          |
| ABDULMALIK, M. ....         | PCA235                                                  |
| Abdulrazak, A. ....         | PCA229*, PCA235                                         |
| Abdur Rouf, M. ....         | PCA258                                                  |
| Abels, M. ....              | PCB221                                                  |
| Abgarmi, S. ....            | PCA260, PCA261                                          |
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| Absi, M. ....               | PCA013                                                  |
| Abubakar, A. ....           | PCA227                                                  |
| Abubakar, I.A. ....         | PCA172                                                  |
| Abubakar, M.B. ....         | PCA087*                                                 |
| Aburima, A. ....            | C039                                                    |
| Adamkov, M. ....            | PCA079                                                  |
| Adams, V. ....              | PCA200                                                  |
| Adan, R. ....               | SA040*                                                  |
| Adedeji, T.E. ....          | PCB228                                                  |
| Adegbite, L.O. ....         | PCA244                                                  |
| Adegghate, E.A. ....        | PCA043, PCA256                                          |
| Adejare, A. ....            | PCA351, PCB323                                          |
| Adeniyi, O.S. ....          | PCB116*                                                 |
| Adewoye, E.O. ....          | PCA247                                                  |
| Adeyemi, W.J. ....          | PCB248                                                  |
| Aduonum, A.D. ....          | PCA270*                                                 |
| Aghoghovwia, A.M. ....      | PCB228                                                  |
| Ahmad, S. ....              | PCA041                                                  |
| Ahmadi-Noorbakhsh, S. ....  | PCA068                                                  |
| Ahmed, A. ....              | C126                                                    |
| Ahmed, M.A. ....            | PCA226*, PCA236*, PCB224                                |
| Ahtiainen, J. ....          | PCB144                                                  |
| Aibara, C. ....             | PCA194                                                  |
| Aigbologa, J. ....          | PCA123                                                  |
| AIKU, A.O. ....             | C128*                                                   |
| Ainslie, P. ....            | PCA176, PCA179, PCA181, PCA316, PCB001, PCB357          |
| Ainslie, P.N. ....          | C040, PCB331                                            |
| Aitken, A.V. ....           | PCB012                                                  |
| Akakin, D. ....             | C067, PCB147                                            |
| Akaniro-Ejim, N.E. ....     | PCB220*                                                 |
| Akarsu, E. ....             | PCA275                                                  |
| Akbulut, H. ....            | PCA260, PCA261                                          |
| Akbulut, K.G. ....          | PCA260*, PCA261*                                        |
| Akindele, O.O. ....         | PCA244                                                  |
| Akinnibosun, O.A. ....      | PCA243, PCB228*                                         |
| Akins, J.D. ....            | PCB357                                                  |
| Akintoye, O.O. ....         | PCA307                                                  |
| Akkoc, R. ....              | PCB230                                                  |
| Aksu, B. ....               | C067, PCB206                                            |
| Al Ali, H.S. ....           | PCA065*                                                 |
| Al Kury, L. ....            | PCA027                                                  |
| Al Owais, M. ....           | PCA061*                                                 |
| Al Saikhan, L.K. ....       | PCA033*                                                 |
| Al Salmani, M.K. ....       | PCB113*                                                 |
| Al-Awadi, M. ....           | PCA252                                                  |
| Al-Bader, M. ....           | PCA252                                                  |
| Al-Hosni, R. ....           | PCB266*                                                 |
| Al-Jaber, M. ....           | PCB346                                                  |
| Al-Shanti, N. ....          | PCA153                                                  |
| Alada, A. ....              | PCB232                                                  |
| Alada, A.A. ....            | PCA218*                                                 |
| Alatorre-Cruz, G.C. ....    | PCB275                                                  |
| Alayande, T.O. ....         | PCA307                                                  |
| Albersdoerfer, L. ....      | SA015                                                   |
| Albrecht, T. ....           | C005                                                    |
| Alda, M. ....               | PCA263, PCB270                                          |
| Aldiss, P. ....             | PCA216*                                                 |
| Alesutan, I. ....           | PCA361, PCA362, PCA363, PCA364, PCB363, PCB366, PCB368* |
| Alexanova, A. ....          | C001                                                    |
| Alexeenko, V. ....          | PCB056*                                                 |
| Alfituri, A. ....           | C104*                                                   |
| Ali, B.H. ....              | PCA019                                                  |
| Ali, E. ....                | PCA242                                                  |
| Ali, F.M. ....              | PCB224*                                                 |
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| Ali, M. ....                | PCB351*                                                 |
| Ali, T. ....                | PCA258                                                  |
| Alitalo, K. ....            | PL006*                                                  |
| Aliyu, M. ....              | PCA227                                                  |
| ALIYU, M. ....              | PCA235                                                  |
| Alkhalil, M. ....           | PCB165                                                  |
| Alkio, M. ....              | C008                                                    |
| Alkner, B. ....             | PCB161                                                  |
| Allam, E.A. ....            | PCB330*                                                 |
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| ALLEN, T.J. ....            | PCA085*                                                 |
| Alleva, C. ....             | PCA115*                                                 |
| Almaas, V. ....             | SA038                                                   |
| AlMarabeh, S. ....          | PCB002*                                                 |
| Almeida, T.S. ....          | PCA075                                                  |
| Almohanna, A.M. ....        | PCA368*                                                 |
| Alobaid, H. ....            | PCA297*                                                 |
| Alomar, F. ....             | PCA256                                                  |
| Alonso, E. ....             | PCB333, PCB334                                          |
| Alonso, L. ....             | C095*                                                   |
| Alsaif, S. ....             | PCB377*                                                 |
| alsalahi, S. ....           | PCB011*                                                 |
| Altarawneh, M. ....         | PCB157                                                  |
| Alter, C. ....              | C014*, PCA054                                           |
| Althaus, M. ....            | C019                                                    |
| Alvarez de la Rosa, D. .... | C108                                                    |
| ALVAREZ MIGUEL, I. ....     | PCB333*                                                 |
| Amabebe, E. ....            | PCA224*                                                 |
| Amann, K. ....              | PCB366                                                  |
| Amat, C. ....               | PCB256                                                  |
| Amati, F. ....              | SA045*                                                  |
| Ameen, A. ....              | PCA271                                                  |
| Ames, S. ....               | PCA098                                                  |
| Amici-Dargan, S.L. ....     | C125*                                                   |
| Amigo-Benavent, M. ....     | C068                                                    |
| Amosah, J. ....             | C029                                                    |
| Andersen, H. ....           | PCB370                                                  |
| Andersen, J. ....           | PCB292                                                  |
| Andersson, O. ....          | PCA350                                                  |
| Andrew R, S. ....           | PCB074                                                  |
| Andrushina, N. ....         | PCA214                                                  |
| Ankney, C.M. ....           | PCA203                                                  |
| Ansdel, P. ....             | PCA300, PCA301*                                         |
| Antignac, C. ....           | C021                                                    |
| Antonyunil, A. ....         | C069                                                    |
| Antoons, G. ....            | PCA031                                                  |
| Anttila, K. ....            | C008                                                    |
| Ao, Z. ....                 | PCA059                                                  |
| Apaijit, K. ....            | PCA149                                                  |
| Apaydin, M. ....            | PCB278                                                  |
| Appadurai, I. ....          | PCB171                                                  |
| Appel, T. ....              | PCB045                                                  |
| Appenzeller, S. ....        | PCA099, PCB100                                          |
| Arabaci Tamer, S. ....      | C057, C067, PCA124*, PCB205, PCB206                     |
| Arendt, J. ....             | PL013*                                                  |
| Arévalo Martínez, M. ....   | PCB334*                                                 |

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Arikawe, A.P. . . . . PCA243\*, PCB228, PCB257  
Arnemo, J. . . . . SA030  
Arnold, C. . . . . C100\*, PCA366, PCA370  
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Arocas, O. . . . . SA004  
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Ashley, Z. . . . . PCA317  
Ashmore, J.F. . . . . C126\*  
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Aslam, M.J. . . . . PCB200\*  
Aslanidi, O. . . . . PCB055  
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Awobajo, F.O. . . . . PCA245  
Awodele, I. . . . . PCA016  
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Aydin, A. . . . . PCA298  
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Aydin, B. . . . . PCB088, PCB090  
Aydin, M. . . . . PCB367  
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Bachmann, A. . . . . PCA369  
Bäcker, V. . . . . SA033, PCA178  
Backs, J. . . . . PCA342  
Bade, M. . . . . PCA017\*  
Badea, R. . . . . PCB286  
Bader, A. . . . . PCA089, PCA129\*  
Baessler, A. . . . . SA015  
Bagley, L. . . . . PCA154  
Bailey, D.M. . . . . PCA164\*, PCA179, PCA180, PCA185, PCA189,  
PCA321, PCB001, PCB171, PCB173, PCB175, PCB176, PCB331

Bailey, M.A. . . . . C020, PCA142\*, PCA144, PCA358,  
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Bailey, T. . . . . PCA060\*  
Baines, D.L. . . . . C035, PCB101, PCB102, PCB107\*, PCB129  
Bajomo, K.F. . . . . PCB228  
Bakker, A.J. . . . . PCA068  
Bakker, E. . . . . SA067\*  
Balanos, G. . . . . PCB011  
Balcarcel, N.B. . . . . PCB212  
Baldin, J. . . . . PCA317  
Baldwin, S. . . . . PCA314\*  
Balentova, S. . . . . PCA079  
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Bang Overgaard, C. . . . . PCB312  
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Bano-Otalora, B. . . . . C015  
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Barak, O.F. . . . . PCB170\*  
Baranyai, D. . . . . PCA036, PCB035\*  
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Bartölke, R. . . . . PCB213\*  
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Baukrowitz, T. . . . . PCB287, PCB288  
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Baumann, I. . . . . C005  
Baur, T. . . . . PCB030  
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Beazley-Long, N. . . . . PCA341  
Becherer, U. . . . . PCA274\*, PCB255  
Becker, A. . . . . PCA089  
Becker, A.K. . . . . PCB303\*  
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| Belova, S. ....         | PCB145*, PCB146                                                        |
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| Bencsik, P. ....        | PCA029                                                                 |
| Bender, F. ....         | C087                                                                   |
| Benedetto, R. ....      | PCA118, PCB077*                                                        |
| Bengtsen, M. ....       | PCB198*                                                                |
| Bennet, L. ....         | PCA066*                                                                |
| Bennett, H. ....        | PCB358                                                                 |
| Bennett, R. ....        | PCB039                                                                 |
| Benson, A.P. ....       | C017, C053, PCB050, PCB055, PCB162                                     |
| Benterkia, F. ....      | PCA007                                                                 |
| Bentzen, B.H. ....      | C003, C018                                                             |
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| Berbari, N. ....        | C028                                                                   |
| Berchtold, M. ....      | PCA062                                                                 |
| Berg, R.M. ....         | PCB175, PCB176                                                         |
| Berger, D. ....         | PCA323, PCB111                                                         |
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| Berthiaume, Y. ....     | PCB024                                                                 |
| Bescos Garcia, R. ....  | C063*                                                                  |
| Bettoni, C. ....        | PCB137                                                                 |
| Bewick, G.S. ....       | C080*                                                                  |
| Beyer, A.N. ....        | C108*                                                                  |
| Beynon, R. ....         | PCB039                                                                 |
| Bhagat, O. ....         | PCA002*                                                                |
| Bhandage, A. ....       | PCA230, PCA231                                                         |
| Bhandari, B. ....       | PCA072                                                                 |
| Bhargava, A. ....       | PCB018                                                                 |
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| Bidasee, K.R. ....      | PCA256                                                                 |
| Biggart, M.G. ....      | PCB102*                                                                |
| Bilal, M. ....          | SA038                                                                  |
| Billeter, R. ....       | PCA188*                                                                |
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| Blasco-Lafarga, N. .... | PCA198, PCB186                                                         |
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| Bleich, M. ....         | PCA135, PCB134, PCB135                                                 |
| Bloksgaard, M. ....     | C097, PCB375*, PCB376*                                                 |
| Blouet, C. ....         | SA027*                                                                 |
| BMRES, B. ....          | PCA166, PCA167                                                         |
| Boachie, J. ....        | C069*                                                                  |
| Boardman, N. ....       | PCB031*                                                                |
| Boas, D. ....           | PCB016                                                                 |
| Bobbe, S. ....          | PCA323, PCA324*                                                        |
| Boccaccio, A. ....      | PCB125*                                                                |
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| Boehm, U. ....          | PCB100                                                                 |
| Boehme, B. ....         | PCA362, PCA363*, PCA364, PCB363, PCB366                                |
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| Bohn, A. ....           | PCB131                                                                 |
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| Bonfiglio, A. ....      | PCA306                                                                 |
| Boon, R.A. ....         | PCB324                                                                 |
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| Bor-Kucukatay, M. ....  | PCB008, PCB061, PCB216, PCB217                                         |
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| Børsting, C. ....       | PCB292                                                                 |
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| Bothe, S.N. ....        | PCB284*                                                                |
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| Brassard, P. ....       | PCA164                                                                 |
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| Breiderhoff, T. ....    | PCB135                                                                 |
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| Brenner, B. ....        | C037                                                                   |
| Brette, F. ....         | PCB051*                                                                |
| Brierley, D. ....       | C058*                                                                  |
| Brinkhus, M. ....       | PCB135                                                                 |
| Brinkmeier, H. ....     | PCA197                                                                 |
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| Broach, J. ....         | PCB157                                                                 |
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| Brooks, D.A. ....       | C010                                                                   |
| Brothers, M. ....       | PCB357                                                                 |
| Brown, A.M. ....        | PCA205*, PCA268, PCB265*, PCB269*                                      |
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| Bruegmann, T. ....      | C016*, PCB033, PCB034, PCB040                                          |
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| Brüggemann, A. ....     | PCB028, PCB065, PCB285                                                 |
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| Heidler, J.        | PCA151, PCA338                                  |
| Heiestad, C.       | PCA055                                          |
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| Hellmuth, M.       | PCA140                                          |
| Hellsten, Y.       | C051, PCB349, PCB350                            |
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| Scheer, A. ....            | PCA233*                                                        | Selescu, T. ....        | PCB286                                     |
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| Schillers, H. ....         | PCA323*, PCA324, PCB111, PCB126                                | Seniuk, A. ....         | PCA014*, PCA067                            |
| Schilpp, C. ....           | C025                                                           | Senol, H. ....          | PCB008, PCB216, PCB217                     |
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| Schiwy, A. ....            | PCB283                                                         | Sentürk, M. ....        | PCB019, PCB202                             |
| Schlueter, K. ....         | PCA035, PCA045*, PCA047*                                       | Sepúlveda-Díaz, J. .... | PCB300                                     |
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| Schmidt, N. ....           | PCA369, PCB110                                                 | Serhatlioglu, I. ....   | PCA241, PCA360, PCB231, PCB296, PCB328     |
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| Schnipper, J. ....         | PCA116                                                         | Sfyri, P.P. ....        | C059*                                      |
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| Schützhold, V. ....        | PCA219*                                                        | Sheppard, D.N. ....     | PCB113                                     |
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| Smith, J. ....                   | C038, PCB166*, PCB352*                                         |
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| Sobrevia, L. ....                | PCA328*, PCA336, PCB353, PCB359                                |
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