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### The Negative Side of Life – The Calcium Activated Chloride Conductances in Vascular Smooth Muscle Cells

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Vascular smooth muscle cells have at least two calcium activated chloride conductances – one is the classical niflumic acid sensitive conductance and one is the cGMP dependent, zinc sensitive conductance. The former is dependent on the chloride channel protein TMEM16A, while the latter is dependent on both TMEM16A and one or more members of the bestrophin protein family. The two conductances are present in smooth muscle cells from all vascular beds but their relative importance varies. Because chloride is actively pumped into smooth muscle cells, an increase of a calcium activated chloride conductance leads to depolarisation and consequent contraction, and TMEM16A and bestrophins are important in this function. The cGMP dependent, zinc sensitive chloride conductance is also important for the coordinated oscillatory activity of smooth muscle cells, which gives rise to vasomotion in rat mesenteric resistance arteries. A model for how vasomotion occurs and how the cGMP dependent, zinc sensitive conductance ensures coordinated activity of the smooth muscle cells will be provided. Bestrophins are placed in close contact with TMEM16A in smooth muscle cells and may potentially constitute a subunit of TMEM16A. Furthermore, TMEM16A is regulating the expression of bestrophins. TMEM16A knockdown may also affect L-type calcium channel expression and several other proteins of importance for vascular function. Therefore, in addition to being a chloride channel and provide a depolarizing conductance, TMEM16A may have additional and still poorly understood roles in controlling the expression of various proteins of importance for vascular smooth muscle function. These additional roles will be discussed.

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### Development of a Novel Angiogenic Score for Early Detection of Hepatocellular Carcinoma among High-Risk Hepatitis C Virus Patients

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Hepatocellular carcinoma (HCC) is often diagnosed at advanced stage where effective therapies are lacking. More than 90% of HCC cases develop in chronically inflamed liver as a result of viral hepatitis including virus C and B. The current diagnostic tools for HCC among high risk patients include clinical, laboratory, imaging, and biopsies which have multiple drawbacks and limitations. Therefore, there is an urgent need to identify more sensitive and reliable serum biomarkers for early non-invasive detection of HCC among high risk patients. One of the notable features of HCC is hypervascularity which open the door for using angiogenic markers for HCC early detection; thus, herein we aimed to develop a novel score based on combination of angiogenic factors including angiopoietin 2 (Ang2), copper, nitric oxide (NO) in addition to routine laboratory tests for early prediction of HCC. Ang2 were assayed for HCC group (80 patients), liver cirrhosis group (65 patients), and control group (40 patients) by enzyme-linked immunosorbent assay (ELISA). Copper and nitric oxide were assayed by simple colorimetric method. Data from all groups were retrospectively analyzed including  $\alpha$ -fetoprotein (AFP), international normalized ratio (INR), albumin and platelet count, transaminases, and participants age. Areas under receiving operating curve (ROC) were used to develop the score. A novel index named Angio-HCC score =  $2.4 [0.45 \times \text{Ang2 (ng/ml)} - 0.01 \times \text{Cu (mg/dL)} + 97 \times \text{NO } (\mu\text{mol/L)} - 9.9 \times \text{Albumin (g/l)} - 0.04 \times \text{Platelets count } [\times 10]^3 \text{ l}^{-1}]$  was developed. Angio-HCC score produced area under ROC curve of 0.97 for discriminating HCC patients from liver cirrhosis with sensitivity of 89% and specificity of 81% at cutoff 1.5 (i.e., less than 1.5 was considered cirrhosis whereas greater than 1.5 was considered HCC). Angio-HCC score is a novel non-invasive diagnostic tool that could replace AFP in HCC screening and follow up of cirrhotic patients.

### Novel Optimized Cell Culture Medium for Growth and Maintenance of Endothelial-Like Cells and Endothelial Progenitor Cells with minimal serum

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Endothelial progenitor cells (EPCs) are crucial for vascular homeostasis & repair; also they are currently used for the cellular therapy of peripheral arterial diseases & cardiovascular diseases. For a better understanding of the EPCs biology & their physiological functions including proliferation, senescence, apoptosis & autophagy, mobilization & migration, tube formation & angiogenic capacity and differentiation, in addition to their isolation from peripheral or umbilical cord blood and/or their induction/derivation from ESCs or iPSCs; there must be a clear picture and knowing all the constituents for the endothelial culture medium used for EPCs and most other EC-Like cells. Unfortunately, most of the formulas for all the commercial basal cell culture medium used for EC-like cells are masked, as well as the growth factors & cocktails of exogenous factors that are added to the basal medium have unknown concentrations. Thus, Herein, we optimized a novel maintenance/growth cell culture medium which is chemically defined for the basal medium and the exogenous factors with the least serum content of 0.7%. We have prepared eight various patches of our BMBD141 media with different ranges of amino acids, inorganic salts, vitamins & miscellaneous compounds. Proliferation of endothelial-like cells including EPCs (both from umbilical cord & adult peripheral blood), HUVECs and hAECs (human aortic endothelial cells) at passages 3–6 were measured using Brdu proliferation assay. The proliferative effect of BMBD141 patches was compared with conventional media (like DMEM/F12, M199, IMDM) & commonly used endothelial growth media (EGM2/Lonza, Endogrow/Merck Millipore, Endothelial Cell Growth Medium 2(EGCM2)/Promocell). Moreover, different ranges of the VEGF, IGF-1, EGF, FGF2, Hydrocortisone, Vitamin C, heparin was optimized and fixed in the Brdu comparison assays. The first six patches of BMBD141 did not work and the cells died after three days of culture. Then the seventh & eighth patches have given a higher proliferative potential (both using 5% & 2% FBS) compared with DMEM/F12, M199, IMDM & ECGM2 and comparable (and in some cases higher) proliferation rates compared with EGM2 & Endogrow. Neither the morphology of all four types of endothelial-like cells nor their ability to express the conventional endothelial surface markers (like CD31, KDR & VE-cadherin) were affected with our novel BMBD141 cell culture media. In conclusion, we have successfully prepared a working EC-like cell culture medium with minimal serum content that we will use to study the metabolic profile, senescence and the rejuvenative capacity of EPCs in various pathological conditions.

### Single Cell Transcriptomic Heterogeneity of Novel Markers for Human Endothelial Progenitor Cells

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Endothelial progenitor cells (EPCs) are promising candidates for the cellular therapy of peripheral arterial diseases & cardiovascular diseases. However, hitherto there is definitive marker(s) for EPCs. We tried to find novel marker(s) of EPCs in peripheral blood. Using Gene ontology and literature survey, we assembled five groups of EPCs' molecules/factors/markers analyses: 72 Illumina gene identifiers were collected in buck transcriptome of different cell origins of ECs (skin, adipose tissue and HUVECs). One way ANOVA was performed comparing EPCs with three types of ECs. Moreover, Functional enrichment on mouse phenotype database was performed, and finally using publicly available single cell RNA-sequencing data, we investigated cell expression heterogeneity of significant markers in healthy donors PBMCs (33000 transcriptomes). BMP2, 4 & ephrinB2 were exclusively highly expressed in EPCs; the expression of neuropilin-1 & VEGF C were significantly higher in EPCs & HUVECs compared with skin & adipose ECs; Notch 1 was highly expressed in EPCs & skin-ECs; MIR21 was highly expressed in skin-ECs; PECAM-1 were significantly higher in EPCs & adipose ECs. Moreover, Functional enrichment of EPCs factors/genes on mouse phenotype database allowed to find significant relations between regulated genes and endothelial functions where ephrinB2, BMP2 and BMP4 molecules were highly expressed in EPCs and were connected to abnormal function (angiogenesis, arterial morphology & vascular development). Single cell analyses has revealed that among the EPCs regulated markers in transcriptome analyses: i-ICAM-1 & Endoglin were weakly expressed in the monocyte compartment of peripheral blood (especially the CD14+); ii-CD163 & CD36 were highly expressed in CD14+ monocyte compartment whereas CSF1R (colony stimulating factor 1 receptor) was highly expressed in CD16+ monocyte compartment; iii-L-selectin & IL6R were globally expressed in the lymphoid/myeloid compartments; iv-Surprisingly, PLAUR/UPAR (Urokinase Plasminogen Activator (UPA) Receptor) & NOTCH2 were highly expressed in the both the CD14+ & CD16+ monocytic compartments. Transcriptomic analyses of chosen markers could optimize the characterization of EPCs from other adult ECs which would aid in a better understanding of EPCs biology especially when further studying the overexpressed genes in EPCs that affect vascular development. Moreover, single cell RNA-sequencing revealed novel endothelial related markers expressed in PBMCs which will be useful to design multi-parametric cytometric experiments to better characterize EPCs sub-population(s) in the blood. In conclusion combining both transcriptomic & single cell analyses could optimize the characterization of novel EPCs-specific markers and studying their associated functional alterations and/or EPCs-biologic/ontogenic hierarchy.

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### Fetal Breathing Movements and Pulmonary Lymphatics Function Together to Prepare the Lung for Inflation at Birth

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Embryonic lungs must be inflated immediately after birth to establish respiration. In addition to pulmonary surfactant, recently we have revealed lymphatic function as a previously unknown mechanical regulator of prenatal lung compliance that prepares the embryonic lung for inflation at birth. It is well-documented that the late gestation embryo performs episodic breathing-like movements called as fetal breathing movements (FBMs), but the physiological importance of these events is not clear. In our current study we aimed to define the physiological role of FBMs in preparation for air inflation. Clp1K/K late gestation embryos develop a progressive loss of spinal motor neurons associated with axonal degeneration and denervation of neuromuscular junctions serving as an ideal genetic model to test the possible role of FBMs. We demonstrated that Clp1K/K newborns show impaired motor function resulting in fatal respiratory failure after birth. Next, we characterized the development of the embryonic lung before air inflation. The alveolar septae are thicker, and the alveolar area is reduced in late gestation embryos lacking FBMs, while the lack of FBMs does not influence molecular lung development. Importantly, pulmonary lymphatic vessels appear to be dilated and the prenatal pulmonary lymphatic function is impaired in embryos lacking FBMs. Our results have revealed the previously unrecognized role of FBMs in prenatal lung expansion, suggesting that FBMs and prenatal pulmonary lymphatics function together to prepare the developing lung for inflation and gas exchange at birth. Stimulating FBMs during late gestation might be an effective way to reduce the risk of the development of neonatal respiratory failure.

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### Endothelial Dysfunction in the Diabetic Patient and the Contribution by Circulating Endothelial Colony Forming Cells

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Endothelial dysfunction is the initiating factor in atherosclerosis, but also implicated in the pathogenesis of inflammatory and metabolic diseases including diabetes, leading to both microvascu-

lar and macrovascular complications. We have shown that patients with diabetes have impaired endothelial colony forming cell (ECFC)/endothelial progenitor cell function, potentially leading to a defective vascular endothelial repair process. This study describes defects in matrix adhesion and metabolic changes in ECFCs isolated from neuroischaemic diabetic patients and show that ECFCs from neuroischaemic patients differ from ECFCs isolated from neuropathic patients. We have generated small molecule glycomimetic drugs with protective effects against endothelial dysfunction and demonstrate restoration of function in neuroischaemic, but not neuropathic ECFCs. We propose that the glycocalyx offers potential as a novel therapeutic target for endothelial repair.

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### Neurovascular Dysfunction and Cognitive Impairment Induced by Chronic Cerebral Hypoperfusion are Ameliorated by Knockout of NADPH Oxidase 2

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**Introduction:** Carotid artery stenosis is a main risk factor for brain injury, with cerebral hypoperfusion being a key feature of vascular cognitive impairment and an early event of Alzheimer's disease. NADPH oxidase 2 (NOX2) is a major source of reactive oxygen species in resident microglia, invading macrophages and endothelial cells. Our research has shown that microvascular inflammation is related to cognitive decline and cerebrovascular dysfunction. Therefore, the aim of this study is to explore the role of NOX2 in chronic cerebral hypoperfusion.

**Materials and Methods:** Bilateral carotid artery stenosis was induced using microcoils in wild type, NOX2 knockout and NOX2 transgenic mice with endothelial- and myeloid-specific overexpression. Using laser speckle contrast imaging in vivo, cerebral blood flow was measured at several time-points before and after surgery, while neurovascular coupling was assessed at the end of experiment. Impairments in working memory were evaluated by radial arm maze, brain injury and markers of neuroinflammation were evaluated ex vivo by histochemical analysis and polymerase chain reaction.

**Results:** Carotid stenosis results in blood flow reductions after microcoil placement and microvascular inflammation compared with sham-control animals. NOX2 deficiency increases neurovascular coupling and reduces the impairment in working memory caused by hypoperfusion. Less marked were the effects of transgenic overexpression of NOX2. We are investigating further the role of NOX2 in models of microvascular amyloid.

**Conclusions:** Altogether, our studies elucidate the importance of NOX2 in neurovascular and inflammatory responses to chronic hypoperfusion, and support its potential use as a target for dementia treatment. (We acknowledge the support of the Alzheimer's Society for funding this project).

## Potential Role for G Protein Coupled Receptor Kinase 2 in Vasoconstrictors-Stimulated Vascular Smooth Muscle Cell Proliferation via MAPK/ERK Signalling Pathway

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**Introduction:** Vascular smooth muscle cell proliferation plays a fundamental role in hypertensive vascular remodelling development [1], a process associated with increased circulating vasoconstrictor levels [2] such as angiotensinII (AngII), endothelin-1 (ET1) and uridine-5'-triphosphate (UTP), leading to continuous activation of their cognate Gαq-coupled/G protein-coupled receptors (GPCR). Additionally, hypertension is associated with elevated G protein coupled receptor kinase 2 (GRK2) expression in vascular smooth muscle cells (VSMC) [3], which negatively regulates Gαq/GPCR signalling [4]. This study aimed to investigate the potential roles that GRK2 plays in VSMC proliferation and its effect on ERK signalling pathway as a possible molecular mechanism underlying GRK2-mediated regulation of VSMC proliferation.

**Methods:** VSMC were prepared from isolated male adult Wistar rat aortae. [<sup>3</sup>H]-thymidine incorporation assays and small molecule GRK2 inhibitor (compound101) were used to determine whether GRK2 played a role in vasoconstrictor-stimulated VSMC growth. Additionally, the MEK inhibitor (PD98059) was used to examine if MAPK/ERK signalling pathway mediate vasoconstrictor-stimulated VSMC proliferation. Furthermore, we examined the effects of GRK2 catalytic activity on AngII, ET1 or UTP stimulated MAPK/ERK signalling pathway.

**Results:** AngII or ET1 stimulation increased ( $P < 0.05$ ) thymidine incorporation in VSMC above that seen with foetal calf serum (FCS) treatment alone. Pre-incubation with compound101 inhibited ET1 and AngII ( $P < 0.05$ ) stimulated VSMCs proliferation. The MEK inhibitor PD98059, significantly inhibited ( $P < 0.0001$ ) both vasoconstrictor- and FCS-induced [<sup>3</sup>H]-thymidine incorporation, suggesting that ERK signalling mediates VSMC growth. AngII, ET1 and UTP induced time-dependent increases in ERK phosphorylation, peaking at 5 min and remaining elevated for 60 min in control cells. Inhibition of GRK2 catalytic activity suppressed the peak, and eliminated the prolonged phase of AngII ( $P < 0.01$ ) and ET1 ( $P < 0.0001$ ) induced ERK phosphorylation. Contrastingly, UTP did not induce VSMC thymidine incorporation, but did activate ERK phosphorylation. Interestingly, compound101-mediated inhibition of GRK2 markedly ( $P < 0.01$ ) increased UTP-stimulated ERK signals.

**Conclusion:** These data suggest that the GRK2 catalytic activity plays a central role to facilitate vasoconstrictor-stimulated VSMC proliferation. Moreover, since ERK signalling is known to mediate vasoconstrictor VSMC growth and as GRK2 is required for AngII and ET1-mediated ERK signalling, this may explain why inhibition of GRK2 activity prevents vasoconstrictor-stimulated VSMC proliferation.

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## Asymmetric Endothelial Adherens Junctions in Angiogenesis

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Collective migration of endothelial cells is required during angiogenesis, and critically relies on cell rearrangements through the remodeling of VE-cadherin-based cell-cell contacts couple to the dynamic actin cytoskeleton. During directional collective migration, an imbalance on pulling forces between cells leads to the formation of asymmetric adherens junctions (AJs) in which the F-BAR protein Pascin2 specifically recognizes the convex membrane generated in the front of follower cells. Our group previously showed that Pascin2 locally inhibits VE-cadherin internalization to promote cell-cell adhesion. However, the mechanism behind junctional Pascin2 signaling and the importance of asymmetric AJs for vascular physiology remain unknown. We have made use of the Pascin2 and its effector knock-out mouse to further investigate the importance of asymmetric AJs in angiogenesis. Our results show that deletion of Pascin2 leads to the formation of aberrant sprouts characterized by the accumulation of endothelial cells, suggesting Pascin2 is needed in endothelial collective behaviors in angiogenesis. Furthermore, we hypothesized that other proteins could be sensing and translating asymmetric forces in the junctions into polarized signals in the cell to control collective cell migration. To investigate that, we are performing a shRNA screening for a large library of proteins and study their implication in this process. Studying the molecular mechanisms responsible for asymmetry sensing would lead to a deeper knowledge of collective process as tissue morphogenesis, wound healing and cancer invasion.

## Coagulation Factor Xa Promotes Metastasis Through Endothelial Cells Activation

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Hypercoagulable state is associated with cancer presence and poor prognosis. In clinical and preclinical models, anticoagulants have been reported to reduce cancer-associated thrombosis and increase survival. Malignant cells can activate the coagulation cascade principally by the exposure of Tissue Factor (TF) on their cell surface and the tumor microenvironment (e.g. endothelial cells), with the subsequent activation of FX and prothrombin. Despite thrombosis being recognized as a principal cancer-related death, the role of the coagulation system on metastasis remains elusive. Herein, we examined the contribution of a hypercoagulable state to the formation of metastasis lesions via the administration of intravenous Coagulation Factor Xa (FXa) accompanied with tail vein injection of B16F10 mouse melanoma cells in C57BL/6 mice. FXa administration increased lung and other organ metastasis in the B16F10 tail vein injection model. The anticoagulant Dalteparin decreases metastasis promoted. In search of a mechanism, FXa did not alter cancer cell proliferation, migration or invasion in vitro. Alternatively, FXa action upon the endothelium increased the inflammatory adhesion molecules ICAM-1 and VCAM-1, promoted cytoskeleton contraction/F-actin stress fibers formation, VE-cadherin membrane ruffling, endothelial-hyperpermeability and increased B16F10 adhesion to an endothelial monolayer (under static and underflow conditions). Moreover, a microarray analysis showed that inflammatory cytokines/chemokines and pro-angiogenic factors genes are up-regulated in FXa-treated endothelial cells. Our results suggest that FXa-increases metastasis via an increase in cancer cell-endothelium adhesion and endothelial hyperpermeability. This study suggests that FXa inhibition could be a strategy in the management of metastasis.

**Methods:** During living-donor renal transplantation, subcutaneous fat biopsies were obtained from the abdominal wall at the incision site from donors (n = 36) and recipients (n = 40) for isolation of resistance-sized arteries to assess endothelial and smooth muscle function using wire myography. In vivo, reactive hyperemia index (RHI) was measured to assess endothelial function with the EndoPAT. Isolated arteries were also collected to detect senescence signature markers P16 and P21, RUNX2 (calcification), sirtuin 1 (endothelial function) and sclerostin (structure) within the artery wall. Additionally, the number of biochemical markers (proinflammatory, remodeling milieu, including signs of stress-related damage in addition to clinical characteristics) were collected to test for possible correlations of interest.

**Results:** The selected groups (CKD patients and donor controls) for the study were similar in age, sex and in respect to other parameters measured, except those related to symptoms of the disease presentation, medications and biochemical characteristics related to the renal failure. There was no difference in vascular function assessed by EndoPAT between patients and controls (RHI;  $2.3 \pm 0.1$ , n = 37 vs  $2.4 \pm 0.1$ , n = 32), however ex vivo investigations of vascular function revealed a significant difference in the contribution of endothelium-derived factors conferring the endothelium-dependent dilatation (e.g. nitric oxide contribution: CKD group  $7.37\% \pm 3.05$  vs controls  $18.37 \pm 2.08$ ; p = 0.03). Moreover, there was a positive correlation between in vivo RHI and ex vivo measurements of endothelial function (r = 0.4; p = 0.02) in the studied subjects (n = 34).

A higher expression of senescence and calcification markers such as P16, P21, RUNX2, and reduced expression of sirtuin1 was seen in CKD patients (n = 40) versus controls (n = 36), while there was no difference in expression of sclerostin. Further studies are ongoing to assess if correlations are present between functional parameters, markers of vascular calcification and senescence, with circulating biochemical markers of interest.

**Conclusions:** The uremic environment has an effect on vascular function by changing the contribution of endothelium-derived factors in patients undergoing renal transplantation. Moreover, the vasculature from CKD patients is characterized by the presence of a "senescence signature" which might confer the development of cardiovascular complications in this specific patient group.

## Resistance Vasculature in Chronic Kidney Disease: Focus on Function, Structure and Senescence Signature

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**Background:** Chronic kidney disease (CKD) is an independent risk factor for cardiovascular mortality and morbidity, and the cardiovascular risk increases with the severity of renal failure. Premature vascular aging in CKD patients is believed to drive the cardiovascular complications.

We hypothesized that peripheral vascular dysfunction in CKD patients is accompanied by a uremia induced senescence phenotype, including the presence of calcification.

## Unraveling the Selective Accumulation of Highly Unusual Liposomes to the Blood Brain Barrier in Embryonic Zebrafish

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Zebrafish have emerged as a powerful tool to study lipid metabolism as virtually all lipid receptors, transporters, apolipoproteins and lipid-processing enzymes are present and functions are highly conserved. In mammals, specialized endothelial cells are known to regulate lipid-transport in organ-specific vascular beds. A main example of this is lipid transport across the blood-brain barrier (BBB). In zebrafish embryos, the BBB is functional, comprises endothelial cells, tight-junction proteins and active transport systems and it is structurally/functionally homologous to the BBB of mammals.

We have identified liposomes (lipid-based nanoparticles) that accumulate at the BBB of embryonic zebrafish with unprecedented selectivity, and are subsequently endocytosed by brain endothelial cells (bECs). Cryo-electron microscopy revealed a highly unusual liposome morphology, characterized by a phase separated lipid droplet, which is essential for BBB targeting. Mechanistic studies revealed that bEC uptake was fully ablated after co-injection with heparin. Heparin is known to release proteins that are non-covalently attached to heparan sulfate proteoglycans (HSPG) on the surface of endothelial cells, including lipoprotein lipase (LPL). LPL is primarily responsible for the tissue specific metabolism of lipids, providing cells with essential and non-essential fatty acids. Pre-injection of a selective endothelial LPL inhibitor (XEN445) also diminished liposome accumulation at the BBB in a concentration dependent manner. However, since LPL cannot itself trigger endocytosis, a secondary membrane receptor is likely to be involved, and we suggest a similar two-step mechanism for targeted BBB delivery that is known to result in the internalization of endogenous very low density lipoproteins (VLDL) and chylomicrons.

Elucidating the complete biological mechanism of liposome BBB-targeting in the embryonic zebrafish might be essential in the successful translation of a new active targeting drug delivery technology, based on lipids only, to diagnostic and/or therapeutic applications in mammals.

## Plasma Membrane Calcium ATPase 4 Gene Expression is Downregulated in Pulmonary Artery Endothelial Cells Treated with Inducers of Pulmonary Arterial Hypertension

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**Introduction:** Pulmonary arterial hypertension (PAH) is a life-threatening disease characterized by a progressive narrowing and occlusion of small pulmonary arteries. It leads to increased pulmonary resistance and finally right ventricular failure. Increased activity of pro-inflammatory cytokines is linked to PAH development. We have analysed the effect of TNF- $\alpha$  and IL-1 $\beta$  in the expression of Plasma Membrane Calcium ATPase 4 (PMCA4) in human pulmonary artery endothelial cells (PAEC).

**Methods:** PAEC were cultured for different times and with different doses of TNF- $\alpha$  or IL-1 $\beta$ . Expression of PMCA4 RNA and protein was determined by qPCR and western blot respectively. PMCA4 expression was silenced using siRNA specific for human PMCA4. Quantification of apoptotic cells was performed by flow cytometry.

**Results:** TNF- $\alpha$  or IL-1 $\beta$  induced a time- and dose-dependent decrease in PMCA4 RNA levels in PAEC. Analysis of PMCA4 RNA levels in the lungs of mice with overexpression of ectopic TNF- $\alpha$  confirmed the *in vivo* relevance of our observations. In agreement with the reduction in RNA, PMCA4 protein expression strongly decreased by treating PAEC with TNF- $\alpha$  or IL-1 $\beta$ . Importantly, silencing PMCA4 gene expression sensitised PAEC to apoptosis, suggesting that PMCA4 protects PAEC to apoptosis induced by pro-inflammatory cytokines.

**Conclusion:** The pro-inflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$  significantly downregulate the expression of the PMCA4 gene in PAEC at the RNA and protein level. Decrease in PMCA4 expression sensitises PAEC to apoptosis, what might play an important role in the apoptotic loss of endothelial cells observed in the pulmonary arterioles of patients with PAH.

### Resveratrol-Loaded Nanostructured Lipid Carriers Restore Dilator Function Following Acute Pressure Elevation, Ex Vivo

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**Introduction:** Resveratrol (RV) is a polyphenolic phytoalexin compound with antioxidant and anti-inflammatory properties, but with poor stability and solubility, leading to limited bioavailability. Nanostructured lipid carriers (NLC) are an emerging platform that can be used for the encapsulation of RV for improved delivery. The aim of this study is to determine the effect of RV-NLCs on vasodilator responses following acute pressure elevation, ex vivo.

**Methods:** NLCs were synthesised and characterised using chemical techniques (including dynamic light scattering, laser doppler micro-electrophoresis (zeta potential), ultraviolet-visible spectroscopy and fluorescence spectroscopy). The effect of RV-NLCs on human coronary artery endothelial cell viability was determined using an alamar blue assay and the generation of mitochondrial superoxide (O<sub>2</sub><sup>-</sup>) was quantified using MitoSOX™. NLC uptake into cells was assessed using fluorescence microscopy. Data are expressed as mean ± SEM.

Endothelial-dependent (acetylcholine, ACh 1.0 nM – 1.0 mM) and independent (sodium nitroprusside – SNP, 100 µM, papaverine – PAPA, 100 µM) responses were assessed in isolated coronary arteries at 60 mm Hg and following acute pressure elevation (150 mm Hg, 30 minutes) in the presence/absence of RV-NLCs/superoxide dismutase, using pressure myography. The influence of RV-NLCs on the dilator component of vessels was assessed using pharmacological inhibition: nitric oxide synthase inhibitor N<sup>ω</sup>-nitro-L-arginine (L-NNA, 100 µM), small and intermediate calcium activated potassium channel blockers apamin (100 µM) and TRAM-34 (1 µM) respectively, as well as the cyclooxygenase (COX)-1 inhibitor, indomethacin (10 µM).

**Results:** Cell viability was unaffected following 24 h incubation with RV-NLCs. RV-NLCs induced a reduction in the generation of superoxide anions, after 30 minutes exposure to hydrogen peroxide. Uptake was confirmed using RV-dye-loaded-NLCs. Endothelium-dependent (ACh) dilation was significantly improved following incubation with RV-NLCs, after elevated pressure (@ ACh 10 µM: 52 ± 8 and 79 ± 17, respectively; p ≤ 0.05) and following incubation in SOD (82 ± 5; p ≤ 0.05). SNP and PAPA responses were unaffected. Whilst incubation in the presence of L-NNA alone led to a significant reduction in ACh dilation (-35 ± 17 vs control 52 ± 8; p ≤ 0.001), this was significantly improved in the presence of L-NNA + RV (25 ± 13; p ≤ 0.01).

**Conclusions:** RV-NLCs have the potential to restore the ROS-mediated attenuation of endothelial-dependent dilator responses following acute pressure elevation, ex vivo. Our findings have important implications for the future design and implementation of anti-hypertensive treatment strategies.

### Determining the Role of the Transcription Factor TBX1 in Endothelial Cell Differentiation

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**Introduction:** Tbx1 expression in endothelial cells (EC) is essential for vascular development in mice. EC-specific inactivation of Tbx1 leads to lymphatic and brain vessel anomalies. Tbx1 regulates two essential vascular genes, Vegfr2 and Vegfr3, in ECs. We are using genetic manipulation in vitro and in vivo to study the interplay between these genes in endothelial cell differentiation and early vascular development. The purpose of this study is to understand better the mechanisms responsible for cell fate transitions in multipotent progenitors during embryonic development to form derivative tissues, in particular endothelial cells.

**Methods:** For the in vitro approaches we are using cultured differentiating murine embryonic stem cells (mESC), using a serum-free procedure that induces cardiomyocyte (CM) and EC differentiation. RNA-seq and ATAC-seq are being used to identify the molecular events involved in EC differentiation and to determine the involvement of Tbx1 in this process.

**Results:** We found that mESC differentiation promoted expression of Tbx1 and several EC-specific markers, including, Pecam1, VE-cadherin, Kdr. RNAseq performed on mESCs between day 2 and day 4 of differentiation, which coincides with the peak of Tbx1 expression, identified 44 angiogenesis genes (GO term/DAVID analysis), suggesting that an the EC transcription program is activated in this time interval. ATACseq data from differentiating mESCs (day 2-day 4) identified two putative enhancers in the Pecam1 and Notch1 genes, both of which are critical for vascular development. The enhancers are being validated by deleting them in mESCs using the Crispr-Cas9 technology. Their requirement in EC differentiation will then be tested in functional assays.

**Conclusions:** The induction of many EC-specific genes indicates that the differentiation protocol in use is a good experimental cell-based model to investigate the molecular events involved in EC differentiation. It also allows us to apply powerful genome-wide approaches to identify candidate Tbx1 interacting genes and tissue-specific enhancers in cells and in murine embryos.

## Effects of Far Infrared Irradiation on Vascular Function in Healthy Subjects

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**Introduction:** Far infrared (FIR) is an invisible electromagnetic wave which is a source of heat. It improves endothelial dysfunction in patients with chronic heart failure. Thus, this study aims to investigate the effects of FIR on endothelial function and vascular compliance in healthy subjects.

**Methods:** 70 healthy, non-smoking volunteers (30 ± 11 years) participated in this study. Participants were assigned blindly into 6 groups (n = 10, except for the first two groups where n = 15). Two groups received FIR on the back with two different testing protocols (protocol-1 and protocol-2), two groups received FIR to the forearm following testing protocol-1 and protocol-2, one group received heat and one placebo-receiving group. The latter two interventions were delivered to the back only following protocol-1. FIR was delivered using a FIR ray device, a heating pad set at 38°C was used for the heat group and for the placebo group, a switched-off FIR device was used. Four 40-minute-session of FIR, heat or placebo were given over 2 weeks. Endothelial function was assessed using laser Doppler imaging with the iontophoresis of endothelial dependent agent, acetylcholine (ACh) and endothelial independent agent, sodium nitroprusside (SNP). Arterial stiffness was measured using pulse wave analysis to determine augmentation index (AIx) using Sphygmocor. Finally, blood was obtained to look at the expression of 9 genes regulated by the nuclear factor erythroid 2-related factor 2 (Nrf2), Nrf2, Kelch-like ECH-associated protein 1 (KEAP1) and endothelial nitric oxide synthase (eNOS) in Peripheral Blood Mononuclear Cells. These assessments were performed following two protocols. Protocol-1: at baseline, immediately after the 1st session and 24-hour after the last session. Protocol-2: at baseline, 4-hour after the 1st session and 4-hour after the last session.

**Results:** In comparison with baseline ACh and SNP responses were significantly improved with FIR treated back (protocol-1) (overall P-value = 0.02 and 0.046 respectively) and greater responses were seen with protocol-2 (overall P-value = 0.005 and 0.05 respectively). AIx significantly improved compared with baseline after FIR session(s) to the back in both protocols (overall P-value <0.001). Few Nrf2 related genes and KEAP1 were significantly expressed in FIR treated back (protocol-1). Significant expression of Nrf2, some Nrf2 related genes and eNOS was seen in FIR treated back (protocol-2). Nrf2 and KEAP1 were significantly expressed in FIR treated forearm (protocol-2).

**Conclusion:** FIR non-thermally improves endothelial function and vascular elasticity in healthy subjects when irradiating large areas i.e. back, possibly through the upregulation of NOS3 and Nrf2.

## Non-Coding RNA Control of Pathological Vascular Remodelling

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My laboratory is interested in the pathological remodelling of the vessel wall in response to injury. For example, we have a long standing interest in the development of a gene therapy strategy to block pathological remodelling in vein grafts that is currently under clinical evaluation targeting matrix metalloproteinase activity after implantation of autologous saphenous veins in to the coronary circulation. We have also had a substantial interest in developing research into the non-coding RNA transcriptome as how it control vascular smooth muscle cell function in the proliferative and migratory phase. We have identified both miRNA (such as miR-21) and long non-coding RNA (e.g. SMILR) that are activated in response to injury. We have developed both mechanistic and translational insight. In particular, SMILR has a novel mechanism of action where it intersects the pathway of cytokinesis to control the proliferation of vascular smooth muscle cells. We have also focused on the endothelial compartment in vascular pathologies and have used different sequencing approaches (deep and single cell) to identify causal pathways that impact upon vascular remodelling. We have a particular interest in the long non-coding RNA miR-503HG and its role in EndMT. Research focusing on these pathways will be presented.

## 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 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 mice. 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 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. To determine whether these paravascular spac-

es are inflow or outflow pathways, fluorescent tracers were injected into the brain parenchyma. Tracers injected directly into the hippocampus dispersed inhomogeneous, with accumulation at border zones between brain parenchyma and CSF, and accumulation and spreading along arteries. This suggests that interstitial fluid drains into the CSF along paravascular spaces, while larger solutes are retained by sieving. Distribution of tracers was drastically enhanced in spontaneously hypertensive rats, indicating enhanced production of interstitial fluid, possibly from capillary leakage. Collectively, these data suggest that paravascular spaces around leptomeningeal arteries form low resistance pathways on the surface of the brain that facilitate CSF flow. Much more narrow paravascular spaces exist around arteries that penetrate the brain and facilitate net outflow of interstitial fluid into the CSF. Most likely, arterial pulsations induce mixing of the fluid in these spaces, which aids the removal of waste products from the brain.

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### Lymph Flow Mediated Postnatal Remodeling of Meningeal Lymphatics is Required for the Uptake and Transport of Macromolecules from the Central Nervous System

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**Introduction:** Until recently, the classical view was that the central nervous system (CNS) lacks lymphatic structures. A paradigm shift occurred when the presence of meningeal lymphatics and their possible role in the pathogenesis of Alzheimer's disease and neuroinflammatory diseases have been demonstrated. However, the possible regulators of the developmental program and function of meningeal lymphatics remain unclear.

**Objectives:** Here, we aimed at characterizing the lymph flow dependence of the developmental program and function of the meningeal lymphatics.

**Methods:** Genetic models including lymphatic reporter strains and *Plcy2*<sup>-/-</sup> mice with impaired lymph flow due to blood-filled lymphatics were used. The developmental program was characterized by immunostaining with lymphatic and blood vessels markers. Lymphatic function was followed by injection of macromolecules into the CNS and the drainage to the lymphatics and lymph nodes was monitored.

**Results:** First, the presence of lymphatics in the dura mater was demonstrated. After intracranial injection of labeled macromolecules, we found tracer in the meningeal lymphatics. The meningeal lymphatics develop during the postnatal period when their structural remodeling occurs, which process coincides with the beginning of the drainage of macromolecules from the CNS to the deep cervical lymph nodes. Importantly, the structural remodeling of the meningeal lymphatics is impaired in the absence of lymph flow in *Plcy2*<sup>-/-</sup> mice. Furthermore, macromolecule uptake and

transport by the meningeal lymphatics from the CNS to the cervical area are also affected in *Plcy2*<sup>-/-</sup> mice.

**Conclusions:** Taken together, our results indicate that the meningeal lymphatic vessels are involved in the uptake and transport of macromolecules injected into the CNS. We also found that a lymph flow-mediated postnatal remodeling of the meningeal lymphatics is required for the drainage of macromolecules from the CNS to the deep cervical lymph nodes. Defining the lymph flow-dependence of the development and function of meningeal lymphatics may lead to better understanding of the pathogenesis of neurological diseases such as Alzheimer's disease and neuroinflammatory diseases including multiple sclerosis.

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### PEMF in REHAB at Extreme Sport – Special Case

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**Introduction:** PEMF-Systems get more evaluated, are certified and are structured used by health-care professionals in supporting preparational treatment before and REHAB after accidents in sports.

**Cases:** Extreme sports athlete on high-mountainering, ultramarathon and biking, standard applying specific PEMF-Treatment on high scale, aged 59 and exercising several sport disciplines for 45 years, underwent 3 mostly lethal bike-crashes in 2016 and 2018. Remaining pathology is still present, as documented by X-Ray and MRI: hernia cervicalis C4, hernia lumbalis L4, double contusion cerebri, Baker's Cystia right knee, injury in supra- and infra-spinalis sinister incl. corpus alineum, multiple cut wounds on face, arms e.d.

Injuries should have led "normally" to a very long-term "standard" REHAB with wheel-chair dependency on total or partial paraplegia.

**Treatment:** Immediately after demission from the half-day hospital ambulatory, a specific PEMF-Treatment at the technical maximal possible Tesla level was administered three times/day/15 minutes; several different applicators were used.

PEMF-Treatment on this extreme level was administered for 2 months, supporting osteopathy on weekly scale, application-set of nutritional supplements and special REHAB-Training at the gym, prescribed by physiotherapist, well extended to 300% on own risk by proband.

After 2 months, the specific PEMF-Treatment was reduced to individual "standard" scale of proband, which is the maximal Tesla-dose on the device.

No medication was administered.

**Results:** 1 week after each of the three crashes, proband was still able to follow training program on ± 70% of his standard level, hernia-precaution included.

3 weeks after each of the crashes, proband could switch to his standard training-program for the following extreme sport event,

including track and field running with 12 kg extra weight as heavy gym-exercises. Summer 2016 up to April 2017 for Marathon des Sables and summer 2018 for extreme mountain-run and bike-challenge.

**Positive Side-Effect:** Specific PEMF-Treatment is applied on a structural level since spring 2016 before starting training and afterwards for regeneration. Proband never suffered any more from cramps, muscle injuries or –pain, joint problems or overload symptoms of the muscle-skeletal system.

**Conclusions and Future Directions:** Specific PEMF-Treatment as single-use-therapy and/or as supporting other therapeutic approaches in extreme sports need to be more tested and evaluated.

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### Specific PEMF-Treatment in Hypertension Therapy – Cases

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**Introduction:** PEMF-Systems get more evaluated, are certified and are structural used by health-care professionals in supporting treatment, single-use or combined with other therapies.

Specific PEMF-Systems are proved to stimulate the Vasomotion and hereby, microcirculation betters.

**Methods:** Several patients with (essential) hypertension without any other patho-mechanism, treated with anti-hypertensiva, whose GP's/PhD's accepted a therapeutical treatment by a specific PEMF-System, as conducted by a certified therapist in function of research nurse. Medicine was stopped and a specific PEMF-Treatment on a standard method was started.

**Results:** After 2 weeks, a significant lower RR systolic & diastolic was stated.

6 months later, GP's/PhD's didn't restart anti-hypertensiva and a structural PEMF-treatment continued under supervision of the nurse and in a scheme of 2 times/day for minimal 8 minutes.

12 months later, the PEMF-Treatment as therapy for hypertension was still being accepted by GP/PhD. Controls on regular scheme shows a continuously stability of the RR in a normal range, diastolic up to max. 87 mm/Hg. No medication was necessary or had to be re-started.

The 18 mths, 24 mths and 30 mths evaluation showed the same results.

**Positive Side-Effect:** Quality of life bettered, home-treatment with own system started and the negative side-effects of anti-hypertensiva were prevented.

**Positive Side Effect on Other Pathology:** One of the patients also suffers from high obesity and DM, type 1.

On treatment day, we asked for security reason, to measure the blood glucose the evening before insulin injection. More security to advice the right dose is obliged. Each treatment day, the results of these measurements were 30% lower than on days without

PEMF Treatment. Thus, the patient adapted the value to the insulin-regime and had to administer a about 30% lower dose.

Hypothetic explanation may be, that this PEMF-System also stimulates-even on a short-time range – the function of the Langerhans Cells and a probably weak insulin-production may be stimulated. More research on this will be necessary.

**Conclusions and Future Directions:** Specific PEMF-Treatment as single-use-therapy and/or as support other therapeutic approaches need to be more evaluated and been updated in Medical Compendia, combined with the specific PEMF-Systems.

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### Beyond Pericytes – PDGF-BB Accelerates Vascular Stabilization by Stimulating the Semaphorin3A/Neuropilin1+ Monocyte Axis

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**Introduction/Objective:** Vascular Endothelial Growth Factor (VEGF) is the master regulator of angiogenesis. However its therapeutic potential is challenged by the need to control both the dose and duration of expression: high and sustained expression causes aberrant angioma-like tumors, but transient delivery shorter than about 4 weeks is insufficient for stabilization and persistence of induced vessels. Therefore, it would be desirable to accelerate new vessel stabilization to enable transient and safe VEGF delivery. We previously found that: 1) increasing VEGF doses impair vessel stabilization by inhibiting the endothelial Semaphorin3A (Sema3A)/Nrp1+ monocytes (NEM)/TGF- $\beta$ 1 paracrine axis; and 2) Platelet-Derived Growth Factor-BB (PDGF-BB) co-expression restores normal angiogenesis despite high VEGF levels. Here we investigated whether and how PDGF-BB accelerates the stabilization of VEGF dose-dependent angiogenesis.

**Methods:** Mouse leg muscles were implanted with monoclonal populations of genetically modified myoblasts homogeneously producing specific VEGF levels that cause normal (low and medium) or aberrant angiogenesis (high), alone or with a fixed 1:3 ratio of PDGF-BB (V+P). VEGF signaling was abrogated by systemic treatment with the receptor-body Aflibercept after 2 or 3 weeks.

**Results:** PDGF-BB did not change the effects of low and medium VEGF, but it greatly accelerated vascular stabilization with high VEGF (80% at 3 weeks vs 0% with VEGF alone). All normal capillaries induced by different V or V+P doses displayed similar pericyte coverage and functional perfusion, despite different stabilization rates. However, PDGF-BB co-expression restored Sema3A production, NEM recruitment and TGF- $\beta$ 1 levels in vivo despite high VEGF, leading to endothelial quiescence. PDGF-BB directly increased Sema3A expression dose-dependently in vivo even without VEGF. Blockade of Sema3A/Nrp1 binding abolished NEM recruitment and greatly reduced vessel stabilization by both V and V+P, but interestingly a Sema3A/NEM-independent fraction of about 30% persisted in all conditions. In vitro VEGF and PDGF-BB had direct and opposing effects on endothelial Sema3a. Co-

expression prevented VEGF-induced loss of Sema3a both in vitro and in ex vivo-isolated endothelium, but did not increase it compared to controls. However, in situ hybridization revealed that PDGF-BB specifically and dose-dependently expanded a non-endothelial source of Sema3A expression.

**Conclusions:** We identified a novel role for PDGF-BB to promote vascular stabilization independently from pericyte recruitment, by regulating the Sema3A/NEM/TGF- $\beta$ 1 axis.

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### SIRT1 Deacetylation of p53 Inhibits Hyperglycaemic Induced Senescence in Vascular Smooth Muscle Cells

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**Introduction:** Accumulation of senescent cells plays a pivotal role in type II diabetes, through senescence-associated secretory phenotype (SASP) tissue damage and metabolic dysfunction. Irreversible growth arrest within the smooth muscle layer occurs in response to cellular stresses, such as telomere erosion, DNA damage or aberrant cell signalling pathways, which become activated during hyperglycaemia. Sirtuin 1 (SIRT1), an NAD<sup>+</sup>-dependant deacetylase, has a beneficial role in insulin sensitivity and glucose homeostasis, suggesting a link between senescence, hyperglycaemia and SIRT1 in diabetes. This study aims to investigate the protective role of SIRT1 against hyperglycaemic-induced senescence.

**Methods:** Diabetic vessels harvested from 10 patients undergoing limb amputation (14/NW/1062) and 6 non-atherosclerotic IMA vessels underwent histological staining. vSMCs were incubated in low (5 mM) or high (25 mM) glucose to reflect a diabetic milieu supplemented with osteogenic factors. SIRT1 was quantified via qPCR and western blotting, following Sirtinol inhibition and SIRT1720 activation. Senescence was confirmed via qPCR analysis, immunofluorescence and  $\beta$ -galactosidase staining (SA $\beta$ -Gal). Acetylation of the p53 promoter region was confirmed via chromatin immunoprecipitation. Proliferation and apoptosis were confirmed by AlamarBlue and caspase 3/7 cleavage respectively.

**Results:** Senescence was increased in diabetic vessels compared to IMA controls, shown by increased p21 staining and lipofuscin accumulation in both the vSMC and endothelial layer. Diabetic vSMCs exhibited decreased telomere length ( $p < 0.05$ ) and a reduction in SIRT1 expression at both mRNA ( $p < 0.005$ ) and protein level ( $p < 0.05$ ) compared to controls. vSMCs cultured in hyperglycaemic conditions demonstrated increased SA $\beta$ -Gal staining and decreased proliferation after four days treatment ( $p < 0.05$ ). SIRT1 inhibition significantly decreased cellular proliferation ( $p < 0.005$ ) and increased cellular senescence ( $p < 0.001$ ), however did not affect apoptosis. Conversely, SIRT1 activation reduced  $\beta$ -galactosidase staining by 50% ( $p < 0.05$ ) and increased proliferation by a third in hyperglycaemic conditions compared to untreated controls ( $p < 0.001$ ). Acetylation of p53 transcription factor increased in vSMCs within hyperglycaemic conditions ( $p < 0.05$ ) compared with control, correlating with the up-regulation of

mRNA markers for the senescent associated phenotype; p16 ( $p < 0.005$ ) and p21 ( $p < 0.005$ ), the latter showed increased translocation to the nucleus within hyperglycaemic conditions. SIRT1 activation reduced p53 acetylation by 15% ( $p < 0.005$ ), inhibiting production of both p21 ( $p < 0.005$ ) and p16 ( $p < 0.005$ ) in hyperglycaemic conditions.

**Conclusions:** A pronounced loss of SIRT1 within diabetic vSMCs may be responsible for the increase in hyperglycaemic-induced senescence and the development of the senescence-associated phenotype within vSMCs. Reactivation of the Sirtuin pathway within this model suggests an essential role for SIRT1 via deacetylation of p53 and a reduction in its downstream markers.

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### Single Cell Transcriptomics Identifies a Hedgehog-Mediated Immunomodulatory Signaling Circuit Between Endothelial and Perivascular Stromal Cells in the Eye Choroid

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The choroid is a highly vascularized layer of the eye localized between the sclera and the outermost retinal layer, the retinal pigment epithelium (RPE). Blood supplied by choroidal circulation is the main source of oxygen and nutrients for the RPE and photoreceptors, as well as the main evacuation route for retinal waste. However, microvascular endothelial cells (ECs) are not just passive conduits for delivering blood. Rather, ECs also play tissue-specific functions by providing highly specialized sets of angiocrine factors at different body locations that maintain tissue homeostasis. In the eye, the specific repertoire of factors expressed by choroid ECs and the identity of their target cells remain unknown. Moreover, the potential role of such intercellular crosstalk in choroid homeostasis has not been studied so far. Herein, we report the first single cell RNAseq analysis of adult mouse RPE/choroid tissue, identifying 13 main cell types including 3 subtypes of ECs. By combining these results with a transcriptomic analysis of tissue-specific ECs, we found a marked enrichment of Indian Hedgehog expression in choroid ECs, particularly in those located in close apposition to the RPE. Using reporter mice, we identified the target of choroidal Hedgehog (Hh) signaling as a large population of stromal, GLI1+ perivascular mesenchymal stem cell-like cells. Stimulation of iso-

lated GLI1+ choroidal cells with a Hh agonist induced profound transcriptional changes related to immune response. Indeed, genetic manipulation of the Hh pathway in vivo induced significant loss of choroidal mast cells, as well as an altered inflammatory response and exacerbated visual function defects after retinal damage. Our studies reveal the cellular and molecular landscape of adult RPE/choroid and uncover a Hedgehog-regulated choroidal immunomodulatory signaling circuit. These results open new avenues for the study and treatment of retinal vascular diseases and choroid-related inflammatory blinding disorders.

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### Effect of Naringin on Type 2 Diabetes-Induced Endothelial Dysfunction and Cognitive Impairment Correlates with AGEs/RAGE and NF- $\kappa$ B Inhibition

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**Introduction/Objective:** Accumulation of AGEs under hyperglycemia and insulin resistance plays an important role in the development of endothelial dysfunction and subsequent cognitive impairment. Moreover, AGEs also accelerate the expression of RAGE and they promote oxidative stress and inflammation in several tissues including brain. Evidence from several studies suggest that naringin, a natural flavonoid compound extracted from grapefruit or Citrus fruits, possessed strong antioxidant and anti-inflammatory properties. Therefore, we investigated the effects of naringin on cerebral endothelial dysfunction and cognitive impairment in type 2 diabetic rat (DM2) and its possible mechanism.

**Methods:** Six weeks old Spargue-Dawley rats were divided into three groups (n = 8/group); normal group (CON), type 2 diabetes group without and with administration of naringin (DM2 and DM2-NG, respectively). To induce DM2, rats were fed with high-fat diet for three weeks followed by single injection of streptozotocin (STZ: 30 mg/kg I.V.). Naringin was administered by gavage feeding (50 mg/kg B.W.). After 12 weeks of naringin administration, fasting blood glucose (FBG), HbA1C and serum insulin (S. insulin) were evaluated. Cognitive function was determined by using Morris Water Maze test. Cerebral blood flow (CBF) was evaluated using a Laser Doppler flowmeter. To examine the endothelial function, leukocytes adhesion (LA) to the venular endothelium was examined. Additionally, levels of AGEs, RAGE, pro-inflammatory cytokines (TNF- $\alpha$  and IL-6), malondialdehyde (MDA), and expression of NF- $\kappa$ B (p65) in the hippocampus were evaluated. The experiments were approved by the ethical committee, Faculty of Medicine, Srinakharinwirot University, Thailand.

**Results:** DM2 rats developed hyperglycemia and insulin resistance. Naringin treatment significantly decreased FBG and S. insulin levels in DM2-NG rats. In addition, in DM2-NG rats, the CBF was significantly greater whereas the LA was significantly less than that in the DM2-rats. Furthermore, performance on Morris water

maze test was improved after naringin administration in DM2-NG rats. Interestingly, naringin treatment reduced the levels of AGEs, RAGE, MDA, TNF- $\alpha$ , IL-6 and expression of NF- $\kappa$ B in hippocampal tissue.

**Conclusion:** The results of this study suggested that naringin treatment attenuates diabetes-induced cognitive impairment via restoring endothelial function through antioxidant and anti-inflammatory properties involvement of AGEs-RAGE-NF- $\kappa$ B system.

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### Vascular Cell Ageing and Senescence

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The most important clinical manifestations of atherosclerosis are myocardial infarction (MI) and thrombotic stroke, predominantly due to plaque rupture or erosion; the processes that regulate stability of advanced plaques therefore represent major targets for therapy. The fibrous cap protects the plaque from rupture, and is synthesised by vascular smooth muscle cells (VSMCs). However, VSMCs in advanced plaques demonstrate poor proliferation, early senescence and apoptosis.

VSMC senescence causes irreversible growth arrest, while both senescence and cell death promote inflammation, both of which promote plaque instability. We have examined the mechanisms and consequences of VSMC senescence in atherosclerosis and vascular ageing, and in particular the role of DNA damage and mitochondrial dysfunction. We find that plaques demonstrate a range of DNA damage and reduced mitochondrial function, and correction of this damage and augmenting mitochondrial function reduces atherosclerosis. Similarly, mitochondrial damage occurs in vascular ageing, and can be reversed by augmenting mitochondrial function. Finally, we have identified novel markers of VSMC senescence, and show the therapeutic possibilities of clearance of senescent VSMCs.

### Characteristics of the Retinal Microvasculature in Association with Cardiovascular Risk Markers in Children with Overweight, Obesity and Morbid Obesity

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**Objective:** To characterize the retinal microvasculature and potential associations with classic and novel (endothelial function and low-grade inflammation) markers for cardiovascular risk, in a cohort of children with overweight and (morbid) obesity.

**Patient and Methods:** Children who started participating in the Centre for Overweight Adolescent and Children's Healthcare program between 2011 and 2015 and from whom fundus images were available at the start of their participation were retrospectively included. Their assessment included, amongst others, fasting blood examination and fundus photography. For the latter, we determined the central retinal arteriolar equivalent (CRAE), central retinal venular equivalent (CRVE) and their ratio (AVR). Body mass index (BMI) was calculated and BMI z scores were obtained using a growth analyzer. In addition, 15 children with normal weight were included as healthy reference point.

**Results:** In total, 226 overweight and obese children (43% boys) with a median age of 13.0 years and a median BMI z score of 3.25 were enrolled. Twenty percent was classified as overweight, 46% obese and 34% morbidly obese. CRAE was significantly lower and AVR significantly higher in children with morbid obesity than in children with overweight and normal weight ( $p < 0.01$ ). CRVE did not differ significantly between the weight categories. A multiple linear regression model with CRAE as dependent variable showed that only diastolic blood pressure (DBP) z-score ( $\beta = -2.85$ ,  $p = 0.029$ ) and plasma glucose concentrations ( $\beta = 6.03$ ,  $p = 0.019$ ) contributed significantly to the variation in CRAE.

**Conclusions:** This is the first study showing in a population of children with overweight and obesity that the retinal arteriolar diameter, but not venular diameter, is aberrant with increasing BMI z-score and that CRAE was significantly associated with several cardiovascular risk markers.

### Endothelial Function and Cardiovascular Stress Markers in ApoE Knockout Rats After a Single Simulated Heliox Dive

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**Introduction:** Compressed gas breathing during diving augments partial pressure of oxygen, causing the oxygen concentration of the blood to increase above normal (hyperoxia). Hyperoxia in combination with gas bubbles that develop during the decompression (ascent) phase, likely causing oxidative stress leading to endothelial dysfunction. The number of aging divers is rising and aging itself is associated with a gradual impairment of endothelial function. These alterations play a central role in the pathogenesis of atherosclerosis and coronary artery disease. While diving and aging are independent modulators of cardiovascular function, little is known about their combined effect. Thus, the central question is: does diving expose already impaired cardiovascular system to further endothelial damage?

**Methods:** ApoE homozygous knockouts (KO) rats that are prone to develop atherosclerosis due to defect in lipoprotein metabolism were used as a model for atherosclerosis. 10 ApoE rats (male and female) were exposed to 500 kPa heliox gas (80% helium/20% oxygen) for 1 hr in a pressure chamber to simulate diving. 10 ApoE rats served as a control group. Endothelial function was examined in-vitro by isometric myography of the pulmonary and mesenteric artery. Oxidative stress biomarkers were measured in plasma (collected from the heart ventricle) and lung tissue via TBARS assay. Relative expression of total and phosphorylated endothelial NO synthase was quantified by Western blot and normalized to loading control, alpha-actin.

**Results and Conclusion:** The results demonstrated that a single dive causes endothelial dysfunction in pulmonary arteries of ApoE KO rats. This seems to be associated with reduced relative contribution of all three major endothelium-dependent relaxing pathways (NO, cyclooxygenase product(s) and endothelium-dependent hyperpolarizing factor). These responses were more aggravated in male than female rats.

## Diabetes Induces Premature Senescence in Retinal Microvascular Endothelial Cells

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**Introduction:** Diabetic Retinopathy (DR) is a highly frequent microvascular complication in diabetic patients. Diabetes is associated with endothelial dysfunction. The ageing process in vascular cells is known as endothelial senescence. The aim of this study is to investigate the senescence profile of retinal microvascular endothelial cells and to elucidate how this is affected by the diabetic milieu.

**Methods:** Human Retinal Microvascular Endothelial Cells (HRMECs) were grown under normal and high glucose conditions (NG: 5 mM D-glucose; HG: 25 mM D-glucose). Senescence-associated (SA)- $\beta$ -galactosidase staining was performed to quantify cellular senescence and growth curves with Population Doubling Level (PDL) to assess for proliferative capacity. Tube formation capacity was studied using 3D-Matrigel angiogenesis assay. Endothelial barrier function was examined through trans-endothelial electrical resistance measurements using the xCELLigence. The Agilent Seahorse XF technology allowed the investigation of the cellular metabolic profiles in HRMECs. To investigate senescence in vivo, the oxygen-induced retinopathy (OIR) and Streptozotocin (STZ) mouse models were used. Retinas were dissected for mRNA extraction and immunohistochemistry. Gene expression profiles were assessed via RT-qPCR.

**Results:** Senescent HRMECs showed significant increase in cellular size and SA- $\beta$ -galactosidase staining. Senescence-Associated Secretory Phenotype (SASP) components, such as IL8, and cell cycle-associated genes, such as CCNB2, were upregulated and downregulated, respectively. After 4 weeks, HG-HRMECs start exhibiting significantly lower PDLs when compared to NG-HRMECs. The number of SA- $\beta$ -Galactosidase positive cells and the expression of SASP components were significantly higher in late-passage HG-HRMECs when compared to age matched NG-HRMECs. Long-term HG treated cells showed less tubulogenic potential and increased barrier permeability than NG-HRMECs. Senescent HRMECs were more glycolytic than early-passage cells. Interestingly, long-term high glucose significantly decreased HRMECs glycolytic potential. There was significantly higher SA- $\beta$ -galactosidase staining in OIR retinas. Both OIR and STZ retinas showed increased gene expression of senescent markers, such as p53 and SASP components, such as IL1 $\beta$ .

**Conclusion:** In summary, our results indicate that diabetic conditions induce premature cellular senescence in retinal endothelial cells in vitro and in vivo. Senescent HRMECs show decreased tubulogenic ability, increased permeability and impaired metabolic potential. Moreover, we show evidence to demonstrate accumulation of senescent cells in the diabetic and ischaemic mouse retina.

## Wnt5A Dependent Revascularisation After Ischemia Is SRPK1 Dependent

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**Introduction:** Vascular insufficiency in peripheral artery disease (PAD) results in tissue ischemia. In response, circulating monocytes produce vascular endothelial growth factor (VEGF-A). Patients with PAD and murine models of metabolic ischemic disease have reduced soluble frizzled related protein (sfrp5) resulting in increased wingless-type MMTV integration site family 5a (Wnt5a) activity and monocytes from both humans and mice overexpress the anti-angiogenic isoform, VEGF-A<sub>165b</sub>. VEGF splicing is regulated by the phosphorylation of serine/arginine splicing factor 1 (SRSF1) by serine-arginine protein kinase 1 (SRPK1). We therefore investigated the effect of SRPK1 inhibition of revascularisation after ischemia in two different transgenic mouse models of Wnt5A hyperactivity.

**Methods:** Wildtype (WT), sfrp5 knockout (Sfrp5<sup>-/-</sup>) and Wnt5a gain of function (LysM-Wnt5a<sup>GOF</sup>) mice underwent left femoral artery ligation. Blood flow to the paw was measured by Moor FLPI-2 Laser Speckle imaging before and after surgery and on post-operative days, 3, 7, 14 and 21. Animals received SPHINX31 (0.8 mg/kg, i.p.) biweekly from day 1. The ratio of the ischemic vs non-ischemic speckle intensity was calculated and plotted against time to determine the blood flow recovery. Muscles were stained to measure capillary and arteriole density.

**Results:** Blood flow recovery in sfrp5<sup>-/-</sup> mice (day 7 55  $\pm$  5%, day 14 54  $\pm$  5%, day 21 46  $\pm$  5.7% of contralateral, N = 5) was slower than the littermate controls (day 7 69  $\pm$  11%, day 14 63  $\pm$  7%, day 21 70.3  $\pm$  10.9%, N = 5). This was associated with a reduction in the density of arterioles after ischemia in the gastrocnemius muscle. This impaired revascularisation was rescued by SPHINX31 immediately after the ligation and twice a week thereafter (day 7 63.6  $\pm$  6.8%, day 14 93  $\pm$  8.1%, day 21 75  $\pm$  6.5%, N = 8). SPHINX31 had no effect on wild type revascularisation (day 7 73  $\pm$  7.8%, day 14 79  $\pm$  6.9%, day 21 80  $\pm$  5%, N = 8).

The impairment was due to an effect of SRPK1 activation on monocyte derived Wnt5a, as the revascularisation in LysM-Wnt5a<sup>GOF</sup> (day 3 19  $\pm$  2.56%, day 7 40  $\pm$  3%, day 14 58  $\pm$  7.7%, N = 7), was significantly slower than littermate WT mice (day 3 70  $\pm$  4%, day 7 80  $\pm$  3%, day 14 91  $\pm$  6.2%). This was rescued by SPHINX31 on day 3 (57  $\pm$  14%), day 7 (62  $\pm$  11%) and day 14 80  $\pm$  10% (p < 0.0001, two-way ANOVA).

**Conclusion:** Increased activity of Wnt5a in two different PAD models result in impaired collateralisation and insufficient angiogenesis. This is reversed with SPHINX31 suggesting that impaired revascularisation in patients with PAD could be SRPK1 dependent, and could be treated with SRPK1 inhibitors.

## Smooth Muscle Cell Plasticity in Atherosclerosis. Role of S100A4

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During atherosclerosis, smooth muscle cells (SMCs) accumulate in the intima and switch from a contractile to a synthetic phenotype. During the two last decades, synthetic SMCs have been considered as beneficial players in atherosclerotic plaque development by essentially contributing to fibrous cap formation that protects the plaque from rupture. However SMCs exhibit a remarkable plasticity depending on environmental cues/signals, even acquiring inflammatory cell markers.

Our research on SMC heterogeneity has been instrumental in deciphering mechanisms involved in SMC phenotypic transition. In our model, we have isolated two distinct SMC populations, spindle-shaped (S) and rhomboid (R), from the porcine coronary artery. The S-phenotype is typical of the contractile phenotype whereas the R-phenotype represents the synthetic phenotype. We identified S100A4 as a marker of activated SMCs *in vitro* and of intimal SMCs both in mouse, pig, and man. In the last years we have investigated the role of S100A4 in atherosclerotic plaque. We observed that, after treatment of S-SMCs with S100A4-rich conditioned medium (collected from S100A4-transfected SMCs), SMCs switched towards R-phenotype associated with acquisition of pro-inflammatory properties.

To study the exclusive role of S100A4 in SMCs phenotypic transition, S-SMCs were treated with multimeric recombinant S100A4, which resulted in partial transition from S- to R-phenotype and NFκB activation. Remarkably, treatment of S-SMCs with multimeric S100A4 and platelet-derived growth factor-BB (PDGF-BB) together induced a complete SMC transition toward a R-phenotype associated with pro-inflammatory properties, likely through toll-like receptor-4 (TLR-4). *In vivo*, we have shown that neutralization of extracellular S100A4 decreased area of atherosclerotic lesions, decreased necrotic core and cholesterol cleft, decreased number of CD68 positive cells and increased number of α-smooth muscle actin and smooth muscle myosin heavy chains-positive cells when compared to control groups. We are developing a SMC lineage tracing mouse model associated with SMC-specific deletion of S100A4 to ascertain whether the depletion of SMC-specific S100A4 will impact the formation, composition and progression of atherosclerotic plaques.

Our results indicate that extracellular S100A4 could be a new target to influence the evolution of atherosclerotic plaque, leading to plaque stabilization.

## Bosutinib Protects against Vascular Leakage by Improving Endothelial Cell-Matrix Adhesion

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**Rationale:** Dysfunction of the endothelial barrier leads to uncontrolled fluid extravasation and edema. We previously demonstrated that the Abl-kinase inhibitor (AKI) imatinib protects against vascular leak via inhibition of Abl-related gene (Arg). Since then, next generation AKIs were developed with broader kinase inhibition and better safety profiles.

**Objective:** The current study aims to evaluate whether combined kinase inhibition as provided by next generation AKIs, provides novel treatment strategies against endothelial barrier dysfunction and vascular leakage.

**Materials:** Endothelial barrier function was assessed in primary human umbilical vein endothelial cells and primary lung microvascular endothelial cells using ECIS<sup>®</sup> and macromolecule passage assays. *In vivo* permeability was determined in an acute lung injury mice model.

**Results:** A screen on second and third generation AKIs revealed that bosutinib (Bosulif<sup>®</sup>) has better protective effects on endothelial barrier, as compared to imatinib and other AKIs. Upon exposure to various inflammatory mediators, bosutinib reinforced the endothelial barrier through enhanced focal adhesion formation and adherens junction stabilization. In mice, bosutinib treatment attenuated alveolar protein leakage and development of pulmonary edema. The protective effects of bosutinib resulted from combined inhibition of mitogen-activated protein kinase kinase kinase 4 (MAP4K4) and Arg. MAP4K4 was identified as important regulator of focal adhesion turnover via spatial distribution of ezrin, radixin and moesin. Combined inhibition of Arg and MAP4K4 completely mimicked the protective effect of bosutinib on barrier function.

**Conclusion:** Bosutinib shows a robust protective effect against inflammation-induced endothelial barrier disruption, acting by combined inhibition of Arg and MAP4K4. MAP4K4 was identified as an important novel kinase involved in endothelial barrier regulation that reduces cell-matrix adhesion via turnover of integrin-based adhesions. Because bosutinib is a clinically available drug, reinforcement of cell-matrix adhesions by bosutinib may be a viable strategy against vascular leakage syndromes.

### Genetic Polymorphism of Apolipoprotein E in Hemorrhagic Stroke: Study Case-Control

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**Introduction:** Our study is the type case-control realized at the Hospital of Constantine.

**Objective:** It discusses the relationship between polymorphism of apolipoprotein E and hemorrhagic stroke.

**Method:** The determination of the polymorphism of apolipoprotein E was carried out by PCR-digestion (polymerase chain reaction) using the enzyme of restriction HhaI. The study population consisted of 81 Algerian patients with hemorrhagic stroke, and 509 control subjects.

**Results:** Three isoforms of apolipoprotein E have been identified. The allelic distribution of apo E in the general population showed a predominance of the allele  $\epsilon 3$  (84.3%) followed distantly by allele  $\epsilon 4$  (10.7%) and  $\epsilon 2$  (5%) respectively. In hemorrhagic stroke patients, allele frequencies of  $\epsilon 4$  and  $\epsilon 2$  are respectively 10.5% and 3.3%. These frequencies are not statistically different as reported in the control group. The assessment of the odds ratio of patient subjects with an allele  $\epsilon 4$ ,  $\epsilon 2$ ,  $\epsilon 3/\epsilon 4$ , and  $\epsilon 2/\epsilon 3$  compared to control subjects with genotype  $\epsilon 3/\epsilon 3$  don't show any statistical association between the polymorphism of the apo E and the hemorrhagic stroke.

**Conclusion:** The distribution of apolipoprotein E allele frequencies in the population of Constantine is similar to that of Southern Europe. The  $\epsilon 2$ ,  $\epsilon 4$  alleles do not appear to be implied in the occurrence of this affection; Nevertheless, large additional studies are necessary to confirm these results.

### N-Acetylcysteine Limits Arterial Medial Calcification But Preserves Bone Formation

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Arterial medial calcification (AMC) involves the deposition of calcium phosphate, as hydroxyapatite, in the tunica media. Characterised by increased vessel stiffness and reduced blood flow, it is a significant risk factor for future cardiovascular events. AMC shares similarities with physiological bone formation; however, emerging evidence suggests there are several key differences between these two processes. For example, antioxidants inhibit AMC

but promote bone formation. N-acetylcysteine (NAC), the acetylated form of L-cysteine, displays direct and indirect antioxidant properties.

This study investigated the effects of NAC on AMC and bone formation, both in vitro and in vivo. Human umbilical artery-derived vascular smooth muscle cells (VSMCs) and mouse osteoblasts were cultured in mineralising medium (1 mM  $\beta$ -glycerophosphate, 1 mM sodium phosphate, 50  $\mu$ g/ml ascorbate) and treated with NAC (0.5–5 mM) for up to 7 and 21 days, respectively. VSMC calcification was assessed by colorimetric assay and bone formation by nodule analysis. The effects of NAC on cell function, survival and gene expression were investigated using colorimetric alkaline phosphatase and cytotoxicity assays, flow cytometry and quantitative RT-PCR. NAC was administered daily (300 mg/Kg) to Wistar rats fed a warfarin/vitamin K1-enriched diet for 10 weeks (n = 9–10). AMC and trabecular/cortical bone parameters were assessed using microcomputed x-ray tomography.

NAC potently reduced VSMC calcification ( $\leq 80\%$ ,  $p < 0.001$ ) but increased bone formation ( $\leq 10$ -fold,  $p < 0.001$ ). NAC reduced VSMC cell death by  $\leq 25\%$  ( $p < 0.01$ ) and VSMC apoptosis by  $\leq 40\%$  ( $p < 0.001$ ), but had no effect on osteoblast survival and viability. Tissue non-specific alkaline phosphatase (TNAP) plays an important role in driving mineralisation processes. NAC had no effect on VSMC TNAP activity but increased osteoblast TNAP activity by  $\leq 40\%$  ( $p < 0.001$ ). Development of AMC is associated with decreased expression of typical VSMC marker genes (e.g.  $\alpha$ -SMA, SMemb, SM22 $\alpha$ ). mRNA expression of many VSMC genes was unaffected by NAC treatment, whereas small increases in Acta2 and Caldesmon levels were observed (1.4 and 3-fold respectively,  $p < 0.05$ ). NAC increased expression of mRNAs for osteoblast-associated genes (Osterix, Osteocalcin, TNAP) by  $\leq 6$ -fold ( $p < 0.001$ ). Expression of the mineralisation inhibitors (NPP1, Ank, Osteopontin) was also decreased by  $\leq 2$ -fold ( $p < 0.001$ ). In vivo, NAC treatment decreased aortic calcification by  $\leq 25\%$  ( $p < 0.05$ ) but trabecular and cortical bone parameters were unaffected. However, a negative correlation between the level of aortic calcification and trabecular bone volume ( $p = 0.0237$ ) was observed.

Collectively, our findings suggest that NAC exerts opposing differential effects on AMC and bone formation and could represent a potential therapy for limiting AMC without negatively affecting the skeleton.

### Vascular Redox Regulation: From Homeostatic Balance to Oxidative Stress

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Vascular inflammation is an important risk factor for cardiovascular diseases and associated with increased formation of reactive oxygen species (ROS) as well as marks of oxidative stress. The specific contribution of ROS to vascular disease is unclear. ROS have broad effects on signaling and each alteration of the cellular homeostasis alters ROS level. Thus, causality for oxidative stress in

specific signaling pathways in general is difficult to establish and identification of specific redox-targets is still challenging. Physiological ROS generation in the microcirculation helps to maintain vascular tone and contributes to endothelium-dependent relaxation. Oxidative processes are also involved in the generation of vasoactive lipids and matrix-formation. In vascular disease processes, numerous enzymatic systems are induced or activated to increase ROS formation which results in inflammatory gene expression and leukocyte recruitment. As the capacity of leukocytes to generate ROS is much greater than that of vascular cells, ROS measured in vascular preparations primarily originate from leukocytes and leukocytes are often identified as the mediator of a ROS-dependent process. In the presentation the above mentioned aspects will be exemplified for several vascular disease conditions and also the different ROS generators active in this process will be discussed.

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### Single Cell Transcriptome Analyses Reveal Novel Targets Modulating Cardiac Neovascularisation by Resident Endothelial Cells Following Myocardial Infarction

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**Aims:** Chronic heart failure as a consequence of left ventricular impairment following acute myocardial infarction (MI) has reached epidemic proportions. However, the pathways that regulate regeneration of the coronary vasculature following MI remain unresolved. We aimed to investigate the origin and clonal dynamics of endothelial cells (EC) associated with neovasclogenesis in the ischemic border zone of the adult mouse heart following MI. Further, we aimed to define the transcriptional signature of pro-regenerative EC in the ischemic heart using single cell RNA-sequencing (scRNAseq), and to confirm the potential relevance of new targets in cardiac tissue samples from patients with ischemic heart disease.

**Methods:** MI was induced in EC-specific multispectral lineage-tracing mice, "Pdgfb-iCreERT2-R26R-Brainbow2.1" by permanent coronary artery ligation. Injured (and uninjured control) hearts were collected at 7 days post-MI and prepared as whole-mounts for quantification of EC clonal proliferation and investigation of cell phenotype. Femoral bone marrow cells were collected

for flow cytometric analysis of reporter fluorophore expression. EC were isolated from the ventricles for scRNAseq (10X Chromium). Immunofluorescence staining for targets identified by scRNAseq was performed on human cardiac tissue from patients with ischemic heart disease (and healthy control hearts).

**Results:** Pdgfb-iCreERT2-R26R-Brainbow2.1 mice had tamoxifen-inducible expression of YFP, RFP, GFP, or CFP specifically in EC. Fluorophore expression was inherited by EC progeny following proliferation, allowing quantitative clonal analysis. Clonal proliferation was observed in healthy hearts, although was significantly increased in the infarct border at 7 days post-MI (cells per clone =  $4.5 \pm 3.3$  versus  $10.3 \pm 10.6$ ,  $P < 0.0001$ ). Minimal Brainbow2.1 reporter expression was observed in femoral bone marrow cells (healthy =  $0.04 \pm 0.02\%$  versus MI =  $0.03 \pm 0.009\%$ ,  $P = 0.79$ ). Ten transcriptionally discrete EC clusters were defined following scRNAseq. GO term enrichment analysis highlighted pathways associated with the top differentially expressed genes in each cluster, informing us as to the putative functional identity of EC in each cluster. We validated our findings at the protein level for multiple novel targets in healthy and ischemic human cardiac tissue sections.

**Conclusions:** Generation of new perfused blood vessels following ischemic injury in the adult mouse heart is predominantly mediated by clonal proliferation of resident EC, and not bone marrow cells. We present a single cell gene expression atlas of resident cardiac EC, and the transcriptional hierarchy underpinning endogenous vascular repair following MI. This resource has identified novel targets that may augment myocardial perfusion post-MI, and thus inform future design of clinical strategies aimed at promoting vascular perfusion in ischemic heart disease.

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### Breast Cancer Metastasis to Bone: The Role of the Perivascular Niche in Regulating Tumour Cell Dormancy

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**Introduction/Objective:** Tumour cell dissemination to bone is an early event in breast cancer. Approximately 30% of breast cancer patients have disseminated tumour cells in their bone marrow at the time of receiving treatment for their primary tumour. However, only a proportion of these patients develop metastatic disease, often following an extended period of dormancy. Currently, there is no cure for metastatic breast cancer. The perivascular niche within bone plays an important role in regulating the dormancy of DTCs, but the cellular and molecular components of this metastatic perivascular niche remain to be clearly defined. Therefore, the objective of this research is to gain a better understanding of the cellular and molecular changes that occur in the bone microenvironment when breast cancer cells emerge from dormancy, outgrow and form metastatic lesions.

**Methods:** We have established two mouse models of bone metastasis, where breast cancer cells arriving in bone either undergo outgrowth (6-weeks old animals with high bone turnover) or enter dormancy (12-weeks old animals with mature skeleton). We use confocal microscopy and qPCR to quantify differences in both the cellular and molecular composition of the bone microenvironment in these two models, with particular emphasis on how cancer cells interact with the perivascular and endosteal niches.

**Results:** The bone microvasculature and perivascular niche are markedly different in these two models. We show that the bone microenvironment of our dormancy-promoting model has a decreased abundance of CD31+ osteogenic vasculature and of osteoprogenitors expressing the markers Osterix and PDGFR. We also show that breast cancer cells colonise and survive in bone microenvironments that are enriched for osteogenic vasculature and osteoprogenitors. Finally, we show that the dormancy of breast cancer cells in bone is correlated with a reduced association with the perivascular niche in favour of the endosteal niche.

**Conclusion:** Our data indicate that tumour cell dormancy in bone is supported by the reduced osteogenic potential of the bone microvasculature, and surrounding perivascular niche, coupled to an increased association of cancer cells with the endosteal niche.

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### Nutritional and Functional Blood Flow in Glabrous and Nonglabrous Skin: Can They Be Separately Measured

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**Introduction:** Different response of microvascular skin blood flow in glabrous and nonglabrous skin to short lasting submaximal incremental cycling (SIC) has been reported when measured by the laser Doppler method. Metabolic needs of the skin in both particular skin sites seems to remain the same and change simultaneously during exercise and in the recovery period. Transcutaneous partial oxygen pressure (tcpO<sub>2</sub>) is a variable which reflects the oxygen availability in the peripheral tissue and hence its metabolic needs. The aim of our study was to compare the changes in tcpO<sub>2</sub> and laser Doppler skin blood flow (LDF) response in glabrous and nonglabrous skin provoked by SIC.

**Methods:** 9 healthy volunteers (age 25.9 ± 3.3, BMI = 23 ± 2.5 kg/m<sup>2</sup>) participated in our study. LDF and tcpO<sub>2</sub> in glabrous and nonglabrous skin, finger arterial blood pressure and ECG were measured simultaneously 10 minutes at rest sitting on the bicycle, during SIC (starting cycling at 40 W, increasing in steps of 50 W every three minutes till the submaximal heart rate (HR) was reached) and in the recovery. Cutaneous vascular conductance (CVC) was calculated. Submaximal HR was determined as 85% of maximal individual HR.

**Results:** CVC in non-acral skin increased during exercise and returned to baseline level after exercise cessation. tcpO<sub>2</sub> re-

sponse in nonacral skin showed the same pattern: changing in positive linear correlation with CVC during exercise as well as in the recovery. On the other hand, CVC in acral skin and corresponding tcpO<sub>2</sub> showed different patterns. CVC decreased during exercise and returned to its resting value during recovery while tcpO<sub>2</sub> increased slightly with exercise, reached its maximum at peak exercise and decreased during the recovery. No correlations were found between tcpO<sub>2</sub> and CVC in acral skin. The responses of tcpO<sub>2</sub> to SIC in both skin sites showed the same pattern.

**Discussion and Conclusion:** Our results confirmed that tcpO<sub>2</sub> reflects the tissue metabolic needs and could be a surrogate of the nutrition circulation to the skin. LDF measures the total blood flow to the skin which is the sum of nutritional and functional, thermoregulatory, contributions. Hence, LDF could be a marker of skin oxygenation only in nonglabrous skin while high LDF in acral skin does not necessary reflect high oxygenation and could be misleading in the diagnostics and treatment of vascular malfunction such as chronic wounds, erythromelalgia, Raynaud disease and others in this skin site.

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### Nutritional and Functional Blood Flow in Glabrous and Nonglabrous Skin: Can We Dissociate Them

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**Results:** CVC in non-acral skin increased during exercise and returned to baseline level after exercise cessation. tcpO<sub>2</sub> response in nonacral skin showed the same pattern: changing in positive linear correlation with CVC during exercise as well as in the recovery. On the other hand, CVC in acral skin and corresponding tcpO<sub>2</sub> showed different patterns. CVC decreased during exercise,

and returned to its resting value during recovery while tcpO<sub>2</sub> increased slightly with exercise, reached its maximum at peak exercise and decreased during the recovery. No correlations were found between tcpO<sub>2</sub> and CVC in acral skin. The responses of tcpO<sub>2</sub> to SIC in both skin sites showed the same pattern.

**Discussion and Conclusion:** Our results confirmed that tcpO<sub>2</sub> reflects the tissue metabolic needs and could be a surrogate of the nutrition circulation to the skin. LDF measures the total blood flow to the skin which is the sum of nutritional and functional, thermoregulatory, contributions. Hence, LDF could be a marker of skin oxygenation only in glabrous skin while high LDF to acral skin does not necessary reflect high oxygenation and could be misleading in the diagnostics and treatment of vascular malfunction as chronic wounds, erythromelalgia, Raynaud disease and others in this skin site.

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### Impact of Circadian Rhythm in Smooth Muscle Cells

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The accumulation of smooth muscle cells (SMCs) in the intimal layer of the arterial wall is a hallmark of atherosclerosis. During this process SMCs switch from a contractile to a synthetic phenotype that is accompanied by structural and functional changes, such as the loss of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and smooth muscle myosin heavy chains (SMMHCs). Our group has previously established a porcine model, in which two populations of SMCs were obtained from normal coronary arteries, the spindle-shaped (S)-SMCs and rhomboid (R)-SMCs that correspond to the contractile and synthetic phenotypes, respectively. R-SMCs are characterized by a strong upregulation of S100A4, a specific marker in vitro of R-SMCs, and in vivo of intimal SMCs in pigs and humans. In addition to S100A4, several other genes and proteins are differentially expressed in R-SMCs compared to S-SMCs, including members of the matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs).

In mammals, many of the cardiovascular processes are controlled by a so-called cell-dependent circadian clock, such as blood pressure, vasodilatation, and basal heart rate, thus opening the possibility that circadian rhythm impacts atherosclerosis. Nonetheless, only a handful of circadian rhythm studies have been performed in the context of SMCs and atherosclerosis. Herein, we have investigated whether, in our model, S- and R-SMCs display a circadian rhythm, and depicted whether their profiles are distinct. Using lentiviral transduction of SMCs with the sequence of two core clock gene (CCG) promoters – *Bmal1* and *Per2* -, coupled with a luciferase reporter, we showed that S- and R-SMCs exhibited different period length for *Bmal1* gene, with an increase pe-

riod in R-SMCs. The same pattern was observed when S-SMCs were treated with fibroblast growth factor 2 (a known activator of the synthetic phenotype). Other CCGs were also tested by qPCR: *BMAL1*, *CRY1*, *CRY2*, *PER1*, *PER2* and *REV-ERB $\beta$*  displayed a phase delay (from 2 to 8 h) in R-SMCs compared to S-SMCs. In addition, *S100A4* and *TIMP-3* (upregulated in R-SMCs), *SMMHCs* and *SM22- $\alpha$*  (downregulated in R-SMCs) were tested and we found that *TIMP-3* exhibited an oscillatory profile in R-SMCs, opposite to what was found in S-SMCs. This suggests that *TIMP-3* may be under the control of the CCGs in this specific phenotype. Our results indicate that the two SMC populations exhibit distinct circadian profiles, which could be of utmost importance in yet unexplored molecular links between circadian oscillators operative in SMCs and regulators of SMC plasticity in atherosclerotic plaque formation.

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### Smooth-Muscle Cell Switch in Atherosclerosis: A Matter of Intracellular S100A4?

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During atherosclerosis, smooth muscle cells (SMCs) are known to accumulate into the intima and to switch from a contractile to a synthetic phenotype. In our model, we isolated two distinct SMC populations, spindle-shaped (S-) and rhomboid (R-) SMCs from the porcine coronary artery; the R-phenotype represents the synthetic phenotype. We further identified S100A4 as a marker of R-SMCs in vitro and of intimal SMCs in both pigs and humans. One of the most interesting features of S100A4 is its dual role whether it is located intra- or extracellularly. Recently we have shown that the extracellular form of S100A4 is essential for the establishment of the R-phenotype associated with pro-inflammatory properties, although the mechanisms responsible for this process need to be clarified.

This study is mainly focused on the intracellular role of S100A4 in SMC phenotypic switch. We have set up two cell models, using HeLa cells and R-SMCs (which express and release S100A4), where S100A4 was depleted via CrispR/Cas9 lentiviral transduction, followed by clonal selection. With this model, S100A4 will be permanently depleted. The effects of its downregulation will be assessed with several parameters: proliferation, migration, cytoskeleton protein expression, and proinflammatory profile.

In vivo, our aim is to decipher the influence of S100A4-expressing SMCs in plaque formation and progression, irrespective of S100A4 produced by macrophages. For this purpose, we are developing a SMC lineage tracing mouse model associated with SMC-specific deletion of S100A4. We have generated the S100A4-floxed mouse strain by using CrispR/Cas9 technology. These mice will be crossed with the *Myh11-CreERT2;R26-YFP* strains to deplete S100A4 solely in SMCs, while labeling them with YFP. To induce atherosclerosis we will also introduce the *ApoE*<sup>-/-</sup> mouse strain, ending up with a quadruple transgenic mouse model that under high-fat diet develops atherosclerotic lesions in the arterial tree. With this model, we will be able to ascertain whether the depletion

of SMC-specific S100A4 will impact the formation, composition and progression of atherosclerotic plaques. This knowledge will ultimately be useful to determine the therapeutic value of this protein in the context of atherosclerosis.

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### Role of Apelin in Vascular Smooth Muscle Cell Phenotypic Transition: A Proatherogenic Factor for Atherosclerosis

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Apelin is a small peptide involved in vascular disease but its role in atherogenesis remains unclear. We have investigated the role of apelin in smooth muscle cell (SMC) phenotypic changes by using two distinct SMC populations isolated from porcine coronary artery, spindle-shaped (S) and rhomboid (R) SMCs. S-SMCs are well differentiated whereas R-SMCs display the features of synthetic SMCs.

We have shown that apelin was highly expressed in R-SMCs compared with S-SMCs. Moreover, a nuclear expression of apelin in vitro in R-SMCs as well as in vivo in intimal SMCs of porcine coronary stent-induced intimal thickening was observed. To determine the effect of apelin cellular localization and its role in the phenotypic switch, two mutated preproapelin-His-tag encoding plasmids targeting apelin into the nucleus (N. Ap) and into the secretory vesicles (S. Ap) were transfected into S-SMCs (devoid of apelin). Both induced a SMC transition towards a R-phenotype, which was associated with increased proliferative activity, down-regulation of  $\alpha$ -smooth muscle actin, and increased expression and release of S100A4 (a marker typical of R-SMCs). Unexpectedly, overexpression of N. Ap, but not S. Ap, led to nuclear localization of S100A4. Stimulation of S-SMCs with platelet-derived growth factor-BB, known to induce a transition toward the R-phenotype, yielded nuclear expression of both apelin and S100A4. After transfecting and overexpressing S100A4 in S-SMCs we did not observe any induction of apelin expression, which suggests that apelin acts upstream of S100A4.

In conclusion, apelin induces a SMC phenotypic transition towards the synthetic phenotype, associated with S100A4 upregulation and release. These results suggest that apelin act as a pro-atherogenic factor. In addition, N. Ap promotes the re-localization of S100A4 into the nuclei, raising the possibility of a complex relationship between apelin and S100A4.

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### 3D Vessel-on-Chip Models: An Approach to Understand Vascular Physiology and Disease

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The vasculature provides the conduits for delivering nutrients and oxygen throughout the body, while simultaneously regulating fluid flow between the intravascular and interstitial compartments. Studying the biology of these processes has been limited because in vivo models are difficult to manipulate and monitor, while many of these functions are not captured by traditional cell culture. Here, I present the use of engineered microfluidic cultures in which endothelial cells line perfused channels that pass through an extracellular matrix. Using these engineered vessel-on-chip platforms, we have begun to expose the complex interplay that occurs between adhesions, force, signaling, and function. We will present ongoing efforts to investigate how shear versus interstitial flow regulates barrier function and endothelial cell remodeling and sprouting, and how these effects are distinctly regulated by blood versus lymphatic endothelial cells.

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### Endothelial C-Type Natriuretic Peptide Acts on Perivascular Mast Cells to Inhibit Postischemic Vascular Inflammation and Thrombosis

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**Introduction/Objective:** Endothelial C-type natriuretic peptide (CNP) participates in the local regulation of vascular tone and barrier integrity. The cyclic GMP forming guanylyl cyclase (GC) B receptor for CNP is expressed in different types of cells within and surrounding the vessel wall but the exact CNP functions and signalling pathways are only partly known. Notably, we observe that GC-B is densely expressed in perivascular resident mast cells, important players both for the initiation and progression of vascular inflammatory and thrombotic diseases. To follow the hypothesis that CNP mediates a local communication between endothelial cells and mast cells (MCs) we generated a novel genetic mouse model with restricted inactivation of the GC-B receptor in connective tissue type MCs (Npr2fl/fl; Mcpt5Cre: MC GC-B KO).

**Methods and Results:** In vitro, in cultured bone marrow (BM) MCs obtained from control mice, CNP increased intracellular cGMP levels and cGMP-dependent phosphorylation of the cytoskeleton-associated protein VASP. Both responses were abolished in GC-B – deficient BMSCs. In vivo, intravital microscopy of the mouse cremaster microcirculation was performed to follow ischemia/reperfusion-induced degranulation of perivascular resident MCs (ruthenium red uptake) and microcirculatory inflammation (FITC-dextran extravasation). Notably, in control mice the local superfusion of synthetic CNP markedly attenuated postischemic MC degranulation and vascular leakage. In MC GC-B KO mice these protective CNP effects were fully abolished. Even more, already under baseline, resting conditions, the number of intact as well as degranulated perivascular resident MCs was markedly increased in such KO mice. To study whether endogenous CNP counterregulates pathological MC activation and inflammation, we used a preclinical model of deep vein thrombosis (DVT), a disease condition in which MC-derived mediators critically participate in endothelial injury and thrombus formation. As demonstrated by ultrasound Doppler and necropsy, partial ligation (stenosis) of the inferior vena cava for 48-hours induced thrombosis in all study mice. Remarkably, the size of these thrombi was significantly increased in the MC GC-B KO mice.

**Conclusion:** Our studies indicate that endothelial CNP counterregulates the activation of resident, perivascular MCs already under baseline and even more under disease conditions. CNP-induced GC-B/cGMP signalling in connective tissue MCs is essential for the maintenance of normal vascular integrity and might be a novel target for the prevention of diseases linked to ischemia and vascular inflammation.

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### Role of Leucine-Rich Alpha-2-Glycoprotein 1 (LRG1) in Diabetes-Related Ischaemic Heart Diseases

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Ischaemic heart diseases (IHD), the leading cause of morbidity and mortality worldwide, presents as a major global healthcare burden and an enormous socio-economic impact. The conventional standard of care includes lifestyle changes, pharmacological and surgical interventions, but these approaches are not universally effective, especially for patients with comorbidities like diabetes. Effective revascularization in IHDs remains challenging given a growing magnitude and the complexity of the disease. Therapeutic angiogenesis, a strategy that promote neovascularization

and reperfusion of ischemic myocardium using angiogenic growth factors, emerged as a potential alternative treatment option. This project aims to investigate the functional role of a novel angiogenic factor, Leucine-rich alpha 2 glycoprotein 1 (LRG1) in cardiac angiogenesis under both normal and diabetic conditions.

Our recent data demonstrated a pro-angiogenic effect of LRG1 on cardiac-specific endothelial cells (EC). We observed increased EC viability, proliferation, migration, and tubule formation, as well as vessel outgrowth from various organ explants, under both normal and diabetic condition. Besides its established role in modulating endothelial TGF $\beta$  signalling, our study also indicated a Smad4-dependent effect of LRG1 on VEGF signalling. Interestingly, both the expression level and the phosphorylation of VEGFR2 and its downstream signalling transducer phosphorylated Akt were induced by the treatment of recombinant human LRG1. Furthermore, the pro-angiogenic effect of LRG1 is attenuated by the treatment with a VEGFR2 inhibitor, Linifanib suggesting LRG1 exerts its pro-angiogenic effect, at least to some extent, by mediating the VEGF/VEGFR2 signalling pathway.

Finally, our study revealed enhanced adverse outcomes in transverse aortic constriction (TAC)-induced cardiac remodelling induced in LRG1-deficient mice under diabetic condition. Our findings demonstrated significant lower survival rates, reduced microvessel density, and increased cardiac hypertrophy and perivascular fibrosis when compared to wild-type counterparts. Taken together, the findings suggest that LRG1 exerts vital cardioprotective under normal and diabetic condition.

In summary, the information extracted from this study provided novel insights into the LRG1-regulated angiogenesis, which may facilitate the development of more effective treatment strategies to treat diabetes-associated IHD.

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### Discovering Potential Acetylcholinesterase Inhibitors Using Molecular Docking Against Alzheimer's Disease and Angiogenesis

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Alzheimer's disease (AD) is one of the most common causes of dementia in the elderly. Recently, a great attention has been paid to the possible role of vascular changes in the pathogenesis of AD, namely vascular dysfunction being the pathological hallmark. Acetylcholinesterase (AChE) inhibitors are widely used for the improvement of AD symptoms. Until now, however, the effects of AChE inhibitors on vascular changes including angiogenesis are not fully understood. In this present work, potential compounds (Curcumin, Bacoside A, Piperine and Chebulinic acid) were targeted against AChE as well as the current FDA approved drugs (Donepezil and Galantamine).

Lately some scientists proposed that antiangiogenic drugs might be able to improve the management of AD and this was observed using donepezil and galantamine. Previous studies have shown that both FDA drugs are able to significantly increase the number of vessels in the chorioallantoic membrane and mitigate oxidative stress in brain endothelial cells. This could be associated

to early angiogenesis restoring blood flow and reduces amyloid beta deposition.

Our data showed that galantamine and curcumin displayed better binding affinity towards AChE at both catalytic action site (CAS) and peripheral anionic site (PAS) locations comparing to bacoside A. This indicates a stable complex that can enable inhibitory enzyme activity of AChE. Moreover, galantamine and curcumin displayed interactions with 2 out of the 3 key amino acids involved in the enzymatic breakdown of acetylcholine while bacoside A only has 1 key amino acid interaction formed. This further supports the findings of unstable complex formation with repulsion forces leading to an unlikely enzymatic inhibition from bacoside A. Besides, piperine also exhibited lower and better binding affinity towards AChE comparing to chebulinic acid. This similar binding affinity was also observed in donepezil. This maybe due to the structural size of chebulinic acid itself, where it is too big to be docked into the AChE active site.

From here, we could ascertain that curcumin and piperine are able to exhibit similar traits as donepezil and galantamine in targeting the AChE active site. Thus, the effects of AChE inhibitors by curcumin and piperine may also have angiogenesis-accelerating properties similar to those in donepezil and galantamine. However, it is too early to draw any conclusion on the relationship between AChE inhibitors (curcumin, piperine, donepezil and galantamine), angiogenesis and AD.

sies, characterized and used for transwell co-culture assays with HUVEC. After 7-days, HUVEC were tested for osteogenic differentiation by calcium stain with Alizarin Red and by mRNA levels of SLUG, MMP-9, CD31 and RUNX-2. Considering that SLUG is a potential target of miR-30 family, we also detected miR-30a/d variations in HUVEC after each culture conditions.

**Results:** SLUG analysis by IHC revealed intense stain in AAA and CP tissues, mostly within inflammatory infiltrates and in endothelial cells. TNF- $\alpha$  and TGF- $\beta$ 3 effectively promoted End-MT, as supported by the spindle-shape morphology and by the increase of SLUG and MMP-9 mRNA. TNF- $\alpha$  also stimulated RUNX-2 expression in HUVEC. In addition, HUVEC demonstrated osteogenic property as shown by mineralization assay and RUNX-2 expression, especially after culture with AAA- and CP-MSC. Interestingly, the HUVEC osteogenic phenotype was accompanied by SLUG up-regulation and slight decrease of CD31. Finally, End-MT and osteogenic differentiation were associated with decreased expression of miR-30a and miR-30d in HUVEC.

**Conclusions:** Our results support the occurrence of the End-MT process in human atherosclerotic disease and highlight that the endothelial cells actively govern the plaque formation as well as the late calcification, by switching into the mesenchymal cell intermediate. Future investigations will address the potential therapeutic strategies targeting the main End-MT mediators in order to inhibit the calcification of the atherosclerotic plaque.

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### Endothelial-Mesenchymal Transition Represents a Key Process During Atherosclerotic Plaque Calcification

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**Study Objective:** The endothelial-mesenchymal transition (End-MT) has been recently proposed as a key process during plaque evolution and calcification. In our hypothesis, microenvironment cues stimulate the endothelial cell conversion into MSC-like cells that lately differentiate toward the osteogenic lineage, contributing to the calcium accumulation within the intima. The main objective of the present study was to investigate the expression of SLUG, a key transcription factor regulating the End-MT, in human atherosclerotic lesions of abdominal aortic aneurysms (AAA) and carotid plaques (CP). Then we addressed the End-MT in endothelial cells, investigating its possible association with the calcification process in presence of inflammatory soluble mediators and in co-culture models with vascular resident cells.

**Methods:** SLUG was analysed in FFPE tissues of AAA and CP patients. End-MT was explored in Human Umbilical Vein Endothelial Cells (HUVEC) in presence of the inflammatory cytokine TNF- $\alpha$  and the members of the TGF- $\beta$  superfamily (the canonical End-MT factor TGF- $\beta$ 1 and TGF- $\beta$ 3) for 7 days. Mesenchymal Stem Cells (MSCs) were ex-vivo isolated from AAA and CP biop-

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### PDE3 and -5 Inhibition Differentially Affects Platelet Function under Hemostatic and Inflammatory Conditions

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**Background:** Platelet-rich thrombi, formed after atherosclerotic plaque rupture, may cause myocardial infarcts and strokes. Current treatment is focused on suppressing thrombus growth by inhibiting platelet activation. However, recurrent events are still occurring in about one third of the patients. Recently, we found that platelets in thrombi remain active for a prolonged time. We hypothesize that platelet secretion is pivotal in mediating not only acute, but also prolonged thrombus activities. The aim of this study is to examine the effect of suppressing platelet secretion, by (in) directly elevating cAMP and/or cGMP, on key functional thrombus activities.

**Methods:** The effect of PDE3/5 inhibitors on platelet  $\alpha$ IIB $\beta$ 3 activation and  $\alpha$ - and  $\delta$ -granule secretion was measured with flow

cytometry. Microfluidics were used to study whole blood thrombus formation at 1000 s<sup>-1</sup> over a collagen type I + tissue factor surface or over a confluent monolayer of human umbilical vein endothelial cells treated overnight with tumor necrosis factor- $\alpha$  (10 ng/ml). Platelet extracellular vesicle (EV) release was measured with a prothrombinase-based assay.

**Results:** Cilostazol (PDE3i, 5  $\mu$ M) significantly inhibited platelet  $\alpha$ Ib $\beta$ 3 activation and platelet  $\alpha$ - and  $\delta$ -granule secretion of collagen-related peptide-treated washed platelets ( $p = 0.0006$ ,  $p = 0.0001$  and  $p = 0.01$ ). Tadalafil (PDE5i, 10 nM) tended to affect these processes ( $p > 0.05$ ). Only cilostazol significantly decreased platelet EV release upon stimulation with convulxin or thrombin. In whole blood perfusion, PDE3 (50  $\mu$ M) or -5 (100 nM) inhibition did not affect platelet adhesion but showed a tendency to decrease thrombus size. PDE3 inhibition significantly increased time to fibrin ( $p = 0.03$  vs.  $p = 0.17$  with PDE5 inhibition). Strikingly, a ten-fold lower dose of cilostazol (5  $\mu$ M) or tadalafil (10 nM) gave a strong and significant decrease in platelet adhesion on inflamed endothelial cells.

**Conclusion:** These data indicate that PDE5, and especially, PDE3 are key controlling enzymes in the regulation of platelet adhesion, activation and secretion. Herein, platelet adhesion and thrombus formation appear to be differentially regulated on collagen versus inflamed endothelium. Interestingly, the effect of PDE3 inhibition is more pronounced on EV release than on  $\alpha$ - and  $\delta$ -granule secretion. Combination of PDE3/5 inhibition with current antiplatelet medication might be the key in the regulation of acute and prolonged thrombus activities.

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### The Effects of Vaccinium Myrtillus Extract on Hamster Pial Microcirculation During Hypoperfusion-Reperfusion Injury

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The Vaccinium myrtillus extract contains about 34.7% of anthocyanins and turns out to be an excellent source of antioxidants. The aim of this study was to assess the effects of oral administration of Vaccinium myrtillus in preventing damage due to cerebral hypoperfusion and reperfusion in the hamster pial microcirculation.

We studied two groups of male hamsters: the first fed with control diet and the other with Vaccinium myrtillus supplemented diet. Hamster pial microcirculation was visualized by fluorescence microscopy through an open cranial window; the hypoperfusion was induced by bilateral common carotid artery occlusion for 30 min; successively, the clamps were removed (reperfusion period, 60 min). Pial arterioles were classified according to Strahler's method.

In age-matched control diet-fed hamsters, hypoperfusion and the reperfusion caused a significant constriction of all arterioles. Microvascular leakage and leukocyte adhesion were markedly enhanced, while perfused capillary length (PCL) decreased. At the end of hypoperfusion, in age-matched Vaccinium myrtillus supplemented diet-fed hamsters, the arteriolar diameter did not sig-

nificantly change compared to baseline. At the end of reperfusion, pial arterioles significantly dilated in 2, 4 and 6 month Vaccinium myrtillus supplemented diet-fed hamsters, compared with the age-matched control diet-fed hamsters. Microvascular leakage and leukocyte adhesion were significantly reduced in all groups according to the time-dependent treatment, when compared with the age-matched control diet-fed hamsters. Similarly, the reduction in PCL was progressively prevented as well as the ROS production.

In conclusion, Vaccinium myrtillus extract protected pial microcirculation during hypoperfusion-reperfusion, preventing the microvascular damages and preserving the endothelium integrity.

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### Metabolic Interactions Between the Endothelium and the Muscle Microenvironment

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Angiogenesis, the formation of new blood vessels from existing ones, is initiated by the secretion of growth factors – the vascular endothelial growth factor VEGF is the best described one – from a hypoxic environment. To grow under low oxygen conditions, ECs have unique metabolic characteristics. Indeed, even though they are located next to the blood stream – and therefore have access to the highest oxygen levels – ECs are highly glycolytic. However, when they need to sprout into avascular areas and form new vessels, they upregulate glycolysis even further to fuel migration and proliferation. Suppression of glycolysis via inhibition of the glycolytic regulator PFKFB3 (phosphofructokinase-2/fructose-2,6-bisphosphatase isoform 3) in endothelial cells prevents blood vessel growth in the retina of the mouse pup and also in various models of pathological angiogenesis. While we now know that ECs are metabolically preconditioned to rapidly form new vessels, it remains an outstanding question whether this also holds true in muscle and whether endothelial metabolism can become a target for the treatment of peripheral artery disease. The Laboratory of Exercise and Health aims to investigate whether muscle endothelial cells need to reprogram their metabolism to promote optimal muscle angiogenesis. Moreover, we try to understand how muscle and the endothelium communicate to ensure optimal nutrient and oxygen delivery into the muscle.

## Partial Inhibition of the Key Glycolytic Enzyme PFKFB3 in Myeloid Cells Impacts Whole-Body Immune Cell and Liver Metabolism, But Not Atherogenesis

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**Background:** Macrophages play a central role in atherosclerosis and depend highly on glycolysis for energy metabolism, specifically upon pro-inflammatory stimulation. Blockage of glycolytic enzyme PFKFB3 reduced both glycolysis and inflammation. We hypothesised that myeloid PFKFB3 deficiency reduces glycolysis and subsequent pro-inflammatory activity in vascular and extravascular macrophages, attenuating atherosclerotic burden in vivo.

**Methods:** LDLr<sup>-/-</sup>-LysMcre<sup>+/+</sup>-PFKFB3<sup>wt/wt</sup> (n = 17, wildtype, PFKFB3<sup>WT</sup>) and LDLr<sup>-/-</sup>-LysMcre<sup>+/+</sup>-PFKFB3<sup>fl/fl</sup> (n = 20, knock-down, PFKFB3<sup>KD</sup>) mice were fed a 0.25% cholesterol diet for 12 weeks. Weight, phenotype and/or gene expression were analysed for liver, kidneys, white and brown adipose tissue. Splenic and circulating immune cells were analysed by flow cytometry. Plaque characteristics were measured in aortic roots.

**Results:** Culture medium of PFKFB3<sup>KD</sup> bone marrow derived macrophages showed increased glucose and decreased lactate, in line with 50% knock-down of PFKFB3 mRNA (p > 0.01). PFKFB3<sup>KD</sup> mice gained 34% more weight during diet (p < 0.05) and fasted glucose was 39% higher (p < 0.0001), despite lower baseline weight (p < 0.01) liver (14%, p < 0.005) and subcutaneous fat (66% p < 0.05) weights were increased compared to controls. PFKFB3<sup>KD</sup> mice livers exhibited more steatosis and inflammation (p < 0.05), increased cholesterol (p < 0.001) and triglyceride content (p < 0.01). Circulating and splenic levels of Ly6C-low monocytes were increased (+2.5 fold and 47%, p < 0.0005 and p < 0.05 respectively), while splenic Ly6C-high monocytes were decreased (p < 0.05). Wildtype and PFKFB3<sup>KD</sup> atherosclerotic plaque area, necrotic core and macrophage count did not differ.

**Conclusion:** Myeloid knockdown of the glycolytic enzyme PFKFB3 did not affect advanced atherosclerotic lesions in mice. However it did induce a whole-body immuno-metabolic phenotype, characterised by weight gain, hepatic steatosis and abundance of Ly6C-low monocytes.

## The Many Faces of Endothelium-Dependent Relaxation in Resistance Arteries from Patients with Residual Cardiovascular Disease

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In resistance-sized arteries of rats and mice, receptor-mediated endothelium-dependent relaxation involves NO synthase (NOS) and endothelium-dependent hyperpolarization (EDH). The NOS-dependent component is usually attributed to endothelium-derived NO stimulating soluble guanylyl cyclase (sGC) in the arterial smooth muscle. In nitrergic neurotransmission, however, the transmitter is proposed to be nitroxyl (HNO) and to resist inactivation by superoxide anions (O<sub>2</sub><sup>-</sup>). Here we evaluated in pericardial resistance arteries from patients undergoing cardiothoracic surgery, the roles of the NOS/NO/sGC pathway and of EDH in responses to bradykinin (BK). Effects of the NO-donor Na-nitroprusside (SNP) were studied as positive control. Isolated vessel segments were first contracted with 32 mM K<sup>+</sup> to mimic myogenic tone and attenuate hyperpolarization including EDH. Relaxing responses to 1 μM BK and 1 μM SNP averaged 62 ± 20 and 58 ± 19%, respectively. Those to SNP were not modified by endothelium-removal, 100 μM L-NAME (NOS-inhibitor) or 3 mM N-acetylcysteine (scavenger of HNO), but were abolished in presence of 300 μM cPTIO (scavenger of NO), 3 mM DETCA (inhibitor of Cu/Zn superoxide dismutase) or 10 μM ODQ (sGC-inhibitor). Those to BK were abolished by endothelium-removal, L-NAME or ODQ, but not modified by cPTIO, N-acetylcysteine or DETCA. Next, BK-induced relaxations were recorded during contraction stimulated by an analogue of thromboxane A<sub>2</sub> (U46619) or by endothelin-1 (ET1). Here, the vessels were more sensitive to BK than during partial depolarization. In presence of U46619, the relaxations were only partly reduced by L-NAME and additionally attenuated by inhibitors of small- and intermediate conductance calcium-activated K<sup>+</sup>-channels. In presence of ET1, BK-induced relaxations were not attenuated by any of the pharmacological inhibitors mentioned and not by an inhibitor of xanthine oxidase or three inhibitors of NADPH oxidases. They were, however, blunted by 0.1 μM iberiotoxin (inhibitor of large conductance calcium-activated K<sup>+</sup>-channels, BKCa) and by 1000 U mL<sup>-1</sup> catalase (scavenger of extracellular H<sub>2</sub>O<sub>2</sub>). Thus, in resistance arteries from patients with residual cardiovascular disease, the endothelium-dependent vasodilator BK can stimulate NOS, sGC and EDH. The link between NOS and sGC does not involve NO or HNO and is resistant to O<sub>2</sub><sup>-</sup>. During agonist-induced contraction, EDH accounts for a part or the entire endothelium-dependent relaxation as observed in presence of U46619 and ET-1, respectively. In presence of ET1, the role of NOS/sGC is taken over by H<sub>2</sub>O<sub>2</sub> stimulating BKCa. The source of this H<sub>2</sub>O<sub>2</sub> is unclear, because inhibitors of NADPH oxidases or Cu/Zn superoxide dismutase did not attenuate the relaxation.

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### The VWF/ADAMTS13 Axis in Cerebral Ischemia/Reperfusion Injury

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Ischemic stroke is caused by a blood clot that occludes one or more cerebral arteries, preventing blood flow to the brain. Treatment is focused on achieving fast reperfusion of the occluded blood vessel, either via pharmacological thrombolysis or via endovascular thrombectomy. However, reperfusion injury can further aggravate brain damage. The pathophysiological importance of von Willebrand factor (VWF) and its cleaving enzyme ADAMTS13 in ischaemic stroke and reperfusion injury has become clear. Inhibition of VWF function results in reduced cerebral ischaemia/reperfusion damage. A brief overview will be given of the experimental evidence that illustrates the crucial role of the VWF/DAMTS13 axis in ischemic stroke, as well as of potential new strategies to inhibit VWF activity.

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### Autophagy in Vascular Smooth Muscle Cells Regulates Large Artery Contractility and Compliance in Young C57Bl6 Mice

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**Introduction:** An independent and early hallmark of cardiovascular disease such as hypertension and atherosclerosis is an increase in stiffness of the large arteries. Because the incidence of cardiovascular disease increases with age and aging is associated with a decline in vascular autophagy, we speculated that autophagy deficiency increases vascular stiffness and thus decreases arterial compliance.

**Methods:** Vascular stiffness of aortic segments isolated from young (2 months old) mice with normal autophagy or an autophagy defect (Atg7 deletion via Cre-loxP technology) selectively in vascular smooth muscle cells (VSMCs) was investigated using an in-house developed Rodent Oscillatory Tension Set-up to study Arterial Compliance (ROTSAC) at physiological pressures (70–150 mm Hg) and frequencies (10 Hz).

**Results:** In basal unstimulated conditions (5.9 mM K<sup>+</sup>) and at normal physiological pressures, the Peterson's modulus (Ep), which is a vessel diameter independent measure for arterial stiffness, was not significantly different in Atg7F/F SM22-Cre mice as compared to Atg7+/+ SM22-Cre control mice. However, at increased distention pressures, Ep values were significantly higher in Atg7F/F SM22-Cre mice. This difference in vascular stiffness was similar after the removal of extracellular Ca<sup>2+</sup>, indicating that passive aortic wall remodeling rather than differences in VSMC tone is responsible for the difference in stiffness, which is in line with histological data showing an increase in aortic medial wall thick-

ness and increased focal adhesion molecules. Differences in vascular stiffness at higher pressures were enhanced by depolarizing aortic segments with high K<sup>+</sup>, suggesting that in these conditions active tonus of the segments also contributed to the different aortic stiffness. These findings were confirmed in a traditional isometric organ bath setup. Aortic segments of Atg7F/F SM22-Cre mice showed elevated contractions and were more sensitive to depolarization (high K<sup>+</sup>).

**Conclusion:** Overall, our data demonstrate that autophagy deficiency in VSMCs plays an important role in arterial wall compliance and suggest that pharmacological induction of autophagy might be a novel approach to treat vascular stiffness.

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### Preserved Cardiovascular Homeostasis Despite Blunted Acetylcholine-Induced Dilation in Mice with Endothelial Muscarinic M3 Receptor Deletion

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Muscarinic acetylcholine receptors (AChMR1-5) mediate cellular responses upon release of acetylcholine (ACh) from parasympathetic nerves. In addition, ACh is the prototypical agonist stimulating endothelium-dependent dilation, but most blood vessels lack parasympathetic innervation, raising the question as to the physiologic function of endothelial AChMR. Global deletion of AChM3R revealed a role in ACh-induced vasodilation in vitro as well as food uptake, but overall cardiovascular homeostasis has not been examined thoroughly. We hypothesized that ACh exerts through these receptors a continuous dilator influence in resistance vessels, thereby affecting blood pressure and a long-term defect herein impacts cardiac function. To characterize the function of endothelial AChM3R in vivo, we deleted AChM3R specifically in endothelial cells using an inducible or a non-inducible Cre-loxP system driven by the promoters VE-cadherin (indEC-M3R<sup>-/-</sup>) or TIE2 (EC-M3R<sup>-/-</sup>). In both EC-M3R<sup>-/-</sup>, ACh-induced dilation was strongly impaired in arterioles in vivo (at 10 μM from 76 ± 2 to 40 ± 4 or 24 ± 4%, indEC-M3R<sup>-/-</sup> and EC-M3R<sup>-/-</sup>, respectively) while responses to other dilators were mostly preserved. However, mean arterial pressure was not altered in ndEC-M3R<sup>-/-</sup> (99 ± 4 vs 104 ± 2 mm Hg in controls) and, additionally, arteriolar tone was also comparable in EC-M3R<sup>-/-</sup> mice and respective controls. Aged EC-M3R<sup>-/-</sup> mice (74–78 weeks) did not differ in body weight, heart weight, cardiac structure or contractile function from controls. We conclude that AChM3R elicits the endothelium-dependent dilation upon ACh also in arterioles in vivo. Despite this prominent role, the endothelial deletion of AChM3R does not affect overall cardiovascular homeostasis. Thus, their physiologic function in endothelial cells remains obscure.

## Differences in Basal Levels of Microcirculation and Reactive Hyperemia in the Forehead and Forearm Skin of Humans

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There are differences in the physiological function of the microcirculation of the forehead and anterior forearm skins. Less is known however regarding the regulation of their microcirculation. We hypothesized the resting perfusion of forehead skin is higher than that of the forearm and therefore an ischemic stimulus elicits less increase in the perfusion in forehead compared to that of forearm skin. Thus we aimed to measure local changes in subcutaneous microcirculation in these two locations. We have used Perimed 5000 Laser Doppler flowmetry (LDF) to continuously measure perfusion (perfusion unit an arbitrary number: PU) of the skin. To achieve ischemia the temporal artery or the upper arm was occluded for 2 min, then released (followed by reactive hyperemia, RH). The resting blood pressure and heart rate were also measured.

In response to occlusion and release of the temporal artery, perfusion changed from control PU:  $73.01 \pm 15.4$  during occlusion:  $51.64 \pm 7.16$ , then after release to  $110.76 \pm 22.56$ . Thus the peak RH was 151.7%. In response to occlusion and release of the upper arm, perfusion in the forearm skin changed from control PU:  $10.4 \pm 1.2$ , during occlusion:  $1.9 \pm 0.5$ , then after release to  $48.8 \pm 25.5$ . Thus the peak RH was 469%. In addition, we have investigated the effect of botulinum toxin (BT) known to paralyze muscle tissues. We have found that BT elicited a temporal biphasic response, then PU returned close to control. Occlusion of temporal artery changed the forehead skin perfusion control PU  $70.63 \pm 16.08$ , during occlusion  $55.73 \pm 6.75$  and after release to  $71.37 \pm 29.22$ . Thus there was no significant RH.

On the bases of these findings we propose that 1) the basal microcirculation is higher in the temporal region of the forehead skin than that of the forearm, 2) ischemia/reperfusion elicit greater reactive hyperemia in the forearm skin than in the temporal skin, 3) botulinum toxin inhibits the development of reactive hyperemia. We suggest that anatomical (e.g. collateral circulation) and physiological (e.g. thermoregulation) mechanisms could be responsible for these findings and their elucidation requires further investigations.

## Transcriptional Regulation of the Blood Brain Barrier

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The microvasculature of the central nervous system includes the blood-brain barrier (BBB), which regulates the permeability to nutrients and restricts the passage of toxic agents and inflammatory cells. Canonical Wnt/ $\beta$ -catenin signaling is responsible for the early phases of brain vascularization and blood-brain barrier differentiation. However, this signal declines after birth and other signaling pathways able to maintain barrier integrity at postnatal stage are still unknown.

Sox17 constitutes a major downstream target of Wnt/ $\beta$ -catenin in endothelial cells and regulates arterial differentiation. In the present paper, we asked whether Sox17 may act downstream of Wnt/ $\beta$ -catenin in inducing BBB differentiation and maintenance.

Using reporter mice and nuclear staining of Sox17 and  $\beta$ -catenin, we report that while  $\beta$ -catenin signaling declines after birth, Sox17 activation increases and remains high in the adult. Endothelial-specific inactivation of Sox17 leads to increase of permeability of the brain microcirculation. The severity of this effect depends on the degree of BBB maturation: it is strong in the embryo, and progressively declines after birth. In search of Sox17 mechanism of action, RNA-Seq analysis of gene expression of brain endothelial cells has identified members of the Wnt/ $\beta$ -catenin signaling pathway as downstream targets of Sox17. Consistently, we found that Sox17 is a positive inducer of Wnt/ $\beta$ -catenin signaling and it acts in concert with this pathway to induce and maintain BBB properties. In vivo, inhibition of the  $\beta$ -catenin destruction complex or expression of a degradation-resistant  $\beta$ -catenin mutant, prevent the increase in permeability and retina vascular malformations observed in the absence of Sox17.

**Conclusions:** Our data highlight a novel role for Sox17 in the induction and maintenance of the BBB and they underline the strict reciprocal tuning of this transcription factor and Wnt/ $\beta$ -catenin pathway. Modulation of Sox17 activity may be relevant to control BBB permeability in pathological conditions.

## Aprotinin Preserves Cremaster Microcirculatory Perfusion, But Not Renal Perfusion Following Cardiopulmonary Bypass in Rats

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**Background:** Cardiopulmonary bypass (CPB) during cardiac surgery impairs microcirculatory perfusion, which is paralleled by microvascular leakage and associated with acute kidney injury.

Thrombin is excessively released during CPB, which leads to endothelial and platelet activation and increased endothelial permeability. Aprotinin (Trasylol), an anti-fibrinolytic, is also suggested to inhibit thrombin-PAR1-induced endothelial hyperpermeability. Therefore, this study investigated whether aprotinin reduces CPB-induced microcirculatory perfusion disturbances in cremaster and kidney and whether this is paralleled by reduced kidney injury and edema formation.

**Methods:** Male rats were anesthetized and subjected to 75 minutes of CPB after treatment with aprotinin (n = 15) or PBS (n = 15) as control. The jugular vein and femoral artery were cannulated and connected to the CPB circuit, consisting of a roller pump and membrane oxygenator. Microcirculatory perfusion was measured in the cremaster muscle using intravital microscopy and in the kidney using contrast echography before CPB, and 10 and 60 minutes after weaning from CPB (post-CPB). Renal perfusion was calculated by multiplying microvascular filling velocity and microvascular blood volume. Wet/dry weight ratios were determined from harvested kidney tissue. Renal capillary endothelial ultrastructure was analyzed with electron microscopy. Plasma kidney injury molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL) were measured by ELISA as markers for kidney injury.

**Results:** Onset of CPB was associated with a decrease in hematocrit levels ( $39 \pm 3$  to  $22 \pm 2\%$ ,  $P < 0.001$ ) and blood pressure ( $88 \pm 15$  to  $73 \pm 9$  mm Hg,  $P = 0.02$ ). In addition, CPB resulted in a 2-fold reduction in the number of perfused capillaries in the cremaster muscle ( $p < 0.0001$ ), which did not restore in the first hour post-CPB. One hour post-CPB, a reduction in renal microvascular filling velocity ( $1.3 \pm 0.4$  to  $0.9 \pm 0.5$ /sec,  $P < 0.001$ ) and renal perfusion ( $258 \pm 173$  to  $135 \pm 88$ ,  $P = 0.03$ ) was observed, paralleled by increased plasma levels of NGAL ( $123 \pm 37$  to  $2621 \pm 577$  ng/ml,  $P = 0.003$ ) and KIM-1 ( $197 \pm 39$  to  $273 \pm 81$  pg/ml,  $P = 0.001$ ).

Aprotinin preserved cremaster perfusion following CPB ( $P = 0.002$ ), whereas renal microvascular filling velocity ( $P = 0.5$ ) and perfusion ( $P > 0.9$ ) were not affected compared to untreated animals. In parallel, no differences were observed in plasma levels of NGAL ( $P = 0.7$ ) or KIM-1 ( $P = 0.2$ ). Aprotinin treated animals required less additional fluids ( $3.9 \pm 3.3$  vs  $7.5 \pm 3.0$  ml,  $P = 0.006$ ) during CPB and reduced kidney wet/dry weight ratios ( $4.6 \pm 0.2$  vs  $4.4 \pm 0.2$ ,  $P = 0.046$ ) post-CPB compared to untreated animals.

**Conclusion:** Treatment with aprotinin preserved cremaster microcirculatory perfusion following CPB, but did not reduce renal perfusion disturbances and renal injury, despite reducing renal edema formation. Future studies should focus on identifying therapeutic strategies to improve renal perfusion and function following CPB.

## Aging Downregulates Lamin A/C Expression in Smooth Muscle Cells: A Potential Mechanism Contributing to Age-Dependent Cardiac Disease

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A-type lamins (lamin A/C) are type-V intermediate filaments of the nuclear envelope that are generated by alternative splicing of a common pre-mRNA transcribed from the LMNA gene. Lamin A/C play important structural roles and regulate a broad range of cellular functions, including signal transduction, gene transcription, chromatin organization, DNA damage response, cell cycle progression, cell differentiation and migration. LMNA mutations or defective posttranslational processing of pre-lamin A cause human diseases termed laminopathies, which include tissue-specific as well as systemic disorders. Aging is the main risk factor for cardiovascular disease; however, whether aging affects lamin A/C expression in the cardiovascular system remains unknown. Here, we examined the effect of aging on lamin A/C expression in arterial tissue, and generated and characterized a mouse model lacking lamin A/C specifically in smooth muscle cells (SMC), a main component of the artery wall that undergoes damage during aging.

We quantified by flow cytometry and western blot (WB) lamin A/C levels in aortas of young (3 week old) and old (aprox. 100 weeks old) mice, and in human coronary artery samples from individuals aged  $\leq 30$  and  $\geq 58$  years. A mouse model with genetic disruption of Lmna specifically in SMCs (Lmna-SMC-KO) was generated by crossing Lmna<sup>flx/flox</sup> mice and SM22 $\alpha$ -CRE transgenic mic. Cardiovascular characterization of control and Lmna-SMC-KO mice was performed by echocardiography, electrocardiography, and histology.

Flow cytometry analysis of aorta demonstrated less lamin A/C expression in SMCs and endothelial cells (EC) of old versus young mice. WB of mouse aorta and human coronary arteries also revealed age-dependent lamin A/C downregulation. Remarkably, Lmna-SMC-KO mice lacking lamin A/C in SMCs died prematurely (average life span: aprox. 5 weeks). Moreover, compared with controls, Lmna-SMC-KO mice exhibited cardiac alterations, including defective conduction and repolarization (revealed by electrocardiography), severe left ventricle and right ventricle dysfunction (revealed by echocardiography), and prominent interstitial fibrosis.

Our findings demonstrate a significant downregulation of lamin A/C in aged mouse and human arterial tissue, due to diminished expression in SMCs and ECs. The relevance of lamin A/C downregulation in SMCs is demonstrated by the severe phenotype of Lmna-SMC-KO mice, which developed cardiac fibrosis, electrocardiographic alterations and cardiac dysfunction, and died prematurely. These results suggest that lamin A/C downregulation contributes to CVD during aging. Future studies will examine the molecular mechanisms underlying these new regulatory functions of lamin A/C in aging and CVD.

## Myeloid HIF-Prolyl Hydroxylase Proteins in Human and Mouse Atherosclerosis: Triplets with Differing Characters

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**Introduction:** HIF-prolyl hydroxylase (PHD) 1, 2 and 3 control hypoxia inducible factor (HIF) activity; a master transcription factor in hypoxia. Atherosclerotic plaques are abundant in hypoxic macrophages and plaque reoxygenation restores efferocytosis. Therefore, we studied myeloid cell-specific PHD deficiency in atherosclerosis.

**Approach and Results:** Immunoreactivity of all PHDs co-localized with macrophages in human carotid plaques and murine BMDMs expressed PHD1, 2 and 3 mRNA. In human carotid plaques of the BiKE cohort (Stockholm, Sweden), expression of PHD mRNA correlated with CD68,  $\alpha$ SMA, HIF1/2 mRNA expression. Furthermore, PHD3 mRNA was increased in plaques of symptomatic compared to asymptomatic patients (+8%,  $p = 0.02$ ) altogether suggesting macrophage PHD involvement in atherosclerosis.

Hence, myeloid PHD knockout (PHDko) mice were created via bone marrow transplantation (PHD1 & PHD3) or LysMCre knockout (cko)(PHD2) and fed 0.25% cholesterol diet for 6–8 weeks to induce atherosclerosis. Flow cytometry analysis at sacrifice showed similar circulating leukocyte subsets. Aortic root plaque characterization showed enhanced plaque size in PHD2cko (+155%,  $p < 0.001$ ) and PHD3ko (+40%,  $p < 0.05$ ), but not PHD1ko mice, compared to respective controls. Additionally, necrotic content was increased in PHD3ko (+53%,  $p < 0.05$ ). Macrophage apoptosis was enhanced in PHD2cko (+133%,  $p < 0.05$ ) and PHD3ko (+112%,  $p < 0.05$ ) mice in situ and in vitro (+55%,  $p < 0.001$ , and +23%,  $p < 0.05$  respectively). This effect was HIF1 $\alpha$  and HIF2 $\alpha$  dependent via BNIP3 induced apoptosis in PHD2cko and PHD3ko BMDMs as shown by siRNA gene silencing.

Additionally, PHD2cko plaques displayed more collagen (+203% Sirius red+ area,  $p < 0.001$ ), possibly stabilizing the plaque. This was a consequence of increased collagen production, not reduced degradation, as macrophage MMP activity remained similar while  $\alpha$ SMA+ plaque area was increased in PHD2cko (+72%,  $p < 0.05$ ) suggesting a paracrine effect of PHD2cko macrophages on  $\alpha$ SMA+ cells. However, proliferation and migration of primary smooth muscle cells (SMC), and fibroblasts (3T3, MEF) cultured

in PHD2cko-conditioned BMDM medium was unchanged compared to wildtype-conditioned medium. Interestingly, independent of hypoxia, PHD2cko-conditioned medium enhanced collagen secretion by fibroblasts, but not SMC, in presence of a collagen-producing stimulus (TGF- $\beta$ 1) (+55%,  $p < 0.05$ ). Indeed, fibroblast-like cells (PDGFR $\alpha$ +, PDGFR $\beta$ +, FSP1+, or FAP+ and CD45-/ $\alpha$ SMA-/VE-cadherin-) were prevalent in human and mouse atherosclerosis. PDGFR $\alpha$ + $\alpha$ SMA- density was increased in PHD2cko plaques, while endothelial-to-mesenchymal transition in vitro was unaffected. Single cell sequencing of CD45- cells from WT and PHD2cko plaques is expected to reveal changes in fibroblast density, heterogeneity and function.

**Conclusion:** Myeloid PHD proteins show autocrine and paracrine effects on murine atherosclerosis, mediated by a new player in atherosclerosis; fibroblast like cells.

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### CCL5 and CXCL4 are Rapidly Internalized in Endothelial Cells

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Interaction between platelets and the endothelium facilitates leukocyte arrest and subsequent transendothelial migration to sites of vascular inflammation. In turn, transmigrated leukocytes can stimulate the endothelium to produce substances that induce platelet activation. Activated platelets are known to release bio-molecules from their granules, and the chemokines CCL5 and CXCL4, abundantly present in alpha-granules, can be deposited onto the endothelial cells where they play an important role in monocyte arrest to endothelial cells, which is an essential early step in the development of atherosclerosis.

In this study we aimed to elucidate the mechanisms behind the leukocyte arresting properties of CCL5 and CXCL4 and the role of platelet-endothelial interplay in vascular inflammation. We focused on the localization of CCL5 and CXCL4 on the cell surface and their internalization to the endothelial cell interior.

HUVECs and the endothelial cell line EA.hy926 were incubated with recombinant human CCL5 or CXCL4 for up to 120 minutes. Cells were stained and analyzed with light-, confocal- or stimulated emission depletion (STED) microscopy. To quantify internalization, whole cell lysates and organelle-fractionated cells were analyzed using ELISA.

Both CCL5 and CXCL4 were rapidly internalized in endothelial cells (<10 min). Whereas CXCL4 remained partly presented on the cell surface, all of the CCL5 was internalized. The chemokines were endocytosed by a process dependent on dynamin and clathrin, as internalization was blocked by inhibitors of these molecules. Cell surface proteoglycans, chemokine binding polysaccharides, had a less definite role in the internalization process, as enzymatic cleavage of heparin- and chondroitin sulfate did not result in a decreased

internalization of CCL5 and CXCL4. Combined incubation of CCL5 and CXCL4 with endothelial cells did not influence the internalization or the localization of either of the chemokines. Localization studies by confocal and super-resolution microscopy suggested that both CCL5 and CXCL4 partly have a nuclear localization which, in some cells, seem to be directed to the nucleoli. These visual observations were supported by cell fractionation experiments where chemokine accumulation was quantified in various cell compartments, which revealed a relatively high nuclear accumulation.

In summary, endothelial cells rapidly and actively internalize CCL5 and CXCL4 by clathrin and dynamin dependent endocytosis, where the chemokines appear to be directed to the nucleus. These findings introduce a potential novel, non-canonical role of alpha-granule released chemokines in the cross-talk of activated platelets and endothelial cells, which could have implications for the mechanisms in which leukocytes are attracted to sites of inflammation.

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### A Disintegrin and Metalloprotease ADAM10 Controls Endothelial Functions in Atherosclerosis

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Through shedding of various membrane molecules, including adhesion molecules and chemokines, A Disintegrin And Metalloproteinase 10 (ADAM10) could regulate endothelial functions e.g. permeability and leukocyte recruitment, critical processes in inflammatory diseases like atherosclerosis. Indeed, proteomic analysis on mouse endothelial cell sheddome revealed  $\pm 300$  differentially regulated proteins upon ADAM10 inhibition, of which 10% appeared involved in permeability and leukocyte transmigration. In this study we evaluated the causal role of endothelial ADAM10 in atherosclerosis development and related endothelial cell functions.

Wildtype or endothelial ADAM10-deficient (ADAM10<sup>fl/fl</sup>/Tie2-Cre; in brief ADAM10<sup>del</sup>) mice were rendered atherogenic by adeno-associated virus-mediated overexpression of PCSK9, resulting in persistent LDL receptor knockdown and hyperlipidemia after high cholesterol diet feeding (HCD). Using RNAi-mediated

ADAM10 knockdown in HUVECs, we assessed the role of ADAM10 in endothelial proliferation, metabolism (Seahorse) and neutrophil adhesion and transmigration under flow.

As expected, in vitro inhibition of endothelial ADAM10 decreased neutrophil transmigration. Surprisingly, after 12 weeks of HCD diet feeding, ADAM10<sup>del</sup> mice showed significantly larger ( $\pm 45\%$ ) and more advanced atherosclerotic lesions. Strikingly, lesions contained intraplaque hemorrhage and neovascularization, features of plaque instability that are rarely observed in wildtype mice. Necrotic core area was increased ( $\pm 87\%$ ) and macrophage content decreased ( $\pm 49\%$ ). No differences were observed in granulocyte and collagen content. In vitro, ADAM10 inhibition was shown to modulate endothelial metabolism, enhancing endothelial proliferation and dysfunction.

In conclusion, this study reveals an unexpected protective effect of endothelial ADAM10 in atherosclerosis development and indicate an important role for ADAM10 in regulating endothelial metabolism and (dys)function.

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### Increased Oxidative Stress Underlies Impaired Endothelial Function and Vascular Reactivity with High Salt Dietary Intake, Independently of Blood Pressure Changes – Translational Studies

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**Introduction:** High salt dietary intake (HS) is known causal factor of endothelial dysfunction, even without increases in arterial blood pressure. The aim of this study was to present our current work on the underlying mechanisms of endothelial dysfunction occurring in HS diet, related to increased oxidative stress, in healthy animals and young healthy humans.

**Methods:** Functional studies on the mechanisms of microvessel's reactivity (endothelium-dependent and endothelium-independent) as well as molecular (mRNA; protein expression) and biochemical analyses (enzymatic activity, flow-cytometry, direct fluorescence) on the biomarkers of vascular and systemic oxidative stress were performed in healthy normotensive young men and women (N = 10–15 (per set; average age 21 yrs) and healthy normotensive Sprague-Dawley male rats 11-weeks old (N = 7–10/per group) on a HS and low-salt (LS) diet.

**Results:** Human: HS diet impairs endothelium-dependent, but not endothelium-independent vasodilation in skin microcirculation (post-occlusive reactive hyperemia (PORH); ACH vs. SNP) and increases TXA2 production in healthy women (J Physiol 2015;593(24):5313–24). HS diet significantly increased basal ROS

production in monocytes. HS diet significantly increased oxidative stress level (TBARS) and decreased antioxidant capacity (FRAP). PORH and FRAP positively correlated, while PORH and TBARS negatively correlated. There was negative correlation between salt intake and FRAP and between salt intake and PORH. 7-day HS diet did not induce significant change in arterial blood pressure, or in body composition or fluid status in young healthy population. Plasma renin activity (PRA) and serum aldosterone level in humans are suppressed by HS diet.

**Animals:** Attenuated flow-induced dilation of cerebral resistance arteries (MCA) is related to increased vascular oxidative stress and NO pathway in rats on a short-term HS diet (J Physiol 2016;594(17):4917–31). HS diet suppresses flow-induced NO production in MCA, which is restored by superoxide scavenging (AJP 2018;315(3):H718-H730). Brain blood vessels antioxidant enzyme expression is decreased in HS diet (Gpx4), while HIF-1alpha and COX-2 protein levels are increased, possibly related to increased oxidative stress caused by HS diet and reversed by TEMPOL in vivo. Oxidative stress in leukocytes isolated from blood and peripheral lymph nodes is increased.

**Conclusions:** HS diet significantly alters microvascular reactivity in young healthy normotensive women, without changes in blood pressure and in animal model of normotensive Sprague-Dawley rat. HS diet significantly reduces antioxidant capacity and increases oxidative stress markers, leading to switch in pathways mediating vasodilation, which can be associated with microvascular dysfunction, confirmed in both types of studies. Microvascular dysfunction precedes changes in blood pressure. Funded by grants from Croatian Science Foundation: RAS-AdrenOX (IP-2016-06-8744, PI Ana Stupin) and VELI-Athero (IP-2014-09-6380, PI Ines Drenjancevic).

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### miR-378a Influences Vascularization in Skeletal Muscles

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**Introduction/Objective:** MicroRNA-378a, highly expressed in skeletal muscles, was demonstrated to affect myoblasts differentiation and to promote tumor angiogenesis. We hypothesized that

miR-378a could play a proangiogenic role in skeletal muscle in physiological conditions and after ischemic injury. We aimed to investigate the role of miR-378a in differentiation and paracrine angiogenic potential of myoblasts and in regenerative neovascularization after femoral artery ligation (FAL) in mice.

**Methods:** As an experimental model murine myoblasts cell line (C2C12) with silenced miR-378a expression and aortic cross sections from miR-378a knockout (-/-) and wild type mice were used. To induce hind limb ischemia mice were subjected to FAL. The local and systemic miR-378a overexpression was obtained by, respectively, intramuscular or intravenous delivery of adeno associated vectors (AAV). In addition, blood from patients with peripheral artery disease (PAD) and healthy donors was analyzed.

**Results:** Silencing of miR-378a in C2C12 did not affect differentiation but impaired angiogenic potential towards aortic endothelial cells. Decreased endothelial sprouting from aortic rings and less CD31-positive blood vessels in gastrocnemius muscle of miR-378a-/- mice was noticed. Interestingly, although fibroblast growth factor 1 expression was decreased in miR-378a-/- muscles and in myoblasts upon miR-378a silencing, this growth factor did not mediate the angiogenic effects exerted by miR-378a. In vivo, miR-378a knockout did not affect the revascularization of the ischemic muscles in both normo- and hyperglycemic mice subjected to FAL. No difference in the level of Vegf nor the number of arterioles or regenerating muscle fibers was detected between miR-378a-/- and miR-378a+/+ mice. Unexpectedly, miR-378a expression declined in ischemic skeletal muscles of wild type mice already on day 3 after FAL and restored following the regeneration process. At the same time, the level of miR-378a was increased in the plasma 3 days after FAL. Noteworthy, elevated expression of miR-378a in the plasma of PAD patients was also evident. Importantly, intramuscular but not systemic delivery of AAV-miR-378a improved reperfusion of the ischemic limb on day 7 after FAL. Interestingly, the number of infiltrating CD45+ cells was higher in the ischemic muscles of miR-378a-/- mice while it was slightly attenuated by AAV-miR-378a.

**Conclusion:** Our study indicates for miR-378a role in physiological angiogenesis. Although it seems dispensable for revascularization of ischemic muscles, its overexpression may enhance this process. Moreover, miR-378a appears to be a marker of ischemic muscle injury and may modulate the leukocyte infiltration into the damaged tissue.

### Induced Pluripotent Stem Cells-Derived Cardiomyocytes Improve Heart Function in Murine Model of Myocardial Infarction

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**Introduction:** It has been estimated that up to 1 billion cardiomyocytes are lost during myocardial infarction (MI) without the possibility to replace them by surviving cells due to their limited proliferative potential. Importantly, no available treatment of MI enables restoration of functional cardiomyocytes in place of fibrotic scar formed in damaged region, and various so called “stem cell” therapies based on the cells lacking capacity to differentiate into cardiomyocyte appear non-effective. Thus, there is an immense need for novel, biologically rational therapies which would allow for recovery of viable myocardium upon MI. Therefore, here we evaluated the therapeutic potential of genetically modified human induced pluripotent stem cells-derived cardiomyocytes (hiPSC-CMs) in the murine model of acute MI.

**Methods:** Peripheral blood mononuclear cells of a healthy donor were used for generation of hiPSCs using non-integrating Sendai vectors. The cells were subsequently transduced with lentiviral vectors expressing GFP, luciferase (Luc), and cardioprotective and proangiogenic factors: either HO-1 or SDF-1 to improve survival and therapeutic potential of transplanted cells. Sorted GFP-positive cells were differentiated into cardiomyocytes with small molecules regulating WNT signaling pathway. Obtained population contained 70–90% cardiac troponin T-positive contracting cells. Next, NOD SCID mice were subjected to either sham operation or permanent ligation of left anterior descending (LAD) coronary artery. Shortly after MI each type of cells ( $5 \times 10^5$  in  $10 \mu\text{l}$ ) or saline were injected into per-infarct zone of the left ventricle wall. The ultrasonography of murine hearts was performed on day 7, 14, 28 and 42 to assess their function whereas the presence of hiPSC-CM was monitored using IVIS Spectrum In Vivo Imaging system upon administration of luciferin.

**Results:** Measurements of luciferase activity revealed the strongest bioluminescent signal in the hearts of mice transplanted with iPSC-CM-HO1 42 days after MI in vivo, indicating the survival of hiPSC-CM in murine myocardium at least six weeks upon administration. Importantly, transthoracic ultrasonography demonstrated that in all mice after hiPSC-CM delivery the improvement of ejection fraction (EF) was very significant in comparison to animals injected with saline after induction of MI (Sham,  $n = 8$ , EF = 57.2%; MI + saline,  $n = 7$ , EF = 19.1%; MI+ hiPSC-CM-Luc-GFP,  $n = 7$ , EF = 36.7%; MI + hiPSC-CM-SDF-1-Luc-GFP,  $n = 8$ , EF = 35.1%; MI + hiPSC-CM-HO-1-Luc-GFP,  $n = 8$ , EF = 37.2%).

**Conclusion:** Obtained results strongly indicate that proposed cell therapy is effective in treatment of MI. Additionally, overexpression of HO-1 in hiPSCs-CM may positively influence their survival upon in vivo delivery in the unfavorable conditions of infarcted tissue.

### Adventitial Interleukin-6 Release is Critical for Neointima Formation

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**Objective:** The aim of this study was to analyze the impact of the adventitial layer on vascular remodeling processes and to define the underlying cellular mechanisms.

**Methods and Results:** The femoral artery of C57BL/6J mice was dilated with a straight spring wire and morphometric analysis of the lesion and immunohistochemical staining for the proliferation marker Ki-67 was performed 7, 14, and 21 days following injury. Formation of a neointimal lesion at 21 days was preceded by high adventitial proliferation rates and massive adventitial thickening at 7 and 14 days (adventitial area:  $0.036 \pm 0.015 \text{ mm}^2$  at 0 d vs.  $0.082 \pm 0.013 \text{ mm}^2$  at 7 d vs.  $0.102 \pm 0.029 \text{ mm}^2$  at 14 d,  $n = 15$ ,  $P < 0.0001$ ).

Complete removal of the adventitial layer prevented neointima formation, attributing pivotal importance to the adventitial layer (luminal stenosis:  $71.73 \pm 3.77\%$  vs.  $7.44 \pm 1.71\%$ ,  $n = 5$ ,  $P < 0.0001$ ). Re-transplantation of the aortic adventitia of ubiquitously GFP expressing C57BL/6-Tg(CAG-EGFP)10sb/J mice around the medial vascular layer of the femoral artery where the native adventitia has been removed completely restored neointima formation. Importantly, only very few GFP+ cells were present in the neointimal layer, indicating that a direct contribution of adventitial cells to the neointimal lesion represents an extremely rare event.

To investigate a potential paracrine effect of the activated adventitial layer, we explanted adventitial transplants 14 days following injury and transplantation and incubated the respective samples in serum-free media for 24 hours. BrdU incorporation assays and scratch wound assays revealed significantly increased proliferation and migration rates of human coronary artery SMCs in response to the supernatant of adventitial transplants compared to the supernatant of control samples or serum-free media. Further secretome analyses of the same adventitial supernatants identified predominantly interleukin (IL)-6 to trigger SMC proliferation and migration. Accordingly, serum-free media incubated with adventitial grafts of IL-6<sup>-/-</sup> mice prevented SMC proliferation and migration. Transplantation of the adventitia of IL-6<sup>-/-</sup> mice into C57BL/6J wild type mice was not sufficient to trigger neointima formation.

**Conclusion:** Acute vascular injury is followed by an expansion of cytokine-producing adventitial cells, whose paracrine function and especially whose release of IL-6 is essential for the subsequent induction of the proliferation and migration of local SMC and thus for neointima formation.

## New Considerations of Lymphatic Endothelial Cells as Key Players of the Pro-Tumoral Microenvironment

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Metastatic dissemination is the main cause of cancer-related death. Cancer cells spread from the primary tumor to distant organs through blood and/or lymphatic vessels. The lymphatic vessels are highly permeable and are often viewed as simple “tubes” where tumor cells passively transit to form metastasis. However, lymphatic endothelial cells (LECs) demonstrate plasticity and heterogeneity that are worth exploring for understanding their implication in tumor microenvironment.

Our hypothesis is that LECs can be activated by tumor cells and therefore act as active stromal players in the complex metastatic process.

The direct confrontation of a LEC monolayer to different human carcinoma cell lines (HaCat II4, HaCat A5RT3) disturbs the endothelium integrity and impairs LEC-LEC junctions (VE-cadherin internalization). Cytokine array and ELISA revealed that tumor cell conditioned medium (TCM) induced significant modification of LEC secretome. A spheroid assay showed an increase of tumor cells migration in presence of LEC. Moreover, we observed an increase of tumor cell proliferation in presence of medium conditioned by activated LECs (previously exposed to TCM). These observations showed that LECs influence tumor cell behavior by stimulating their migration and proliferation.

LECs can be activated by tumor cells to secrete cytokines and that, in turn provide a permissive microenvironment for tumor cells. Beyond the intravasation of tumor cells into lymphatics, our work sheds a new light on reciprocal tumor cells-LEC crosstalk and identified them as key actors of the tumor microenvironment.

## Improving Stratification of Cardiovascular Risk in Systemic Lupus Erythematosus using Endothelial Microvesicles as Novel Biomarkers

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**Introduction:** Systemic Lupus Erythematosus (SLE) is an autoimmune disease that confers increased risk of a major cardiovascular event; up to 50-fold in female patients <45 years. Traditional risk prediction algorithms do not include SLE and the lack of relevant biomarkers prevents accurate stratification of patient risk. Endothelial microvesicles (EMVs) impact on, and offer a snapshot of, vascular health through transference of microRNA (miRNA). This project aims to evaluate the relevance of a new risk algorithm, QRISK3, and the use of EMVs as novel biomarkers for cardiovascular risk in SLE.

**Methods:** Risk scores were calculated for 109 patients and 29 controls, and correlated with clinical markers of inflammation and vascular health. Circulating EMVs were enumerated using flow cytometry and their miRNA content analysed by quantitative PCR. Predicted miRNA targets were identified using gene ontology techniques before miRNA overexpression and sponge lentiviral constructs were generated using Gateway cloning technology. Effects of miRNA expression on vascular calcification were elucidated using alizarin red staining and qPCR.

**Results:** Patients with SLE demonstrated higher average QRISK3 scores than controls (5% vs 0.3%;  $p < 0.001$ ) and scores were significantly elevated in patients when using QRISK3 compared to QRISK2 and Framingham score ( $p < 0.001$ ). QRISK3 identified an additional 21 patients at high risk, who were also found to have dysregulated cardiovascular and inflammatory measurements compared to low risk patients. Furthermore, EMVs were elevated in SLE ( $p = 0.001$ ) and in missed patients ( $p = 0.019$ ), and correlated with QRISK3 score ( $p = 0.001$ ) as well as vascular and inflammatory markers. 7 vesicular miRNAs were associated with SLE; of these, miR-3148 and miR-126-3p were elevated in SLE patient plasma ( $p = 0.009$  and  $p = 0.048$  respectively). Overexpression of miR-3148 in vascular smooth muscle cells resulted in decreased calcium deposition ( $p = 0.002$ ), which was associated with reduced RUNX2 expression; effects of miRNAs on endothelial cells is ongoing. Gene ontology identified VE-PTP (PTPRB; implicated in vascular instability) as a shared target for miR-3148 and miR-126-3p, and RUNX2 as a target for miR-3148; confirmation of binding is underway.

**Conclusions:** QRISK3 is more representative of cardiovascular risk in SLE and supports the use of EMVs as novel biomarkers of

vascular health. Examination of the miRNA content of patient EMVs opens multiple opportunities for personalised medicine, such as improved risk stratification and the identification of novel therapeutic pathways. Further understanding of the interactions at the vascular interface in SLE will improve wellbeing and prevent premature mortality in the future.

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### Unaltered Sublingual Microcirculatory Function Throughout ECMO Treatment: A Prospective Longitudinal Observational Study in Critically Ill Neonatal and Pediatric Patients

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**Introduction:** With extracorporeal membrane oxygenation (ECMO) mechanical cardiorespiratory support is offered to neonatal and pediatric patients with acute cardiorespiratory failure. It remains unclear, however, whether recovery of global systemic hemodynamics through ECMO treatment is sufficient to also improve microcirculatory function.

**Objective:** To study the sublingual microcirculation during ECMO treatment in neonatal and pediatric patients to assess change over time and the predictive value of microcirculatory parameters for survival.

**Methods:** We performed a prospective longitudinal observational study at a pediatric ICU, including consecutive patients treated with ECMO. The sublingual microcirculation was assessed with the handheld vital microscope Cytocam. Daily measurements were performed before, during and after ECMO treatment. Parameters of vessel density, perfusion, and flow quality were assessed for all vessels (diameter <100 µm) and small vessels (<20 µm). Mixed models and logistic regression models were built to assess change over time and important covariates. With ROC curves the predictive value of microcirculatory parameters was assessed for successful weaning from ECMO and overall survival.

**Results:** The study population comprised 34 patients with a median age of 0.27 years (IQR 2.22), of whom 16 neonates (47%) and 16 females (47%). Twelve patients were treated with venovenous (VV) ECMO and 22 with veno-arterial (VA) ECMO. Sixty-five percent was successfully weaned from ECMO and 56% survived until discharge. None of the microcirculatory parameters changed significantly over time, before, during and after ECMO treatment. No associations were found between microcirculatory parameters and global systemic hemodynamics, except between microcirculatory flow index (MFI), a parameter of flow quality, and mean arterial pressure (MAP). The odds for a normal MFI (MFI >2.6) increased with the increase of MAP (OR: 1.050, 95% CI: 1.008–1.094). Microcirculatory parameters did not differ between VV and VA ECMO or between survivors and non-survi-

vors. None of the microcirculatory parameters could predict successful weaning from ECMO or overall survival.

**Conclusion:** In this heterogeneous study population the sublingual microcirculatory parameters did not change throughout ECMO treatment and could not predict survival. Future research should focus on determining which ECMO patients could benefit from microcirculatory monitoring.

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### Cerebral Microvascular Impairment Contributes to Ischemic Injury in the Aging Brain

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Aging is the most important independent risk factor for the incidence and prevalence of ischemic stroke. Aging also accelerates infarct growth and significantly predicts poor patient outcomes. Our hypothesis posits that cerebral microvascular impairment in the old brain contributes to the age-related exacerbation of ischemic brain injury. Therefore our research focuses on the impact of age on the structure and reactivity of the cerebral microvasculature to metabolic and ischemic challenges.

Our experimental work has revealed that the cerebral microvascular network displays typical alterations in the aging brain, including basement membrane and pericyte pathology, which may compromise the fine regulation of microvascular tone. In addition, the rarefaction of the cortical arteriolar tree is thought to provide insufficient support to react with flow elevation in response to metabolic challenges. Indeed, functional hyperemia in response to spreading depolarization proves to be insufficient in the aging brain. Moreover, spreading depolarization is frequently coupled with potassium concentration-related flow reduction (i.e. inverse neurovascular coupling) rather than flow elevation in the old brain under ischemic stress. While the cerebral blood flow response involves the young cortical surface in a homogeneous fashion, the spatial pattern of the flow response becomes typically inhomogeneous in the old brain. Finally, flow compensation following ischemia onset is impaired in the old brain, partially due to the inability of cerebral arterioles to dilate, and also to the failure to recover from the inverse flow response evolving with spreading depolarization.

On the basis of our accumulating data we propose that (mal) adaptation of cerebral microvascular function with aging reduces ischemic tolerance of the nervous tissue, which is implicated in the intensified expansion of ischemic damage in the old brain.

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## Atypical Adhesion Molecule T-Cadherin Is a Novel Regulator of Pericyte Function

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**Introduction:** Pericytes are mural cells which play a key role in regulation of angiogenesis, endothelial permeability, maintenance of endothelial quiescence and survival. Pericytes also possess progenitor/stem-like cell properties and ability to differentiate into various cell types, thus contributing to tissue repair. Cadherins are a adhesion molecules which mediate Ca<sup>2+</sup>-dependent homophilic intercellular interactions. To date, N-cadherin is the only cadherin described in pericytes. It forms adherens junctions between endothelial cells and pericytes and takes an important part in vessel stabilization during angiogenesis. Here we demonstrate that another member of the superfamily, atypical GPI-anchored T-cadherin is expressed in pericytes and regulates pericyte function.

**Methods:** Pericytes from human placenta and adipose tissue were isolated as CD146+/CD34- cell population and used to achieve T-cadherin overexpression or silencing by lentivirus-mediated transduction. Cell behavior was analyzed using CyQuant proliferation assay, invasion in fibrin gel, time-lapse videomicroscopy, mixed endothelial-pericyte spheroid assay, angiogenesis in Matrigel and microfluidic chamber. Gene and protein expression in cultured cells was evaluated by immunoblotting, immunocytochemistry and qPCR. Protein expression in human and mouse fresh-frozen tissue was evaluated by immunohistochemistry. Pericyte differentiation was studied using flow cytometry analysis of human stromal-vascular fraction (SVF).

**Results:** T-cadherin is expressed by cultured CD146+/CD34- placental and adipose-tissue pericytes and NG2+ cells from the heart, brain and adipose tissue. and pericyte-like cells from human aorta. T-cadherin regulates pericyte proliferation, migration and invasion, differentiation and causes actin cytoskeleton remodeling. In cocultures with human endothelial cells from umbilical vein (HUVEC) T-cadherin overexpression promotes and silencing delays pericyte-dependent regulation of endothelial network formation. T-cadherin overexpression modulates expression of pericyte genes relevant for differentiation and angiogenesis.

**Conclusions:** T-cadherin is a novel regulator of pericyte function which mediates endothelial-pericyte interactions during angiogenesis and pericyte differentiation status. The novel T-cadherin-dependent signaling pathway might be relevant for progression of (patho)physiological conditions involving activation of angiogenesis and tissue regeneration.

## Barcoding in Obese: Who's at Increased Risk for the Development of Cardiovascular Disease?

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**Introduction/Objective:** Obesity predisposes to cardiovascular diseases (CVD); however, not all obese subjects will develop CVD. In search of predictive biomarkers to stratify the obese, we considered bioactive lipids. Secreted by adipose tissue, these lipids regulate key processes of atherosclerosis, including inflammation. In this study, we set out to profile lipids in plasma from a prospective cohort (Cohort On Diabetes and Atherosclerosis Maastricht, CODAM) of obese subjects that suffered or did not suffer a CVD event during 7-year follow-up.

**Methods:** To this end, we included baseline (1999) plasma samples of 92 individuals. The samples were divided into three groups: obese patients with CVD after 7-year follow up (2006, n = 39), obese patients with no CVD after 7-year follow-up (2006, n = 39) and healthy lean controls (n = 10) and were matched for age, sex, smoking status, type 2 diabetes and waist size. Total lipids were extracted from plasma and lipidomics was performed using low chromatography mass spectrometry.

**Results:** Features were extracted from the data, aligned between samples and further identified as lipids. 868 lipids were identified according to their exact mass-to-charge ratios and their retention time. Not all lipid constituents were detectable in all patient's samples either due to sub-threshold concentrations or due to technical issues, resulting in 32% of missing values. Using a novel machine learning approach, we generated a code to make use of incompletely observed data. We produced 160.000 datasets on 6 imputation value threshold (ranging from 20 to 70% imputation of missing values) and assessed these with logistic regression and c-statistics. Imputation of missing values did not introduce bias in our dataset, as the AUCs generated remained similar amongst all different thresholds (threshold 0.8: 20% missing values included, AUC: 0.529. Threshold 0.4: 60% missing values included, AUC:0.545). To avoid overfitting, we further performed feature selection on all imputed datasets. Univariate feature selection reduced the feature set from 868 to an average of 50 features, dramatically improving the prediction model (average AUC: 0.89). Subsequent multivariate feature selection further reduced the dataset to an average of 20 lipids mostly composed of triglycerides, sphingolipids and ceramides\* (average AUC of 0.96). Importantly, our model significantly outperforms classical CVD risk prediction in obese population (using systolic blood pressure, total cholesterol and fasting plasma glucose levels, AUC:0.53).

**Conclusion:** Taken together, we have identified a plasma lipid signature with unprecedented high predictive power for cardiovascular events in obese adults.

\*For intellectual property reasons, lipid names are not revealed.

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### MMP9 Associates with Endothelial Glycocalyx Degradation During Haemorrhagic Fever with Renal Syndrome

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**Introduction:** Haemorrhagic fever with renal syndrome (HFRS) is characterized by fever, hypotension, vascular leakage, thrombocytopenia and renal failure. HFRS in Sweden is caused by the Puumala hantavirus and is spread by viral-infested droppings from bank voles. The health care system has little to offer these patients since there is no antiviral treatment and as of yet there is no vaccine prophylaxis available. We previously showed that a marker of endothelial glycocalyx degradation (Syndecan-1) was associated with disease severity and disseminated intravascular coagulation during HFRS (Connolly-Andersen et al., 2014, Open Forum Infect Dis.).

**Methods:** We analysed the levels of other endothelial glycocalyx degradation markers (heparan sulfate, soluble thrombomodulin, albumin), a potential “shedase”: Matrix Metalloproteinase 9 (MMP9) and neutrophil activation/tissue damage (neutrophil gelatinase-associated lipocalin, NGAL) in patient plasma from 44 HFRS patients collected consecutively following disease onset. We used the generalized estimating equation to analyse the association between endothelial glycocalyx degradation, MMP9 levels, neutrophil activation/tissue damage and HFRS disease outcome (need for oxygen, transfusion with blood components, need for intensive care unit (ICU) treatment and renal damage).

**Results:** 44 HFRS patients were included in this study (29 females (66%)); need for oxygen: 11 (25%); transfusion with blood components: 3 (7%) and stay at ICU: 2 (5%). The levels of MMP9 were significantly associated with all markers of endothelial glycocalyx degradation. Neutrophil activation/tissue damage (NGAL) was also significantly associated with MMP9 and endothelial glycocalyx degradation markers (apart from albumin ( $p = 0.053$ )). In addition degradation of endothelial glycocalyx associated with HFRS disease outcome.

**Conclusion:** Degradation of the endothelial glycocalyx could be a potential mechanism of HFRS pathogenesis, and potentially MMP9 could contribute to degradation of the endothelial glycocalyx.

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### Endothelial Cells Exert Stimulus-Specific Paracrine Effects on Co-Cultured Cardiomyocytes During Inflammation

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**Introduction:** We sought to determine in vitro if endothelial activation during inflammation affects cardiomyocyte (CM) function (i.e. contractility). Interestingly many studies have demonstrated the ability of endothelial-derived factors such as nitric oxide or endothelin-1 to influence CM survival, morphology and contractility. However, it is still unclear how this basal signalling is regulated in health and disease where plasticity of endothelial cells (EC) is synonymous with a quantitative/qualitative change of paracrine activity. The aims of this study were therefore to emulate the remodelling of EC during inflammation, using a cocktail of three pro-inflammatory cytokines, and investigate indirect paracrine effects on co-cultured CM.

**Methods:** Human cardiac microvascular EC and adult rat ventricular CM were co-cultured in a transwell system for 4 hours (pore size: 0.4  $\mu\text{m}$ ; Greiner Bio-One). EC were first pre-conditioned for 24 hours with Cytomix (1 ng/ml TNF- $\alpha$ , 1 ng/ml IL-1 $\beta$  and 25 ng/ml IL-6 R $\alpha$ /IL-6 chimera). The technique of optical mapping was used to characterise the response of CM to paracrine signals produced by EC: calcium transients were recorded using Fluo-4. Finally, we performed secretome analysis of co-culture supernatants using a Cytokine Profiler Array (R&D).

**Results:** When pre-conditioned by Cytomix, EC induced a significant shortening of calcium transients in co-cultured CM compared to untreated EC, indicating a cardiac lusitropic response (pro-relaxation). However, amplitude and time to peak were unaffected by the inflammatory pre-conditioning. Analysis of supernatants revealed an enrichment of multiple analytes in Cytomix pre-conditioned co-cultures, including CXCL5 and CXCL1, when compared to untreated conditions. Pre-conditioning of EC with individual cytokines (TNF- $\alpha$ , IL-1 $\beta$  or IL-6 R $\alpha$ /IL-6 chimera) instead of Cytomix did not significantly change calcium transient morphology in CM but they were all associated with unique paracrine profiles, highlighting the complexity of cytokine response in inflammation and the value of co-treatments on EC.

**Conclusions:** This study proposes a novel role of the endothelium on cardiac function. Characterising the importance of multicellularity in disease modelling in vitro, these findings suggest that remodelling of cardiac EC in response to inflammation may adapt their paracrine profile to promote relaxation in neighbouring CM. Finally, the type of inflammatory pre-conditioning of EC was a clear determinant of their paracrine activity in subsequent co-cultures. This suggests the paracrine effects of EC on CM may be regulated by the origin, severity and stage of myocardial inflammation.

## Endothelial Cell Orienting

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The establishment of a functional patterned vascular network is crucial for development, tissue growth and homeostasis. The mis-patterning or dysfunction of this network is associated with cardiovascular diseases. The formation of a functional vascular network requires two distinct processes – formation of primitive vascular plexuses through sprouting angiogenesis; and vascular remodelling, which reorganizes the primitive plexuses into a hierarchical network of arteries, capillaries and veins. Both morphogenic processes are driven by extensive cell migration and cell rearrangements.

Here, we show that blood flow-induced wall shear stress and VEGF-induced junctional tension are two mechanical forces that direct collective endothelial cell polarity. We demonstrate that blood flow and VEGF compete to orchestrate patterns of endothelial cell polarity at the network-level. We establish that the competitive nature of this interaction defines the transition between two distinct morphogenic events, vascular sprouting and vascular remodeling. Accordingly, manipulation of VEGF gradients or blood flow in vivo compromises normal polarity patterns, resulting in delayed or premature remodeling of blood vessels. At the molecular level, we show that mechanotransduction at adherens junctions is key for VEGF-induced polarization and negatively regulates flow-dependent collective polarization.

Our results highlight how the information relayed by two key organizers of cell polarity is integrated to coordinate collective cell behavior in blood vessels, providing a novel concept for the etiology of cardiovascular diseases.

## Novel Optical Instrument for Comprehensive Microcirculation Analysis: The Relation to Cardiovascular Risk Factors

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**Objective:** The objective was to study the relation between comprehensive microcirculatory parameters and the common cardiovascular risk factors obesity and smoking habits. The microcirculatory parameters are local hemoglobin oxygen saturation (SO<sub>2</sub>), tissue fraction of red blood cells (RBC), and speed resolved perfusion.

**Methods:** The parameters were retrieved using the new PF6000 EPOS system (enhanced perfusion and oxygen saturation, Perimed AB, Sweden), which is a non-invasive optical instrument based on diffuse reflectance spectroscopy and laser Doppler flowmetry. The

instrument measures in a single point using a fiber optic-based probe. With the recent advances in bio-optical modeling, the microcirculatory parameters from the instrument are given in quantitative and absolute units.

The study was performed within the Swedish CARDioPulmonary bioImage Study (SCAPIS), a world unique study including detailed imaging and functional analysis of heart, vessels and lungs in 30,000 men and women in Sweden to predict and prevent cardiovascular disease and chronic obstructive pulmonary disease. Microcirculatory measurements were performed on one of the six University hospitals connected to SCAPIS. The first 1,765 subjects are included in this study, aged 50–64 years from the Linköping general population. The measurement protocol involved measurements at 2 Hz on forearm skin during a 20 minutes occlusion-release provocation (5 min baseline, 5 min brachial occlusion to 250 mm Hg, and 10 min reperfusion). Study subjects were grouped on obesity (BMI >30) and smoking habits (currently smoking regularly or occasionally, or never smoked), respectively. Mean and standard deviation of microcirculatory parameters were calculated during baseline and during reperfusion peak. Statistical differences were assessed by t-tests.

**Results:** Significant differences between obesity groups were found during baseline in for example SO<sub>2</sub> (lower for obesity group,  $p < 0.001$ ), RBC (higher,  $p < 0.001$ ), perfusion for speeds 1–10 mm/s (lower,  $p = 0.001$ ). At reperfusion peak, significant differences were found for SO<sub>2</sub> (lower,  $p < 0.001$ ), RBC (higher,  $p < 0.001$ ) and perfusion for speeds <1 mm/s (higher,  $p = 0.002$ ).

There were significant differences between smokers ( $n = 157$ ) and non-smokers ( $n = 869$ ) in baseline RBC (higher for smokers  $p = 0.02$ ). At peak there were significant difference in SO<sub>2</sub> (lower,  $p < 0.001$ ), perfusion for speeds 1–10 mm/s (lower,  $p = 0.01$ ), and perfusion for speeds >10 mm/s (lower,  $p < 0.001$ ).

**Conclusion:** The new microcirculatory parameters are related to obesity and smoking. Those are two well known risk factors for cardiovascular disease. Thus, the results encourage deeper analysis of the relation between the microcirculation and cardiovascular disease.

## Endoplasmic Reticulum Stress in Vascular Smooth Muscle Cells Results in Contractile Phenotype Loss and Regulates Calcification Mediated by Grp78-Loaded Extracellular Vesicles

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**Introduction:** Vascular calcification (VC) is the process of hydroxyapatite deposition in the blood vessel wall, which leads to vascular stiffening and heart failure. It is a health problem common in ageing populations, diabetes and chronic kidney disease. VC is a regulated process mediated by vascular smooth muscle cells (VSMCs), which show a high degree of phenotypic plasticity.

In healthy vessels VSMCs are quiescent and maintain vascular tone and are termed 'contractile'. In disease states or VSMCs become 'synthetic', they secrete matrix proteins, migrate and proliferate in response to injury. Recently it has been shown that synthetic VSMCs release increased amounts of extracellular vesicles (EVs). The endoplasmic reticulum (ER) is involved in folding of proteins. ER stress occurs as a result of unfolded protein accumulation and has been implicated in EV release in other cell types. The aim of this study was to investigate the link between ER stress, phenotypic modulation of VSMCs and VC.

**Methods:** Human primary aortic VSMCs were treated with tunicamycin (TM) and thapsigargin (TG) to induce ER stress and elevated Ca<sup>2+</sup> and PO<sub>4</sub><sup>2-</sup> to induce calcification. Contractile marker expression was measured using western blotting. Calcification was quantified with the o-cresolphthalein assay. Exosomes were captured on beads using an anti-CD63 antibody and detected with FACS using an anti-CD81 antibodies. ER stress activation was measured based on Grp78 and Grp94 chaperone expression by western blotting and immunohistochemistry.

**Results:** Induction of ER stress in VSMCs was associated with decreased expression of contractile markers p-MLC, CNN1, SM22 $\alpha$  and an increase in EV release, which are hallmarks of contractile phenotype loss. Additionally, ER stress increased VSMC calcification. ER stress-induced calcification of VSMCs was blocked by inhibitor of SMPD3, an enzyme involved in EV release. EVs released from ER stress-treated VSMCs were enriched in ER stress chaperone Grp78. Grp78 has previously been shown to facilitate calcium deposition on collagen matrices, but not in the context of VSMC calcification. Therefore, we carried out Grp78 siRNA knock-down and showed that it decreased calcification of VSMCs. Additionally, EVs from VSMCs treated with ER stress inducers had more Grp78 than from control VSMCs and had increased calcification capacity. In sections of calcified human aorta and carotid artery Grp78 and Grp94 accumulated extracellularly in a punctate pattern consistent with previous observation of EVs.

**Conclusions:** ER stress modulates VSMC phenotype, which leads to increased secretion of Grp78-loaded EVs and calcification.

signalling by O<sub>2</sub><sup>-</sup>. Nox4 produces H<sub>2</sub>O<sub>2</sub>. Evidence indicates that: (i) Nox2 activates NF- $\kappa$ B-dependent proinflammatory program; (ii) Nox4 induces Nrf2-targeted antioxidant and anti-inflammatory genes; (iii) Nox2/NF- $\kappa$ B and Nox4/Nrf2 systems regulate each other's expression negatively and are upregulated vs. downregulated, respectively in CVD; (iv) NO may regulate Nox4/Nrf2 positively and Nox2/NF- $\kappa$ B negatively. The imbalance between Nox2/NF- $\kappa$ B and Nox4/Nrf2 systems may underpin the mechanism of the vascular inflammation.

We hypothesized that the endothelial NO secures the balance between vascular Nox2/NF- $\kappa$ B and Nox4/Nrf2 systems.

The studies consisted of three steps: (i) In vivo experiments in rats (diabetes induction, exercise training, feeding animals with nitrite (50 mg l<sup>-1</sup>) by 7 weeks) and pharmacological inhibition of eNOS (L-NAME, 40 mg kg<sup>-1</sup> by 7 days); (ii) perfusion of isolated hearts, measurement of nitrite in the blood and the cardiac O<sub>2</sub><sup>-</sup> production; (iii) biochemical measurements in cardiac tissue activity and expression of the Nox1/Nox2/NF- $\kappa$ B and Nox4/Nrf2 systems.

Rats with 7-wk streptozotocin-diabetes had: (i) decreased plasma nitrite concentration and cardiac eNOS expression; (ii) increased cardiac O<sub>2</sub><sup>-</sup> production, Nox activity, 8-isoprostane and 3-Nitrotyrosine concentration; (iii) increased cardiac and renal Nox1 and Nox2 expression and decreased Nox4 expression; (iv) increased cardiac and renal activation of NF- $\kappa$ B and the expression of iNOS and VCAM-1; (v) decreased cardiac and renal activation of NF- $\kappa$ B and the expression of eNOS, HO-1 and SOD1/2/3. These diabetic changes were prevented or attenuated by exercise training or supplementation of nitrite. eNOS inhibitor mimicked the diabetic effects but nitrite resulted in partial normalization of plasma nitrite. Nitrates had no impact on eNOS expression and systemic homeostasis of NO and did not restore the balance between Nox2/NF- $\kappa$ B and Nox4/Nrf2.

Concluding, the NO derived from functional eNOS but not from nitrite, secures the balance between Nox2/NF- $\kappa$ B and Nox4/Nrf2 pathways in diabetes and preventing the development of vascular oxidative stress and inflammation.

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### Exercise Training and Dietary Nitrite Prevent and L-NAME Mimics Diabetes-Mediated Upregulation of Nox2/NF- $\kappa$ B and Repression of Nox4/Nrf2 in Rat Heart and Kidney

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Reactive oxygen species (ROS) and endothelial nitric oxide (NO) are signalling molecules governing life processes through redox-modification of specific cellular effectors, including transcription factors NF- $\kappa$ B and Nrf2. This signalling is disrupted in cardiovascular disease (CVD), as manifested an overproduction of ROS, endothelial dysfunction and inflammatory vascular phenotype. NADPH oxidase (Nox) is a major source of ROS. Of the Nox's expressed in rodents, Nox1 and Nox2 generate superoxide (O<sub>2</sub><sup>-</sup>) and may signal via O<sub>2</sub><sup>-</sup>, H<sub>2</sub>O<sub>2</sub> and the disruption of NO

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### Effect of Dual Purinoreceptor-Dependent Strategy on Endothelial Permeability and Cells Condition

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**Introduction:** In recent years the relevance of purinoreceptors to vascular disease pathophysiology, the effect of existing agonists/antagonists of these receptors as well as research and synthesizing new compounds have become an area of intense interest, especially because they provide a target for future therapeutic manipulation. In particular, approaches based on the combination of different biochemical pathways to enhance the synergistic effect seem

to be interesting. To assess the potential of therapy, in addition to estimating the immediate result, the effect on the condition of the endothelium, which indirectly regulates many processes in the system, must be examined.

**Method:** These studies focused on (designed in our team) dual purinoreceptor-dependent strategy based on the activation of adenosine P1 receptors with the simultaneous inhibition of P2Y12 receptors. In our current work, we decided to check the effect of a dual approach on the modulation of vascular permeability, the changes of which may underlie cardiovascular dysfunctions. In vitro studies were performed on Human Microvascular Endothelial Cell (HMEC-1). A2A selective agonists (He-Neca; MRE0094; UK-432,097) and non-selective (NECA) were used in combination with P2Y12 antagonists (cangrelor or metabolite of prasugrel). Real-time endothelial permeability and proliferation measurement were applied using the Electric Cell-Substrate Impedance Sensing (ECIS®), which impedance-based cells monitoring technology. Changes in the secretion of vascular endothelial growth factor (VEGF), which plays various roles in numerous biochemical pathways, indirectly also in the change in permeability, were measured by enzyme-linked immunosorbent assay (ELISA).

**Results and Conclusions:** Interesting results were observed within 6 hours of administration of the tested compounds. Individually, P2Y antagonists did not cause significant changes relative to the control, whereas in combination with P1 receptor agonists, they modulated endothelial permeability. The measure of the effect depended on the compound used. Tested purinoreceptor agonists demonstrated a variety of responses in endothelial cells, some of them in a simple approach significantly (even twice) increase the release of VEGF (e.g. MRE0094; NECA), while in the dual concept this effect is abolished. The results suggest a protective nature of this strategy for the condition of the endothelium. Significant changes in VEGF secretion can also cause modulation of the permeability as a result of the following changes in cells over the long term after the application of therapeutic agents. Our investigations demonstrates that modulating the function of purinoreceptors trigger the entire sequence of various biological events that culmination may be protective for the cardiovascular system.

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### Influencing Microcirculation in Horses

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The objective was to investigate to what extent the use of „The BEMER VET“ system, which signal is a sinusoidal waveform with variable amplitude, influences the behavior of horses under different conditions. In a 12-month study and a Placebo-controlled study, improvements in microcirculation could be demonstrated.

BEMER VET is a battery powered therapy device for horses. It consists of an adjustable horse blanket, which is an application module for full body treatment and a cuff for targeted treatments (on neck or leg). The BEMER VET signal is a sinusoidal waveform with variable amplitude. It operates by inducing electromotive force in conductive targets such as tissue of the horses. This inductive coupling is established by using different electrical coils. The

user can select three different programs, in which the duration and signal intensity increase from P1 as the program with the lowest intensity to P3 as the most intense and longest program. Control software for the BEMER VET system resides in the signal controller.

Studies prove the effectiveness for an observational study the blanket was used over a period of approximately 12 months on 49 horses of different ages that were ridden in training as well as in competitions. The therapy blanket was applied to the horses for at least 10 minutes and no longer than 15 minutes both before riding (warmup) and for regeneration afterworking. All horses accepted the application of the blanket without problems and relaxed significantly during the treatment, which was expressed by an almost sedated attitude. It was possible to unambiguously determine that horses that started out “stiff” could begin work faster; horses that were agitated at the beginning became more relaxed. Horses also became less anxious when being prepared for surgical operations. Of note was the fact that the calming, sedating effect intensified with increased intensity (P3). This shows a very recognizable effect.

A further test was carried out in a horse clinic using a placebo as well as a verum blanket preceding surgical operations under general anesthesia. The test horses with the verum blanket behaved significantly less frightened during the preparation for the operation and during the post-operative phase when attempting to stand. In operations with a duration longer than a certain period, horses with the verum blanket showed a smaller increase in their Creatine kinase (CK) level.

For the author, these observations are a clear sign of improved microcirculation.

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### Markers of Microvascular Dysfunction Are Associated with Incident Depressive Symptoms: The Maastricht Study

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**Background and Objectives:** The etiologic factors of late-life depression are still poorly understood. Microvascular dysfunction has been suggested to play a role in the etiology of depression, but direct evidence of this association is scarce. Therefore, we investigated whether measures of microvascular dysfunction are prospectively associated with depressive symptoms in a population-based cohort study.

**Methods:** We used data from The Maastricht Study (n = 2763; 257 with incident clinically relevant depressive symptoms, mean age 59.6 ± 8.2 years, and 49.5% were women). Depressive symptoms were assessed with the 9-item Patient Health Questionnaire (PHQ-9) at baseline and during annual follow-up over a period of four years. At baseline, we measured microvascular function by use of flicker light-induced retinal vessel dilation response (Dynamic Vessel Analyzer), heat-induced skin hyperemic response (laser-Doppler flowmetry), and plasma markers of microvascular endothelial dysfunction (sICAM-1, sVCAM-1, sE-selectin, and vWF). Individual markers were transformed into z-scores to facilitate direct comparisons. Analyses were performed by use of Cox regression and generalized estimating equations, adjusted for demographical-, cardiovascular- and lifestyle factors.

**Results:** After full adjustment, a one standard deviation higher retinal arteriolar dysfunction and endothelial dysfunction at baseline was associated with a higher risk for incident depressive symptoms (PHQ-9 >10, HR 1.23 per SD (1.04; 1.45), p = 0.018 and HR 1.19 per SD (1.05; 1.35), p = 0.007, respectively). Retinal venular dysfunction and microvascular dysfunction measured in the skin were not significantly associated with incident depressive symptoms (HR 1.10 per SD (0.94; 1.28), p = 0.251 and HR 1.20 per SD (0.98; 1.46), p = 0.076, respectively). Furthermore, we found an association between a higher composite score of overall microvascular dysfunction with an increase of depressive symptoms over time after full adjustment (RR 1.02 (1.01;1.04)).

**Conclusions:** Markers of microvascular dysfunction are associated with the incidence of depressive symptoms and an increase of depressive symptoms over time. These findings support the idea that microvascular dysfunction is involved in the etiology of late-life depression and might help in finding new potential targets for the prevention and treatment of late-life depression.

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### Effect of 3D Dynamic Culture on Human Stromal Vascular Fraction Cell Composition

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Adult stem cell-based therapies aiming to restore the damaged microcirculation and induce the release of pro-survival factors hold great promise in the treatment of chronic cardiac ischemia, principally by means of paracrine-released factors. Compared to other sources of adult stem cells, adipose tissue-derived stromal vascular fraction (SVF) presents a higher angiogenic potential, due to its heterogeneous composition of vascular progenitor cells, besides stromal mesenchymal cells. Previous findings showed that in contrast to static condition, perfusion culture of human SVF-based three-dimensional (3D) constructs significantly increased (i) the number of pericytes (4.5-fold-increase compared to freshly isolated SVF); (ii) the release of angiogenic factors; and (iii) the vascularization and cell survival upon subcutaneous implantation in nude rats. In the present study, we aimed to identify paracrine-

mediated molecular mechanisms regulating enrichment of SVF-derived pericytes under dynamic culture. In particular, we focused on the effects of connective tissue growth factor (CTGF), which was previously shown to support pericyte (CD146+) growth in vitro. We hypothesized that supplementation with human recombinant CTGF during static culture would promote survival and proliferation of pericytes (identified as CD146+, CD34-, CD45-). SVF-based constructs were cultured in static for 5 days in either perfusion-conditioned or CTGF-supplemented media (0, 10 and 100 ng/ml). CTGF release and CTGF+ cells were significantly higher in perfused constructs compared to static condition. Static constructs did not show any CD146+CTGF+ cell, which instead represented the 33.1 ± 0.3% of the total SVF after perfusion. Compared to the initial SVF population, the number of CD146+ cells did not increase, neither with CTGF supplementation nor with the use of perfusion-conditioned medium. These results suggest that hydrodynamic shear-stress associated to perfusion culture might be responsible for the activation of signaling pathways enhancing pericyte enrichment, rather than CTGF-based mechanisms. The lack of increase in the pericyte population, even following the use of perfusion-conditioned medium, confirm that mechanotransduction might be playing a key role in promoting in vitro pericyte survival and proliferation by specific signaling cues, such as the activation of the ERK pathway. Phosphorylated ERK was indeed upregulated (3-fold increase) in SVF cells cultured under perfusion, compared to static. Further studies will focus on the role of pericytes and their secretome in the generation of SVF-based constructs as a possible treatment for cardiovascular diseases, by promoting vascular remodeling and cardiomyocyte survival.

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### Modification of Lymph Node Microenvironment During Tumor Progression

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Lymphangiogenesis, defined as the outgrowth of new lymphatic vessels from pre-existing ones, is correlated with cancer progression and tumor cell dissemination. The lymph node (LN) status (presence/absence of tumor cells) is a strong prognostic factor for cancer patients. Clinicians use this LN status to adapt their therapeutic decisions for patients with carcinomas such as breast and cervical cancers among others. The role of LN metastasis in further progression of the disease has been the subject of a long debate. Nevertheless, recent data in pre-clinical models provided evidence that LN metastasis could promote tumor cell dissemination to distant organs.

To better characterize the role of lymphatic endothelial cells (LEC) in the sentinel LN, we performed a proteomic analysis of the marginal sinus of sentinel and non-sentinel human LN (without tumor cells) by laser-capture microdissection. We identified 16 differential proteins, including periostin (POSTN), an extracellular matrix protein, which can mediate communication between

cells and their microenvironment. These data demonstrate molecular changes in LN lymphatic vasculature before tumor cell arrival and support the formation of a “pre-metastatic niche” in human LN of patients with cervical cancer.

Specific objectives are to analyze the immunosuppressive features and the matrix environment that could contribute to tumor progression and metastases.

We developed an original *in vivo* model, the “ear sponge assay” that reproduces the pre-metastatic lymphangiogenic niche in LN. Briefly, a gelatin sponge soaked with tumor cells is implanted between the two mouse ear skin layers. Two weeks post-implantation sentinel LN is resected and analysed by histological staining. We observed a modification of the lymphatic spatial distribution (LYVE-1 staining) in pre-metastatic LN versus control (sponges soaked with medium). Although lymphatic vessels are localized near the tissue border in control LN, they are infiltrating deeper in sentinel LN of mice bearing a sponge populated with tumor cells. POSTN staining revealed fibers differently distributed in control and pre-metastatic LNs. While POSTN was confined near to the LN border in control LNs, it was present deeper in pre-metastatic LNs. *In vitro*, a POSTN gradient promotes LEC migration.

At (pre)-metastatic stages, LNs present an immunosuppressive environment and an increase density of POSTN and HEV (high endothelial venules) (preliminary data).

In the continuation of this work, we will explore the implication of POSTN in tumoral lymphangiogenesis and deeper the microenvironment modification.

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### Dynamic Molecular Signatures of Endothelial Cells During Postnatal Development of the Blood-Brain Barrier

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The blood-brain barrier (BBB) is a highly specialized vasculature that separates circulating blood and extracellular fluid from the brain parenchyma. Endothelial cells form the BBB by tightly regulating a series of specific transporters that control the passage of nutrients, energy metabolites, and other essential molecules. In addition, brain endothelium regulates the clearance of metabolic waste products from the brain's interstitial fluid and prevents the entry of pathogens and neurotoxins into the brain parenchyma. Thus, the BBB is vital for brain hemostasis. Experimental and clinical studies have eloquently shown that BBB dysfunction correlates with numerous diseases of the central nervous system (CNS) such as neurological disorders, stroke and vascular malformations. While many cellular and molecular interactions have been identified to better understand the function and structure of the BBB, a deep comprehensive understanding of the molecular mechanisms, spatio-temporal dynamics and networks involved in the postnatal development of BBB remains elusive. In our study, we applied an RNA-sequencing approach to

compare the expression profiles of mouse brain endothelial cells at six developmental stages from birth to adulthood. We evaluated six different time points (P1, P4, P9, P13, P21 and P35) from both cerebrum and cerebellum to better understand the maturation profile of the BBB. We observed that the development of the BBB is differentially regulated in the cerebellum and cerebrum. The cerebellum shows a delayed maturation when compared to the cerebrum based on their gene expression profiles. Our aim is to further explore the different signaling pathways involved during BBB maturation and their dynamic changes contributing to BBB development and maintenance. We aim to further expand our study by investigating the link between disruption of the BBB maturation and subsequent development of pathologies that affect the CNS.

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### Association Between Sural Nerve Conductance Amplitude and Skin Microvascular Responsiveness in Individuals with and without Type 2 Diabetes Across the Subclinical Neuropathy Range

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Microvascular and neural dysfunction contribute to diabetes related clinical complications (e.g. retinopathy, nephropathy and neuropathy). This study aims to examine the relationship between nerve function and microvascular responsiveness in individuals with and without type 2 diabetes (T2DM) across the subclinical neuropathy range.

Individuals without clinical neuropathy (defined by monofilaments) with nerve function and microvascular responsiveness data were identified from the Exeter SUMMIT cohort. Sural nerve conductance velocity (SNCV) and amplitude (SNAP) were non-invasively assessed (right leg, DPN-Check device). The peak skin perfusion response to iontophoretically delivered acetyl choline (ACH) and sodium nitroprusside (SNP) were used to represent endothelial dependent and independent microvascular responsiveness, respectively.

79 individuals were included (58%M, 52% T2DM, age range: 46–85 yrs). Median (25th, 75th quartiles) SNCV and SNAP were 46 (42–49) m/s and 7 (4,11)  $\mu$ V, respectively (63% normal nerve conductance, 8% mild and 29% moderate subclinical neuropathy, DPN-Check reference range). Median peak response to ACH and SNP were 432 (334,538) and 333 (263,425) PU, respectively. SNAP was associated with age, BMI and total cholesterol. SNAP positively correlated with ACH ( $R_s = 0.223, p = 0.048$ ) and SNP ( $0.392, <0.001$ ). The relationships remained after adjustment for known confounders (age, BMI, gender, diabetes status, total cholesterol, mean arterial blood pressure) and iontophoresis delivery voltage (ACH standardised  $\beta: 0.276, p = 0.028$ ; SNP standardised  $\beta: 0.315, p = 0.014$ ).

Sural nerve conductance amplitude, but not velocity, was positively associated with microvascular responsiveness in individuals with and without type 2 diabetes across the subclinical neuropathy range.

### Tumour Vascular Normalisation Through Inhibition of LRG1 Improves the Microenvironment and Augments Immunotherapy

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**Introduction:** Tumour vessels are typically disorganised, leaky and poorly perfused; properties that contribute to a pro-oncogenic environment and limited delivery of systemically administered drugs and immunotherapeutics. It has therefore been proposed that prevention or reversal of this dysfunctional vascular state, a phenomenon termed vessel normalisation, may improve the tumour microenvironment and enhance the delivery of therapeutics. The aim of this study was to determine whether deletion of the gene coding for the secreted glycoprotein, leucine-rich alpha-2-glycoprotein 1 (LRG1), or blockade of its action through function-blocking antibody treatment, improves tumour vascular function and the efficacy of immunotherapeutics.

**Methods:** Subcutaneous B16/F0 mouse tumours were generated in wild type (WT) or *Lrg1*<sup>-/-</sup> mice or in WT mice treated with 15C4, an anti-LRG1 blocking antibody. Tumour growth was monitored, and post-mortem analysis of vascular structure and function undertaken. The effect of blocking LRG1 function on the efficacy of adoptive T cell therapy and immune checkpoint inhibition was determined.

**Results:** In *Lrg1*<sup>-/-</sup> mice, or following functional blockade of LRG1 in WT animals, there was a significant reduction in B16/F0 tumour growth, a small reduction in vessel density and an improved pericyte-endothelial cell association in persisting vessels. In these 15C4 treated tumours we observed an increase in the proportion of perfused vessels, and a reduction in tumour hypoxia. We also saw increased expression of the junction-associated adhesion junction molecule VE-cadherin and a reduction in vascular permeability. We next evaluated the effect of blocking LRG1 on adoptive T cell and immune checkpoint inhibition therapy. Combination of 15C4 with adoptive T cell therapy resulted in a significant reduction in tumour volume compared with monotherapy alone. The infiltration of both donor and host T cells was also significantly enhanced. Combined 15C4 and anti-PD1 therapy also resulted in a significant decrease in tumour growth, a substantial increase in CD8<sup>+</sup> T cell infiltration and increased tumour cell killing over monotherapy alone.

**Conclusion:** These data show that LRG1 promotes dysfunctional vessel growth, and that therapeutic targeting of LRG1 normalises tumour vascular function and enhances the efficacy of immunotherapy.

### The Photodynamic Effects on the Microvasculature Vessels

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**Introduction:** Microcirculation (MC) disorders in a tumor and surrounding tissues are of great importance in the mechanism of photodynamic therapy (PDT) on tumors. The purpose of this study is to evaluate the MC disorders after photodynamic therapy when using various photosensitizers (PS).

**Material and Methods:** The experiments were conducted on male Wistar rats 250–300 g. Objects of study: venules of the small intestine mesentery and microcirculation in the skin. Measurement of the blood flow velocity in the venules was performed using intravital microscopy with a high-speed digital camera. The blood flow in the skin was observed using laser fluorometry.

**Laser Irradiation:** semiconductor lasers (662 nm, 635 nm, 530 nm), the radiation dose for mesentery vessels 30–36 J/cm<sup>2</sup>, for skin vessels – 50, 300 J/cm<sup>2</sup>.

Photosensitizers: Radachlorin (RC), – 5 mg/kg 3 hours before irradiation, Coproporphyrin (CP) – 10 mg/kg 3 hours before irradiation, Bengal rose (BR) – 17 mg/kg 1 hour before irradiation. All photosensitizers were administered intravenously. The accumulation of PS in the vessel wall of the MC was evaluated by the confocal laser scanning microscopy; subcutaneous mast cell film preparations were stained with toluidine blue.

**Results:** In experiments with BR a progressive slowing down of blood flow up to stasis began 3–4 minutes after irradiation, in experiments with CP the changes were the same but less obvious. In experiments with RC, blood flow slowed down and sludge formation was observed in 1–2 minutes after the start of irradiation. Irradiation of the skin at a dose of 50 J/cm<sup>2</sup> against the background of pre-administered CP and BR did not cause a significant reduction in blood flow either immediately or one hour after exposure. Irradiation at a dose of 300 J/cm<sup>2</sup> led to a perfusion decrease by 32 and 66% respectively ( $p < 0.05$ ). Unlike other PSs, RC caused a decrease in perfusion immediately after irradiation at both 50 and 300 J/cm<sup>2</sup> by 63 and 71% respectively ( $p < 0.01$ ). Changes in blood flow developed against the background of pronounced mast cell degranulation, especially in experiments with RC. All studied PSs were detected in the vascular wall, however, BR showed affinity to the endothelium.

**Conclusion:** The differences in the effects of the PS at the MCs seem to be related to the peculiarities of their physicochemical properties, as well as to different accumulations in the vascular endothelium. The most pronounced violations of the MC observed when using RC.

### **mDia1 is Crucial for Advanced Glycation End Product-Induced Endothelial Hyperpermeability**

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**Background/Aims:** Disruption of endothelial barrier integrity in response to advanced glycation end products (AGEs) stimulation contributes to vasculopathy associated with diabetes mellitus. Mammalian diaphanous-related formin (mDia1) has been reported to bind to the cytoplasmic domain of the receptor for advanced glycation end products (RAGE), which induces a series of cellular processes. This study directly evaluated the participation of mDia1 in AGE-induced hyperpermeability and revealed the precise intracellular signal transductions of this pathological process.

**Methods:** Human umbilical vein endothelial cells (HUVECs) were used in the *in vitro* studies. Trans-endothelial electric resistance and permeability coefficient for dextran (Pd) were measured to analyze cell permeability. Western blotting, immunofluorescence staining and flow cytometry assay were performed to investigate the underlying mechanism. Dextran flux across the mesentery in mice was monitored to investigate *in vivo* microvascular permeability.

**Results:** we found that AGEs evoked Nox4 membrane translocation, reactive oxygen species production, phosphorylation of Src and VE-cadherin, dissociation of adherens junctions and eventual endothelial hyperpermeability through RAGE-mDia1 binding. Cells overexpressing mDia1 by recombinant adenovirus infection showed stronger cellular responses induced by AGEs. Down-regulation of mDia1 by infection with an adenovirus encoding siRNA or blockade of RAGE-mDia1 binding by transfection with RAGE mutant plasmids into HUVECs abolished these AGE-induced effects. Furthermore, knockdown of mDia1 using an adenovirus or genetical knockout of RAGE in C57 mice rescued AGE-evoked microvascular hyperpermeability.

**Conclusion:** Our study revealed that mDia1 plays a critical role in AGE-induced microvascular hyperpermeability through binding to RAGE.

### **The NADPH Oxidase Nox4 Promotes Differentiation from Induced Pluripotent Stem Cells into Endothelial Cells**

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Nox4 is the only constitutively active NADPH oxidase and directly produces H<sub>2</sub>O<sub>2</sub>. In endothelial cells, Nox4 contributes to cellular homeostasis and quiescence. Therefore we investigate the role of Nox4 in the process of endothelial differentiation out of induced pluripotent stem cells (iPSC).

Mouse embryonic fibroblasts (MEF) from wildtype (WT) and Nox4<sup>-/-</sup> mice, as well as human dermal fibroblasts, were reprogrammed into induced pluripotent stem cells. Induction of first me-

sodermal lineage commitment was achieved with bone morphogenic protein 4 and afterwards endothelial cell differentiation was induced with vascular endothelial growth factor.

In the course of differentiation Nox4 expression in WT cells was increased. Online available RNA-sequencing data were analyzed and revealed a positive correlation between Nox4 expression and endothelial specific genes, whereas pluripotency markers correlated negatively.

Our data show that knockout of Nox4 results in prolonged expression of pluripotency markers and in diminished expression of endothelial markers, such as vascular endothelial growth factor receptor 2 (VEGFR-2), platelet endothelial cell adhesion molecule (PECAM-1) and endothelial nitric oxide synthase (eNOS). Not only is there decreased endothelial marker expression in the Nox4<sup>-/-</sup> but also the functionality of the iPSC-derived cells is impaired. Nox4 negative iPSC derived endothelial cells present with more apoptosis, less tube formation and sprouting capacity, as well as in an impaired integration into a newly formed vascular network in a matrigel plug in mice.

We identified a mechanism by which Lysine 27 methylation of histone 3 (H3K27me3) increased in Nox4<sup>-/-</sup> cells. An increase in this mark represses the expression of VEGFR2 and PECAM-1. The histone demethylase Jumonji Domain-Containing Protein 3 (Jmjd3) reduces the methylation of H3K27me3. Although no role of Nox4 was obvious in Jmjd3 expression was observed, nuclear translocation of the demethylase was reduced in the absence of Nox4. Interestingly, oxidation of Jmjd3 was Nox4 dependent and oxidized Jmjd3 was predominantly found in the nucleus. Therefore we propose that Nox4 oxidizes Jmjd3 which allows its nuclear translocation and demethylation of H3K27me3, which consequently promotes endothelial differentiation.

### **Mitochondria in Skeletal Muscle Microvascular Endothelial Cells and the Influence of Glucose Availability**

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**Introduction:** We 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 developed a novel method to determine mitochondrial respiration and ROS production in a limited number of microvascular endothelial cells isolated from skeletal muscle samples of rats and, examined the response to high and regular glucose conditions.

**Method:** Muscle endothelial cells were isolated from the lower limb of rats by use of antibody coated magnetic beads. The H<sub>2</sub>O<sub>2</sub> emission (ROS) was measured fluorometric in intact and permeabilized 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<sub>2</sub>O<sub>2</sub> production of approximately 200,000 cells in the chamber. The cells were incubated with high (11 mM) or regular physiological (5.5 mM) glucose levels either acutely during measurement in the Oxygraph or after 3-days incubation 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.

There was no effect of acute high glucose incubation on respiration or ROS production. With 3 days of prior incubation with high glucose levels, ROS production was 3.7-fold higher in intact cells compared to cells incubated for 3 days in regular glucose levels in the presence of 5 mM glucose. Furthermore, after the 3-day incubation, the ROS production had increased by 2.8–3-fold in permeabilized endothelial cells. The increase occurred at Complex I+II, complex II alone, and when ADP was added to achieve maximal respiration.

**Conclusion:** Our data show, that mitochondrial respiration and ROS formation can be determined in microvascular endothelial cells isolated from rat skeletal muscle. Moreover, whereas an acute elevation of glucose does not appear to influence respiration or ROS formation, a high, yet physiologically relevant, glucose concentration leads to increased ROS production/oxygen consumed. We propose that elevated plasma glucose levels lead to endothelial dysfunction in part through increased mitochondrial ROS formation.

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### Autonomic Actions of Physical Vascular Therapy in Ischemic Heart Disease Patients by Heart Rate Variability Analysis

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**Introduction:** Physical vascular therapy (BioElectroMagnetic Energy Regulation, BEMER) increases the spontaneous pulsation of microvessels beyond autonomic actions-regulated arterioles and at the small venules excluding capillaries by low-frequency electromagnetic field resulting in improved microcirculation. Heart rate variability (HRV) analysis is considered a non-invasive method to investigate autonomic effects on cardiovascular regulation.

**Methods:** Chronic ischemic heart disease patients (n = 22, 59.8 ± 8.6 years, 8 female) on rehabilitation were enrolled following ethical approval and informed consent of each patient. HRV analysis was performed on the first III. generation BEMER (B.Box Professional with B.body Pro applicator, Triesen, Lichtenstein) treatment at 3.5–35 µT average flux density for 20 min in supine position after 15 min postural adaptation. Six-minute records were done right before and right after the start of treatment, before the end and right after treatment, one hour after the treatment. A

hand-held ECG data acquisition device and custom HRV analysis software developed at our laboratory (L.H.) were used. Non-invasive blood pressure measurement was performed in the middle of each epoch. Results were compared by Friedman and post hoc Wilcoxon test with Holm-Bonferroni correction.

**Results:** Mean RR-interval (RRI) significantly increased during the treatment, standard deviation of RRI (SDNN) tendentiously increased during and after treatment, root mean square of successive RRI-differences (RMSSD) tendentiously increased just at the start of treatment. Guzik index significantly increased just at the beginning of the treatment. Blood pressure did not change during the study. Respiration frequency by Fourier analysis of the tachograms did not show significant changes.

**Conclusion:** This is the first preliminary study to prove systemic autonomic actions of physical vascular therapy by means of HRV analysis. Further studies are encouraged on a higher number of patients and with recording several other physiological parameters to elucidate the mechanism of action.

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### Flow-Mediated Vasodilatation in the Mouse Involves Unliganded Membrane, But Not Nuclear, Estrogen Receptor Alpha

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**Objective:** Flow-mediated dilation (FMD) of resistance arteries (RAs) plays a key role in tissue perfusion. A reduced FMD is the hallmark of the endothelium dysfunction occurring early in cardiovascular and metabolic disorders. Estrogen therapy improves FMD in diseased conditions without major effect in healthy subjects. Nevertheless, estrogen receptor alpha (ERα) mutation in a man was associated with severe and selective FMD reduction.

**Methods:** Thus, we evaluated the role of ERα in FMD in healthy young male mice. FMD was determined in vitro in mesenteric RAs isolated from mice lacking ERα (ERα<sup>-/-</sup>), the ERα nuclear activation function AF2 (AF2-ERα<sup>-/-</sup>) or the ERα palmitoylation site (C451A-ERα<sup>-/-</sup>, inactivation of the membrane effects or ERα), compared to littermate wild-type (WT or +/+) mice.

**Results:** We found that FMD was significantly reduced in ERα<sup>-/-</sup> compared to ERα<sup>+/+</sup> male and female (ovariectomized or not) mice. Exogenous ERα stimulation or blockade did not affect FMD. FMD was not affected in AF2-ERα<sup>-/-</sup> mice whereas it was significantly decreased in C451A-ERα<sup>-/-</sup> mice. NO-synthesis blockade with L-NNA strongly reduced FMD in WT and AF2-ERα<sup>-/-</sup> mice without major effect in ERα<sup>-/-</sup> and C451A-ERα<sup>-/-</sup> mice. Acetylcholine-mediated relaxation, not affected by the absence of ERα was similarly reduced by L-NNA in all groups. After ROS reduction in vivo, NO-dependent FMD was equivalent in C451A-ERα<sup>-/-</sup> and C451A-ERα<sup>+/+</sup> mice.

**Conclusion:** Thus, membrane ERα is important for FMD through activation of eNOS activity and reduction of ROS production, possibly in a ligand-independent manner

### The Mitochondrial Fission Factor Dynamin Related Protein 1 Limits VEGF/VEGFR2 Endocytic Trafficking and Angiogenesis

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Because of the mostly glycolytic nature of endothelial cell metabolism, the role of mitochondria and mitochondrial shape in angiogenesis, the new blood vessel formation from existing vasculature, has not been studied. Here we show that the mitochondrial fission factor Dynamin related protein 1 (Drp1) unexpectedly limits endosomal VEGFR2 signaling and hence angiogenesis. Drp1 levels were reduced when Human Umbilical Vein Endothelial Cells (HUVECs) were activated, and angiogenesis was accordingly stimulated in HUVECs where DRP1 was silenced. In vivo, inducible Drp1 ablation in endothelial cells increased early stage postnatal retina vascular sprouting. Mechanistically, upon VEGF stimulation Drp1 interacted with the internalized VEGFR2 and its early endosome partner Rab5 at the endosomal VEGFR2 signaling platform. Drp1 deletion unleashed VEGFR2 activation and its downstream signaling, indicating that the VEGFR2-Rab5-Drp1 interaction limits VEGFR2 mediated angiogenesis. Our data reveal an unexpected extramitochondrial function of Drp1 in endothelial cells, where it localizes also at the endosomes to constrain the endosomal VEGFR2 signaling platform.

### The Protective Function of Intracranial Endothelial Cells in Vascular Cognitive Impairment

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**Introduction:** Cardiovascular diseases are increasingly recognized as an important mediator of cognitive dysfunction. Cerebral hypoperfusion and vascular dysfunction are common underlying mechanisms that link both pathologies, although molecular mechanisms remain largely unknown. Based on our earlier data in experimental animals, we postulate that endothelial cells (ECs) that line the intracranial vessels display an endogenous protection mechanism that limits vascular damage. Defining molecular players that control such pathways may lead to the identification of ways to boost this endogenous route, thereby reinstating vascular function and cognition. Moreover, identification of biomarkers that reflect vascular damage will assist in proper diagnosis of ongoing endothelial damage and subsequent vascular cognitive impairment.

**Methods:** We used laser capture microdissection to isolate ECs of human postmortem intracranial and extracranial arteries (of 11

patients). We performed RNA sequencing, immunofluorescence microscopy and in vitro functional assays to reveal the unique profile of these cells.

**Results:** We found that ECs of intracranial arteries have a different gene expression profile compared to ECs of extracranial arteries. Strikingly, we found that intracranial ECs display a molecular signature that limits endothelial inflammation and in parallel enhances junctional markers. To find new candidates to improve endothelial function upon cerebral hypoperfusion, we have selected eight genes, out of the 900 differentially expressed genes, that are involved in perfusion and endothelial damage. Currently, we are studying the functional relevance of these eight genes in brain endothelial damage induced by cerebral hypoperfusion. Furthermore, we study the presence of the eight gene products in EC-derived extracellular vesicles, to find possible biomarkers.

**Conclusion:** We expect to find the role of the eight genes in intracranial EC damage caused by cerebral hypoperfusion and their possibility to function as biomarker. This will give us crucial insights in and targets for protection of the brain to cerebral hypoperfusion associated cognitive impairment.

### Development of An Ex Vivo Human Kidney Model to Study The Role of The Endothelium During Sepsis-Induced Acute Kidney Injury

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**Introduction:** Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection and associated with a high mortality rate. One frequent complication of severe sepsis is the development of acute kidney injury (AKI). During septic AKI, a systemic inflammatory process is paralleled by altered microcirculation and endothelial cell activation in affected organs. We and others have shown in mice and human kidney tissue that during sepsis the renal microvasculature becomes activated and expresses endothelial adhesion molecules. Likewise microvascular integrity is lost. Therefore we hypothesized that the endothelial cells of the renal microvasculature plays an important role in the development of sepsis induced AKI. The aim of this study is to generate an ex vivo AKI model using human precision-cut kidney slices (PCKS) to elucidate the role of early endothelial responses during septic AKI development.

**Methods:** Slices were prepared from human renal cortical tissue obtained from tumor nephrectomies. Slices were incubated with 1 µg/ml LPS or 50 ng/ml TNFα for 4 hours to study the re-

sponse to known inflammatory stimuli. RT-qPCR was performed to assess gene expression of adhesion molecules, pro-inflammatory cytokines and renal injury markers (TIMP-2, IGFBP7, NGAL, KIM-1 and KLOTHO). To detect the location of microvascular responses, expression of blood vessel marker CD34 and adhesion molecule E-selectin studied by immunohistochemistry (IHC).

**Results:** Slices from two human kidneys showed after 4 hours incubation with LPS or TNF $\alpha$  an increased mRNA expression of adhesion molecules E-selectin, VCAM-1 and ICAM-1 as compared to control slices. Moreover, we observed an increased expression of the cytokine IL-6 as well as TIMP-2 and IGFBP7, two molecules associated with renal injury following exposure to LPS or TNF $\alpha$ . Interestingly, NGAL mRNA level decreased upon LPS exposure while TNF $\alpha$  induced NGAL mRNA expression. Furthermore, IHC showed that exposure to LPS or TNF $\alpha$  in slices augmented the glomerular expression of E-selectin as compared to untreated slices.

**Conclusions and Future Perspectives:** These pilot experiments show that in human kidney slices the microvasculature responds to pro-inflammatory stimuli. This ex vivo model thus reproduces endothelial activation observed in in vitro and in vivo in animal models. The induced expression of molecules associated with AKI corroborates other studies in the field. Thus our human precision-cut kidney slice model will be further developed and employed for AKI research by exposing the slices to plasma from patients with sepsis-induced AKI and monitoring the molecular responses.

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### A Role for Mineralocorticoid Receptors in Vascular Smooth Muscle Cell Stiffness and Adhesion During Aging

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**Background:** Alterations in the mechanical properties of arteries, including enhanced stiffness, are known to occur as a function of aging. Increased vascular stiffness is independently associated with increased risk of cardiovascular disease. Changes in vessel stiffness involve alterations to both the vascular wall extracellular matrix (ECM) and intrinsic properties of vascular smooth muscle cells (VSMC). We previously demonstrated that male mice lacking the mineralocorticoid receptor (MR) in smooth muscle cells (SMC) were protected from vascular stiffening with aging. Vascular gene expression profiling in these mice revealed that SMC-MR deletion modulated expression of the receptors for fibronectin (Fn).

**Hypothesis:** In the present study we hypothesized that the SMC-MR contributes to age-related changes in stiffness by modulating adhesion of SMC to the ECM protein, Fn.

**Methods:** Using atomic force microscopy (AFM) cortical stiffness and adhesion properties were assessed at the single cell level in aortic SMC obtained from MR-intact and SMC-MR-KO mice.

Parallel studies were performed using aortic vascular (V)SMCs obtained from young and aged human donors.

**Results and Conclusions:** In 18 month old mice, intrinsic stiffness of aortic smooth muscle was significantly increased in MR-intact compared to that of SMC-MR-KO mice. SMC stiffness was increased in female compared to male mice of both genotypes. VSMCs from aged humans (males), compared to young adults, showed elevated intrinsic stiffness and increased adhesion to Fn. Aged human VSMCs showed increased expression of mRNA for alpha 5 integrin. Further, using AFM, Fn-mediated adhesion was shown to be attenuated in the presence of a function blocking anti alpha 5 integrin antibody. The binding of Fn-coated beads (5  $\mu$ m) to human VSMCs was also shown to be associated with cortical actin accumulation to the adhesion site. Treatment of human VSMCs with the MR antagonist, spironolactone (10–6 M, 24 hrs), caused a decrease in intrinsic cortical stiffness as measured by AFM. Collectively, the data implicate a role for the MR in VSMC in age-related changes in intrinsic VSMC stiffness and adhesion to Fn. Further, age-related changes in the mechanical properties of VSMCs may be associated with enhanced alpha 5 integrin-mediated adhesion.

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### Leucine-Rich Alpha-2-Glycoprotein-1 Disrupts Vessel Maturation in Developmental Retinal Angiogenesis

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**Introduction:** Previous work from our laboratory has shown that leucine-rich alpha-2-glycoprotein-1 (LRG1) is not involved in developmental angiogenesis but is induced in disease leading to disruption of the normal angiogenic process. Accordingly, LRG1 contributes to dysfunctional blood vessel growth in mouse models of ocular neovascularisation such as oxygen-induced retinopathy and laser-induced choroidal neovascularization. Here, we tested the hypothesis that introducing LRG1 during developmental angiogenesis will corrupt the process and drive dysfunctional vessel formation.

**Methods:** We examined the effect of exogenously introduced LRG1 or LRG1 overexpression during vascularization of the retina, which occurs in the first 3 postnatal weeks in mice. Human recombinant LRG1 or a viral overexpression vector for LRG1 were injected intravitreally in C57BL/6 pups on postnatal day 2. Control eyes were injected with denatured LRG1 or transfected with a null vector. LRG1 treated eyes were harvested on postnatal day 5, and transfected eyes were harvested on postnatal day 16. Retinal flatmounts were immunostained for endothelial markers, collagen IV and pericyte markers.

**Results:** A feature of vessel maturation is the tight association with pericytes. High magnification confocal images and overlap analysis of endothelial and pericyte immunostaining showed significantly reduced pericyte coverage of the capillaries in LRG1 protein treated and LRG1 overexpressing eyes compared to controls. Likewise, the overlap of the endothelium with collagen IV, a basement membrane marker, was reduced. LRG1 treatment or overexpression also alters the vascular growth pattern. The total vessel

length and number of vessel junctions were significantly reduced, indicating a lower retinal vessel density.

**Conclusion:** These results indicate that the introduction of LRG1 during retinal blood vessel development leads to structurally abnormal vessels with reduced pericyte coverage, supporting the hypothesis that LRG1 is a vascular disrupting factor. This also highlights the therapeutic potential of LRG1 inhibition in the treatment of ocular diseases which are characterized by the growth of structurally and functionally abnormal blood vessels.

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### Effect of L-NMMA on Microvascular Blood Flow and Glucose Metabolism After an Oral Glucose Load

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**Objective:** The aim of this study was to investigate whether the effects on local blood flow and metabolic changes observed in the skin after an endogenous systemic increase in insulin is mediated by the endothelial nitric oxide pathway, by local administration of the nitric oxide synthase inhibitor NG-monomethyl L-arginine (L-NMMA).

**Methods:** Microdialysis catheters, perfused with L-NMMA and with a control solution, were inserted intracutaneously in 12 human subjects, who received an oral glucose load to induce a systemic hyperinsulinemia. During microdialysis the local blood flow was measured by urea clearance and by laser speckle contrast imaging (LSCI), and glucose metabolites were measured.

**Results:** After oral glucose intake both microvascular blood flow and glucose metabolism were significantly suppressed in the L-NMMA catheter compared to the control catheter (urea clearance:  $p < 0.006$ , glucose dialysate concentration:  $p < 0.035$ ). No significant effect of L-NMMA on microvascular blood flow was observed with LSCI ( $p = 0.81$ ).

**Conclusion:** Local delivery of L-NMMA to the skin by microdialysis suppresses tissue glucose metabolism after an oral glucose load. This is likely the effect of reduced insulin-dependent vasodilation, limiting the delivery of insulin itself to the tissue.

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### Simultaneous Inhibition of Glycolysis and Multikinase Receptor Activity Can Have an Additive Effect on Endothelial Cell Proliferation and Migration

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Activated endothelial and many cancer cells prefer glycolysis to obtain energy for their proliferation and migration. Therefore, blocking of glycolysis can be a promising strategy against cancer progression and metastasis. Inactivation of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (PFKFB3), a key enzyme in the control of glycolysis can suppress glycolysis level and contribute to decreased proliferative and migratory activity of endothelial and cancer cells. Selective inhibitor of PFKFB3 activity 1-(4-pyridinyl)-3-(2-quinolinyl)-2-propen-1-one (PFK15) was found to decrease glucose uptake into endothelial cells and significantly reduce fructose-2,6-bisphosphate concentration. In our present study we investigated consequences of simultaneous inhibition of glucose metabolism and growth factor receptor activity. We hypothesize that different primary endothelial cell types with reduced level of glycolysis are more sensitive to antiangiogenic action of the multikinase inhibitor sunitinib.

Human umbilical vein endothelial cells (HUVECs) and bovine aortic endothelial cells (BAECs) were treated with different doses of glycolysis inhibitor PFK15 and multikinase inhibitor sunitinib alone or in combination. Effects of PFK15 on cell proliferation were evaluated by the MTT assay and cell migration was quantified by the wound healing assay in both cell types. Changes in cell cycle were analyzed by flow cytometry and capillary tube formation was determined by tubulogenesis assay on Matrigel in BAECs.

Our results showed that both PFK15 and sunitinib administered individually decreased HUVECs and BAECs proliferation in a dose-dependent manner. Combined administration of PFK15 with sunitinib showed an additive suppressive effect on HUVEC's proliferation and migration compared to inhibitors applied individually. Interestingly, this effect was not observed in BAECs at the same inhibitor concentrations. Simultaneous administration of these two compounds applied in lower concentrations showed stronger effect on capillary like structures formation on Matrigel compared to inhibitors alone also in BAECs. Analysis of cell cycle phase's distribution in BAECs did not show any significant changes 16 hours after the treatment.

PFK15 is an effective glycolysis inhibitor that efficiently blocks pathological angiogenesis. Our results showed stronger effect of simultaneous administration of PFK15 and sunitinib on cell proliferation and migration. The effect depends on concentration and can differ in different cell types. Obtained results suggest that re-

duced glycolytic activity of endothelial cells in combination with growth factor receptor blocking can be an effective antiangiogenic treatment.

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### Characterization of Germinal Matrix Vasculature in the Early Postnatal Mouse Brain

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**Introduction:** Intraventricular hemorrhage (IVH) affects 12,000 premature births yearly in the United States. It most often initiates in the germinal matrix (GM), a region of rapid angiogenesis, and is thought to be driven by hypoxia secondary to the metabolic demands of replicating neuronal and glial precursor cells. Blood vessels in the GM are suggested to have fewer associated pericytes than vessels in subcortical white matter; this paucity of pericytes is thought to play a role in the pathogenesis of IVH. IVH is more common following very premature birth; it is unclear, however, that pericytes are less abundant earlier in fetal development.

**Objective:** Determine how pericyte coverage of GM vasculature changes during development, and compare vascular morphology and pericyte coverage in wild-type and “double-reporter” mice, that is, mice expressing DsRed via the NG2 promoter (i.e. pericytes) and Flk1-GFP (i.e. endothelial cells). We hypothesize that pericyte coverage will increase with post-natal age, and that pericyte coverage and vascular morphology will not differ between double-reporter and wild-type mice.

**Methods:** Wild-type C57BL/6 mouse brains were harvested on post-natal days 1 (P1) and 7 (P7), and then sectioned and immunostained for CD31/PECAM-1, an endothelial marker, platelet-derived growth factor receptor- $\beta$  (PDGFR- $\beta$ ), a pericyte marker, and Phospho-Ser10 on Histone H3 (PH3), a mitotic marker. Brains from double-reporter mice were harvested and sectioned similarly. Images were resolved using confocal fluorescent microscopy. Blood vessel length, area, branch point density, and lacunarity were quantified and analyzed using three-factor regression ANOVA with post hoc pairwise comparisons.

**Results:** In both wild-type and double-reporter mice, the subventricular zone was hyper-cellular with many PH3-positive cells, and its blood vessels formed a densely-branching sub-ependymal plexus. This plexus was surrounded by diffuse PDGFR- $\beta$  signal in wild-type mice. Periventricular vessels had significantly increased branch point density. Double reporter mice had significantly increased average vessel diameter and branch point density. Pericyte coverage of the GM vessels was difficult to assess in wild-type mice due to the diffuse periventricular PDGFR- $\beta$  signal; it was not grossly different between P1 and P7 double reporter mice.

**Conclusion:** The increased branch point density in vessels within the subventricular zone is consistent with increased VEGF signaling and concomitantly increased angiogenesis in the setting of hypoxia. Despite its increased branch point density and vessel diameter, the double-reporter mouse reasonably approximates the vascular morphology of the wild-type GM. Pericyte coverage does not appear to increase with postnatal age.

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### Polydatin Ameliorates Barrier Function in LPS-Induced Endothelial Injury by Enhancing the Activation of SIRT3

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Endothelial barrier disruption has been implicated in the pathogenesis of sepsis. We previously demonstrated the protective role of SIRT3 in acute kidney injury in septic rats. Here, we showed that SIRT3 also exerts protective effects against LPS-induced endothelial hyperpermeability. In primary human umbilical vein endothelial cells (HUVECs), LPS caused a decrease in SIRT3 deacetylase activity. Furthermore, a decrease of short form SIRT3 protein expression and a transient increase of long form SIRT3 were observed in response to LPS challenge. SIRT3 activation with polydatin (PD) or overexpression by an adenovirus alleviated LPS-induced F-actin rearrangement, cadherin-catenin complex dissociation, and subsequent endothelial barrier disruption. In contrast, SIRT3 inhibition with 3-TYP or targeted siRNA abolished the barrier protective effects of SIRT3. In LPS-induced septic mice, SIRT3 was also found to prevent from vascular leakage in multiple organs. Through the detection of cellular and mitochondrial reactive oxygen species (ROS) level and mitochondrial permeability transition pore (mPTP) opening, we verified that SIRT3-mediated deacetylation of mitochondrial superoxide dismutase (SOD2) and cyclophilin D (CypD) prevents from mitochondrial dysfunction and subsequent endothelial barrier dysfunction. In addition, we also revealed an involvement of receptor for advanced glycation end products (RAGE) in LPS-regulated SIRT3 signaling. Our study provides a novel mechanism that compromised mitochondrial function due to LPS-mediated SIRT3 suppression accounts for increased endothelial permeability.

### The Oxidative Stress Profile and its Relation to Vascular Function in a Bi-Ethnic Cohort: The SABPA Study

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**Introduction/Objective:** Oxidative stress and inflammation has been implicated in arterial stiffness, and hypertension development. Oxidative stress may interfere with normal vascular function by inactivation of NO and/or the production of endothelin-1. Limited information exists regarding the link of NO and endothelin-1 with oxidative stress markers in black South Africans, a population known for early vascular compromise and increasing prevalence of hypertension. Our objective was to compare variables implicated in vascular function namely NO metabolites, endothelin-1 and markers of inflammation and oxidative stress in a bi-ethnic South African population. We also investigated the association of endothelin-1 with markers of oxidative stress implicated in arterial stiffness and endothelial function as well as if an increase in endothelin-1 levels over time predict a change in markers implicated in vascular function.

**Method:** Data from the SABPA study was analysed both cross-sectionally and prospectively. The participants were stratified by race (black n = 194, white n = 204). Cardiovascular measurements included blood pressure (BP) and pulse wave velocity (PWV). Biochemical variables include endothelin-1, NO metabolites, citrulline, reactive oxygen species (ROS), glutathione reductase (GR), glutathione peroxidase (GPx), C-reactive protein (CRP), interleukin-6 (IL-6) and urine albumin creatinine ratio (UACR) which were determined by recognised biochemical methods.

**Results:** Blacks had higher blood pressure and pulse wave velocity ( $P < 0.01$ ) and higher plasma levels of endothelin-1 (only in men,  $p < 0.001$ ), CRP ( $p < 0.003$ ), interleukin-6 ( $p < 0.03$ ), UACR ( $p < 0.001$ ), ROS ( $p < 0.003$ ) and GR ( $p < 0.001$ ) while citrulline was lower ( $p < 0.001$ ) and NO metabolites similar ( $p = 0.28$ ). In single, partial (adjusted for age, BMI, Total energy expenditure and anti-hypertension medication) and forward stepwise multiple regression an independent positive association of endothelin-1 with GR was found (adj.  $R^2 = 0.10$ ,  $\beta = 0.232$ ,  $p = 0.02$ ) and with GR-to-GPx ratio (adj.  $R^2 = 0.051$ ,  $\beta = 0.191$ ,  $p = 0.05$ ) in black men. In the total group an increase in endothelin-1 correlated positively with a change in pulse pressure over a period of three years ( $r = 0.168$ ;  $p = 0.041$ ) and with multiple regression a correlation was found only in the black group (adj.  $R^2 = 0.092$ ,  $\beta = 0.278$ ,  $p = 0.036$ ).

**Conclusion:** Higher levels of inflammation and oxidative stress in the black population may lead to increased endothelin-1 production and the uncoupling of eNOS with the resultant production of superoxides instead of NO and citrulline. Decreased endothelial function could explain increased levels of UACR while the up-regulation of GR and increased GR-to-GPx ratio indicates increased anti-oxidant enzyme activity in an attempt to maintain the redox balance. The prospective correlation of endothelin-1 with pulse pressure is in line with these results.

### A Novel Macrophage Long Non-Coding RNA Expressed in Human Atherosclerosis

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**Introduction:** Mechanisms of atherosclerotic plaque development and progression to instability and rupture remain unclear. Long non-coding RNAs (lncRNAs) are a recently discovered class of non-coding RNA that perform diverse functions in vascular health and disease. Development of novel therapeutics and biomarkers based on manipulation of this novel class represents an exciting opportunity for innovation in the field of atherosclerosis.

**Aims:** To identify novel long non-coding RNAs expressed in human atherosclerosis and uncover their pathophysiological significance.

**Methods:** To identify novel candidates, high-depth Illumina RNA sequencing was performed in stable and unstable human atherosclerotic plaque. Patients with stroke or transient ischaemic attack (TIA) were included if 18F-NaF PET/CT imaging demonstrated high uptake in vivo, indicating microcalcification and plaque instability. Following carotid endarterectomy, plaques were macroscopically dissected into unstable, haemorrhagic and adjacent stable regions prior to RNA isolation and sequencing. Candidate lncRNAs were selected according to differential regulation, cellular expression in vitro and genomic locus. Function of the candidate lncRNAs was analysed by GapmeR knockdown in high-throughput, fluorescent reporter assays, chosen for critical roles in atherogenesis, such as phagocytosis, reactive oxygen species production, lipid uptake, and apoptosis. Plaque and cell-type localisation of the candidate lncRNA was confirmed by in-situ hybridisation, with clinical correlation using lncRNA levels in blood from patients with acute myocardial infarction.

**Results:** We found 1760 protein coding and 202 lncRNAs transcripts were differentially regulated (false discovery rate  $< 1\%$  and fold change  $> 2$ ) in 4 patients (paired samples) by RNA sequencing. Candidate lncRNA LINC01272 was chosen for further investigation based on upregulation in unstable plaque (fold change 2.2,  $p < 0.0001$ ) and high expression (mean FPKM 6.6). LINC 01272 was selectively and highly expressed in human primary peripheral blood-derived (PBMC) monocyte/macrophage cells compared to fibroblasts, smooth muscle and endothelial cells. The transcript was exclusively nuclear. Selective knockdown (60%) by GapmeR in human PBMC-derived macrophages caused a reduction in phagocytosis of fluorescent latex beads ( $> 2$  fold,  $p$ -value  $< 0.001$ ), while apoptosis, cell number and other functions were unchanged. In-situ hybridisation confirmed localisation to unstable, macrophage-rich regions of the atherosclerotic plaque. Expression of LINC01272 was observed in RNA isolated from blood of patients with acute myocardial infarction.

**Conclusion:** LINC01272 is a novel long non-coding RNA transcript, upregulated in unstable human atherosclerotic plaque, ex-

pressed in the nucleus of monocytes and macrophages, and present in plasma. Reduction of phagocytosis upon knockdown suggests a functional role for this lncRNA in human atherosclerosis.

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### Defining Vegfr3-Tbx1 Interactions in Brain Vascular Development

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**Introduction:** The transcription factor TBX1 is responsible for most of the phenotype associated with 22q11.2 deletion syndrome (22q11.2DS), characterized by multiple congenital anomalies and brain-related clinical problems. Using genetic experiments in mice, we found that loss of function Tbx1 mutations specifically in endothelial cells (EC) cause cerebral vessel hyperplasia, perinatal lethality and functional brain vascular defects that lead to brain hypoxia (Cioffi, 2014). We have shown that Tbx1 exerts these effects via the regulation of Vegfr3 and Dll4 expression in ECs. Like Tbx1, both of these genes have anti-angiogenic functions in the mouse brain (Tammela 2011; Suchting 2007).

**Methods:** In order to gain insights into the mechanism by which the Tbx1-Vegfr3 interaction affects brain vascular development, we used cell fate mapping analysis to label Tbx1-expressing cells and their progeny in mouse brain and anti-Vegfr3 immunostaining to evaluate the distribution of double positive ECs during embryogenesis. We also generated a transgenic mouse that expresses Vegfr3 upon Cre recombination.

**Results:** Cell fate mapping showed that Tbx1-expressing cells and their descendants contribute to most brain vessels. We examined the brain vasculature of Tbx1cre/+;R26Rmt/mG embryos between embryonic stage E8.5-E18.5 and we found that the first GFP-positive cells localized to blood vessels within the hindbrain neuroepithelium at E10 indicating that Tbx1 is required for early brain vascular development. Furthermore, the majority of the labeled vessels also expressed Vegfr3. Then we used forced expression of Vegfr3 from a Tbx1Cre-activated transgene to test whether increasing/restoring Vegfr3 expression in Tbx1-expressing ECs was able to rescue the brain vessel hyperplasia and we observed a partial rescue of the phenotype.

**Conclusions:** Our study indicates that Tbx1 and Vegfr3 are both regulators of brain vasculature and transgenic expression of Vegfr3 can rescue the Tbx1 mutant phenotype.

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### Regulation of Cardiac Lymphatic Development by Tbx1

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**Introduction:** Tbx1 is the major gene implicated in 22q11.2 deletion syndrome. The complex clinical phenotype includes vascular anomalies and a very recent report has presented new cases of primary lymphedema and other lymphatic anomalies in 22q11.2DS patients.

In mice, loss of Tbx1 is associated with a strong reduction in lymphatic vessel density in most tissues and downregulation of Vegfr3. Our data demonstrate a strong genetic interaction between Tbx1 and Vegfr3 in cardiac lymphangiogenesis that surprisingly is more pronounced in the ventral versus dorsal cardiac lymphatic networks (unpublished). We are currently addressing where and when Tbx1 and Vegfr3 interact to promote cardiac lymphangiogenesis and established whether Tbx1 is active in different cardiac lymphatic progenitors.

**Methods:** We first evaluated the distribution of Tbx1-expressing cells and their descendants in the heart of Tbx1Cre/+; Rosa-mT-mG embryos at E18.5, where these cells were labelled by Tbx1Cre-activated expression of a GFP reporter. We then used  $\beta$ -gal staining and Lyve-1 immunostaining on E18.5 Tbx1lacZ/+ hearts to identify Tbx1-expressing cells and lymphatic vessels respectively, and through this establish the earliest time point of Tbx1 expression in cardiac lymphatic vessels. Finally, we used timed-conditional inactivation of Tbx1 to determine the time window in which Tbx1 is required for cardiac lymphangiogenesis. We injected tamoxifen between E10.5 and E16.5 to inactivate Tbx1 at different times and analyzed embryonic hearts at E18.5.

**Results:** The results show that Tbx1-expressing cells and their descendants populate the majority of cardiac lymphatic vessels at E18.5. Tbx1 is expressed in the earliest forming cardiac lymphatic vessels (E14.5) and timed-conditional inactivation of Tbx1 indicate that it is required between E10.5 and E14.5, with the major effect at the earlier time points.

**Conclusions:** Tbx1 is required early in cardiac lymphatic development, perhaps in lymphatic EC progenitors, prior to the formation of mature lymphatic vessels.

## High Fat Feeding Decreases Vascular Density in Rat Mesenteric Perivascular Adipose Tissue

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Visceral adipose tissue receives a rich blood supply, however, the vascular density in mesenteric perivascular adipose tissue (PVAT) and the effects of high-fat feeding on vessel density in PVAT has not been reported. We hypothesized that vascular density would be decreased in PVAT surrounding mesenteric resistance arteries after 16 weeks of high-fat feeding (HFF) in male Sprague-Dawley rats. To test this hypothesis, we labeled endothelial cells in PVAT with rabbit anti-CD-31 (PECAM) 1<sup>0</sup> antibodies/AlexaFluor594-conjugated donkey-anti-rabbit 2<sup>0</sup> antibodies, *ex vivo*. Vessel staining density (fraction occupied by CD-31-labeled structures) was computed from thresholded, background subtracted, maximum intensity z-projections of image stacks. We found that vessel density as assessed by CD-31 staining density was  $0.15 \pm 0.02$  ( $n = 11$ ) in PVAT from control fed animals. HFF increased PVAT adipocyte diameter from  $39 \pm 0.5 \mu\text{m}$  ( $n = 672$ ) to  $72 \pm 0.6 \mu\text{m}$  ( $n = 365$ ,  $p < 0.0001$ ). Concomitant with the increase in adipocyte diameter, HFF resulted in a decrease in vessel density to  $0.07 \pm 0.01$  ( $n = 12$ ,  $p = 0.0006$  vs. Control). Our data indicate that rat mesenteric PVAT has a well-developed blood supply and that HFF results in a substantial reduction in vessel density that is proportional to the increased size of the adipocytes induced by HFF. The reduction in vessel density has significant implications for oxygen and nutrient delivery, as well as the trafficking and targeting of immune cells to PVAT in obesity.

## Nucleoside-Modified VEGFC mRNA Induces Organ-Specific Lymphatic Growth and Reverses Experimental Lymphedema

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Lack or dysfunction of the lymphatics leads to secondary lymphedema development that seriously reduces the function of the affected organs and results in degradation of quality of life.

Currently, there is no definitive treatment option for lymphedema. Here, we utilized nucleoside-modified mRNA encapsulated in lipid nanoparticles (LNPs) encoding murine Vascular Endothelial Growth Factor C (VEGFC) to stimulate lymphatic growth and function and reduce lymphedema in mouse models. We demonstrated that administration of a single low dose of VEGFC mRNA-LNPs induced durable, organ-specific lymphatic growth and active lymphatic function in newly formed vessels. Importantly, VEGFC mRNA-LNP treatment reversed experimental lymphedema without inducing any obvious adverse events. Collectively, we present a novel application of the nucleoside-modified mRNA-LNP platform, describe a model for identifying the organ-specific physiological and pathophysiological roles of the lymphatic system, and propose an efficient and safe treatment option that may serve as a novel therapeutic tool to reduce lymphedema.

## Antioxidant and Anti-Inflammatory Effects of Alpha-Mangostin on Cerebral Endothelial Dysfunction and Blood Brain Barrier Leakage in Type 2 Diabetic Rats

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**Introduction:** Insulin resistance and hyperglycemia, a hallmark of type 2 diabetes (DM2) could increase oxidative stress and pro-inflammatory cytokines which ameliorate cerebral endothelial function and blood brain barrier integrity. Alpha-mangostin (alpha-MG) is the main xanthone purified from mangosteen (*Garcinia mangostana* Linn.), known as anti-oxidative and anti-inflammatory properties. The aim of this study was to investigate the protective effect of alpha-MG against oxidative stress and inflammation on type 2 diabetic rat model induced endothelial dysfunction and blood brain barrier (BBB) leakage.

**Methods:** Male Sprague-Dawley rats were divided into three groups ( $n = 8/\text{group}$ ): normal control (CON) and diabetes with or without alpha-MG supplementation (DM2 and DM2-MG, respectively). DM2 rat was induced by feeding high fat diet for three weeks followed by an I.V. injection of low dose streptozotocin. Daily gavage feeding of alpha-MG (200 mg/kg BW) was performed for 12 weeks. The effect of alpha-MG on blood glucose (BG), HbA1C, serum insulin were investigated. Cerebral blood flow (CBF) was evaluated using a laser Doppler flowmeter. To examine the endothelial function, leukocytes adhesion (LA) to the venular endothelium, and responses of cerebral arterioles to endothelium-dependent (acetylcholine; ACh) vasodilators were evaluated. Evans blue dye was used to assess the changes of BBB permeability. Additionally, levels of cerebral malondialdehyde (MDA), pro-inflammatory cytokines (TNF- $\alpha$  and IL-6) and expression of eNOS, ICAM-1 and tight junction proteins (claudin-5, occludin and zonula occludens-1 (ZO-1)) were evaluated by Western blots anal-

ysis. The experiments were approved by the ethical committee, Faculty of Medicine, Srinakharinwirot University, Thailand.

**Results:** The elevated of BG, HbA1c, and S. insulin were observed in DM2 rats. The alpha-MG supplementation was able to reduce BG, HbA1c and S.insulin. Furthermore, DM2 rats had significantly decreased CBF, but statistically increased MAP and number density of LA compared with control rats. In the DM2-MG rats, the CBF perfusion was significantly increased but the MAP and LA were decreased, as compared to DM2 rats. Additionally, the magnitude of vasodilation to ACh was restored to near normal range in DM2-MG rats. Alpha-MG supplementation effectively reduced leakage of the BBB, promoted expression of claudin-5, occluding, ZO-1 and eNOS and reduced expression of ICAM-1. Moreover, alpha-MG supplementation was able to decrease MDA, TNF- $\alpha$  and IL-6 in brain tissue of DM2-MG rats.

**Conclusion:** The results of present study suggested that alpha-mangostin supplementation could improve cerebral endothelial function and BBB integrity of DM2 rats. These effects are primarily mediated through an enhanced antioxidant and anti-inflammatory properties.

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### Cardiac Macrophages in Myocardial Infarction – Role of Heme Oxygenase 1

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Myocardial infarction (MI) results in cardiomyocyte hypertrophy and cardiac remodeling progression leading to systolic and diastolic dysfunction. After ischemic episode, monocyte-derived macrophages (MDMs) represent the major cardiac macrophage population and play an important role in clearing the damaged tissue and initiating cardiac repair. Inefficient clearance of dying cells prolongs inflammation and impairs healing. Heme oxygenase 1 (HO-1) is a heme-degrading enzyme that exerts important cytoprotective effects and is able to modulate the monocyte/macrophage-mediated immune responses. We have recently shown a more potent deterioration of post-MI heart function and cardiomyocyte hypertrophy in HO-1 knockout (HO-1 KO) when compared to wild type (WT) mice. This was accompanied by higher, than in WT mice, numbers of classical inflammatory Ly6Chigh monocytes in the peripheral blood (PB) and increased monocyte chemoattractant protein (MCP)-1-dependent infiltration of myocardium by MDMs.

The aim of this study was to better understand the role of HO-1 in suppression of post-MI inflammation.

HO-1 KO and WT mice were subjected to a permanent ligation of left anterior descending coronary artery. At day 4 after MI PB classical monocytes (CD45+CD11b+Ly6C++CD43+) and cardiac macrophages (CD45+CD11b+CD64+) were sorted and used for TaqMan low-density array (TLDA) miRNA profiling. Out of

641 analyzed mouse miRNAs, 65 were expressed by classical monocytes and 71 by cardiac MDMs. Among them, 60 miRNAs were commonly expressed in both cell populations. Statistical analysis identified 21 differentially expressed (DE) miRNAs. Among them, 6 miRNAs were significantly upregulated in HO-1 KO MDMs vs. WT MDMs (miR-126a-3p, miR-146a-5p, miR-146b-5p, miR-186-5p, miR-19a-3p, and miR-222-3p). In case of classical monocytes, there were no DE miRNAs when HO-1 KO and WT mice were compared, but we found 17 miRNAs to be down-regulated in MI HO-1 KO classical monocytes vs. sham classical monocytes and 12 miRNAs to be up-regulated in MI HO-1 KO MDMs vs. sham/MI HO-1 KO classical monocytes. Pathway enrichment analysis identified Hippo, AMPK, and TGF $\beta$  signalling pathways, and endocytosis to be significantly enriched. Interestingly, transplantation of bone marrow cells from HO-1 KO/GFP+/+ donors to lethally gamma radiated WT/GFP-/- recipients and their exposure to MI did not, per se, additionally impair post-ischemic heart function.

In conclusion, HO-1 deficiency affects the post-MI miRNA profile in cardiac MDMs and in this way can influence their functions. The cross-talk between cardiac macrophages and other cells in the heart can be responsible for the adverse cardiac remodeling in the absence of HO-1.

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### Identification of a Novel Redox-Sensitive Kinase That Controls Endothelial Cell Permeability

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**Objective:** To understand signalling mechanisms that control endothelial cell permeability in response to cytokine stimulation.

**Introduction:** The endothelium provides a regulated semipermeable barrier between the blood/lymph and the underlying interstitium. Endothelial cell-cell junctions can become disassembled after inflammation leading to paracellular gap formation, vascular leakage and potentially life-threatening oedema, such as during Acute Respiratory Distress Syndrome (ARDS). TNF- $\alpha$ , secreted from leukocytes, is a major cytokine that drives ARDS. TNF- $\alpha$  induces endothelial cell permeability through phosphorylation (and therefore activation) of the ezrin/radixin/moesin (ERM) proteins, leading to actin cytoskeletal rearrangements and paracellular gap formation. However the mechanisms by which TNF- $\alpha$  induces ERM activation, and conversely by which the endothelium can suppress excessive ERM activation, are poorly understood.

**Methods:** We performed a kinome-wide screen in *Drosophila* to identify novel kinases phosphorylating ERM proteins. Using Human Umbilical Vein Endothelial Cells (HUVEC), we characterised kinase involvement in endothelial permeability using assays such as: siRNA/inhibitor studies, Western blotting for ERM activation, transwell permeability assays, spinning disk confocal and widefield microscopy. An in vivo paw swelling model of TNF- $\alpha$  induced vascular leakage was also performed. In vitro ki-

nase assays and PEG-switch assays were used to elucidate the role of reversible cysteine oxidation in kinase regulation.

**Results:** We identified a MAP4K7 as a novel kinase required for TNF- $\alpha$  induced ERM activation, paracellular gap formation and permeability. MAP4K7 directly phosphorylates ERM. MAP4K7 activity is switched off by hydrogen peroxide, which induces (reversible) cysteine oxidation, likely resulting in the formation of an intramolecular disulphide bond. In the endothelium this redox signalling is likely driven by endogenous reactive oxygen species (ROS) producing enzymes, with hydrogen peroxide functioning as a second messenger.

**Conclusions:** MAP4K7 is required for TNF- $\alpha$  induced endothelial permeability through direct ERM phosphorylation. Excessive ERM activation is prevented through reversible hydrogen peroxide signalling and oxidation of MAP4K7 controlled through ROS-producing enzymes. Thus redox signalling in the endothelium can maintain a protective barrier. MAP4K7 inhibition may provide a therapeutic target against excessive oedema.

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### Role of Non-Coding RNAs in Atherosclerosis

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The development, progression, and destabilisation of atherosclerotic plaques underlies myocardial infarction and stroke, the leading causes of morbidity and mortality worldwide. The pathogenesis of atherosclerosis is characterised by haemodynamic-induced changes in endothelial cell function, the accumulation of inflammatory cells such as macrophages, and the concomitant loss of vascular smooth muscle cells. Moreover, in response to a variety of stimuli, the interaction between biochemical and biomechanical mechanisms affect the behaviour and function of these multiple cell types, promoting atherosclerotic plaque progression. Accumulating evidence has highlighted microRNAs (miRs) as prominent regulators and micro-managers of key cellular and molecular pathophysiological processes involved in the development and progression of atherosclerosis, including recruitment and retention of inflammatory cells, modulation of vascular smooth muscle cell phenotype and endothelial cell function, lipid metabolism, generation of inflammatory mediators, and dysregulated proteolysis. Human pathological and clinical studies have aimed to identify select microRNA which may serve as biomarkers of atherosclerosis and disease progression. In addition, a plethora of in vivo investigations have been undertaken to examine the modulation of distinct microRNA on the pathophysiology of atherosclerosis, and to identify key microRNAs which drive disease progression and are of subsequent therapeutic interest. Collectively, clinical and animal studies have begun to unravel the complex and often diverse effects microRNAs and their targets impart during the development of cardiovascular diseases and revealed promising therapeutic strategies through which modulation of microRNA function may be applied clinically.

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### Oxygen Saturation in Skin Microcirculation as a Predictor for Cardiovascular Disease

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**Objective:** The objective of this study was to assess microvascular function in terms of local oxygen saturation at reactive hyperemia to predict and prevent cardiovascular disease and chronic obstructive pulmonary disease and includes in total 30,000 men and women 50 to 64 years, extensively characterized using detailed imaging and functional analyses of heart, vessels and lungs. Microcirculatory measurements were performed on 3,809 subjects at Linköping University Hospital (recruited from the Linköping general population) and this study includes the first 1765 subjects.

**Methods:** The study was conducted within the Swedish CardioPulmonary bioImage Study (SCAPIS). SCAPIS has the overall aim to predict and prevent cardiovascular disease and chronic obstructive pulmonary disease and includes in total 30,000 men and women 50 to 64 years, extensively characterized using detailed imaging and functional analyses of heart, vessels and lungs. Microcirculatory measurements were performed on 3,809 subjects at Linköping University Hospital (recruited from the Linköping general population) and this study includes the first 1765 subjects.

Skin microcirculation was measured with the PF6000 EPOS system (Enhanced Perfusion and Oxygen Saturation, Perimed AB, Sweden). The non-invasive probe system integrates diffuse reflectance spectroscopy and laser Doppler flowmetry to estimate microcirculatory oxygen saturation, red blood cell tissue fraction and speed resolved perfusion in absolute units. Measurements were performed on the volar forearm and the protocol included a 5-min baseline, 5-min occlusion of the brachial artery with a standard blood pressure cuff set to 250 mm Hg, and a 10-min reperfusion phase. Peak microcirculatory oxygen saturation was calculated as the maximum value after release of the cuff.

Based on peak oxygen saturation, the subjects were divided into tertiles (T1 to T3). Peak oxygen saturation in T1 was 79.9% (57.2–84.3%), in T2 86.8% (84.3–89.1%), in T3 92.4% (89.1–100%), numbers representing mean (range). To test significant differences in characteristics between the three groups, one-way ANOVA was used for continuous variables and chi-square for categorical. A p-value below 0.001 was considered significant.

**Results:** There were significant differences in age (mean T1 = 58.1 years, T2 = 57.9 years, T3 = 57.0 years), BMI (mean T1 = 27.7, T2 = 27.1, T3 = 25.9), gender (% female T1 = 39%, T2 = 50%, T3 = 61%), waist circumference (men: mean T1 = 100.6 cm, T2 = 98.4 cm, T3 = 95.0 cm. Women: mean T1 = 90.6 cm, T2 = 89.2 cm, T3 = 86.3 cm), smoking habit (% no never/yes regularly/yes occasionally/have quit T1 = 49/11/6/33%, T2 = 57/3/4/34%, T3 = 61/4/2/31%), diagnosed high blood pressure (T1 = 30%, T2 = 22%, T3 = 15%), HDL cholesterol (mean mmol/L T1 = 1.56, T2 = 1.65, T3 = 1.76).

**Conclusion:** Peak oxygen saturation in skin microcirculation is strongly associated with virtually all established risk factors for cardiovascular disease. The potential of oxygen saturation as a marker of dysfunction in the microvascular bed and as a bio-marker for cardiovascular disease will be further explored by evaluating the future cardiovascular events in the cohort.

### Cardiac Microvascular Endothelial Cells Improve Cardiomyocyte Contractile Function: The Role of Pro-Inflammatory Stimulation and Empagliflozin Treatment

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**Background:** Heart failure with preserved ejection fraction (HFpEF) composes 50% of HF cases. The paradigm of HFpEF suggests a systemic low-grade pro-inflammatory state leading to cardiac microvascular dysfunction and cardiac diastolic impairment. The EMPA-REG trial's positive findings on HF outcome suggest a direct effect of a sodium-glucose co-transporter 2 inhibitor, Empagliflozin, on the heart, indicating the potential of this drug for HFpEF treatment.

**Hypothesis:** Cardiac microvascular endothelial cells (CMECs) directly regulate cardiomyocyte (CM) systolic and diastolic properties. Impairment of CMEC function by pro-inflammatory stimulation abolishes the beneficial effect of CMECs on CM function, which can be restored by Empagliflozin.

**Results:** We set up a co-culture system to evaluate the effect of CMECs on CM contractility. Using a novel method to assess CM mechanics, we showed that CMECs enhanced CM systolic and diastolic function. The positive effect of CMECs was abolished by L-NAME and the NO quencher cPTIO, suggesting nitric oxide (NO) as an important factor mediating the effect of CMECs. The beneficial effect of CMECs on CMs was abrogated after pre-incubation of CMECs with pro-inflammatory cytokine TNF $\alpha$ , which was accompanied by enhanced reactive oxygen species (ROS) and reduced CMEC NO levels. Simultaneous administration of TNF $\alpha$  and Empagliflozin reduced TNF $\alpha$ -induced ROS level in CMECs and coincided with restoration of NO release and the positive effect of CMECs on CM contractility. Empagliflozin showed no direct antioxidant capacity, suggesting that it activates intracellular mechanisms in CMECs that cause reduced ROS and enhanced NO level.

**Conclusion:** CMECs positively regulates not only CM systolic but also diastolic function. This effect requires NO and is abolished after pre-incubation of CMECs with TNF $\alpha$ , which can be restored by Empagliflozin treatment. Empagliflozin shows no direct radical scavenging capacity, indicating that it activates intracellular pathways leading to reduced ROS production, increased NO bioavailability and improved cardiac contractility.

### iPSC-Derived Endothelial Cells with Mutation in HNF1A as Model of Maturity Onset Diabetes of the Young

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Maturity onset diabetes of the young (MODY) is an autosomal dominant monogenic diabetic disease typically affecting individuals before the age of 25 and closely resembling type 2 diabetes. The most common form of the disease is caused by mutations in hepatocyte nuclear factor 1A (HNF1A) gene. Patients with HNF1A-MODY were associated with abnormalities in endothelial function, microvascular complications like retinopathy, and increased risk factor for cardiovascular diseases. However, up to date there is no clear relation between the mutation in HNF1A gene and endothelial dysfunction.

As the clinical phenotype of HNF1A-MODY diabetes varies considerably in the patients, we used commercially available induced pluripotent stem cell (iPSC) line from control individual for introduction of mutation in the HNF1A gene. The isogenic cell lines were created using HNF1A guided RNA CRISPR vectors from GenScript. Derived clones had confirmed monoallelic insertion at the site recognized by applied guided RNA, leading to frame shift and occurrence of premature stop codon. Subsequently, control iPSC and two HNF1A clones were differentiated toward endothelial cells (ECs) and different markers/functions were compared.

The created isogenic cell lines had similar expression of pluripotency markers like Oct4, TRA-1-60, Nanog and SSEA-4 and no significant difference in the differentiation efficiency toward ECs. Differentiated cells (iPS-ECs) from all cell lines showed 90–100% expression of CD31 (PECAM1) and Tie-2, whereas around 70–80% of the cells expressed VE-cadherin. Importantly, there was a clear difference in the expression level of VE-cadherin (CD144), which is endothelial specific adhesion molecule located at the junctions of the cells. Even though the iPS-ECs with mutated HNF1A gene had lower expression of VE-cadherin, no difference in the location of the protein was found. The expression pattern of other ECs markers like phospho-eNOS, angiotensin 1 and angiotensin 2 was similar. Isogenic cell lines responded similar to stimulation with pro-inflammatory cytokine TNF-1 $\alpha$  with increase in ICAM-1 (CD54) on the cell surface. Additionally, all tested iPS-EC showed expected angiogenic response in a tube-like formation assay.

Summarizing, monoallelic mutation of HNF1A in iPSC-derived ECs leads to decreased expression levels of VE-cadherin, without affecting the protein localization. Other tested endothelial markers were similar in the isogenic cell lines as well as their response to pro-inflammatory cytokine.

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### Dissecting the Roles of Altered Cell Type Composition and Cell Specific Responses to Proatherogenic Stimuli in Contributing to Global Gene Expression Changes in Atherosclerotic Aorta

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Coronary artery disease (CAD) is the leading cause of death globally. There is clear consensus that endothelial cells (ECs), macrophages and smooth muscle cells (SMCs) play important but different roles in the disease progression. Despite growing appreciation of the cell type specific contributions, the fundamental mechanisms by which these cell types respond to the proatherogenic environment and contribute to the establishment of the complex pathogenesis of CAD are not well understood and present a major impediment for the development of effective cures.

In this study, we sought to determine the contribution of endothelial cells, macrophages and smooth muscle cells on the global gene expression changes identified in bulk atherosclerotic tissues. Using single cell sequencing of mouse tissues and deconvolution analysis of human atherosclerotic lesions, we provide evidence for altered cell type composition. In addition, we investigated the transcriptional responses of HAECs, HASMCs and CD14+ macrophages to proatherogenic stimuli in order to decipher the cell type specific contributions to CAD-associated pathways and to identify novel lncRNAs that could serve as markers of disease state. Finally, mechanisms underlying cell type specific gene expression were studied by analyzing the contributions of master transcription factors to cell type specific open chromatin regions and sites of enhancer RNA production using scATAC-Seq and GRO-Seq.

### Optimising the Myocardial Homing and Therapeutic Efficacy of Mesenchymal Stem Cells Using 3D Culture and Specific Sub-Populations of Bone Marrow-Derived Cells

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**Background:** Although systemic injection of bone marrow (BM)-MSCs is a promising approach to treat myocardial infarction, clinical success has been limited. BM-MSCs exist as a heterogeneous population, varying in differentiation and immunomodulatory capabilities and their anatomical location within the BM. A failure to identify the most therapeutic sub-population has like-

ly hampered clinical success. Furthermore, culture conditions may also affect myocardial homing and subsequent retention. We therefore determined whether 2D vs 3D culture influenced myocardial homing of two different BM-MSC sub-populations and their ability to modify the inflammatory cytokine response to injury.

**Methods:** Human BM-MSCs tested were: (i) tri-lineage (OAC) differentiating, CD317- and perivascular Y201 cells or (ii) poorly differentiating, CD317+ and endosteal Y202 cells. MSCs were cultured using conventional 2D or 3D hanging drop methods. Intravital microscopy imaged the homing kinetics of CFSE-labelled MSCs to sham or ischaemia-reperfusion (IR) injured beating hearts in anaesthetised mice (ketamine/medetomidine; ip). A Luminox<sup>®</sup> multiplex immunoassay was used to quantitate serum levels of 23 inflammatory cytokines. The ability of MSCs to reduce oxidative stress in H<sub>2</sub>O<sub>2</sub> damaged endothelial cells [ECs] in vitro was also assessed using 8-OHdG immunostaining.

**Results:** Although increased homing of 3D-Y201 and 3D-Y202 MSCs to sham hearts was observed when compared to 2D cultured cells, only 3D-Y201 demonstrated significantly ( $p < 0.05$ ) increased adhesion. No differences in free flowing cells were observed in IR injured hearts, but significantly increased adhesion of both 3D-Y202 ( $p < 0.05$ ) and 3D-Y201 ( $p < 0.05$ ) MSCs was observed, with adhesion greater than that observed in sham hearts. Interestingly, all 3D-MSCs adherent within coronary microvessels appeared smaller than 2D-MSCs. 3D-Y201 MSCs significantly ( $p < 0.05$ ) reduced the serum presence of 12 pro-inflammatory cytokines (e.g. IL-1 $\alpha$ , IL-6, IL-8, IFN $\gamma$ , MCP-1 etc.) whilst 2D-Y201, 2D-Y202 and 3D-Y202 only reduced 0, 6 and 4 factors respectively. Furthermore, 2D-Y201 ( $p < 0.01$ ) and 3D-Y201 ( $p < 0.05$ ) MSCs significantly reduced oxidative stress in ECs.

**Conclusion:** This novel study shows for the first time that 3D culture improved MSC myocardial retention. More importantly, perivascular CD317- Y201 MSCs with high differentiating capacity demonstrated better anti-inflammatory and anti-oxidative and effects in vivo and in vitro respectively. Clinical studies routinely utilise heterogeneous, non-sorted populations of BM-MSCs. The inclusion of a cell fraction of non-differentiating CD317+ MSCs in cell therapies could contribute to poor tissue regenerative responses in clinical interventions. These results strengthen the case for better identification and selection of therapeutic MSC sub-populations for myocardial applications.

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## Intravital Imaging of Vasculoprotective Effects of Haematopoietic Stem/Progenitor Cells Following Myocardial Ischaemia-Reperfusion Injury in the Beating Mouse Heart

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**Introduction:** The in vivo kinetics of haematopoietic stem/progenitor cell (HSPC) homing to the injured heart, and their ability to confer vasculoprotection within injured myocardial microcirculation, is not known. This has been limited by an inability to directly image these events at a cellular level in a beating heart environment in real-time. This study performed confocal based intravital microscopy on the anaesthetised mouse beating heart to image these coronary microcirculatory events.

**Methods:** Myocardial ischaemia-reperfusion injury (IRI) was induced by LAD coronary artery ligation (45 min) followed by reperfusion (2 hr) in anaesthetised mice (Ket/Med; i.p.). A 3D-printed stabiliser was attached to the beating left ventricle to enable the imaging of HSPCs (CFSE-labelled), neutrophils (PE+anti-Gr1b), platelets (APC+anti-CD41ab) and/or monocytes (FITC-labelled microspheres or using transgenic hCD68GFP reporter mice). Myocardial perfusion was detected using FITC-BSA to assess changes in functional coronary capillary density. Full field laser speckle microscopy was performed for the first time on beating hearts to monitor ventricular blood flow.

**Results:** IRI induced increases in neutrophils ( $p < 0.001$ ), monocytes ( $p < 0.05$ ) and microthrombi ( $p < 0.01$ ) within coronary capillaries. Interestingly, slow moving or 'patrolling' neutrophils were occasionally noted within coronary capillaries in both sham and IRI hearts. Capillary, but not larger blood vessel, perfusion was impaired as indicated by multiple areas devoid of FITC-BSA which were interspersed with regions of flow. Although local retention of trafficking HSPCs was poor, neutrophil, monocyte and microthrombus presence was reduced resulting in improved microcirculatory perfusion. Flow cytometric studies revealed the coronary endothelium was an early target of oxidative stress post-IRI and more susceptible to injury than cardiomyocytes. HSPCs also reduced this endothelial oxidative damage and ICAM-1/VCAM-1 expression as determined using flow cytometry. Interestingly, laser speckle of beating hearts demonstrated a sustained reactive hyperaemia ( $p < 0.001$ ) in response to IRI which was not affected by HSPCs.

**Conclusion:** We present a novel approach to intravital image multiple microcirculatory perturbations in a beating heart with laser speckle quantification of overall ventricular blood flow. The post-reperfusion hyperaemic response could easily have been interpreted as flow being adequately re-established. However, intravital it was clear that this was poorly transmitted to capillaries, and thus did not correspond to adequate myocardial perfusion at a microvascular level. Hence this study revealed a great mismatch between a "global hyperaemic response" during reperfusion, and microcirculatory heterogeneity. We further show that, despite poor local retention, HSPCs prevented thromboinflammatory events and thus benefitted blood perfusion at a microvascular level.

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## Improving Senescent Endothelial Cell Function by Partial Reprogramming

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The angiogenic and regenerative response after acute or chronic myocardial damage is severely reduced in senescent endothelial cells (EC). Partial reprogramming by a timely restricted activation of Yamanaka-factors (Oct3/4, Sox2, Klf4, c-Myc) by genetic tools has been shown to reverse cellular senescence and rejuvenate aged cells.

The aim of this study was to establish a pharmacological, non-genetic partial reprogramming strategy that induces a timely restricted activation of Yamanaka-factors, leading to a reversal of EC senescence. Such a strategy may allow a translational approach towards the clinical setting. A functional recovery of EC may thereby support the revascularization and tissue regeneration after myocardial damage.

Methods to characterize senescent EC and the effects of pharmacological reprogramming included quantitative real-time PCR (qRT PCR), Western Blot analysis, measurement of the cell and nuclear size, immunofluorescence staining of specific senescence markers, quantification of senescence associated  $\beta$ -galactosidase activity and measurement of telomere length in human umbilical vein EC. Further, angiogenic properties, proliferation, migration and stress resistance of EC were studied in vitro. Senescence of EC was induced by cell replication and followed for 16 passages. Senescent EC showed a time- and passage-dependent phenotype with enlarged cell size and nuclei, reduced proliferation and migration and increased expression of cell cycle regulators p16ink4a and p14arf as well as pro-inflammatory activation on mRNA as well as protein level. Further, senescent EC showed shortened telomeres and a high expression of DNA damage markers. In addition to replicative senescence, senescence was also induced by genotoxic (Etoposide) or inflammatory (Angiotensin II) stimuli, leading to similar characteristics. The application of a pharmacological reprogramming cocktail of FDA-cleared drugs (Valproic acid, lithium carbonate and Galunisertib) resulted in a robust but timely restricted activation of Oct3/4, Sox2, Klf4 and c-Myc in replicative senescent EC ( $P < 0.0001$ ). This was associated with a significant reduction of senescence markers p16ink4a, p14arf, TNF $\alpha$ , IL-1b, IL-6 and CD44 ( $P < 0.001$ ). Moreover, the telomere length was stabilized. Importantly, pharmacological reprogramming also significantly enhanced functional properties of senescent EC as proliferation, migration, sprouting and tube formation ( $P < 0.001$ ).

By applying a specific pharmacological cocktail to senescent endothelial cells the Yamanaka-factors Oct3/4, Sox2, Klf4 and c-Myc can be activated in a timely-restricted manner. This activation is sufficient to partially reverse the senescent phenotype and to restore functional and regenerative capacities of senescent EC. Thus, pharmacological reprogramming might represent a promising strategy to enhance endothelial function in age-related cardiovascular pathologies.

## Adipose Derived Mesenchymal Stem Cell Delivered Endothelial Protection During Graft Vasculopathy

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**Background:** Vascularized composite allotransplantation (VCA) is a promising treatment for composite tissue defects. Current clinical regimen includes life-long immunosuppression (IS) treatment post-transplantation that temporarily reverses the inevitable occurrence of graft rejection and dysfunction. A hallmark of rejection is graft vasculopathy (GV), characterized by vascular inflammation and intimal hyperplasia. Mesenchymal stem cells (MSC) carry both immunomodulatory and endothelium protective properties. This study aims at attenuating GV with the use of MSC to achieve a final goal of reducing the administration of IS.

**Methods:** A rat model of hind limb transplantation from Brown Norway to Lewis was used as a model for VCA. Post-transplant IS was stopped after 7 days. Upon grade II rejection (swelling/redness), therapy was initiated as follows. Group1 received IS therapy with Tacrolimus and Dexamethasone (4 mg/kg BW) to mimic a clinical situation. Adipose derived-MSC (AD-MSC) were administered systemically or locally in two doses of  $1 \times 10^6$  cells within three days in Group2 (n = 7) and Group3 (n = 7), respectively. Group4 received saline (n = 9). Eight days of therapy or progression to grade III rejection (epidermolysis) were defined as endpoints. Skin and femoral artery samples were stained with Van Gieson stain to determine intimal hyperplasia and vWF as a marker for endothelial cell activation.

**Results:** IS therapy in Group1 provided graft survival in all animals. In the absence of IS, Group4 animals suffered from grade III rejection within 3–5 days. Treatment with AD-MSC, significantly reduced progression of rejection (6/7 and 4/7 animals reaching endpoint in Group2 and 3, respectively). The efficacy of AD-MSC in attenuating intimal hyperplasia was predominant in the skin in arterioles  $>40 \mu\text{m}$  (Group4 vs Group3 ( $p < 0.005$ )) and in arterioles  $<40 \mu\text{m}$  (Group4 vs Group3 ( $p < 0.0001$ ) and Group 4 vs Group 2 ( $p < 0.05$ )). Intimal hyperplasia in the femoral artery showed no differences between the groups. The vWF staining showed contrasting results with no difference between the groups in skin, but a significant decrease in Groups 2 & 3 vs Group 4 ( $p < 0.05$ ) in femoral artery. Interestingly, vWF expression in the femoral artery of group 1 was similar to that for Group4.

**Conclusions:** IS treatment reverses acute rejection probably in the absence of endothelial cell protection thus accounting for recurring episodes of rejection. AD-MSC attenuates acute graft rejection and in addition, might elicit endothelial cell protection. Analyzing other markers for endothelial damage and changes in the vascular glycocalyx is warranted before AD-MSC can be used to relieve patients from long-term IS regimen.

## Machine Learning to Automatically Identify and Evaluate Intimal Hyperplasia in Histological Sections

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**Introduction:** Deep learning utilizes artificial neural networks to automatically learn from datasets as opposed to conventional, task-specific algorithms. In this study, we employed deep learning to automate the evaluation of intimal hyperplasia and the classification of vasculopathy in histological sections.

**Methods:** Tissue specimens of skin, muscle and arteries were harvested from rats after experimental hind-limb allotransplantation. Digital images of tissue sections stained with Elastica van Gieson were employed in two deep learning approaches. In the first approach, Cognex ViDi Suite software analyzed the images in three steps: 1. Vessel recognition; 2. False positive vessel elimination; 3. Segmentation of vessel wall into intima and media layers. This process was performed with 46 images for training and 20 images for validation. Furthermore, ViDi measured the surface area of segmented vessel walls to obtain a ratio. This ratio was compared to the conventional gold standard technique of calculating intimal hyperplasia. In the second approach, a custom-made Python algorithm using TensorFlow was implemented to classify vasculopathy into five stages of severity. Here, 50 images were used for training, and 94 images were used for validation.

**Results and Discussion:** ViDi could successfully recognize potential vessels (F-score = 86.5), eliminate false positive vessels (F-score = 82.8) and segment vessel walls into intima (F-score = 79.3) and media (F-score = 77.9) layers. The intimal hyperplasia determined by ViDi was generally lower than that determined by the gold standard technique. The automated classification of images into stages of severity by TensorFlow was achieved with an F-score from 12.0–52.1.

**Conclusion:** ViDi offers an evaluation platform for identifying vessels and vessel walls. The lower values of intimal hyperplasia calculated by ViDi indicate the consideration of wall irregularities. TensorFlow allows the automatic classification of vasculopathy, although extensive training is required to achieve expected outcomes. To conclude, this study recognizes the potential of deep learning in the histological assessment of vasculopathy.

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### Targeted Liposomal Delivery of NF- $\kappa$ B siRNA to Endothelial Cells Abrogates Anti-MPO Antibody-Induced Glomerulonephritis

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Endothelial cells in the microvasculature of the kidney are attractive targets for therapeutic intervention because of their involvement in the pathology of inflammatory kidney diseases. Vasculitis induced by anti-neutrophil cytoplasmic autoantibodies is an inflammatory disease and can result in necrotizing crescentic glomerulonephritis (NCGN). We hypothesize that NF $\kappa$ B in glomerular endothelial cells mediates NCGN and that preventive inhibition of NF $\kappa$ B reduces the progression of glomerulonephritis. We have developed a liposome-based siRNA delivery system (SAINT-O-Somes, SOS) that is targeted to inflamed microvascular endothelial cells in the kidney and efficaciously releases its siRNA intracellularly. To create specificity for activated glomerular endothelial cells the SOS were harnessed with anti-E-selectin antibodies. Previously we have shown that anti-E-selectin SOS effectively homed to glomerular endothelial cells. To interfere with disease associated endothelial cell activation of NF $\kappa$ B, p65 siRNA was encapsulated into the SOS. In a murine anti-MPO antibody induced glomerulonephritis model, a single injection of anti-E-selectin SOS containing NF $\kappa$ B-p65 siRNA resulted after 7 days in diminished albuminuria and protection against glomerular necrosis and crescent formation, while leukocyte influx in the kidney was significantly lower than in control siRNA treated mice. Our studies demonstrate that kidney endothelial cell specific delivery of NF $\kappa$ B-p65 siRNA results in local anti-inflammatory effects and attenuation of disease progress.

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### Functional Studies on the EPHB4 Signalling Pathway in Patients with Generalised Lymphatic Dysplasia

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**Introduction/Objective:** Lymphatic Endothelial Cells (LECs) have been shown to express EPHB4, a receptor tyrosine kinase which signals via the EphrinB2 ligand. Two loss of function mutations in EPHB4 were reported to be associated with a type of Generalized Lymphatic Dysplasia. The receptor was also identified as a critical regulator of lymphangiogenesis. This study aims to further investigate the role of EPHB4 in lymphangiogenesis and the mechanisms by which the two specific mutations interfere with EPHB4 signalling and dysregulate lymphangiogenesis.

**Methods:** To study the effect of the two mutations on downstream signalling pathways after EphrinB2 stimulation, receptor stimulation experiments are being carried out in LECs and activa-

tion of downstream signaling targets in the cascade investigated. Moreover, cells were treated with the specific inhibitor NVP-BHG712 to assess the effect of EPHB4 downregulation on LEC proliferation, migration and tube formation. In parallel, siRNA was used to silence EPHB4 expression in LECs.

**Results:** EPHB4 kinase-inactivating mutations may alter MAPK signalling in response to EphrinB2 stimulation in LECs. EPHB4 silencing supports this observation, resulting also in cytoskeleton changes in LECs. Impairment of LEC migration and tube formation was also observed after chemical inhibition of EPHB4.

**Discussion:** The initial results identify that the specific mutations disrupt EPHB4 signalling and suggest alterations on MAPK signaling and cytoskeletal dynamics as the possible mechanism for the lymphatic phenotype. Furthermore, they give new insights into the pathophysiological role of EPHB4 in lymphangiogenesis. Our future work will provide a better understanding of human lymphatic development and the role of EPHB4 in this process.

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### Formation of Diverse Occlusive Arterial Thrombi in a Novel Two-Channel Microfluidic System

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Arterial thrombi are traditionally described as white thrombi, consisting largely out of platelets and fibrin. However, patient' arterial thrombi greatly differ in the amount and distribution of platelets, fibrin, and red blood cells [1–4]. We hypothesize that current microfluidic models for thrombus formation insufficiently address this variation. The aim of this research is to develop a microfluidic system to mimick the variation in pathophysiological arterial thrombus formation.

A transparent polycarbonate parallel-plate perfusion chamber, consisting of a single inlet that bifurcates into two separate channels and merges before the outlet, was designed. The coverslip of the occluding channel (width: 1.5 mm, height: 50  $\mu$ m) was coated with collagen with(out) tissue factor. The coverslip of the non-occluding channel (width: 1.5 mm, height:100  $\mu$ m) was blocked with bovine serum albumin and acts as a pressure relief system for the other channel. Citrate-anticoagulated whole blood was recalcified prior to entering the perfusion chamber and perfused through both channels at a calculated arterial shear rate of 1000 s<sup>-1</sup> in the occluding channel.

On a collagen surface, thrombi reached a height of 50  $\mu$ m (T1) after approximately 14 minutes, and occluded the channel (T2) after approximately 38 minutes. T1 and T2 decreased to respectively 9.5 and 34 minutes upon perfusion of whole blood over a combined collagen-tissue factor surface. Occlusive thrombi formed as follows: after initial adhesion of platelets to the thrombogenic surface, fibrin fibers were generated. Fibrin fibers trapped new platelets, but few red blood cells, in the developing thrombus. After T1 was reached, an increasing number of RBC's adhered, which formed a shell around the existing platelet and fibrin thrombi. In contrast, thrombi did not become occlusive (T2 > 60 minutes) upon perfusion of citrate anti-coagulated whole blood, over collagen and tissue factor, through an one-channel flow chamber.

The latter thrombi often did not reach T1 and mainly consisted out of platelets and fibrin, with little RBC's.

This novel two-channel microfluidic device allows for occlusive thrombus formation under arterial shear conditions. Occlusive thrombi consist out of a platelet and fibrin core with a RBC shell versus non-occluding thrombi that are composed of platelets and fibrin. The pressure relief system is essential for the growth of an occlusive thrombus as it diverts blood flow from the occluding channel to the non-occluding channel once pressure rises due to thrombus formation.

Ultimately, this device may aid in monitoring the efficiency of currently used and novel antiplatelet and anticoagulant therapies.

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### Injury-Induced Vascular Changes and Thrombus Activity After Aspirin or Rivaroxaban Treatment in Mice

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The COMPASS clinical study investigated the effect of combined therapy of ASA and 2.5 mg bid rivaroxaban on patients with coronary or peripheral artery disease. However, little is known about the effects of this treatment on the vasculature.

The aim of this research was to study the effects of aspirin and/or rivaroxaban on thrombus activities and on injury-induced vascular remodelling in mice.

C57BL/6 mice (n = 12/group) were either treated with vehicle, aspirin (5 mg aspirin/kg/day) and/or rivaroxaban (12 mg/g chow). Vascular injury was induced by temporary ligation of the carotid artery. Vascular stiffening was monitored for two weeks by non-invasive ultrasound imaging. Two weeks after ligation, platelet-leukocyte aggregates (PLA) and vascular changes were assessed. Whole blood thrombus formation and thrombus-induced cleavage of dye-quenched collagen were studied with microfluidics.

Treatment with aspirin with(out) rivaroxaban decreased the number of adhered platelets per PLA (P < 0.05) in unstimulated blood and blood stimulated with stable ADP analogue or PAR4 peptide (flow cytometry). Importantly, treatment with aspirin with(out) rivaroxaban protected the arteries against ligation-induced stiffening (P < 0.05), while rivaroxaban alone showed a trend herein (P = 0.08). Histological analysis indicated that ligation of the carotid artery resulted in local intima-media thickening, which was reduced by treatment with aspirin or rivaroxaban (P <

0.01). Treatment of mouse blood with aspirin and/or rivaroxaban did not alter the size of thrombi formed ex vivo. Interestingly, thrombus-induced collagenolytic activity ex vivo was reduced (P < 0.05) upon treatment with aspirin or rivaroxaban.

In conclusion this study provides new insight into the vascular-directed effects of aspirin and/or rivaroxaban treatment after thrombotic injury. Treatment with aspirin significantly antagonised, and rivaroxaban showed potential to inhibit, vascular stiffening, intima-media thickening, PLA formation and thrombus-collagenolytic activity.

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### Bradykinin Signaling Regulates Solutes Permeability and Cellular Junctions Organization in Lymphatic Endothelial Cells

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**Introduction:** Some forms of angioedema (AE) – like the rare hereditary angioedema (HAE) or the more frequent ACE inhibitor (ACEi) induced AE are mainly mediated by increased levels of the tissue hormone bradykinin (BK). In spite of the broad knowledge about BK and its receptors, the form of appearance of bradykinin mediated AE is not yet fully understood.

**Objective:** To determine the effect of BK in solutes permeability and in cellular junctional proteins in human dermis microvascular endothelial cells (HDMEC).

**Methods:** Commercial HDMEC were characterized by immunofluorescence and fluorescence-activated cell scanning (FACS). BK effects on macromolecular (MM) transport through HDMEC monolayers were monitored by permeability assays of dextran and albumin molecules. Whereas the effects of BK at the cellular junctions proteins were determined by immunoblot analyses of VE-cadherin and claudin-5. Intracellular calcium (Ca<sup>2+</sup>) and cyclic adenosine monophosphate (cAMP) levels were additionally evaluated. Furthermore, BK-induced regulation of endothelial target genes was analyzed by quantitative real-time PCR (qRT-PCR).

**Results:** HDMEC comprise more than 95% lymphatic endothelial cells (LEC). BK increases the permeability to dextran via BK-2-receptor (B2R) in a dose-dependent manner, while it reduces the permeability to albumin. BK treatment down-regulates VE-cadherin expression and induces a significant dephosphorylation of VE-cadherin residue Tyr731. It also down-regulates claudin-5 expression at the transcriptional level. In addition, BK exposure lead to an increase of the intracellular Ca<sup>2+</sup> and to a reduction of the cAMP levels. Finally, BK stimulation up-regulates the mRNA levels of the endothelial growth factors, vascular endothelial growth factor C (VEGF-C) and angiopoietin-2 (Ang-2), and of the major endothelial nitric oxide synthase (eNOS) in HDMEC. Secreted VEGF-C protein was found moderately increased in BK-induced HDMEC culture supernates.

**Conclusions:** HDMEC represent a model of LEC that express B2R and change their solute permeability upon BK stimulation. BK-induced B2R signaling in LEC promotes cellular junctions re-organization and homeostasis destabilization through most likely a Ca<sup>2+</sup>-activated NO-cGMP pathway leading to the up-regulation of endothelial target genes VEGF-C, Ang-2 and eNOS, all of which are key modulators of lymphatic vessels function and integrity. To our knowledge we are the first to discuss a potential influence of lymphatic endothelial cells in bradykinin mediated edema, and whether lymphatic vessels and tissue might play a role in the clinical course of bradykinin induced angioedema.

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### Unravelling the (Sub)cellular Mechanisms of Low Frequency Electromagnetic Stimulation as Ischemic Stroke Therapy

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**Background:** Neuroprotection for the treatment of acute ischemic stroke has been unsuccessful in clinical practice. We explored low frequency electromagnetic stimulation (LF-EMS) as an emerging safe and non-invasive neuroprotective therapy for stroke. Previous data demonstrated that LF-EMS ameliorates neurological outcome in rats subjected to global cerebral ischemia which was likely mediated by nitric oxide (NO). However, the mechanism by which NO production is induced remains unknown. Here we studied whether LF-EMS enhances NO production via activation of the endothelial nitric oxide synthase (eNOS) pathway in endothelial cells (EC). In addition, the therapeutic response of LF-EMS in a focal experimental stroke mouse model was investigated.

**Methods:** EC were stimulated with LF-EMS (13.5 mT, 60 Hz) for 20 min or left unstimulated. cGMP levels were evaluated as indirect measurement for NO production via ELISA. eNOS phosphorylation (peNOS), phosphorylation of Akt (pAkt) and  $\beta$ -catenin levels were assessed by Western Blot. Distal middle cerebral artery occlusion (dMCAO) was induced in C57BL/6J mice to determine the effect of LF-EMS on infarct volume. dMCAO operated mice were subjected to sham treatment or LF-EMS for 20 min during 4 days. After 7 days, animals were sacrificed and brain slices were stained with TTC.

**Results:** eNOS activation is regulated by phosphorylation on multiple amino acid residues. peNOS at Thr495 attenuates eNOS function, while phosphorylation at Ser1177 enhances eNOS activity. LF-EMS significantly increased peNOS at Ser1177 ( $p = 0.049$ ,  $n = 7$ ) and decreased Thr495 phosphorylation ( $p = 0.005$ ,  $n = 10$ ) in EC, suggesting enhanced eNOS activation. Additionally, increased cGMP levels were observed in LF-EMS treated EC ( $n = 2$ ). pAkt and  $\beta$ -Catenin were investigated as possible upstream eNOS targets. pAkt (Ser473) was significantly induced by LF-EMS ( $p =$

0.026,  $n = 12$ ), while  $\beta$ -catenin levels were only moderately increased ( $p = 0.076$ ,  $n = 11$ ). In the experimental stroke model, LF-EMS stimulated dMCAO mice showed a trend towards a reduction of 25% in infarct size compared to sham treated dMCAO mice ( $p = 0.078$ ,  $n = 9$ ).

**Conclusion:** Our findings indicate that LF-EMS enhances eNOS activation by modulating its phosphorylation status. Furthermore, the phosphorylation of the possible upstream kinase Akt was increased in response to LF-EMS. Future experiments using pathway inhibitors and eNOS siRNA will be performed to elucidate the pathway(s) activated by LF-EMS. Data obtained from the dMCAO model suggest that LF-EMS results in reduced infarct volumes compared to sham treated mice. In conclusion, this study provides more insight into the subcellular mechanisms of LF-EMS, which aids its clinical translation as a new effective therapy for ischemic stroke.

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### Vascular Cognitive Impairment in Experimental Hypertension: The Microglial Culprit

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**Objective:** Hypertension is a major risk factor for cerebral small vessel disease, which is the most prevalent cause of vascular cognitive impairment. We reported earlier that hypertension induced by a prolonged AngII infusion is associated with focal blood brain barrier (BBB) leakages, microglial activation, myelin loss, and short-term memory impairment (Foulquier et al., Hypertension Research 2018). In this study, we aimed to decipher the contribution of microglia in this pathological cascade by a pharmacological loss-of-function study.

**Methods:** Adult Cx3Cr1gfp/wtxThy1yfp/0 reporter mice were generated to visualize microglia and neurons. Mice were infused for 12 weeks with Angiotensin II (AngII; 1000 ng/kg/min s.c.) or saline via minipumps; and treated with chow laced with a highly selective CSF-1R inhibitor (PLX5622; 1200 ppm) or control chow over the same period of time. Systolic blood pressure (SBP) was measured via tail-cuff and carotid pulsatility index by flow velocity doppler. Short- and long-term spatial memory was assessed during an Object Location and Morris Water Maze task (MWM), respectively, during the last 2 weeks of treatment. At the end of the study, mice were either perfused to perform flow cytometry on

brain, or injected with 70 kDa dextran-Texas Red prior to sacrifice to assess BBB integrity during histological analyses.

**Results:** SBP, heart weight and carotid pulsatility were increased by AngII but were not affected by PLX5622 treatment. MWM performance was not affected by AngII and/or PLX5622 treatment. Short-term memory was significantly impaired by AngII, and this effect was blunted in AngII mice treated with PLX5622. PLX5622 had no impact on memory performance in saline group. Microglia cell density was sharply reduced in PLX5622 treated mice as judged from Cx3Cr1+Iba-1+ staining (pPLX5622 <0.0001) and flow cytometry analysis (CD45intCx3Cr1hiCD11bhi; pPLX5622 <0.0001). In addition, perivascular macrophages (CD206+), a subset of tissue resident macrophages located along large penetrating cortical vessels, were partially depleted by PLX5622 treatment (33–56%; pPLX5622<0.0001). Number and size of BBB leakages were increased by AngII, but not altered by PLX5622.

**Conclusion:** Microglia depletion abrogates short-term memory impairment observed after prolonged AngII infusion, independently of any changes in cardiovascular status and BBB permeability. These results support the hypothesis that microglia are involved in the pathophysiology of hypertension related cognitive impairment.

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### Imaging Hypoxia in Living Cells

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Oxygen homeostasis is crucial for the survival of all eukaryotic organisms. Evolutionarily highly conserved genetic mechanisms govern self-regulation and adaption to local oxygen availability. At the core of this regulation is set of transcription factors, the hypoxia-inducible factors or HIFs, which sense oxygen on a post-translational level and are present in all annotated metazoan genomes. Hypoxia reflects insufficient oxygen delivery to a given tissue, resulting from either acute vascular occlusion or chronic failure to establish appropriate vascular supply. The resulting metabolic state is characterized by increased glycolytic activity, the induction of angiogenesis and frequently pro-inflammatory endothelial activation.

Several strategies have been developed to label hypoxic cells in vivo, which is most efficiently accomplished by 2-nitro-imidazole components (pimonidazole, hypoxyprobe) that covalently bind cellular components in hypoxic cells. Isotope-labeled variants allow intravital imaging with low resolution, while antibody-based detection of neo-epitope adducts is limited to post mortem analysis. Various nanoparticle-based probes deliver excellent signals but their access to the non-perfused space of the body may be significantly limited. Circumventing issues of probe administration and tissue access by genetically encoded GFP/RFP-hypoxia reporters under the control of appropriate promoter elements provided sub-optimal results, since all GFP and RFP variants require oxygen for their chromophore to fully mature. Taking advantage of the recently described oxygen-independent fluorescent protein UnaG from Japanese freshwater eel (*Anguilla japonica*), we have developed a novel family of genetically encoded hypoxia reporters, which ex-

press various destabilized versions of UnaG upon cellular hypoxia. We demonstrated the functionality of this reporter family in a mouse model of glioma using orthotopic transplantation of tumor cells (Erpaneedi et al. (2016) EMBO J. 35:102–113).

A universally applicable, transgenic mouse model for the spatiotemporal visualization of hypoxia during development and in various relevant disease settings in vivo is presently not available. In our hands, pronuclear injection of UnaG-based reporters yielded transgenic founder animals, none of which however expressed UnaG, suggesting a selection for transcriptionally inactive integration sites due to interference with embryonic development. We therefore decided to take a conditionally inducible approach, in which the reporter will be activated in a defined spatiotemporal manner through removal of a transcriptional stop cassette. We will report on this approach to generate UnaG-based hypoxia reporter mice and describe the development of test systems to validate such mice in various developmental and disease settings.

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### A CXCR4 Agonist Protects Brain Vascular Endothelial Cells from Radiation-Induced Senescence and Damage

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Radiation and other anticancer agents cause high levels of DNA damage, leading to endothelial cell apoptosis or senescence, which plays a role in injury of many tissues, including the intestine and the brain. SDF-1 and its main receptor, CXCR4, is a chemokine/chemokine receptor pair that play a critical role in the regulation of endothelial cell function, including proliferation, migration and differentiation during cardiogenesis, angiogenesis and re-endothelialization after injury. In this study, expression of CXCR4 and SDF1 was decreased by radiation treatment and with aging. Recombinant SDF1 protein treatment increased cell proliferation and decreased several senescence phenotypes in the senescent brain endothelial cells, such as upregulated expression of p53 and p21 and increased SA- $\beta$ -Gal activity, likewise SDF1 recovered damaged endothelial cells with radiation treatment by CXCR4-dependent signaling. In addition, the recovery of senescent or damaged cells with SDF1 was confirmed that activation of ERK and STAT3 plays an important role. ATI2341, a CXCR4 agonist, protected brain endothelial cells from radiation-induced damage. In the gastrointestinal syndrome and vessel damage by irradiation, ATI2341 treatment decreased cell death in the crypt of small intestine and SA- $\beta$ -Gal activity of arterial tissue. By ischemic injury experiment, blood flow was decreased by irradiation and was not in ATI2341 administrated mice during irradiation. Radiation was specifically down-regulated expression of CXCR4 and SDF1 in brain vessels of mice, resulted in inducing the abnormal cognitive ability of mice, but ATI2341 treatment partially restored the cognitive ability of irradiated mice. These results demonstrate that a CXCR4 agonist may be a potential drug for

the recovery and repair of the damaged tissues during chemotherapy or radiotherapy, particularly by protection of vascular endothelial cells.

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### **Nrf2 and Ageing Affect the Nanomechanics of the Aorta: In the Pursuit of Possible Mechanisms**

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**Introduction:** Senescence, a permanent state of cell cycle arrest is a predominant mechanism in response to cellular stress and may lead to the onset of early vascular ageing. The Nrf2 transcription factor, a master regulator mediating adaptive stress response by anti-oxidative gene expression, was demonstrated to maintain functional endothelial phenotype and regulate ageing. We aimed to investigate Nrf2-dependent nanomechanical properties of the aorta.

**Methods:** Young (2–3 months) and old (12–14 months) Nrf2 wild-type (WT) and transcriptional-knockout (tKO) mice were used. Senescent phenotype, nonmechanical properties of aortas and related mechanisms were studied by qPCR, HPLC, atomic force microscopy, VEVO ultrasound imaging as well as immunohistochemistry and flow cytometry.

**Results:** Inhibition of Nrf2 transcriptional activity in murine aorta does not lead to the oxidative damage manifested by lack of lipid peroxidation, advanced glycation end products and unchanged expression of antioxidant genes but results in the senescent phenotype confirmed by SA- $\beta$ -gal-positive staining and elevated p21 protein. Moreover, tKO aortas exhibit an elevated level of protein S-nitrosylation, together with the significant downregulation of mRNA for denitrosylating enzymes. No differences in the number of infiltrating immune cells between genotypes are seen. Nrf2 tKO aortas are characterized by increased aortic stiffness with the concomitant higher level of collagen content, but no differences in vasodilation function.

**Conclusion:** Nrf2 may affect nanomechanical properties of the aorta, what is associated with induction of S-nitrosylation and premature senescence.

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### **Myeloid SOCS3-Deficiency Regulates Angiogenesis via Enhanced Apoptotic Endothelial Cell Engulfment**

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Mononuclear phagocytes, such as macrophages and microglia, are key regulators of organ homeostasis including vascularization processes.

In the current study, we investigated the role of the suppressor of cytokine signaling protein 3 (SOCS3) in myeloid cells as a regulator of mononuclear phagocyte function and their interaction with endothelial cells in the context of sprouting angiogenesis.

As compared to SOCS3-sufficient counterparts, we found that SOCS3-deficient microglia and macrophages displayed an increased phagocytic activity towards primary apoptotic endothelial cells. This phenomenon was associated with an enhanced expression of the opsonin growth arrest-specific 6 (Gas6), a major pro-phagocytic molecule.

Furthermore, we found that myeloid SOCS3-deficiency significantly reduced angiogenesis in an ex-vivo mouse aortic ring assay, which could be reversed by the inhibition of the Gas6 receptor Mer. Together, SOCS3 in myeloid cells regulates the Gas6/Mer-dependent phagocytosis of endothelial cells and thereby angiogenesis-related processes.

Our findings provide novel insights into the complex crosstalk between mononuclear phagocytes and endothelial cells and may therefore provide a new platform for the development of new angiogenesis therapies.

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### **Three-Dimensional Tumor Model Based on Alginate Beads In Vitro**

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In two-dimensional (2D) culture systems, cells are grown as monolayers on flat solid surfaces lacking the cell-cell and cell-matrix interactions which are present in native tissue. In contrast, three dimensional (3D) culture systems offer the opportunity to culture one or several cell types imitating the in vivo tissue micro-environment. These interactions cause 3D-cultured cells to acquire morphological and cellular characteristics existing in tissues in vivo. Hydrogels are often used materials as 3D scaffolds due to their cytocompatibility and simple handling. Furthermore, they

are able to mimic the native extracellular matrix (ECM) and allow sufficient diffusion of nutrients and metabolites to support cell viability. In this study we focussed on developing a pre-vascularized 3D tumor model for in vitro testing of chemotherapeutic agents.

We used alginate (alginic acid) as a scaffold due to its almost instant gelation properties when in contact with ionic calcium solutions, tuneable viscosity, shear-thinning behaviour, low price and biocompatibility. To use these 3D constructs as tumor model, angiogenesis is essential for the development of tailored, tissue-engineered organs and tissues. To pre-vascularize the constructs, endothelial cells and supporting cells are embedded in the scaffold.

To produce small tumor-like structures, we compared different alginate and CaCl<sub>2</sub> concentrations to form beads. Pulmonary tumor cells (A549, PC-9), endothelial cells and different supporting cell types were embedded to induce and compare tumor angiogenesis. The tumor models were incubated for different periods of time (between 5 and 14 days) and analysed by two-photon laser scanning microscopy and immunohistochemistry. We evaluated different approaches according to the parameters length, area, volume and branching point numbers of the tubular structures. Our findings and data provide the first step towards understanding the pre-vascularization properties of different supporting cell types and sources for tissue engineering of 3D cell cultures and the application as in vitro tumor model.

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### Obesity, High Blood Pressure and Physical Activity Determine Vascular Phenotype in Young Children: The EXAMIN YOUTH Study

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Cardiovascular disease often develops during childhood but the determinants of vascular health and disease in young children remain unclear. The study aimed to investigate the association of obesity and hypertension as well as physical fitness with retinal microvascular health and large artery stiffness in children. In this cross-sectional study, 1171 primary school children (aged 7.2 ± 0.4 years) were screened for retinal arteriolar (CRAE) and venular diameters (CRVE), pulse wave velocity (PWV), body mass index (BMI), blood pressure (BP) and cardiorespiratory fitness (CRF) by standardised procedures for children. BP was categorised according to the reference values of the population-based German KiGGS study and the American Academy of Pediatrics guidelines. Overweight (mean (95% CI); CRAE: 200.5 (197.9–203.2) μm; CRVE: 231.4 (228.6–234.2) μm; PWV: 4.46 (4.41–4.52) m/s) and obese children (CRAE: 200.5 (196.4–204.7) μm; CRVE: 233.3 (229.0–237.7) μm; PWV: 4.51 (4.43–4.60) m/s) had narrower CRAE, wider CRVE and higher PWV compared to normal weight children (CRAE: 203.3 (202.5–204.1) μm, *p* < 0.001; CRVE: 230.1 (229.1–230.9) μm, *p* = 0.07; PWV: 4.33 (4.31–4.35) m/s, *p* < 0.001). Children with high-normal BP (CRAE: 202.5 (200.0–205.0) μm; PWV: 4.44 (4.39–4.49) m/s) and BP in the hypertensive range (CRAE: 198.8 (196.7–201.0) μm; PWV: 4.56 (4.51–4.60) m/s) showed narrower CRAE as well as higher PWV compared to normotensive

peers (CRAE: 203.7 (202.9–204.6) μm, *p* < 0.001; PWV: 4.30 (4.28–4.32) m/s, *p* < 0.001). With each unit increase of BMI and systolic BP, CRAE decreased and PWV increased significantly. Children with the highest CRF had wider CRAE, narrower CRVE and lower PWV compared to least fit children. Childhood obesity and hypertension, even at preclinical stages, are associated with micro- and macrovascular impairments in young children. Primary prevention programs targeting physical activity behaviour may have the potential to counteract development of small and large vessel disease early in life.

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### Impact of Diabetes on Hypertension-Induced Cerebral Small Vessel Disease

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**Introduction:** Cerebral small vessel disease (cSVD) is a disabling cerebral microangiopathy and is the leading cause of vascular dementia. In addition to ageing, hypertension and diabetes are the main risk factors associated with the development of cSVD. The brain structural abnormalities found in cSVD patients, such as White Matter Lesions, lacunar infarcts, microbleeds and enlarged perivascular spaces, are thought to disturb neuronal network function and thereby impair the cognitive function of patients. While these lesions are triggered by diabetes and hypertension, our understanding of the underlying mechanisms is limited.

**Objective:** While most studies focus on the role of hypertension alone in cSVD, there is an urgent need to study the impact of combined risk factors, as in case of the metabolic syndrome, on cSVD progression. We aim to assess, whether type 2 diabetes influences hypertension-induced cerebrovascular dysfunction.

**Methods:** Obese ZSF-1 (Ob) and lean ZSF-1 (Lean) rats were studied over time and sacrificed at 18 weeks and 21 weeks of age (*n* = 5–6/group). Blood pressure, measured by tail-cuff, and plasma fasting glucose were assessed over time. Brain coronal slices were analyzed by immunohistochemistry for the study of vascular density (IgG), and myelin content (Myelin Basic Protein). Vascul-

lar density was obtained by automatic analysis using Angiotool software. Myelin content and lateral width of the corpus callosum were measured by a blinded experimenter.

**Results:** Body weight was increased in Ob compared to Lean ( $525 \pm 7$  vs  $386 \pm 23$  g,  $p < 0.0001$  at w21). Fasting glucose levels were elevated in Ob starting from w13 and reached  $179 \pm 7$  mg/dL at w20 vs  $124 \pm 16$  mg/dL in Lean ( $p < 0.01$ ), confirming the development of type 2 diabetes solely in the Ob animals. Systolic blood pressure was already elevated in Ob and Lean at w5 and reached  $161 \pm 18$  mm Hg at w20 in Ob vs  $181 \pm 16$  mm Hg in Lean, showing that both the Lean and Ob animals were hypertensive. While myelin content was unchanged in the corpus callosum of Ob compared to Lean, its width was decreased ( $-8\%$ ,  $p < 0.05$ ). Furthermore, cortical vascular density was decreased in Ob vs Lean ( $-48\%$ ,  $p < 0.0001$ ).

**Conclusion:** Diabetes decreases vascular density in the cortex of hypertensive rats. This was associated with a reduced size of the corpus callosum. These first data suggest that diabetes may have an additive deleterious effect on hypertension-related cerebrovascular dysfunction. Investigations are ongoing to further characterize the impact of diabetes on hypertensive brains at several time-points.

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#### Changes in Skin Microcirculation in Response to Ischemia and Heat in Trained and Untrained Individuals

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Skin microcirculation has two main tasks: one is to supply the skin with sufficient oxygen and nutrients, the other is to contribute to the thermoregulation of the organism. Adaptation of skin microcirculation to heat is important to achieve high levels of physical performance during sport activities. We hypothesized that increases in perfusion of skin microcirculation is greater in response to local heat stimulus than to local ischemic/hypoxic challenge. Also, heat-induced perfusion changes are greater in trained than non-trained individuals. Thus, we aimed to measure local changes in subcutaneous microcirculation in various conditions. We have used Perimed 5000 Laser Doppler flowmetry (LDF) to continuously measure perfusion (perfusion unit, PU), temperature (T), and  $pO_2$  of the skin. To achieve ischemia, the upper arm was occluded for 2 min, then released (followed by reactive hyperemia), whereas temperature of the skin was increased locally from control to  $44^\circ\text{C}$  by a special LDF probe. Resting blood pressure and heart rate were also measured.

In response to occlusion and release, perfusion (PU) (an arbitrary number) increased (from  $10.4 \pm 3.7$  to  $48.8 \pm 8.5$  by 469.23%, T did not change, whereas  $pO_2$  decreased to low levels. Increasing local T (from  $32 \pm 1$  to  $44^\circ\text{C}$ ) elicited great increases in PU (from  $10.4 \pm 3.7$  to  $162.6 \pm 24.4$  by 1563.5%. Changes in perfusion in re-

sponse to heat were correlated with, whereas the ischemic responses were independent of resting heart rate of individuals.

On the bases of these findings we propose that thermoregulation is more important physiologically than maintenance of skin blood flow and heat-induced regulation of skin microcirculation is better developed in trained than in untrained individuals.

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#### Role of Synergism Between Pressure and Flow Activated Cerebrovascular Mechanisms and Mechanotransduction Signaling in the Autoregulation of Cerebral Blood Flow

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Cerebrovascular diseases rank at third after coronary and malignant diseases in the Western world: the most important devastating diseases are stroke (ischemic and hemorrhagic) and vascular dementia. Regulation of cerebral blood flow (CBF) is the result of complex, multilevel and interrelated mechanisms that ensure appropriate gas exchange between blood and cerebral tissue and provides the supply of nutrients for active neuronal tissue. At the same time, it has to comply with the limited space available in the cranium, a requirement, which is ensured by the so-called autoregulation of CBF. Previous studies provided evidence for a pivotal role of pressure-sensitive myogenic response is known to play. However, in vivo increases in pressure are accompanied by increases in flow; yet the effects of flow on the vasomotor tone of cerebral vessels are less known. Recently, we have demonstrated the existence and importance of a flow-sensitive constrictor mechanism in the middle cerebral arteries (MCA) isolated from rat and human brains. Also, we have showed that the two constrictor mechanisms additively reduce the diameter of arteries suggesting that both mechanisms contribute to the autoregulation of CBF. Among others, transient receptor potential (TRP) channels and the arachidonic acid metabolite, CYP450-derived 20-hydroxy-5,8,11,14-eicosatetraenoic acid (20-HETE) 20 HETE and thromboxane-prostanoid receptors and large-conductance  $Ca^{2+}$ -activated  $K^+$  (BK) channels mediate the vascular responses. Maladaptation of

these mechanisms, for example during aging and hypertension may contribute to the development of cerebrovascular disorders. Future studies are warranted to explore the mechanotransduction signaling processes and the in vivo importance of these mechanisms.

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### Going with the Flow in Microcirculation. Malpighi Award 2019 Lecture

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One of the great achievements of Malpighi was to discover the capillaries and thereby connecting the arterial and venous circulation, which were in his time still believed to be separate. He was looking at everything through via the microscope, discovered just a few decades ago. I have used the same approach looking at the living microcirculation for hours, try to interfere with it and waiting for something to happen. During investigation of a serial occlusion of an arteriole (to induce reactive hyperemia following release) I have noticed that during the occlusion, in the parallel arterioles flow velocity increased, which were followed by substantial dilations. Nowadays, this response is called “flow dependent” dilation. Yet, the real underlying stimulus is an increase in wall shear stress (WSS) and the dilation brings back WSS close to control, revealing a negative feedback mechanism. On a greater scale regulation of WSS contributes to the optimal power dissipation in the circulatory system. This is important for example in exercise; increase in WSS reduces peripheral resistance (arteriolar and venular) within seconds allowing great increase in cardiac output without great increase in blood pressure. Obviously in hypertension, when the diameter of vessels are reduced WSS is higher, and thus responsible for higher systemic blood pressure and greater power dissipation. Importantly changes in WSS is sensed by the endothelium, which in response releases – dependent on the tissues and organs – nitric oxide, prostaglandins, endothelium derive hyperpolarizing factors, H<sub>2</sub>O<sub>2</sub>, etc. Many diseases impair this endothelial function, in which one of the important steps is an increased oxidative stress. Thus there is still great interest to restore endothelial function by changes in life style or with pharmaceutical means. But what about cerebral circulation, which is enclosed in a rigid cranium? Can the flow/volume increase freely there? Addressing this question, our experiment revealed that increases in flow elicit constrictions both in small interbrain arteries of animals and humans. This was quite opposite to our previous concept, but in the brain the constant volume is extremely important, which is achieved by the autoregulation, previously believe to be regulated only by the pressure sensitive myogenic mechanism. In the new schema pressure- and flow-mechanisms by superimposing constrictions maintain cerebral blood flow close to constant in a wide range of systemic pressure. The primary mediator of flow-induced

constriction is the CytP450-derived 20 HETE. Importantly it seems that when this mechanism is impaired, for example due to traumatic brain injury or aging, there is an increased prevalence of stroke and vascular dementia. Still, many other aspects of flow sensitivity of vessels are not yet revealed and waiting for young investigators to challenge nature.

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### S-Nitrosylation-Dependent Protein Aggregation in Senescent Nrf2-Deficient Endothelial Cells: Is There a Role for ATM Kinase?

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**Background:** Premature senescence is conducive to ageing and cardiovascular diseases. Nrf2 is a stress-responsive transcription factor which has emerged as a guardian of endothelial homeostasis. Ageing was shown to impair the ability of vascular cells to mount an effective Nrf2-dependent antioxidant defence. The role of Nrf2 is mostly attributed to the transactivation of gene expression. However, age-associated mechanisms for declined Nrf2 expression and activity remain unrecognized. We observe that depletion of Nrf2 in endothelial cells leads to premature senescence accompanied by protein aggregation. The latter was recently shown to be regulated by ATM (Ataxia telangiectasia, mutated), a key player in DNA damage response.

**Aim:** To elucidate the mechanism of Nrf2-related premature senescence of endothelial cells (ECs) and verify if DNA damage is responsible for induction of senescence, to understand the role of ATM kinase in the determination of ECs fate.

**Methods:** In vitro experiments were performed on primary human aortic endothelial cells with siRNA-mediated depletion of Nrf2. The animal study was done on aortas isolated from Nrf2 transcriptional knock out mice. Quantitative PCR, fluorescent stainings, Western blotting, flow cytometry, comet assay were used in the study.

**Results:** Although Nrf2 is considered as a master regulator of redox status, its depletion in ECs does not lead to oxidative imbalance. Nevertheless, Nrf2 deficiency is associated with potent S-nitrosylation of proteins, which, however protective from oxidative detriment, redirects ECs to premature senescence. In the basal state, we do not observe DNA damage, what confirms maintenance of redox homeostasis. However, upon exposure to DNA damaging agents, Nrf2-deficient ECs have impaired and delayed DNA damage response. The most prominent changes are observed when it comes to ATM-dependent pathway. As ATM was recently shown to be a regulator of proper homeostasis in the

cells, we hypothesized that it may be a key player in Nrf2-related protein aggregation. Interestingly, depletion of ATM from Nrf2-deficient cells inhibits protein aggregation. Additionally, pharmacological induction of S-nitrosylation does not lead to protein aggregation in cells lacking ATM.

**Conclusions:** We confirm the novel role of ATM as a regulator of protein homeostasis in cells. Modification of ATM, possibly through S-nitrosylation, leads to protein aggregation in Nrf2-deficient cells.

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### Keap-1-Dependent Interplay Between S-Nitrosylation and Protein Aggregation in Ageing Endothelium

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**Background:** Loss of proteostasis has been proposed as one of the hallmarks of ageing. However, while the role of protein aggregates is well established in respect to neurological disorders, little is known about its impact in the vascular system. Protein deposition seems to be one of the causes of endothelial cell dysfunction, and the impairment of cell response to aggregating proteins occurs in e.g. atherosclerosis and diabetes.

**Aim:** We aimed to elucidate if ageing favours protein aggregation in endothelial cells (ECs) and verify its molecular mechanism.

**Methods:** The study was performed on primary human aortic endothelial cells (HAECs) isolated from young (<30 y.o) or old (>60 y.o) donor. RNA interference was employed to decrease Nrf2 expression, whereas inhibition of Nrf2 transcriptional activity was achieved with adenoviral vectors. The animal study was done on aortas taken from Nrf2 transcriptional knockouts together with appropriate controls. Senescent phenotype, protein aggregation and related mechanisms were investigated using mass spectrometry, biotin switch assay, immunofluorescent stainings, flow cytometry, protein and RNA expression.

**Results:** We evidence that age favours protein aggregation in endothelial cells. Interestingly, aggresomes colocalize with Keap1. Depletion of Keap1 decreases protein aggregation and enhances proliferation of aged ECs. A complementary phenotype is discovered in Nrf2-deficient endothelial cells. Lack of Nrf2 results in significant changes in the cell proteome, especially among the regulators of protein homeostasis, which are additionally modified by S-nitrosylation. It leads to an increased number of protein aggregates. Depletion of cytoplasmatic Nrf2 is associated with an overabundance of Keap1, what causes massive S-nitrosylation, protein

aggregation and the onset of senescence of ECs. Inhibition of S-nitrosylation abolishes Keap1-dependent cytoplasmatic protein aggregation.

**Conclusions:** Keap1 is a key regulator of both replicative and stress-induced senescence. The overabundance of unrestrained Keap1 in the cytoplasm determines the fate of ECs through regulation of S-nitrosylation and protein aggregation. We propose that Keap1 may serve as a component of enzymatic machinery for S-nitrosylation in mammalian cells. The overabundance of S-nitrosylated proteins causes their aggregation. The role of Nrf2 is related to the ability to sequester Keap1, therefore we unveil its non-canonical function, independent of transcriptional activity.

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### Dynamic Retinal Vessel Analysis in Arterial Hypertension: How Different Parameters Create a Whole Picture

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**Objective:** Dynamic retinal vessel analysis is usually defined as non-invasive clinical observation of the reaction of retinal vessels to a physiological stimulus. The data which is thus generated may be further analysed with respect to dynamic time-dependent and spatial vessel behaviour. We assume that assessment of different aspects of retinal vessel dynamics highlights different peculiarities of the underlying disease and allow for specific and sensitive characterisation of vascular pathology. We proof the principle of integrated analysis of pathologic dynamic retinal vessel behaviour on the example of arterial hypertension.

**Methods:** Retinal arteries of 15 untreated patients with essential arterial hypertension (age 47.0(43.5–61.5) years [median (1.quartile – 3.quartile)]) and of 15 age matched anamnesticly healthy volunteers were examined by Dynamic Vessel Analyser (DVA, IMEDOS Systems). After baseline assessment a monochromatic flicker (530–600 nm; 12.5 Hz; 20 s) was applied to evoke retinal vasodilation. Unstimulated vessel wall behaviour and pulse wave propagation were evaluated using mathematical signal analysis. Additionally differences in amplitude and frequency of spatial vessel diameter changes along the measured segments were analysed to assess the microstructure of retinal vascular blood columns.

**Results:** Four different aspects of dynamic retinal vessel behaviour were evaluated. Additionally to the previous result on lower retinal arterial dilation following flicker in systemic hypertension significantly different measures for unstimulated vessel wall oscillations, pulse-wave velocities in arteries and vessel wall conformation were found between both groups. Particularly, in arteries there was a difference in the duration of a single low-frequency

temporal oscillation between both groups: 3.6(1.5–5.5) s in arterial hypertension vs. 7.7(4.6–10.5) s in the control group ( $p < 0.05$ ). Longitudinal sections of retinal arteries in systemic hypertension showed pronounced spatial waves with a period of 694–833  $\mu\text{m}$  during vessel dilation ( $p < 0.05$ ), while in the control group the longitudinal sections were similar at all stages of arterial reaction. Retinal pulse wave velocities were significantly higher in systemic hypertension group as in the control group: 1400(1090–1650) RU (relative units)/s vs. 970(700–1250) RU/s ( $p < 0.05$ ).

**Conclusions:** Dynamic vessel analysis includes information beyond conventional DVA evaluation of temporal vessel reaction to flicker which allows for further understanding of the vascular status and underlying disease pathology. We found significant differences in several structural and functional parameters of retinal vessels in systemic hypertension which might indicate alterations in the vascular endothelium, smooth musculature and vessel wall rigidity in systemic hypertension, leading to impaired perfusion and regulation following metabolic demand.

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### Endothelial to Mesenchymal Transition and Endothelial Cell Plasticity

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As an underappreciated phenomenon, the endothelium is capable of remarkable cellular plasticity. One of the major biologic programs underpinning this endothelial plasticity is endothelial to mesenchymal transition (EndMT) – a process whereby an endothelial cell undergoes a series of molecular events that lead to a change in phenotype toward a mesenchymal cell (e.g. myofibroblast, smooth muscle cell). Recently, a great deal of effort has gone into dissecting and understanding the molecular basis of endothelial plasticity and EndMT. As a result, we now understand that during development, primitive endothelial cells exhibit plasticity and differentiate to acquire vascular fates. Also, certain endothelial cells undergo EndMT to give rise to mesenchymal cells necessary for cardiac development, and also some endothelial cells give rise to hematopoietic stem and progenitor cells. In the adult, mounting evidence indicates that EndMT is involved in adult cardiovascular diseases (CVDs), including atherosclerosis, pulmonary hypertension and valvular disease. Therefore, the targeting of EndMT may hold great therapeutic promise for treating CVD.

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### Deletion of Connexin 40 but not KIR2.1 Eliminates the Microvascular Response to Oxygen in Mouse Skeletal Muscle

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Mechanisms that fine-tune the delivery of red blood cells (RBCs) and oxygen to capillaries are critical to tissue function. Elevating RBC flow is intimately tied to the endothelium, the sensing of oxygen and the initiation of upstream responses in terminal arterioles. Studies have implied the endothelial KIR2.1 channels drive oxygen sensing whereas gap junctions, comprised of connexin 40, enable electrical signals to conduct upstream. This provocative assertion was tested in mouse deletion models (KIR2.1<sup>-/-</sup> and connexin 40<sup>-/-</sup>) where we controlled the local oxygen environment of skeletal muscle. Under anaesthesia (urethane and  $\alpha$ -chloralose), the extensor digitorum longus muscle from endothelial KIR2.1<sup>-/-</sup> and connexin 40<sup>-/-</sup> mice was prepared for intravital microscopy; a custom stage insert permitted oxygen control at the tissue surface. Second-by-second microcirculatory responses were recorded as oxygen levels were: 1) oscillated between 2% and 12% (1 cycle/min); or 2) reduced from 7% to 2% or 0% for 3 min. Chamber oxygen at 2% induces microvascular responses without diminishing mitochondrial function while 0% initiates additional metabolic responses. Endothelial KIR2.1<sup>-/-</sup> mice reacted normally to oxygen, albeit an oscillation or a sustained decrease (from 7% to 2%), with a rise of RBC supply rate (52%), velocity (28%) and hematocrit (16%). Likewise, the microcirculatory response to 0% oxygen was similar among control and endothelial KIR2.1<sup>-/-</sup> mice. In striking contrast, the microvasculature of connexin 40<sup>-/-</sup> mice failed to react to oxygen challenges. Cumulatively, our work shows that microvascular oxygen responses depend on coordinated electrical signaling and that endothelial KIR2.1 channels don't drive the initiating electrical event. These findings broaden our mechanistic understanding of functional hyperemia by defining the key channel subunits underlying its manifestation.

### The Effect of High Salt Dietary Intake and Angiotensin II Infusion on Activity of Antioxidative Enzymes in Serum of Sprague-Dawley Rats

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**Objective:** The renin-angiotensin-aldosterone system (RAAS) regulates blood pressure, fluid volume and sodium-potassium balance, thus being one of the most important hormonal mechanisms in controlling hemodynamic stability. The systemic RAAS is under a heavy influence of dietary salt intake and is suppressed by a high-salt diet. In addition, previously, we (J Physiol 2016;594(17):4917–31; AJP 2018;315(3):H718–H730) and others (Am J Physiol Heart Circ Physiol. 2010 Oct;299(4):H1024–33.) demonstrated that suppression of angiotensin II (ANG II), with high salt diet leads to increased vascular oxidative stress due to decreased expression of antioxidative enzymes in blood vessels' tissue. The aim of the present study was to determine the effects of HS diet on activity of antioxidative enzymes in serum.

**Methods:** 9–11 weeks old healthy male Sprague-Dawley rats were divided in 3 groups, low salt (LS; 0.4% NaCl for 7 days, N = 5), high salt (HS; 4% NaCl for 7 days, N=5) and high salt+angiotensin II group (HS+ANG II, 4% NaCl for 7 days, infused with ANG II via osmotic minipump from 4th-7th days (100 ng/kg/min/3 days), N = 6). Following dietary protocol, rats were anesthetized with ketamine (75 mg/kg) and midazolam (2.5 mg/kg) and sacrificed by decapitation. All experimental procedures were in compliance with the European Guidelines for the Care and Use of Laboratory Animals (directive 86/609) and were approved by institutional Ethical Committee. Antioxidative enzymes activity assay in serum samples was assessed by spectrophotometry. Data were analyzed by One Way Analysis of Variance test, presented as mean±SD. Statistical significance level was set to  $p < 0.05$ .

**Results:** Serum activity (U/mgP) of catalase (LS:  $0.94 \pm 0.23$  vs. HS:  $0.50 \pm 0.1$ , vs. HS+ANG II  $0.46 \pm 0.11$ ) and superoxide dismutase (LS:  $2.22 \pm 0.16$  vs. HS:  $1.17 \pm 0.25$ , vs. HS+ANG II:  $1.04 \pm 0.16$ ) was significantly decreased in HS and HS +ANG II group compared to LS group, while glutathione peroxidase (LS:  $0.17 \pm 0.01$ ) was significantly increased in HS+ANG II group ( $0.19 \pm 0.03$ ) compared to HS group ( $0.12 \pm 0.04$ ).

**Conclusions:** High salt dietary intake decreased the activity of antioxidative enzymes, while low-dose ANG II infusion restored the activity of serum glutathione peroxidase, suggesting the physiological importance of ANG II in maintaining systemic antioxidative capacity.

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### Heterogeneity of Perivascular Cells in the Kidney

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Our data indicates that perivascular cells are a major source of myofibroblasts driving kidney fibrosis and chronic kidney disease progression. The heterogeneity of perivascular kidney cells is unclear. We have used scRNA-seq to dissect the heterogeneity of the kidney perivasculature in homeostasis and fibrosis from mice and humans. This data indicates that only a small subset of perivascular cells is driving kidney fibrosis progression.

### Protein Tyrosine Kinase Activity Profiling Reveals Novel Signal Transduction Pathways of LPS-Induced Inflammatory Responses in Endothelial Cells In Vitro

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**Introduction:** Sepsis and sepsis-induced organ dysfunction are common problems in critically ill patients. The endothelium plays a major role in the pathophysiology of sepsis. Lipopolysaccharide (LPS) is a cell wall component of Gram-negative bacteria that mimics part of the disease pathophysiology by activating dedicated protein kinase signalling pathways. For successful pharmacological intervention, knowledge about the identity and the kinetics of activation of the full network of tyrosine kinases during LPS-mediated endothelial activation is crucial. Our study aims to unmask this complex kinase-signalling network in endothelial cells upon LPS exposure, using Protein Tyrosine Kinase (PTK) arrays, and to use this new knowledge to evaluate the potential of specific inhibitors of the identified kinase pathways to reduce endothelial inflammatory responses to LPS.

**Methods:** Phosphorylation profiles of human umbilical vein endothelial cells (HUVEC), challenged with LPS for different time periods, were analysed on PamGene PTK arrays using the Pamstation12. Peptide phosphorylation patterns were analysed using PamGene's BioNavigator software to identify active kinases involved in signalling. Three kinases that were identified as being involved in LPS-mediated signal transduction were further studied by inhibiting their activity in HUVEC. 30 min prior to LPS exposure, cells were treated with inhibitors (0–7  $\mu$ M). Effects on inflammatory responses were assessed by RT-qPCR analysis of inflammatory adhesion molecules E-selectin, VCAM-1, and ICAM-1, and cytokines IL-6 and IL-8. PECAM-1 (CD31) served as reference gene and GAPDH as housekeeping gene.

**Results:** Peptide phosphorylation profiles in endothelial cells changed within minutes after start of exposure to LPS. FAK14, ALK, and Axl were identified as putative activated kinases in relaying LPS intracellular signalling. FAK14 inhibitor inhibited the expression of all three cell adhesion molecules and IL-8 in a concentration-dependent manner (IC<sub>50</sub> ~1–3 μM), while at lower concentrations it induced the expression of IL-6. ALK inhibitor behaved similar to the FAK14 inhibitor, except that IL-6 mRNA expression was not induced at low inhibitor concentrations. Axl inhibition resulted in partial inhibition of adhesion molecules and cytokines at the highest concentration of the inhibitor.

**Conclusions:** In endothelial cells exposed to LPS, a multitude of kinases becomes activated in a time-dependent manner. Using Protein Tyrosine Kinase (PTK) array technology we identified FAK14, ALK and Axl as molecular targets in these cells, and evaluated their role by demonstrating effects of pharmacological inhibition on inflammation-associated gene expression. Effects on endothelial cell function, as well as consequences of interfering after the activating stimulus has been given will be further investigated.

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### Angiocrine Signals in Ageing and Disease

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Bone marrow provides supportive microenvironments for long-lived Hematopoietic Stem Cells (HSCs), Mesenchymal Stem Cells (MSCs), and certain immune cells. Skeletal ageing is associated with the decline in HSC function and increased incidences of bone metastasis. However, the role of the ageing bone marrow microenvironment in regulating the stem and cancer cell behavior remains elusive. Here, we find that the aged bone marrow microenvironment perturbs the quiescence of HSCs and MSCs, and metastatic cancer cells in bone. In particular, the aged bone marrow microenvironment exhibits a decline in secreted factors that induce and maintain quiescence. Intriguingly, both radiation and chemotherapy induces bone-specific upregulation of quiescence promoting factors. Cell-specific screening identified the involvement of pericytes in promoting quiescent microenvironments in bone in response to radiation and chemotherapy. Administration of PDGFR-beta inhibitor – sunitinib malate alongside radiation or chemotherapy led to the decline in quiescent cancer cells, and increase in the susceptibility to these therapeutic strategies. Thus, our study provides a framework for targeting pericytes to manipulate the bone marrow microenvironment in therapeutic interventions to manage bone metastasis.

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### Calcium Phosphate Bions Promote Intimal Hyperplasia in Intact Arteries Even Upon Normolipidemic Conditions by Causing Endothelial Injury Through Lysosome-Dependent Cell Death

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**Introduction:** An association between high serum calcium/phosphate and cardiovascular events or death is well-established. However, the mechanistic explanation of this correlation is lacking. Here we aimed to investigate the role of calcium phosphate bions (CPB, alternatively termed calciprotein particles), nanoscale bodies forming in human blood through supersaturation with calcium and phosphate, in the development of endothelial injury, a mandatory trigger of atherosclerosis.

**Methods:** CPB were synthesised artificially in a serum-supplemented culture medium to recapitulate their formation in blood. To examine whether CPB are able to aggravate pre-existing endothelial injury or even cause endothelial injury per se, we intravenously administered these particles to normolipidemic Wistar rats either upon balloon angioplasty or without any surgical intervention. Cytotoxic and pro-inflammatory effects of CPB were verified on primary human endothelial cells isolated from either coronary artery susceptible to atherosclerosis or internal thoracic artery resistant to atherosclerosis. Mechanism of CPB-driven cell death was explored utilising lysosomal inhibitors bafilomycin A1 and chloroquine. An ability of CPB to enhance production of reactive oxygen species was assessed by fluorogenic indicators of oxidative stress and addition of antioxidant enzymes superoxide dismutase and catalase.

**Results:** Intravenous administration of CPB to normolipidemic Wistar rats provoked intimal hyperplasia and concomitant adventitial inflammation in both balloon-injured and intact aortas. CPB-induced pre-atherosclerotic lesions in rats shared similar histological features with those in hyperlipidemic mice and patients including an increased synthetic phenotype in vascular smooth muscle cells and macrophage infiltration. Studies on primary human endothelial cells isolated from coronary and internal thoracic artery showed significant and specific endothelial toxicity of CPB mediated by lysosome-dependent cell death and excessive release of the pro-inflammatory molecule interleukin-6. Treatment with lysosomal inhibitors bafilomycin A1 and chloroquine partially rescued endothelial cells from CPB-triggered death. In contrast, measurement of reactive oxygen species within endothelial cells and thiobarbituric acid reactive substances in supernatant from these cultures did not show any differences between mock- and CPB-treated cells. Along similar lines, superoxide dismutase and catalase did not rescue endothelial cells after CPB exposure, suggesting that respiratory burst is not involved in CPB-induced cell death.

**Conclusion:** Formation of CPB causes endothelial injury through lysosome-dependent cell death and induction of interleukin-6 secretion, eventually promoting intimal hyperplasia even in normolipidemic conditions. An ongoing investigation of serum propensity to generate CPB in patients with coronary artery disease and cerebrovascular disease is aimed to define the clinical significance of CPB formation in blood and key determinants of this phenomenon.

## Gender Differences and Similarities in the Resolution of Intraluminal Thrombosis in a Rat Aneurysm Model

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**Introduction:** Intracranial aneurysms (IAs) are degenerative deformations of the arterial wall resulting in enlargement of the vessel lumen. IAs are mostly asymptomatic but when rupturing they induce hemorrhagic stroke potentially leading to severe brain damage and death. Women are twice as much as men affected by IAs, however the underlying factors that induce female predominance are unknown. Two explanations have been proposed, i.e. hormonal effects or hemodynamic causes, but no convincing evidence supports one or the other hypothesis.

**Methods:** We used the Helsinki side-wall aneurysm surgical model. A total of 204 female, male and ovariectomized female Wistar rats (10–12 weeks old) were used. Parental artery and aneurysm size was followed by magnetic resonance imaging at implant and 1, 2 and 4 weeks after surgery. No aneurysm rupture occurred. Aneurysmal histological sections were stained with Hematoxylin-Eosin, Martius Scarlet blue (for red blood cells and fibrin), Masson-Trichrome (for total collagen) and Victoria blue (for elastin) or immunolabelled with antibodies recognising  $\alpha$ -smooth muscle actin, CD68 or CD31. Twenty-six unruptured human saccular IA samples were obtained during microsurgery by resecting the aneurysmal dome after clipping of the aneurysmal neck and stored in the AneuX biobank.

**Results:** In female rats, until 2 weeks after aneurysm creation, the thrombus was mainly composed of red blood cells and fibrin. Around 14 days post-surgery, macrophages and smooth muscle cells started to invade the thrombus leading to the removal of red blood cells and deposition of collagen and elastin. Similar profiles of thrombus re-organization were observed in male and ovariectomized female rats. However, collagen content was higher in thrombi of male rats than in female rats ( $46 \pm 6\%$  vs  $23 \pm 2\%$ , mean  $\pm$  SEM,  $P < 0.05$ ), and vessel wall inflammation was higher in aneurysms of male rats. More aneurysm growth was observed in ovariectomized female rats compared with female or male rats (20% (12/31) vs 1% (-3/6) and -6% (-13/11) respectively, median (IQ range),  $P < 0.05$ ). Thrombus coverage by endothelial cells was lower in ovariectomized female than in female or male rats (33% vs 83% and 60% respectively,  $P < 0.0001$ ). Finally, analysis of human IA domes showed that endothelial cell coverage was lower in men and post-menopausal women in comparison to younger women ( $P < 0.0001$ ).

**Conclusion:** Aneurysm growth and intraluminal thrombus resolution show important gender-dependent differences. While certain processes (endothelial cell coverage and collagen deposition) point to a strong hormonal dependence, others (wall inflammation and aneurysm growth) seem to be influenced by both hormones and parental artery size.

## Primary Cilia Affect the Endothelial Response to Aneurysmal Wall Shear Stress

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**Introduction:** Intracranial aneurysm (IA) is an arterial disease resulting in a balloon-like enlargement of the vessel lumen. The pathogenesis is not completely understood. From their specific locations in the circle of Willis it is generally assumed that the initiation of IAs involves a high wall shear stress (WSS) gradient and that growth of wide-neck IAs may result from low WSS. Low WSS may be sensed by endothelial cells (ECs) via specialized structures called primary cilia. People affected by polycystic kidney disease (PKD) have no or abnormal primary cilia and are more prone to develop IAs. Here, we test the hypothesis that defective primary cilia may alter EC behavior, which, in turn, may affect the pathogenesis of IA.

**Methods:** We used embryonic aortic ECs from wild-type (WT) and Tg737orpk/orpk (ORPK) mice, a transgenic model for PKD that lack primary cilia, and exposed them to physiological or aneurysmal WSS (30 and 2 dynes/cm<sup>2</sup>, respectively) for 48 h using an Ibidi flow system. Unbiased transcriptomic analysis was performed by RNAseq. Results were confirmed at protein level by immunofluorescent stainings. Expression of ZO-1 was reduced with siRNA. Monolayers of ECs were cultured in Transwells to measure transendothelial electrical resistance and permeability for 4 kDa FITC-dextran.

**Results:** The percentage of WT ECs with primary cilia was similar under physiological and aneurysmal flow, identifying that the WT and ORPK EC model is suited to decipher the role of primary cilia in WSS-mediated EC response. RNAseq transcriptome analysis (fold change  $\geq 3$ ;  $p \leq 0.001$ ) revealed 296 genes differentially expressed for ORPK ECs against 58 genes in WT ECs when comparing physiological to aneurysmal flow. Further gene analysis revealed an enrichment of cell adhesion/tight junction pathways. We observed that monolayers of ORPK ECs have a lower transendothelial resistance and higher permeability for FITC-dextran than WT ECs. In addition, we confirmed increased expression of ZO-1, ZO-2, Catenin- $\alpha 1$  and Claudin-3 in WT ECs. Interestingly, Catenin- $\alpha 1$  and Cx43 junctional location seemed perturbed in ORPK ECs. Preliminary experiments with siRNA-mediated knock-down of ZO-1 in WT ECs also showed dispersion of junctional Catenin- $\alpha 1$  and Cx43 immunosignals.

**Conclusion:** The response to aneurysmal WSS in ORPK ECs involved 5-to-6-fold more genes than in WT ECs, suggesting that an important role for primary cilia in ECs may be to dampen the pathological response to aneurysmal (low) WSS. ORPK ECs displayed increased permeability compared to WT ECs, which may be due to a disturbed ZO-1 junctional interactome in these cells.

## Docosahexaenoic Acid-Derived D-Series Resolvins Direct the Regenerative Functions of Human Endothelial Colony Forming Cells

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Endothelial cell (EC) dysfunction is associated with the pathogenesis of age-related diseases and characterised by low-grade persistent inflammation, impaired anti-oxidant defence, and reduced tissue repair capacity, a process dependent upon the angiogenic functions of vascular ECs and endothelial progenitor cells. The D series resolvins (RvDs) are derived from the n-3 polyunsaturated fatty (PUFA), docosahexaenoic acid, and are key to inflammation resolution. We have shown that dietary lipoproteins carrying n-3 PUFAs limit EC inflammation and improve anti-oxidant defence but whether n-3 PUFA-derived mediators influence endothelial repair and the mechanisms involved are undefined. The objective of this study was to investigate the EC-directed pro-repair actions of RvDs and their potential role in mitigating age-related EC dysfunction.

Endothelial colony forming cells (ECFCs) were isolated from human peripheral blood and rendered replicatively senescent through serial passaging. Proliferation was assessed by nuclear staining (DAPI), angiogenic potential by tube formation on extracellular matrix, and migration using a monolayer wounding assay. Gene expression was quantified by qRT-PCR and protein expression by immunofluorescence/Western blotting.

Resolvins D1, D2 and D3 stimulated tubulogenesis by ECFCs. Only RvD3 increased proliferation whilst RvD2, D3 and D4, but not RvD1, enhanced ECFC migration. The pro-angiogenic activities of RvD1, D2 and D3, but not VEGF or Resolvin E1 (derived from eicosapentaenoic acid), were attenuated by the selective formyl peptide receptor 2 (FRP2)/ALX antagonist WRW4 and mimicked by MMK1 (FPR2/ALX agonist). Replicatively senescent ECFCs are significantly larger and exhibited high beta-galactosidase activity, reduced sirtuin-1 expression and greatly increased cyclooxygenase-2 expression compared to matched donor low passage ECFCs (n = 4). Senescent ECFCs showed a lower basal rate of migration and less tube formation than low passage ECFCs. RvD2, D3 and D4 increased migration and all RvDs enhanced tubulogenesis by senescent ECFCs; these responses were proportionally similar to those of low passage ECFCs. Studies in vessel ECs showed that the angiogenic response to RvD1 was inhibited by antagonising peroxisome proliferator-activated receptor (PPAR)  $\beta/\delta$  (GSK0660) or sirtuin-1 (EX-527) and that RvD1 increased PPAR $\beta/\delta$  transcriptional activity, augmented heme-oxygenase-1 expression and reduced cytokine-induced EC activation.

Collectively these studies identify a potential role for D-series resolvins in facilitating repair by targeting ECs to enhance cytoprotection and angiogenic function through FPR2/ALX and PPAR $\beta/\delta$ -dependent mechanisms. Dysfunctional senescent ECFCs showed preservation of angiogenic activity in the presence of resolvins, suggesting a potentially important role for these n-3 PUFA-derived lipid mediators in diminishing age-related endothelial dysfunction.

## The Effects of Methotrexate on the Vascular Endothelium

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**Introduction:** Anti-inflammatory therapy has been recently validated as a treatment option for patients with cardiovascular disease (CVD). The anti-inflammatory drug methotrexate (MTX) reduces cardiovascular risk in patients with systemic inflammatory rheumatic diseases; possibly by improving endothelial function. However, the molecular basis of the drug's anti-inflammatory and potential anti-atherogenic effects on the vasculature is not well described. Therefore, our goal was to characterize the actions of MTX on vascular endothelial cells (EC).

**Methods:** MTX-treated human umbilical vein (HUVEC) or aortic endothelial cells (HAEC) were analyzed by quantitative real-time PCR (qRT-PCR), immunoblotting and phospho-kinase activity arrays. EC were treated with tumor necrosis factor (TNF)-alpha to induce pro-inflammatory activation. EC viability and cell cycle were evaluated by flow cytometry using Annexin V and/or propidium iodide staining. For selected experiments, EC were exposed to laminar (LSS) or oscillatory shear stress (OSS) using a parallel plate model.

**Results:** Under static conditions, MTX independently increased the activity of multiple kinases in quiescent and TNF-activated EC. MTX-activated kinases included the mitogen-activated protein kinase (MAPK) p38 and Akt. MTX did not inhibit TNF-induced nuclear factor kappa B (NFkB) transcriptional activation, signaling or target gene expression. However, MTX induced pro-inflammatory markers such as vascular cell adhesion molecule (VCAM)-1 in an additive manner with TNF. Functionally, MTX did not induce apoptosis but caused S-phase cell cycle arrest, which, along with p38 and Akt activation, could be abrogated by supplementation with folic acid.

Findings to date in EC subjected to shear stress are somewhat different. MTX had no or a mild inhibitory effect on kinase signaling in EC under LSS and OSS respectively. MTX did not cause cell cycle arrest nor affect baseline or OSS-induced VCAM-1 expression in EC under shear stress. Further experiments will investigate the mechanisms underpinning the modulation of MTX signaling by shear stress.

**Conclusion:** In static EC, low-dose MTX caused cell cycle arrest through folate depletion, which is a known mechanism of action in other cell types. Of note, this response was not seen in EC preconditioned by shear stress and emphasizes the impact of biomechanical forces on endothelial phenotype and response to exogenous stimulation. This is the first report to study the effects of MTX on EC under shear stress, which will be crucial in understanding its molecular actions on the vasculature.

## High-Level Nrf2 Activation Promotes Endothelial Detachment – Implications for Acute Coronary Syndromes Triggered by Endothelial Erosion of Plaques

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Endothelial erosion of plaques trigger approximately 30% of heart attacks and is most frequently observed in smokers. The haemodynamic environment significantly regulates both plaque development and endothelial function, therefore we determined the haemodynamic environment permissive for plaque erosion.

To investigate possible factors that influence plaque erosion in patients, we determined the haemodynamic environment permissive for plaque erosion by reconstructing the coronary artery geometries from 17 heart attack patients with thrombi overlying intact fibrous caps (OCT-defined erosion) and performed computational fluid dynamic analysis. This identified that OCT-defined erosion most frequently occurs in areas of stenosis, exposed to very elevated flow.

We created an in vitro model of erosion by culturing human coronary artery endothelial cells under elevated flow and exposing them to aqueous cigarette smoke extract and TNF $\alpha$ . This induced significant endothelial detachment, which was enhanced by strong activation of the antioxidant system controlled by transcription factor Nrf2 (NFE2L2). The Oxidative Stress Growth INhibitor genes OSGIN1 and OSGIN2 were both maximally upregulated under these conditions and also in the aortas of mice exposed to cigarette smoke. Adenoviral overexpression of OSGIN1+2 in static culture resulted in cell cycle arrest in S-phase (5.5-fold increase,  $p = 0.003$ ), with a significant increase in the number of multinucleated cells (4.5-fold,  $p \leq 0.001$ ). Immunocytochemical analysis indicated loss of focal adhesions and stress fibres, dysregulation of

autophagy and induction of senescence in HCAEC, with a significant increase in senescence-associated  $\beta$ -galactosidase staining (6.7-fold,  $p \leq 0.001$ ) and P16 expression (3.2-fold,  $p = 0.035$ ). Importantly, overexpression of either Nrf2, or OSGIN1+2 induced cell detachment, which was independent of apoptosis, and could be partially rescued by inhibition of HSP70 nucleotide binding site using VER-155008, or AMPK activation using Metformin.

In summary, we have identified that smoking-induced hyperactivation of Nrf2 may promote endothelial cell detachment, contributing to plaque erosion overlying stenotic plaques, through the combined upregulation of OSGIN1 and OSGIN2. This highlights a completely novel mechanism potentially contributing to 30% of acute coronary syndromes and suggests possible therapeutic avenues for further investigation.

## Regulation of Spinal Cord Vascularization and Regeneration

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The vascular network closely associates with the neuronal network throughout embryonic development, in adulthood and during tissue regeneration. Close association of vessels and nerves allows reciprocal cross-talk involving diffusible molecules, which is important for physiological functions in both domains. Disturbances herein associate with neurodegenerative and cardiovascular diseases. Blood vessel growth and maturation of the nervous system occurs at approximately the same time as different neuronal cell types develop. The parallel development of vessels and nerves suggests local communication processes and raises several questions: how do nerves instruct vessels to ensure adequate vascularization and nutrient supply, and can vessels provide signals influencing neuronal differentiation, and function. Neuronal tissue repair requires local restoration of the vasculature and regeneration involves recruitment of neutrophils and (tissue resident) macrophages/microglia coordinating critical steps of axonal connectivity and arborization across the injury site. To study neurovascular interactions we use the zebrafish model system as it possesses the unique capability to regenerate organs including the heart and neuronal tissue. Using genetic approaches based on CRISPR/Cas9, knock-in strategies and tissue specific loss and gain of function approaches substantiated by high resolution in vivo imaging, we recently discovered a unique organ specific mechanism that allows neurons to precisely time the onset, extent and positioning of the spinal cord vascular network. We uncovered a thus far unknown form of sprouting, termed “tertiary sprouting” involving different molecules, and cellular behaviors when compared to other angiogenesis forms. Our current research focuses at targeting these processes in the context of neuronal differentiation and spinal cord regeneration.

## The Impact of Early Physical Exercise on Brain Microcirculation, Synaptic Proteins and Cognitive Function in a Chronic Model of Cerebral Hypoperfusion

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**Introduction:** The decrease in cerebral blood flow is an important risk factor for brain neurodegenerative dysfunctions. Currently, physical exercise has been proposed as an effective intervention to promote brain function improvement. However, the mechanisms involved in the anti-inflammatory and neuroprotective effects of exercise are not yet clear.

**Objective:** The aim of this study was to investigate the impact of early intervention with physical exercise on cognition, brain microcirculatory and inflammatory parameters in an experimental model of chronic cerebral hypoperfusion induced by permanent bilateral occlusion of the common carotid arteries (2VO).

**Methods:** Wistar rats aged 12 weeks were randomly divided into four groups (n = 10 per group): (1) Sham-sedentary group (Sham-Sed); (2) Sham-exercised group (Sham-Ex); (3) 2VO-sedentary group (2VO-Sed) and (4) 2VO-exercised group (2VO-Ex). The early intervention with physical exercise started three days after 2VO or Sham surgery. Physical exercise consisted of sessions of 30 min, 3 times per week during 12 weeks at 60% of maximal exercise test. After 12 weeks, the brain functional capillary density and endothelial-leukocyte interactions were evaluated by intravital video-microscopy, cognitive function was evaluated by open field test and hippocampus proteins: postsynaptic density protein 95 (PSD-95), synaptophysin and brain-derived neurotrophic factor (BDNF) were evaluated by western blotting. Statistical analyses were performed by analysis of variance (ANOVA) using the Bonferroni as a post-test. All procedures were approved by the Animal Care and Use Committee of the Oswaldo Cruz Foundation (L-002/2016).

**Results:** The 2VO-Sed group showed a decrease in brain functional capillary density compared to Sham-Sed group ( $295.0 \pm 15$  vs  $363.6 \pm 24$  capillaries/mm<sup>2</sup>, respectively;  $p < 0.05$ ). This effect was accompanied by an increased in cerebral rolling leukocytes ( $6.4 \pm 0.5$  vs  $3.7 \pm 0.6$  cells/min/100  $\mu$ m;  $p < 0.01$ ) and reduction of cognitive function. The early intervention with exercise during 12 weeks was able to decrease leukocyte-endothelium interaction compared to 2VO-Sed group ( $3.9 \pm 0.7$  vs.  $6.4 \pm 0.5$  cells/min/100  $\mu$ m, respectively;  $p < 0.05$ ), to improve hippocampus synaptic proteins (synaptophysin: 2VO-Sed:  $0.29 \pm 0.11$  vs 2VO-Ex:  $1.18 \pm 0.2$ ;  $p < 0.01$  and PSD-95: 2VO-Sed:  $0.42 \pm 0.11$  vs 2VO-Ex:  $1.96 \pm 0.28$  AU/ $\beta$ -actine;  $p < 0.01$ ) and restore cognitive function.

**Conclusions:** Microcirculatory and inflammatory changes in the brain appear to be involved in triggering a cognitive decline in animals with chronic cerebral ischemia. Therefore, early intervention with physical exercise may represent a preventive approach to neurodegeneration caused by chronic cerebral hypoperfusion.

## Vascular Smooth Muscle Cell Contraction and Relaxation as a Critical Regulator of Large Artery Compliance

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Stiffening of the large arteries is a hallmark of the aging process and the root cause of increased cardiovascular morbidity and mortality. The mechanisms of arterial stiffening are complex and incompletely understood but it is generally assumed that not only passive, but also endothelial and vascular smooth muscle cell (VSMC) components are important.

We recently found that in basal conditions, elastic, but not muscular, arteries produce high amounts of the relaxing factor NO. This suggests the presence of an evolutionary pressure to keep the VSMC tone of the aorta low. This is in line with epidemiological studies showing that reduced NO bioavailability is correlated with arterial stiffness and raises the question why, in a large range of vertebrates and invertebrates with a closed circulatory system, these contractile cells are evolutionary conserved in the aorta.

Because cyclic stretch is known to be crucial for eNOS activity, we used the Rodent Oscillatory Tension Set-up to study Arterial Compliance (ROTSAC) to investigate the role of VSMCs in the mouse aorta. This set-up is an in-house developed organ bath that allows the acquisition of pressure-diameter loops and the study of aortic segments at physiological pressure and frequencies.

At normal frequency (10 Hz) and pressure (80–120 mm Hg), the Peterson modulus (Ep, a diameter-independent measure of aortic stiffness) was  $293 \pm 4$  mm Hg (n = 5). Upon  $\alpha$ 1-adrenergic stimulation with 1  $\mu$ M phenylephrine (PE), Ep increased by 30% to  $381 \pm 33$  mm Hg. However, when in PE-stimulated segments NO production was blocked with 300  $\mu$ M L-NAME, Ep increased further by 80% to  $527 \pm 9$  mm Hg, confirming the important role of basal NO production in maintaining low VSMC tone.

At very high pressure (180–220 mm Hg), Ep was increased 6-7-fold to  $1923 \pm 148$  mm Hg. Interestingly, when VSMC tone was increased with PE, Ep decreased to  $1060 \pm 52$  mm Hg. Even more interesting, Ep did not change significantly upon addition of L-NAME ( $1011 \pm 33$  mm Hg).

These observations confirm that the NO pathway in the mouse aorta is – at least in vitro – a major determinant of its biomechanical properties. The effect of VSMC tone on Ep at high pressure is completely opposite from the effect at low to normal pressure. This suggests a physiological role for aortic VSMC tone in maintaining optimal hemodynamics, instead of merely a pathological phenomenon typically associated with vascular aging.

### Postocclusive Reactive Hyperemia of the Cutaneous Microcirculation: Impact of Different Mechanisms

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**Objective:** Postocclusive reactive hyperaemia (PORH) has been used as a clinical test to evaluate endothelial dysfunction. However, other mechanisms contribute to PORH, including myogenic, metabolic, and axon reflex. The contribution of separate mechanisms to the PORH phenomenon in skin microcirculation is sparsely investigated and results inconclusive. Our aim was to elucidate the impact of some manoeuvres, implicating specific mechanism, on PORH.

**Methods:** In 30 healthy volunteers (aged  $22.57 \pm 0.63$  yr.), laser Doppler flux (LDF) was assessed on two specific areas with different vascular control mechanisms: the volar forearm and the finger pulp, using laser Doppler fluxmetry. PORH was induced by a transient 3-min occlusion of the brachial artery. Indices of PORH were obtained in 5 separate experiments: in basal conditions, during handgrip exercise to assess the impact of skeletal muscle activity, during mental arithmetic test to assess the impact of the sympathetic nervous system, after the application of EMLA cream to elucidate involvement of axon reflex and after simultaneous inhibition of cyclooxygenase (COX) and endothelial nitric oxide (NO) synthase (eNOS) to inhibit the production of NO and prostacycline (PGI<sub>2</sub>). Simultaneously, we measured arterial blood pressure (BP), and the heart rate (HR).

**Results:** Handgrip exercise shortened t<sub>max</sub> ( $p = 0.006$ , Wilcoxon rank test) and increased the peak LDF of PORH (LDF<sub>max</sub>,  $p = 0.03$ ) in the forearm, showing also a trend of smaller area under the curve (AUC) and shorter duration of PORH (t<sub>dur</sub>) whereas in the pulp, PORH exhibited trends of shorter t<sub>max</sub> and smaller AUC. During mental stress, HR and BP significantly increased ( $p < 0.05$ ), and trends of shorter t<sub>max</sub> and t<sub>dur</sub> in the pulp were found ( $p = 0.06$ ), with no significant differences in the PORH parameters on the forearm. 40-min-EMLA application significantly decreased AUC ( $p = 0.02$ ) without affecting other parameters. COX and NOS inhibition significantly diminished AUC ( $p = 0.02$ ) in the forearm.

**Conclusion:** The mechanisms contributing to PORH depend on the measuring site. Handgrip exercise reduces the PORH response, implying the 'stealing phenomenon' of skeletal muscles. Mental stress reduces PORH in the pulp suggesting impact of the sympathetic nervous system. Decreased PORH response after EMLA confirms the role of axon reflex. Only partial inhibition of PORH after blockade of endothelial NO and PGI<sub>2</sub> suggests either important contribution of other endothelial vasodilators or significantly stronger impact of metabolic, myogenic and axon-reflex mediated component. Accordingly, PORH in skin microcirculation should be interpreted cautiously when considering endothelial (dys)function in clinical practice, as other mechanisms might prevail in the PORH phenomenon.

### Prediabetes and Type 2 Diabetes are Associated with Wider Retinal Arterioles and Venules

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**Background:** Retinal vascular calibers are biomarkers of cardio-metabolic risk. Previously, it has been shown in population-based cohort studies that wider retinal arterioles are associated with incident diabetes and impaired fasting glucose. However, the association of wider retinal venules and diabetes was found in non-Caucasian ethnicities only. The aim of the present study was to investigate the association of (pre)diabetes with retinal arteriolar and venular diameters in a predominantly Caucasian population.

**Methods:** In a population-based cohort study with oversampling of type 2 diabetes mellitus (T2DM;  $n = 1363$  normal glucose metabolism (NGM),  $n = 366$  prediabetes and  $n = 610$  T2DM, 50.1% men, aged  $59.7 \pm 8.2$  years; 98.5% Caucasian), we determined retinal microvascular diameters [measurement unit (MU)] (RHINO software) and glucose metabolism status (oral glucose tolerance test). Associations were assessed with multivariable regression analyses adjusted for age, sex, waist circumference, smoking, systolic blood pressure, lipid profile, the use of lipid-modifying and/or blood-pressure-lowering medication, eGFR, albuminuria, and prior cardiovascular diseases (CVD).

**Results:** Fully adjusted analyses showed a wider central retinal arteriolar equivalent (CRAE) and a wider central retinal venular equivalent (CRVE) in prediabetes (B [95% CI] CRAE: 0.93, [-1.43 to 3.30]; CRVE: 2.94 [-0.78 to 6.66], MU) with further deterioration in T2DM (CRAE: 3.47 [1.06 to 5.88]; CRVE: 3.88, [0.08 to 7.68], MU) versus normal glucose metabolism.

**Conclusions:** T2DM is independently associated with both wider retinal arterioles and venules in a predominantly Caucasian population. These retinal microvascular changes may occur to a lesser extent even in prediabetes. These findings support the concept that microvascular dysfunction is an early phenomenon in impaired glucose metabolism.

## Role of 5'GluCTC-tRNA Half in Human Cardiac Microvascular Endothelial Function

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**Background and Aim:** Heart failure with a preserved ejection fraction (HFpEF) is a growing, non-curable and worldwide clinical problem, frequently seen in patients with metabolic syndrome (diabetes, obesity plus hypertension). Metabolic disease-associated inflammation and consequent microvascular endothelial dysfunction are thought to be underlying pathophysiological mechanisms driving cardiac dysfunction. Improving endothelial cell (EC) functions could be a therapeutic target for HFpEF.

Non-coding RNAs (ncRNAs) have emerged as important regulators of EC functions and are interesting therapeutic targets. Recently, tRNAs have been shown to be actively processed via ribonucleases into ncRNAs of different sizes, including tRNA-halves. We identified specific tRNA-derived ncRNAs that are expressed and differentially regulated in human cardiac microvascular ECs (HMVECs) exposed to metabolic challenges, including the 5'GluCTC-31nt tRNA half. The biological role of 5'GluCTC-31nt is largely unknown. We hypothesized that 5'GluCTC-31nt has a role in metabolic disease-associated EC dysfunction and may be a treatment target for HFpEF.

**Methods and Results:** Using Northern blot and Sanger sequencing, we identified the 5'GluCTC-31nt tRNA half in human heart tissue and HMVECs. Exposure to a pro-inflammatory stimulus, TNF $\alpha$ , lowered the levels of endogenous 5'GluCTC-31nt in HMVECs, whereas exposure of HMVECs to shear stress by pulsatile flow for 48 hr resulted in a significant increase of 5'GluCTC-31nt levels compared to static HMVECs (>2-fold to static,  $p = 0.001$ ). Overexpression of a 5'GluCTC-31nt mimic (40 nM) in HMVECs promoted angiogenesis, as evidenced by an increase in tube density (+20%,  $p = 0.001$ ), average tube length (+74%,  $p = 0.001$ ) and junction density (+59%,  $p = 0.002$ ) compared to scrambled control. Finally, we observed a non-significant increase in neutrophil transmigration in TNF $\alpha$ -treated 5'GluCTC-31nt overexpressing HMVECs compared to scramble control (+13%,  $p = 0.09$ ).

**Conclusions:** Collectively, we addressed the regulation and functional roles of a specific tRNA-half with previously unknown roles in ECs. We found that this ncRNA is induced by pulsatile

flow and that its overexpression in HMVECs had a pro-angiogenic effect. Future work will be aimed at further elucidating the role of this tRNA fragment in endothelial cells, particularly in cardiac microvascular dysfunction and the development of HFpEF.

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### The Effects of Acute Exercise on Thermal Sensory Function

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Cutaneous thermal sensation plays a critical role in behavioural thermoregulation. Exercise results in various responses to ensure optimal metabolic, cardiovascular and thermoregulatory function. The effects of acute exercise on thermal sensation are not clear, however, with equivocal findings from studies with low subject numbers and varied assessment sites (acute vs. inactive limbs). The aim of this study was to assess the effect of exercise intensity on thermal sensory function of active and inactive limbs.

In a randomised and counterbalanced manner 13 healthy young male participants ( $25 \pm 6$  yr,  $1.8 \pm 0.1$  m,  $77 \pm 6$  kg) conducted; 1) 30 minutes low (50% heart rate maximum, HRmax; LOW) intensity, 2) 30 minutes high (80% HRmax; HIGH) intensity cycling exercise and 3) 30 minutes seated rest (CONTROL). Before, immediately and 1-hour after each 30 minute intervention thermal sensory function of the non-dominant dorsal forearm and posterior calf were examined by increasing local skin temperature ( $1^\circ\text{C/s}$ ) to assess heat sensitivity (detection of a change in skin temperature) and pain (detection of discomfort) thresholds.

Exercise increased heart rate (LOW;  $101 \pm 4$ , HIGH;  $147 \pm 7$  beats.min<sup>-1</sup>,  $P < 0.05$  vs. Pre) in an intensity dependent. Forearm skin temperature decreased during exercise ( $32.2 \pm 0.9$  vs.  $31.5 \pm 0.8$  °C,  $P < 0.05$ ) whereas calf skin temperature subtly increased during exercise ( $31.3 \pm 0.9$  vs.  $31.8 \pm 0.8$  °C,  $P = 0.20$ ) with both returning to baseline at 1-hour.

There was a significant trial\*stage interaction for forearm heat sensitivity thresholds ( $P = 0.029$ ). Relative to pre-exercise, forearm heat sensitivity thresholds were not different immediately ( $34.6 \pm 0.7$  vs.  $34.3 \pm 0.6$ °C,  $P = 0.20$ ) but were elevated 1-hr ( $35.1 \pm 0.8$ °C,  $P = 0.02$ ) after LOW. Relative to pre-exercise, forearm heat sensitivity thresholds were increased immediately ( $34.5 \pm 0.5$  vs.  $35.6 \pm 1.2$ °C,  $P = 0.001$ ) and 1-hr ( $35.9 \pm 1.2$ °C,  $P = 0.001$ ) after HIGH. There were no changes during CONTROL. There was a significant main effect of stage for calf heat sensitivity thresholds ( $P = 0.001$ ). Relative to pre-exercise, calf heat sensitivity thresholds were elevated immediately ( $38.1 \pm 0.7$  vs.  $37.2 \pm 0.6$ °C,  $P = 0.06$ ) and 1-hr ( $38.5 \pm 0.8$ °C,  $P = 0.001$ ) after exercise. There was no main effect of trial ( $P = 0.64$ ). There were no changes in calf or forearm heat pain thresholds after exercise in either LOW or HIGH (main effect of stage, Forearm  $P = 0.31$ ; Calf  $P = 0.26$ ) or between trials (main effect of trial, Forearm  $P = 0.44$ ; Calf  $P = 0.44$ ).

These results suggest that cutaneous thermal sensitivity function of a previously active limb is elevated after exercise indepen-

dent of intensity. Cutaneous thermal sensitivity function of an inactive limb is elevated only after higher intensity exercise. Exercise does not affect heat pain sensitivity in either active or inactive limbs.

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### The Regulation and Function of Non-Coding RNAs in Vascular Disease Development

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Dr. Maegdefessel's Molecular Vascular Medicine labs, both, at the Technical University Munich, Germany and the Karolinska Institute in Stockholm, Sweden are interested in the contribution of non-coding RNAs to vascular disease development and progression. Within vascular pathologies, his research team focuses on atherosclerotic plaque vulnerability and expanding aortic aneurysms, utilizing a variety of translational approaches to discover novel treatment and detection methods on a molecular basis. Candidate non-coding RNAs and their putative gene (mRNA) targets and proteins are profiled and detected through different transcriptomic (bulk and single cell), proteomic, epigenomic and genetic analyses applications. For these studies, the Munich lab utilizes human tissue specimens of carotid artery plaques and aortic aneurysms. Discoveries from human profiling studies are extensively investigated in pre-clinical models of vascular diseases, allowing the team to better understand the physiological and pathological function and deregulation of non-coding RNAs. In vitro studies, including artery-on-a-chip systems that utilize adult primary cells and iPSCs from actual vascular disease patients and single cell sequencing are deployed for in-depth mechanistic studies in disease-relevant cell subsets and conditions. Recent discoveries involve disease-contributing roles for miRs-201 and -21, as well as MALAT1 and CHROME in plaque vulnerability. In aortic aneurysm disease, the lab has identified a crucial role for the lncRNA H19 in disease progression by mediating survival of smooth muscle cells during aortic dilatation. The talk at the EVBO meeting will include other examples of long non-coding RNAs with relevance to vascular remodelling and disease development.

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### Vascular Bed-Specific Mechanisms of Lymphatic Development and Disease

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Lymphatic vessels not only provide a structural framework for transportation of interstitial fluid and tissue derived immune cells and antigens, but they can also directly modulate immune cell activation due to lymphatic endothelial cell presentation of antigens and expression of immunoregulatory molecules. In addition, the

lymphatic vasculature plays an essential role in the uptake of dietary fat and has been implicated in clearance of cholesterol from peripheral tissues. Reflecting their tissue-specific functional specialisation, lymphatic vessels within different organs show a remarkable heterogeneity. In addition, lymphatic endothelial cells have multiple organ-specific developmental origins, which may contribute to the development of the diverse lymphatic vessel and endothelial functions in the adult. The talk will discuss our recent and ongoing work aiming to understand heterogeneity within the vascular system, considering the organ-specific functional and molecular specialisation of lymphatic endothelial cells, their developmental origins and differential response to genetic alterations underlying (tissue-specific) vascular diseases.

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### Cytochrome P450 Reductase Is Important for Endothelial Function

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Besides having key physio-pharmacological roles, such as lipid and drug metabolism, cytochromes P450s (CYPs) are important sources of ROS production. Its redox partner, the cytochrome P450 reductase (POR), has an essential role in transferring electrons to CYPs and other oxidases such as hemoxygenase-1, which drives these systems and their redox capabilities. A previous project from our group identified POR as the main source of the signal found in NADPH-stimulated membrane assays, which were wrongly assumed to measure Nox activity. As this enzyme shares structural and biological features with Noxes-both contain flavin domains and consume NADPH for ROS production – it became our aim to investigate the roles of POR in cell signaling and vascular function. By using the DHE/HPLC method, it was observed that treatment of a7r5 cells (rSMC) with AngII (100 µM, 4 h) led to an increase of superoxide production in control cells which was abrogated not only by p22phox<sup>-/-</sup> but also by POR<sup>-/-</sup>. Proliferation rate was reduced in both POR<sup>-/-</sup> and p22phox<sup>-/-</sup> cells in comparison to the wild type counterpart. Activation of p38 induced by AngII was assessed by western blot and results indicated that both POR and Noxes contribute to p38 phosphorylation. Endothelial cell specific and conditional knockout mice of POR led to higher blood pressure, observed through telemetry and tail-cuff experiments, and an impaired vessel relaxation after AngII, observed through organ bath experiments, indicating an important contribution of POR for vessel homeostasis. Thus, our recent data show that POR indeed overlaps with Nox function which suggests a potential crosstalk between both systems and that part of the biological effects originally attributed to Nox enzymes are potentially mediated by the cytochrome P450/POR system.

## Stress and Neurovascular Coupling: A Red Flag? The SABPA Study

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**Introduction and Objectives:** Neuronal hyperactivity mediates communication between neurons and blood vessels (neurovascular coupling) and increases susceptibility for retinal ganglion cells functionality. Contrastingly, monoamine depletion and hypo-activity of cortisol reflected major depression, which may also disturb neurovascular coupling. Hence our objectives were to assess: 1) trajectories of two major stress hormones (norepinephrine and cortisol) over 3 yrs; and determine 2) associations between the retinal vasculature and stress hormone changes and responses during flicker-light-induced-provocation (FLIP).

**Methods:** A teachers' cohort (N = 275; 45 ± 9 years), having similar socio-economic status, was followed for 3 years. Prospective changes for depression (Patient-Health-Questionnaire-9), stress hormones [urinary norepinephrine:creatinine ratio (NE:Cr), serum hypothalamic-pituitary-adrenal axis (HPA-axis: adrenocorticotrophin; cortisol)] and high-density-lipoprotein (HDL) were determined. At 3 yr-follow-up, retinal microvascular calibres were quantified from digital images in the mydriatic eye. A novel approach was applied to obtain salivary cortisol and  $\alpha$ -amylase (adrenergic activity marker) samples directly prior to and post-FLIP.

**Results:** An interaction term (ethnicity x NE:Cr tertiles) was fitted for faster arteriolar vasoconstriction and venular maximal dilation in NE:Cr tertile groups. Over 3 yrs, the low-tertile group presented with chronic depression and adrenocorticotrophin hypo-activity. They had a 111% relative increase in norepinephrine over 3 yrs ( $p < 0.001$ ) with cortisol and HDL decreases. Their venules were wider ( $p < 0.05$ ) and they presented 4.4% retinal microbleeds compared to the high-tertile group (1.1%). Faster arteriolar dilation and -vasoconstriction as well as delayed venular dilation and recovery to baseline were related to  $\alpha$ -amylase and norepinephrine increases, respectively. Stroke risk markers (venular wid-

ening, low HDL) were associated with serum and saliva cortisol hypo-activity in the low-tertile group only ( $p < 0.05$ ). Norepinephrine increases, low HDL and venular widening were associated with chronic depression ( $p < 0.05$ ). These associations were not found in the mid- or high-tertile groups.

**Conclusions:** In reaction to low norepinephrine concentration, upregulation occurred with concomitant HPA-axis hypo-activity; resembling chronic stress. Norepinephrine hyperactivity increased vascular resistance and impaired myogenic control. Stress-induced disturbance of neurovascular coupling can be a red flag for greater stroke susceptibility.

**Clinical Considerations:** Tricyclic anti-depressants which down-regulate norepinephrine should raise some concern.

## Establishing Human Diabetic and Cardiovascular Disease Models in a Petri Dish Through Cell Reprogramming

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The complications arising from macrovascular and microvascular disease can be a major source of mortality in diseases such as diabetes and cardiovascular disease (CVD). Diabetes is a major cause of heart attacks, stroke, lower limb amputation, kidney failure and blindness, all of which are devastating conditions. The healthy and normal function of the endothelium is of utmost importance, against the development and progression of vascular disease. Induced pluripotent stem (iPS) cell-derived endothelial cells (iPS-ECs) have shown notable therapeutic potential in pre-clinical studies, which includes the ability to incorporate into and re-endothelialize damaged vasculature as well as to inhibit neointimal and inflammatory responses to vascular injury. In addition, they have shown great functional promise in providing opportunities for disease modelling. Consequently, iPS cells in regenerative medicine show great potential today as they can be used to generate patient-specific cells and personalised therapies. We have established expertise in generating specific ECs from iPS cells from non-diabetic volunteers and diabetic donors (Type 1 and 2) with or without vascular complications. iPS-ECs from both sources have been fully characterised and show EC morphology, high purity, and typical marker expression. Interestingly, iPS-ECs from diabetic donors demonstrated abnormal capillary permeability versus those from non-diabetic donors, as assessed in EC monolayers exposed to VEGF. In parallel, iPS-ECs from diabetic donors also displayed an impaired tube formation capability in vitro and blood flow recovery in vivo was significantly diminished when they were tested in an in vivo mouse model of limb hind ischemia. In order to start elucidating the underlying mechanisms, RNA Sequencing (RNA-seq) analysis was performed. Interestingly, we observed an impaired balance between pro- and anti-angiogenic growth factors, and genes related to impaired angiogenesis, EC death, mitochondrial dysfunction and oxidative stress to be drastically changed in iPS-ECs derived from diabetic donors in comparison

to the controls. It can be concluded that iPSC-ECs from diabetic donors, even when they are assessed in non-diabetic in vitro and in vivo settings carry an imprint of the disease milieu and which continues to alter normal EC function. Therefore, these diabetic and vascular complications models are capturing the phenotype of the disease in a petri dish and they have now opened the horizon for drug screening and cell based therapies to benefit people with diabetic and improve the quality of life.

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### Unexpected Effects of Photontherapy and Protontherapy on VEGFC Production and Tumor Aggressiveness in Different Medulloblastoma Subgroups

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Medulloblastoma (MB) is the most common malignant cerebellar pediatric tumor. MB is a heterogeneous pathology composed of four molecular subgroups, with variable prognoses depending on the subgroup. MB treatment includes surgical resection of the tumor, craniospinal X-ray irradiation (the reference treatment of most cancers) and adjuvant chemotherapy. Despite this treatment, 30% of the patients relapse. Their progression is marked by the fatal appearance of metastases within 5 years. According to the current dogma, the major actors of metastatic dissemination are the lymphatic vessel growth factor, VEGFC, and its receptor, VEGFR3, in most cancers. Protontherapy, which allows higher precision of tumor targeting while reducing side effects and radio-induced cancers, is now emerging as an alternative treatment of MB. In this work, we meant to demonstrate the differential biological effects of protontherapy and photontherapy on MB, as a model of both brain and pediatric tumors. We repeatedly irradiated MB cell lines by high doses (8 Gy) of X-rays, low-energy protons or high-energy protons (Proteus<sup>®</sup> One equipment, IBA, Belgium), thus generating resistant cells for each form of irradiation. We measured the production of VEGFA (angiogenesis), VEGFC (as a read-out of lymphangiogenesis programming), and ELR+CXCL cytokines (inflammation). We also determined the aggressiveness of the cells by proliferation, migration, and pseudo-vessel formation assays. Finally, we measured the epithelial-to-mesenchymal transition status of the cells and the apparent stemness of the cells after irradiation. We demonstrated that X-ray resistant cells presented an increase in VEGFC production, thus pleading for a cell programming in favor of the generation of new lymphatic vessels. Unexpectedly, however, these cells showed lower aggressiveness than their naïve counterparts. Moreover, we demonstrated that, opposite to the admitted higher effect of protontherapy over photontherapy (EBR = 1.1), proton beams were less harmful to MB cells than photon beams, when these cells were irradiated in the same conditions as patients. Hence, our results

present striking new evidence that i) VEGFC is a negative regulator of irradiation-induced MB aggressiveness; ii) opposite to the common dogma, proton irradiation reduces irradiation-induced damage to MB cells.

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### Investigating the Role of KIF11 in the Lymphatic Function of MCLID Patients

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**Introduction:** Mutations in KIF11 are causative of microcephaly with or without chorioretinopathy, lymphoedema, or intellectual disability (MCLID); a rare autosomal dominant disorder involving a variable spectrum of central nervous system, ocular developmental anomalies and lymphoedema (mostly bilateral in the lower limbs and sometimes restricted to the feet). EG5 (kinesin-5, encoded by the KIF11 gene) is a motor protein that participates in various kinds of spindle dynamics during cell mitosis but nothing is known about its specific role in lymphatic endothelial cells (LECs) and how mutations in KIF11 lead to MCLID. We hypothesise that EG5 could be participating in VEGFR3 intracellular trafficking and resultant defects in VEGFR3 signalling could lead to insufficient lymphangiogenesis and consequently lymphoedema in these patients.

**Methods:** Lymphoscintigraphy by subcutaneous injection of a tracer was used to image and assess lymphatic dysfunction in MCLID patients. With KIF11 haploinsufficiency as the likely underlying key disease mechanism in these patients, LECs were treated with siRNA or Ispinesib to block EG5 function. The effect of EG5 inhibition on LECs was analysed by in-vitro lymphangiogenic assays and the role of EG5 in VEGFR3 signalling investigated by western blot.

**Results:** Lymphoscintigraphy revealed the absence of radioactive isotope uptake from the web spaces between the toes, indicating the failure of initial lymphatics to absorb the tracer. LECs treated with Ispinesib, a specific inhibitor of EG5, displayed abnormal, monopolar spindles visualised by  $\alpha$ -tubulin staining. Inhibition of EG5 function impaired proliferation, migration and tube formation in LECs and the contribution of several signalling pathways is being investigated.

**Conclusions:** The lymphoscintigraphy results and initial functional assays suggest that EG5 clearly has a specific role in LECs. The similarities in the phenotype and lymphoscintigraphy results between Milroy patients (VEGFR3 mutations) and MCLID patients (KIF11 mutations) could suggest a common mechanism for lymphatic dysfunction.

## Human Vascular Pericytes Express a Circadian Rhythmicity

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**Introduction/Objective:** Circadian rhythms regulates several physiological phenomena of the human body such as food intake, hormonal regulation and sleep-awake cycles. The central pacemaker is located in the suprachiasmatic nuclei (SCN) but peripheral tissues also show endogenous 24-h rhythms. At a molecular level, circadian rhythmic gene expression relies on the core clock BMAL1 and CLOCK heterodimer rhythmically driving the expression of their own repressor genes *Period* and *Cryptochrome*. The dysregulation of the circadian rhythm due to lifestyle choices or pathology, profoundly affects the human cardiovascular functionality leading to increased risk of cardiovascular events and tumorigenesis (Morris CJ et al. 2016; Loning Fu et al. 2014). Among several affected mechanisms, angiogenesis plays an important role in the homeostasis and repair of the tissues. The interplay and cross-communication between endothelial cells and pericytes is crucial to the correct development of new blood vessels and the maintenance of the functionality of the existing ones (Campagnolo et al. 2010). While the circadian nature of endothelial cells has been established (Takeda et al. 2007), very little evidence has been published on pericytes and the endothelial/pericyte interaction in regards to the circadian rhythm. The aim of this study is to define the existence of circadian rhythm in pericytes and elucidate the influence of the circadian oscillations on the formation of new blood vessels.

**Methods:** Primary human umbilical vein endothelial cells (HUVECs) and primary patient-derived saphenous vein pericytes (SVPs) were analysed for the expression of the principal components of the circadian clock (BMAL1, PER2, CLOCK and REV-ERBa) upon serum shock synchronisation at different time points. Alongside, bioluminescence readings were collected continuously for 72 hours in cells expressing luciferase reporter under control of circadian genes specific promoters.

**Results:** Real time PCR and Lumicycle results showed that the human primary pericytes present a rhythmic expression of the circadian genes PER2, BMAL1 and REV-ERBa. Importantly, the expression pattern of BMAL1 is shifted by approximately 4-h as compared to PER2, supporting the existence of a feedback loop mechanism. As expected, the expression of CLOCK does not follow a circadian rhythmicity over the 24-h.

**Conclusion:** This preliminary study provides the basis for the future study of how vascular cells influence each other's circadian rhythm and the implications of the circadian cycle synchronisation on physiological and therapeutic angiogenesis.

## Angiotensin II Supplementation by Miniosmotic Pumps Reduces the Level of Oxidative Stress Induced by High Dietary Salt Intake in Rat Microcirculation

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**Introduction:** Previously, we demonstrated that suppressed levels of angiotensin II by high salt diet (HS) impaired endothelium-dependent dilation and reduced nitric oxide (NO) bioavailability due to increased endothelial levels of reactive oxygen species (ROS) in middle cerebral arteries (MCAs) of rats (J Physiol 2016;594(17):4917–31; AJP 2018;315(3):H718–H730). Present study aimed to determine the effect of angiotensin II supplementation on vascular NO and superoxide/ROS production and vascular antioxidative enzymes' mRNA expression.

**Methods:** 9–11 weeks old healthy male Sprague-Dawley rats were randomly assigned to 3 groups (n = 6–8 rats/group): low salt-diet group (LS group, 0.4% NaCl in rat chow); HS group (4% NaCl in rat chow for 1 week) and HS+angiotensin II group (HS diet for 7 days, additionally 4th–7th day subpressor doses of angiotensin II via osmotic minipump (100 ng/kg/min/3 days). Following dietary protocol, rats were anesthetized with ketamine (75 mg/kg) and midazolam (2.5 mg/kg) and sacrificed by decapitation. MCA were isolated and cannulated on pressure myograph, under flow ( $\Delta$ 80 mm Hg) or no flow conditions. Endothelial NO and superoxide/ROS production (determined by the nonfluorescent 4,5-diaminofluorescein and with dihydroethidine, respectively) were measured by direct fluorescence microscopy. In a separate group of experiments, all surface brain blood vessels (BBVs) were isolated and collected for rtPCR expression measurements of mRNA of Cu/Zn SOD, MnSOD, EC-SOD, GPx1 and GPx4 and catalase (CAT). All experimental procedures were approved by the local Ethical Committee and conformed to the EU Directive 86/609. Data were analyzed using One-Way ANOVA, presented as mean $\pm$ SD. p < 0.05 was considered significant.

**Results:** Basal NO production in no-flow condition was similar among groups. L-NAME blocked the production of NO in each group (basal vs. L-NAME LS:35.4  $\pm$  7.09 vs. 22.8  $\pm$  8.01; HS: 32.4  $\pm$  14.5 vs. 14.2  $\pm$  2.58; HS+ANG II 35.22  $\pm$  4.67 vs. 12.23  $\pm$  3.27). Flow-induced NO production was significantly lower in HS group (21.34  $\pm$  1.37) compared to LS (31.26  $\pm$  7.20) and HS+angiotensin II (40.80  $\pm$  8.22) groups. L-NAME blocked flow-induced NO production similarly among groups (p > 0.05). No-flow superoxide/ROS levels in the BBVs endothelium were not significantly different among the groups (p > 0.05). However, flow increased level of superoxide/ROS in the HS group (9.16  $\pm$  1.52) compared with the other groups (LS 7.30  $\pm$  1.13 and HS+angiotensin II 6.44  $\pm$  1.56).

There was significantly higher expression of only of EC-SOD and GPx4 in HS+angiotensin II (EC-SOD 1.75  $\pm$  0.43, GPx4 2.15  $\pm$

0.41) group compared to LS (EC-SOD  $0.60 \pm 0.20$ , GPx4  $0.82 \pm 0.17$ ) and HS group (EC-SOD  $0.51 \pm 0.11$ , GPx4  $0.64 \pm 0.07$ ).

**Conclusions:** Suppressor doses of angiotensin II restored NO production by decreasing flow-induced superoxide/ROS production in MCAs, due to increased antioxidant capacity of vasculature.

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### Development of an Ex-Vivo Model of Vascular Remodelling

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**Introduction:** Coronary Artery Disease (CAD) continues to have the highest morbidity and mortality rates among Cardiovascular Diseases (CVDs). Treatments include coronary angioplasty, stent insertion and coronary artery bypass grafting (CABG). All these interventions lead to accelerated atherosclerosis following the intervention. While natural, age-dependent atherosclerosis progresses for decades, vascular interventions cause accelerated neointimal hyperplasia and plaque formation that occurs within months to years. With over 16,000 CABG surgeries performed in the UK alone in one year (2015), an ex vivo model recapitulating the complications of vascular interventions and providing a 3D dynamic platform to study the associated mechanisms could provide key solutions to improve the long-term success of vascular interventions. This would help the development of therapeutic solutions to reduce the number of life-threatening complications and the resulting in re-hospitalisation and/or death.

**Aim:** In this project, an ex-vivo model has been developed, mimicking the vascular environment, to study the effects of vascular interventions and gain a better understating of the progression of the pathology.

**Methods:** Porcine carotid arteries were excised and mounted on a 3D printed flow chamber and cultured in controlled conditions (flow rate, dissolved gasses, pH) for up to 7 days. Freshly obtained samples and corresponding cultured tissues were used for downstream analysis to assess the health of the vessel.

**Results:** Histological analysis of the samples at day 0, 1 and 7 showed overall satisfactory cell coverage and viability. However, results indicated reduced endothelialisation as demonstrated by loss of CD31 staining. Concurrently, during culture the vascular smooth muscle layer underwent significant remodelling as shown by reduced smooth muscle actin expression and cellular organisation, extracellular matrix remodelling, and increased expression of synthetic metabolism markers. These results taken together suggest that our culture conditions closely resembles pathologically activated blood vessels and might be exploited to study the mechanisms of vascular complications ex vivo.

**Conclusions:** This work lay the basis for future investigations into the pathological remodelling of blood vessels, by providing a robust and controlled culture system for the maintenance of porcine blood vessels in culture.

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### Human Cardiac Microvascular Endothelial Cell Stiffness and Adhesion to Extracellular Matrix Proteins in Type 2 Diabetes

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**Objective:** Previous data from our laboratory show that T2DM coronary resistance microvessels (CRMs) undergo inward hypertrophic remodeling associated with reduced vessel stiffness and reduced vascular smooth muscle cell stiffness. Therefore, the objective of this study was to test the hypothesis that human cardiac microvascular endothelial cells (hCMVECs) from T2DM patients are less adhesive and less stiff compared to normal hCMVECs.

**Methods:** hCMVECs from normal and T2DM patients were obtained from Lonza and cultured in endothelial cell growth medium containing either low glucose (1 g/L) for normal cells or high glucose (4.5 g/L) for diabetic cells. Experiments were performed at passage 6 (n = 2–4 for all groups). Using an atomic force microscope with probes coated with either fibronectin (FN), laminin (LM), collagen I, or collagen IV adhesion to the various extracellular matrix (ECM) substrates was measured and cellular stiffness was measured. The data were analyzed using mixed-effects ANOVA.

**Results:** Normal hCMVECs were most adhesive to FN and least adhesive to collagen IV (normal FN:  $58.92 \pm 1.79$  vs. normal collagen IV:  $39.13 \pm 1.98$  pN,  $p < 0.0001$ ). The diabetic hCMVECs were most adhesive to collagen I and least adhesive to collagen IV (diabetic collagen I:  $70.91 \pm 2.878$  vs. diabetic collagen IV:  $27.11 \pm 1.71$  pN,  $p < 0.0001$ ). There were no statistical differences in adhesion to FN between normal and diabetic hCMVECs, nor were there any difference in adhesion to LM and collagen I between normal and diabetic hCMVECs. Surprisingly there was a significant decrease in adhesion to collagen IV in diabetic hCMVECs compared to normal VSMCs (normal:  $39.13 \pm 1.98$  vs. diabetic:  $27.11 \pm 1.71$  pN,  $p = 0.003$ ). Elastic modulus, a measurement of cellular stiffness, was not different between normal and diabetic hCMVECs (normal:  $3.29 \pm 0.54$  kPa vs. diabetic:  $2.84 \pm 0.79$  kPa,  $p = 0.64$ ).

**Conclusions:** Overall both normal and diabetic hCMVECs displayed different adhesion force to various ECM components with diabetic cells being significantly less adhesive on collagen IV compared to normal cells on collagen IV, suggesting that differences in integrin binding may be involved. Future studies will investigate this possibility. Interestingly, there was no significant difference in cellular stiffness between normal and diabetic hCMVECs; therefore the endothelial cells do not appear to be responsible for the decreased diabetic coronary microvascular stiffness.

## Aortic Valve Regurgitation Causes a Mild Improvement in Coronary Flow Velocity Reserve in the Type 2 Diabetic db/db Mouse

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Patients with type 2 diabetes mellitus (T2DM) often present with cardiovascular complications, including aortic regurgitation (AR). Our laboratory and others have previously established that T2DM db/db mice and Ossabaw pigs exhibit coronary microvascular disease (CMD), which is accompanied by reduced coronary flow. Together with our recent observation that some db/db mice have mild AR, the goal of the present study was to test the hypothesis that aortic regurgitation in T2DM mice would improve coronary flow since the coronary ostia lie directly next to the aortic leaflet. To examine this, control Db/db and T2DM db/db mice were subjected to high frequency Doppler echocardiography at 24 weeks of age for the measurement of aortic and coronary flow at both 1% (baseline) 3% (hyperemia) isoflurane. Aortic flow was evaluated for regurgitation across the aortic valve using color Doppler in db/db mice with (DR) and without (DN) regurgitation. Coronary flow data were analyzed automatically using our recently published MATLAB algorithm. Data are represented as mean  $\pm$  standard error and a one-way ANOVA was used to determine statistical significance. Analysis of the aortic flow showed that 15 of 29 (52%) presented with AR. Similar to our previous reports, coronary flow velocity reserve (CFVR) was significantly decreased for T2DM mice without AR relative to control animals ( $2.31 \pm 0.22$  vs  $2.91 \pm 0.12$ ;  $p = 0.033$ ; respectively). In contrast, CFVR of diabetic mice with AR were not significantly decreased from the controls ( $2.56 \pm 0.17$  vs  $2.91 \pm 0.12$ ;  $p = \text{NS}$ ; respectively). The Doppler echocardiogram also showed the coronary flow pattern was significantly changed in diabetic mice without AR vs control mice, however aortic regurgitation appears to alleviate some of these observed changes; including hyperemic diastolic slope (control:  $2709 \pm 157$  mm/ms/beat, DN:  $2190 \pm 108$  mm/ms/beat, and DR  $2404 \pm 115$  mm/ms/beat; Control vs DN  $p = 0.044$  and Control vs DR  $p = \text{NS}$ ) and hyperemic decay slope 1 (control:  $-1617 \pm 143$  mm/ms/beat, DN:  $-814 \pm 66$  mm/ms/beat, and DR  $-1206 \pm 120$  mm/ms/beat; Control vs DN  $p = 0.0003$  and Control vs DR  $p = \text{NS}$ ). Taken together, these data suggest that the hemodynamic changes associated with the retrograde aortic flow observed in AR have a direct effect on mildly increasing coronary filling as has been observed in patients. Future studies will focus on investigating the extent of the effect caused by AR on the coronary microcirculation.

## Collagen IV is Required for Regulation of Endothelial Cell Mediated Vascular Function

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**Introduction:** Missense mutations in COL4A1 and COL4A2 (collagen IV alpha chain 1 and 2) that affect protein composition cause familial forms of cerebrovascular disease including cerebral small vessel disease, intracerebral haemorrhage and porencephaly. Interestingly, altered collagen IV levels have also been implicated in genetic forms of cerebrovascular disease and common variants in COL4A1/2, also likely to affect collagen gene expression, are associated with arterial stiffness, coronary artery disease and sporadic intracerebral haemorrhage in the general population. Delineating the molecular mechanisms by which collagen IV causes disease is therefore an important question to address.

**Materials and Methods:** We employed a novel heterozygous Col4a2 knockout mouse model to determine the pathogenicity of reduced collagen IV expression, combined with a mouse model with a missense mutation (Col4a1+/SVC) that closely mirrors its human disease counterpart. Histopathological examination determined intracerebral haemorrhaging, while vascular function was determined using wire-myography in mesenteric resistance vessels. Western blotting and qRT-PCR was employed to investigate molecular pathways.

**Results:** Col4a2+/- animals have a 50% reduction in Col4a2 mRNA levels. Surprisingly, in contrast to Col4a1+/SVC mice, no gross morphological defects in the eye and kidney and no obvious signs of intracerebral haemorrhaging in the brain were detected in 6-month-old Col4a2+/- mice. However, wire-myography revealed substantial changes in endothelial function. Incubation with increasing doses of carbachol identified enhanced endothelium-mediated relaxation. Treatment of vessels with N( $\omega$ )-nitro-L-arginine methyl ester (L-NAME) and TRAM-34 and apamin-sensitive calcium-dependent potassium channels (KCa) uncovered reduced nitric oxide-mediated vasodilation combined with increased Kca channel-mediated relaxation, highlighting an upregulation of endothelium-dependent hyperpolarisation (EDH). In Col4a2+/- mice this was associated with increased expression of KCa3.1. Comparative analysis between Col4a2+/- and Col4a1+/svc uncovered a delayed progression of the phenotype in Col4a2+/- with mesenteric arteries from 6-month-old Col4a2+/- reacting similar to 3-month-old Col4a1+/svc. Furthermore, while Col4a1+/svc develop ER stress and matrix defects, the absence of ER stress in Col4a2+/- indicates that reduced collagen levels in the basement membrane are associated with the vascular dysfunction.

**Conclusion:** Phenotypic and mechanistic analysis revealed that reduced COL4A2 expression results in a much milder phenotype compared to missense mutations, suggesting that the Col4a2+/- model represents a model for multifactorial cerebrovascular disease. Our data also identify an important role for collagen IV and the basement membrane in maintaining vascular and endothelial cell function. Therefore, Col4a2+/- may represent a powerful animal model to delineate milder more common, likely adult onset diseases associated with collagen IV.

## The Effect of Caffeine on Cutaneous Microcirculation in Women

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**Introduction:** Although coffee is one of the most widely enjoyed beverages, the effect of caffeine on the arterioles is not thoroughly understood. In majority of people caffeine causes mental stimulation and raises blood pressure. Blood pressure increase in males is mainly due to the effect of caffeine on systemic vascular resistance. This effect is attenuated by regular coffee drinking. However, acute coffee ingestion may be harmful to some people. There are many differences in activity of the small arteries of males and females. Therefore, the aim of this study is to elucidate some effects of caffeine on the cutaneous microcirculation in females, separately in the follicular and luteal phase of the menstrual cycle.

**Methods:** We measured the electrocardiogram, arterial blood pressure, cutaneous laser-Doppler (LD) flux and skin temperature on index fingers in young, healthy women (N = 19; 21.90 ± 0.24 years; BMI 21.39 ± 0.57 kg/m<sup>2</sup>). The measurements were carried out at rest and during cooling of one hand, before and after ingestion of 200 mg caffeine. Measurements in each female were performed twice – once in the follicular and once in the luteal phase of the menstrual cycle. The study was approved by the National Medical Ethics Committee; written informed consent was obtained from each subject.

**Results:** After ingestion of caffeine blood pressure significantly increased. In the follicular phase systolic blood pressure increased from 116.94 ± 3.43 to 132.26 ± 2.60 mm Hg and diastolic blood pressure from 84.64 ± 2.02 to 95.84 ± 1.64 mm Hg (p < 0.05); in the luteal phase systolic blood pressure increased from 115.09 ± 2.67 to 128.64 ± 3.22 mm Hg and diastolic blood pressure from 81.54 ± 2.11 to 92.31 ± 2.23 mm Hg (p < 0.05). LD flux significantly decreased after caffeine ingestion: in the follicular phase from 228.86 ± 22.95 to 136.44 ± 16.63 PU and in the luteal phase from 208.86 ± 32.35 to 131.70 ± 24.58 PU (p < 0.05). The RR interval in all measurements during rest significantly increased after ingestion of caffeine. The increase was from 895.55 ± 33.6 to 971.07 ± 35.6 in the follicular and from 891.01 ± 32.11 to 942.01 ± 32.52 in the luteal phase. The skin temperature after caffeine ingestion was significantly lower. During local cooling of the hand the drop of LD flux was significantly greater after administration of caffeine (p < 0.05).

**Conclusion:** Besides increased RR interval, systolic and diastolic blood pressure we observed a vasoconstrictive effect of caffeine during rest at the level of microcirculation in young, healthy women in both follicular and luteal phase of the menstrual cycle. During local cooling there were augmented LD flux and temperature decrease after ingestion of caffeine.

## Functional High-Throughput Screening Identifies a Role for microRNA-26b in the Pro-Survival of Endothelial Cells and in Vessel Calcification

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**Introduction:** MicroRNAs (miRNAs) are small non-coding RNAs that orchestrate genetic networks by modulating simultaneous gene expression and regulates vascular function. Hence, miRNAs are extremely attractive targets for therapeutic regulation of angiogenesis in human disease. Despite recent accumulation of experimental data and the emergence of functional models, the complexity of miRNA-based regulation is still far from being well understood. In particular, regarding the basic function of ECs our knowledge of which individual miRNAs are at play is still insufficient. Thus, we set to identify miRNAs that are able to influence vascular endothelial cell (ECs) function, by performing high-throughput phenotypic screening. miR-26b emerged from the screen as a top candidate for ECs proliferation.

**Objective:** The aim of this study is to characterize how miR-26b can influence vascular function and identify the subsequent gene targets and molecular pathways involved.

**Methods:** In this study, we have used HCS, in-vivo miR-26b delivery and miR-26b knock-out mice to prove our hypothesis

**Results:** The overexpression of miR-26b significantly increased EC proliferation, migration and survival. Furthermore, miR-26b mimics increased EC tube formation and branching morphogenesis. HCS identified both Phosphatase and Tensin homolog (PTEN), a gene critical in cell survival and Bone Morphogenetic Protein Receptor Type 1B (BMPRI1B; ALK6), important in calcium homeostasis, as direct targets of miR-26b. Under ischemic conditions in-vivo, miR-26b levels were reduced and overexpressing miR-26b after ischemic injury increased the survival capacity of ECs through PTEN regulation. Confirming a link between miR-26b, PTEN and the increased cell survival associated with miR-26b in ECs. To investigate the clinical relevance of miR-26b, we analysed diseased vessel and muscles from below knee amputee patients. The levels of miR-26b was decreased in the muscle biopsy from the less perfused section of the amputated leg. Moreover, miR-26b was downregulated in the calcified tissue portion of the diseased vessels in comparison to the soft tissue. MiR-26b knock-out mice also demonstrated an increased susceptibility to calcification when challenged with warfarin. In-vitro and in-vivo studies have confirmed that up-regulation of BMPRI1B by miRNA-26b inhibition affects vessel calcification.

**Conclusion:** Taken together these findings demonstrate an important role for miR-26b in both vessel survival and calcification.

### Short Term Blockade of Angiotensin II Type 1 Receptor by Losartan Effects on Blood Leukocyte Subpopulations' ROS and RNS Levels in Sprague-Dawley Rats

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**Introduction:** Endogenously produced reactive oxygen (ROS) and nitrogen (RNS) species in leukocytes primarily act as signaling molecules with the capacity to alter their functional status towards either activation or apoptosis. For example, perturbation of ROS/RNS and antioxidants equilibrium can result in T-cell hyper- or hyporesponsiveness, leading to the development of various pathologies.

**Objective:** To determine the level of intracellular ROS and RNS production in various blood leukocyte subpopulations and antioxidant enzymes' gene expression in cerebral blood vessels (CBV) from Sprague-Dawley (SD) rats following short term blockade of angiotensin II type 1 receptor by losartan.

**Methods:** 10 weeks old, healthy male SD rats were fed low salt diet for 7 days (0.4%NaCl; LS group) or LS diet+losartan (40 mg/day in water ad libidum). Following dietary protocol, rats were anesthetized with ketamine (75 mg/kg) and midazolam (2.5 mg/kg) and sacrificed by decapitation for immediate blood sampling and total surface CBV collection. The intracellular hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and peroxynitrite (ONOO<sup>-</sup>) production or superoxide production were determined by flow cytometry with dichlorofluorescein diacetate (DCF-DA) or dihydroethidine (DHE), respectively. mRNA of SOD isoforms (Cu/ZnSOD, MnSOD, ecSOD), glutathione peroxidase 1 and 4 (GPx1,4) and catalase (CAT) was performed by quantitative rtPCR in CBV. Results are presented as mean ± SD (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 Ethical Committee.

**Results:** There were no changes in DCF-DA expression in all leukocyte subpopulations except reduced DCF-DA expression in peripheral blood lymphocytes in LS+losartan group compared to LS group (124.9 ± 21.7 vs. 76.7 ± 9.81; p = 0.002). In CBV, mRNA levels of SOD1 (1.09 ± 0.18 vs. 0.734 ± 0.20) and GPx1 (1.24 ± 0.38 vs. 0.63 ± 0.25) genes were significantly lower in LS+Losartan group compared to LS group, while CAT (0.87 ± 0.11 vs. 4.89 ± 1.55), SOD2 (0.97 ± 0.21 vs. 2.53 ± 0.66) and SOD3 (0.61 ± 0.20 vs. 1.22 ± 0.34) genes were significantly upregulated in LS+Losartan group, and the GPx4 gene expression remained unchanged.

**Conclusion:** Blockade of angiotensin II type 1 receptors by losartan resulted in alteration of ROS/RNS levels in lymphocytes but no other peripheral blood leukocytes subpopulations. Additionally, antioxidant genes' expression profile was altered in in CBV. The functional and signalling effect of these changes in peripheral leukocytes and their fine interplay with the endothelium are yet to be elucidated. Grants: This work has been supported by Croatian Science Foundation under the project #IP-2014-09-6380.

### Angiotensin II-Type 1 Receptor Blockade with Losartan Affects the Expression of Enzymes Involved in Vascular Reactivity and Balance of Vascular Oxidative Stress of Sprague-Dawley Rats

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**Introduction:** It is well known that RAS (Renin-angiotensin system) mediates its signal transduction and functions via ANGII and ANGII receptors interaction and suppression of ANG II levels with high salt diet leads to increased vascular oxidative stress. Our recent study in healthy rats showed that losartan (angiotensin II type 1 (AT-1R) receptor antagonist) impaired vasodilator response of middle cerebral arteries, increased vascular and systemic oxidative stress, decreased activity of antioxidative enzymes and increased antioxidative enzymes gene expression in vessels. Present study evaluated the effects of losartan on protein expression of AT1 and AT2 (angiotensin II type 1 and 2) receptors, enzymes involved in the mechanisms of vascular reactivity (iNOS, COX-1, COX-2), antioxidative enzymes (SOD-1, SOD-2, SOD-3, GPx4, CAT) and NOX1.

**Methods:** Healthy male Sprague-Dawley 9–11 weeks old rats (n = 36) were divided to a control (LS, n = 18) and LS+Losartan group (n = 18), both fed with standard rat chow with 0, 4% NaCl. Rats from LS+Losartan group received 40 mg of Losartan (daily dose) in drinking water for 7 days. Following dietary protocol, rats were anesthetized with ketamine (75 mg/kg) and midazolam (2.5 mg/kg) and sacrificed by decapitation. Protein expressions were determined by Western blot in surface cerebral vessels. Due to small amount of tissue, brain blood vessels from two animals were pooled for one sample. Images were processed and analyzed with ImageJ software, data were analyzed by t-test and presented as mean±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:** Protein expression of GPx4, AT1 and iNOS was significantly decreased in LS+losartan group (GPx4 0.48 ± 0.34, AT1 0.58 ± 0.13, iNOS 0.84 ± 0.20) vs. LS group (GPx4 1.03 ± 0.46, AT1 1.44 ± 0.43, iNOS 1.18 ± 0.23). Expression of COX-2 and NOX1 was significantly increased in LS+losartan group (COX-2 2.99 ± 0.79, NOX1 1.14 ± 0.19) compared to LS (COX-2 1.92 ± 0.96, NOX1 0.92 ± 0.17).

**Conclusions:** This data confirmed our previous observations of increased oxidative stress (possible due the increased NOX1 expression and activity) and impaired vascular reactivity (decreased iNOS expression).

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### Cardiotrophin-1 Deficiency Abrogates Atherosclerosis Progression

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**Introduction:** Atherosclerosis is an inflammatory disease characterized by the build-up of subendothelial deposition of cholesterol and the formation of leukocyte-rich plaques in the intimal layer of arteries. Cardiotrophin-1 (CT-1) is a member of the interleukin (IL)-6 family of cytokines. CT-1 has been shown to stimulate inflammatory and proatherogenic molecules expression and accelerate the development of atherosclerotic lesions. To further investigate the role of CT-1 in the development of atherosclerosis, we have generated a double knockout mouse Apoe<sup>-/-</sup>CT-1<sup>-/-</sup>.

**Methods:** 11-week old Apoe<sup>-/-</sup> C57Bl/6 or Apoe<sup>-/-</sup>CT1<sup>-/-</sup> mice were fed a normal chow diet for 16 weeks (earlier atherosclerosis) or a high-cholesterol diet (advanced atherosclerosis) for 11 weeks. After sacrifice, serum triglycerides, total cholesterol, low-density lipoprotein cholesterol (LDL-C), free fatty acids have been measured. Aortic sections were stained with Oil Red O and the lipid content was quantified. Single-cell suspensions were obtained from the spleen and lymph nodes for flow cytometry analysis of immune cells populations.

**Results:** Apoe<sup>-/-</sup>CT1<sup>-/-</sup> mice fed a high-cholesterol diet (HCD) showed 1.6-fold ( $p < 0.01$ ) increase in the percentage of Treg cells in the lymph nodes and 6.8-fold ( $p < 0.0001$ ) higher percentage of Bregs cells in the spleen in comparison to Apoe<sup>-/-</sup> mice on HCD. In comparison to Apoe<sup>-/-</sup> mice on HCD, Apoe<sup>-/-</sup>CT1<sup>-/-</sup> mice on HCD exhibited a significant reduction of Bregs-produced TGF-beta of 7.8-fold ( $p < 0.0001$ ) and of 8.7-fold ( $p < 0.0001$ ) in the lymph nodes and in the spleen, respectively. In advanced atherosclerosis, Apoe<sup>-/-</sup>CT1<sup>-/-</sup> mice show an 8.7-fold increase ( $p < 0.001$ ) in the percentage of atheroprotective B1a cells in the spleen versus Apoe<sup>-/-</sup> mice. In addition to the immunomodulatory effect of CT-1 deficiency in advanced atherosclerosis, we observed a 1.6-fold ( $p < 0.0001$ ) and 1.7-fold ( $p < 0.0001$ ) reduction of total cholesterol and LDL-Cholesterol respectively, in Apoe<sup>-/-</sup>CT1<sup>-/-</sup> mice versus Apoe<sup>-/-</sup> mice on HCD. Finally, the LDL-C calculated by Friedewald formula is diminished by 1.6-fold ( $p < 0.0001$ ) in Apoe<sup>-/-</sup>CT1<sup>-/-</sup> mice in comparison to Apoe<sup>-/-</sup> mice on HCD. In advance atherosclerosis, all these effects resulted in a reduction of 1.2-fold ( $p < 0.0001$ ) in the lesion area percentage in the aortic root and 3.2-fold ( $p < 0.0001$ ) in the abdominal aorta in Apoe<sup>-/-</sup>CT1<sup>-/-</sup> mice versus Apoe<sup>-/-</sup> mice.

**Conclusion:** CT-1 appears as a key cytokine controlling atherosclerosis development as demonstrated by smaller lesion size and reduction of atherosclerosis progression. CT-1 deficiency in Apoe<sup>-/-</sup> mice during advanced atherosclerosis suggests anti-inflammatory and atheroprotective effects as well as an ability to reduce the levels of cholesterol.

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### Endothelial Function in the Human Cutaneous Microcirculation in Primary Open Angle Glaucoma

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**Objective:** The vascular dysregulation in the optic nerve head in primary open-angle glaucoma (POAG) may be a consequence of vascular endothelial dysfunction. Although endothelial dysfunction has been shown in the peripheral macro-circulation in POAG, investigation of the endothelial function of the peripheral microcirculation has been limited. The aim of this study is to determine the responses of the skin microcirculation to iontophoresis of endothelial-dependent (acetylcholine-ACh) and endothelial-independent (sodium nitroprusside-SNP) vasodilating agents using Laser Doppler Flowmetry in patients with POAG and controls.

**Methods:** We studied 17 POAG patients (48–84 years), and 23 control subjects (40–68 years). The vasodilator responses to iontophoresis of ACh and SNP performed at the finger and forearm skin were determined, with skin microcirculatory blood flow being expressed as cutaneous red cell flux, (RCF) as measured by Laser Doppler Flowmetry.

**Results:** Baseline RCF levels between POAG patients and controls were not different at the finger and forearm skin sites, ( $p > 0.05$ ). ACh and SNP induced significant increases in RCF from baseline ( $p < 0.001$ ) at the finger and forearm skin in POAG patients and controls, but there was no difference in vasodilation between the subject groups. Within POAG patients for both ACh and SNP, the baseline RCF (mean  $\pm$  SD) was higher in the finger  $16.7 \pm 15.7$  PU, and  $22.4 \pm 11.9$  PU, respectively, than in forearm skin  $7.1 \pm 4.0$  PU and  $7.1 \pm 2.5$  PU, respectively ( $p < 0.05$ ). Similarly, in controls, for both ACh and SNP, the baseline RCF was higher in the finger  $18.8 \pm 15.5$  PU and  $19.1 \pm 8.6$  PU, respectively, than in forearm skin  $6.8 \pm 2.9$  PU and  $7.8 \pm 5.0$  PU, respectively ( $p < 0.05$ ). Additionally, the vasodilation to ACh in the finger was higher than in forearm skin in POAG (mean RCF:  $103.4 \pm 49.4$

versus  $70.3 \pm 25.6$ ) and in controls (mean RCF:  $101.8 \pm 53.3$  versus  $69.7 \pm 34.3$ ). For SNP, vasodilation in the finger was also higher than in forearm skin in POAG (mean RCF:  $85.6 \pm 51.9$  versus  $53.2 \pm 28.6$ ) and controls (mean RCF:  $82.4 \pm 35.3$  versus  $49.2 \pm 16.9$ ); ( $p < 0.05$ ) for all differences.

**Conclusions:** The vasodilatory responses to acetylcholine in this sample of POAG patients suggests normal microvascular endothelial function. The differences in baseline RCF and vasodilatory responses between the finger and forearm skin sites may reflect the difference in vascularity between these sites.

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### Cigarette Smoke Impairs the Anti-Oxidative Response by Reducing Heme Levels and Inducing BACH1

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**Introduction:** Cigarette smoke is a major risk factor of cardiovascular diseases. Heme regulates the activity of enzymes and transcription factors including Bach1. Bach1 competes with Nrf2 transcriptional activity and inhibits the expression of anti-oxidative enzymes. However, the molecular mechanism of Bach1 activation and its impact on the anti-oxidative response after exposure to cigarette smoke is not clear.

**Objective:** We aim to understand the mechanism through which cigarette smoke impairs the anti-oxidative response.

**Methods:** Primary human umbilical vein endothelial cells (HUVEC) were exposed to a cigarette smoke extract (CSE) (3 mg/ml nicotine) under static and unidirectional shear stress conditions. Shear stress was induced using the ibidi pump system and cone-and-plate viscometer. Levels of reduced glutathione (GSH) were measured using the fluorescent probe o-phthalaldehyde. Heme levels were measured after oxalic acid extraction. Gene and miR expression was quantified by RT-qPCR. Immunofluorescence imaging was performed on fixed HUVEC. MiR-125b overexpression was achieved by transfecting mimics. Bach1 was inhibited using siRNA transfection. Apoptosis was assessed by caspase 3/7 assay. Human umbilical veins as an ex vivo model were exposed to CSE under static conditions.

**Results:** Exposure to CSE induced the expression of BACH1 ( $1.90 \pm 0.18$ ;  $p < 0.05$ ) and reduced total heme levels ( $0.30 \pm 0.04$ ;  $p < 0.001$ ) in HUVEC. It also reduced miR-125b expression in HUVEC ( $0.39 \pm 0.09$ ;  $p < 0.001$ ) and umbilical veins ( $0.72 \pm 0.12$ ;  $p < 0.05$ ), whereas the expression of miR-125b target AhR repressor (AHRR) increased ( $14.93 \pm 4.08$ ;  $p < 0.05$ ). Inhibiting BACH1 rescued miR-125b expression ( $1.02 \pm 0.45$ ;  $p > 0.05$ ) and reduced AHRR expression ( $0.66 \pm 0.12$ ;  $P < 0.05$ ). Acute exposure to CSE led to a high oxidative stress load. This observation was supported by a 2-fold reduction in glutathione levels, an increase in nuclear:cytosolic ratio of the upstream transcription factor Nrf2 ( $1.33 \pm 0.05$ ;  $p < 0.01$ ) and an increase in expression of anti-oxidative HMOX1 ( $18.18 \pm 1.8$ ;  $p < 0.001$ ) and NQO1 ( $16.12 \pm 1.99$ ;  $p < 0.001$ ). CSE-induced apoptosis ( $1.23 \pm 0.04$ ;  $p < 0.001$ ) was reversible by pre-conditioning with 1 mM N-acetyl cysteine

( $0.99 \pm 0.02$ ;  $p > 0.05$ ) or overexpression of miR-125b ( $0.99 \pm 0.07$ ;  $p > 0.05$ ).

**Conclusions:** These results show that cigarette smoke-induced Bach1 impairs the anti-oxidative response through inhibiting miR-125b in the human endothelium. Reduced heme levels could be involved in the upregulation of Bach1 activity.

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### uPARAP/Endo180 Receptor, a Gatekeeper of Lymphatic Branching and Organization During Pathological Lymphangiogenesis

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The development of new lymphatic vessels occurring in pathological conditions is mainly known to be driven by vascular endothelial growth factor-C (VEGF-C) via its lymphatic endothelial receptors VEGFR-2 and VEGFR-3. Nevertheless, our understanding of the molecular mechanisms controlling lymphangiogenesis and lymphatic network formation is still in its infancy. Here, we identified uPARAP (urokinase plasminogen activator receptor-associated protein), an endocytic receptor involved in cell migration and collagen remodelling whose implication in vascular biology has never been reported, as a novel regulator of pathological lymphangiogenesis. Genetic ablation of uPARAP in adult mice had no effect on lymphatic vasculature in homeostatic conditions, but resulted in hypersprouting and hyperbranched lymphatic vessels in inflammatory corneal lymphangiogenesis and tumor xenografts. Despite this abnormal organization, the vessels were fully functional and this hyperbranched phenotype was even beneficial in a lymphedema model, leading to a reduced edema volume in the affected limb. After analyzing the effect of several growth factors on this phenotypic switch, we determined that uPARAP controlled specifically the VEGF-C-induced lymphangiogenesis. As uPARAP deletion did not affect the lymphangiogenesis induced by VEGF-C156S (a mutated form of VEGF-C which only binds VEGFR-3/R-3 homodimers), uPARAP requires the formation of VEGFR-2/R-3 heterodimers to trigger its effects. Moreover, the hyperbranched phenotype observed in uPARAP<sup>-/-</sup> mice was restored by inhibiting Rac1. In summary, in addition to identifying an unexpected molecular regulator of lymphangiogenesis, our study has uncovered a novel signaling pathway, implicating at least VEGFR-2/R-3 heterodimers and Rac1 and controlling the proper branching of lymphatic network during VEGF-C-driven pathological lymphangiogenesis.

### ADAM10-Mediated Cleavage of ICAM-1 is Required for Neutrophil Dissociation from the Endothelial Surface During the Final Diapedesis Step

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To efficiently cross the endothelial barrier during inflammation, neutrophils first firmly adhere to the endothelial surface. The endothelial adhesion molecule ICAM-1 plays a crucial role in this step. To continue to the final diapedesis step, neutrophils need to be released from ICAM-1. While Integrin LFA1/Mac1 de-activation is one step that leads to this, direct cleavage of ICAM-1 from the endothelium may represent a second option. We found that A Disintegrin And Metalloprotease 10 (ADAM10) cleaves the extracellular domain of ICAM-1 from the endothelial surface. Silencing or inhibiting endothelial ADAM10 increased the surface expression of ICAM-1 and promoted the number of adherent neutrophils. Moreover, the migration distance and velocity of the neutrophils on the endothelial surface was increased. Despite increased number of adherent neutrophils, silencing of endothelial ADAM10 impaired the efficiency of neutrophils to cross the endothelium under flow conditions, suggesting that neutrophils use endothelial ADAM10 to dissociate from ICAM-1. Indeed, when measuring transmigration kinetics, neutrophils took almost twice as much time to finish the diapedesis step when ADAM10 is silenced. Importantly, we found neutrophils that had crossed the control, but not ADAM10-deficient, endothelium, to be positive for the extracellular domain of ICAM-1 derived from the endothelium. Based on these findings, we conclude that endothelial ADAM10 plays an active part in neutrophil transendothelial migration by cleaving ICAM-1, thereby regulating the release of neutrophils from the endothelium during the final diapedesis step.

### Atherosclerotic Plaque Macrophages Re-Programming Towards an Anti-Atherogenic Phenotype

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**Background and Aims:** Atherosclerosis is a chronic inflammatory disease with severe clinical outcome such as stroke or myocardial infarct. Macrophages are one of the critical drivers of atherosclerotic plaque inflammation which in early disease stages may be beneficial. At later stages, they rather act detrimental by destabilizing the plaque. The latter effects could potentially be halted by re-instructing plaque macrophages towards an anti-inflammatory and inflammation-resolving phenotype. Therefore, we aim to identify key genes in atherosclerosis that skew macrophages from

pro- towards an anti-inflammatory as well as an anti-atherogenic phenotype.

**Methods:** Microarray data from stable plaque segments obtained from patients after carotid endarterectomy were analyzed using weighted gene co-expression analysis (WGCNA). Modules were correlated to plaque characteristics and in particular anti-inflammatory Arg1+CD68+ macrophage content. To narrow down the number of genes of interest, module members were ranked based on 1. correlation to the Arg1+CD68+ staining, 2. centrality in the module and 3. relevance to macrophages. The top 20 genes were analyzed for specificity of expression in major plaque cell types.

**Results:** WGCNA generated a network encompassing 58 gene modules. Two modules strongly correlated with Arg1+CD68+ macrophage (p value = 0.05; 0.003) presence, and interestingly inversely correlating with pro-inflammatory iNOS+CD68+ macrophages. Gene ontology analysis showed module enrichment in fatty acid and glucose metabolism genes. The majority of candidates were selectively expressed by macrophages over endothelial and smooth muscle cells. Silencing of STAT5B and to a lesser extent SNTB2, HNMT, RABGGTB and ARHGEF7 expression in primary human macrophages caused significant changes in the expression of the other candidates, indicating a central role of STAT5B in the co-expression network. Finally, the presence of phosphorylated STAT5 in GM-CSF-stimulated macrophages underpinned the candidate's relevancy for macrophage phenotype.

**Conclusions:** Our data suggest that STAT5 might act as a hitherto unknown key regulator of plaque macrophage phenotype.

### Essential Role for Plasma Membrane Calcium ATPase 1 in Angiogenesis

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Recently, plasma membrane calcium ATPase 4 (PMCA) has been established as a novel mediator of angiogenesis (Baggott et al. (2014). *Arterioscler Thromb Vasc Biol* 34:2310–20). In addition to PMCA4, both PMCA1 and PMCA2 are expressed in endothelial cells but their contribution to angiogenesis remains unknown. Therefore, we aim to establish whether PMCA1 modulates the formation of new blood vessels by altering endothelial cell behaviours.

Transient knockdown of PMCA1 was achieved in human umbilical vein endothelial cells (HUVECs) using siRNA. HUVEC viability, proliferation and rate of apoptosis was assessed and live cell imaging performed to evaluate migration of cells. Tubule formation was evaluated using the Matrigel assay and fluorescent activated cell sorting was used to determine cell-cycle distribution. Loss of PMCA1 significantly reduced HUVEC viability without a concomitant increase in apoptosis or reduction in proliferation; however, these cells had a higher percentage of cells in S-phase with fewer in G2/M-phase compared to controls. Additionally, loss of PMCA1 significantly reduced HUVEC migration and tubule formation compared to scrambled siRNA controls. Molecu-

larly, transient knockdown of PMCA1 lead to a significant increase in basal levels of intracellular calcium and following vascular endothelial growth factor (VEGF) stimulation, an increase in expression of the pro-angiogenic gene regulator of calcineurin 1.4 (RCAN1.4).

Given that transient knockdown of PMCA1 has adverse effects on HUVEC viability, migration and tubule formation, suggesting that PMCA1 is essential for in vitro angiogenesis, it is important to question whether loss of PMCA1 from endothelial cells has a detrimental impact on angiogenesis in vivo. To begin to address this, a novel Tie2CreTg/PMCA1fl/fl mouse line was generated. These mice, which are viable and display no overt phenotype under physiological conditions, were subject to transverse aortic constriction (TAC). TAC was performed under general anaesthetic (2% isoflurane, v/v) and mice were monitored for adverse effects 1 week post-TAC, followed by functional, biochemical and histological analyses. Following 1 week TAC, both Tie2CreTg/PMCA1fl/fl and wild type littermate controls show an increased heart weight/tibia length ratio and display significantly reduced fractional shortening and ejection fraction. Histological analysis shows there is an increase in cardiomyocyte cell size area following TAC, but this increase is only significant in wildtype littermates.

Overall, loss of PMCA1 impairs in vitro angiogenesis suggesting PMCA1 is required for efficient vessel formation. However in contrast to the established role of PMCA4 in angiogenesis, the mechanism by which PMCA1 may mediate angiogenesis appears to be RCAN1.4-independent.

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### **Nutrient-Overload Induces Leptin Production by Skeletal Muscle Pericytes: Implications for Capillary Remodelling**

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The adipokine leptin enhances key functions within skeletal muscle such as the metabolism of glucose and lipids. Recently, we reported that leptin receptor mutant (db/db) mice exhibit reduced skeletal muscle capillarity and decreased levels of vascular endothelial growth factor (VEGF)-A protein, leading us to propose that leptin is a physiological regulator of capillary network in skeletal muscle. Furthermore, we detected leptin transcripts within skeletal muscle indicating the presence of leptin producing cells. The purpose of this study was to 1) investigate a potential role for leptin as a regulator of VEGFA production and 2) define the cellular source and regulation of leptin synthesis within skeletal muscle. In support of a role of leptin in maintaining the capillary network in skeletal muscle, leptin stimulated the production of VEGFA in cultured skeletal myocytes. This response was abrogated by pre-treatment with PI3K and JAK inhibitors. Leptin positive staining colocalized with perivascular platelet derived growth factor (PDGFR)- $\beta$ + cells in murine skeletal muscle. In isolated PDGFR $\beta$ + cells, leptin transcripts were detected and increased in mice fed a

high fat diet, coinciding with increased mRNA levels of the pre-adipocyte commitment factor zfp423. Notably, PDGFR $\beta$ + cells co-expressed the gene encoding NG2 (Cspg4), indicating that pericytes are a source of leptin within skeletal muscle. To investigate the production of leptin within human skeletal muscle, we first isolated stromal cells and confirmed the absence of myogenic or endothelial cells by immunofluorescence. In these cells, leptin transcripts were detected at relatively low levels, but increased with both palmitate (+362%) and insulin (+378%) treatments ( $P < 0.05$ ), consistent with the regulation of local leptin production by nutrient-overload. In contrast, neither treatment altered levels of adipogenic regulators C/EBP $\alpha$  and PPAR $\gamma$ . By flow cytometry analysis, PDGFR $\beta$ + cells comprised ~52% of the stromal cell population and ~68% of PDGFR $\beta$ + cells were adipogenic, based on co-expression of PDGFR $\alpha$  (a marker for adipogenic precursor cells). Adipogenic (PDGFR $\alpha$ -high) and non-adipogenic (PDGFR $\alpha$ -low) PDGFR $\beta$ + cells (Pericytes) were separated by fluorescence activated cell sorting and will enable further assessments of basal and nutrient-inducible levels of leptin within these cells. These data demonstrate a plausible role for pericytes as energy-sensing cells within skeletal muscle, through their production of leptin in response to nutrient excess. In turn, leptin stimulates myocyte VEGFA production, increasing muscle capillarity.

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### **Lactate Oxidation in Endothelial Cells: A Feature of All Endothelial Cells?**

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Metabolism of endothelial cells is a topic that has gained an increasing interest in the last years. This is due to their role in the angiogenic process, which is pathologically upregulated in several diseases, such as retinopathies, diabetes and cancer. Glycolysis, among other metabolic routes, has been found to be essential for triggering the angiogenic switch. Additionally, it has been seen that endothelial cells are able to take up lactate from the extracellular media, for example in the case of the tumor microenvironment, where cancer cells would have secreted high amounts of this metabolite. Endothelial cells would oxidize this lactate for obtaining energy, but lactate can also act as a signaling molecule for the angiogenic process. However, experiments to determine the molecular fate of lactate have been performed using only macrovascular endothelial cells. The aim of the present work is to prove whether microvascular endothelial cells are also able to take up and oxidize lactate. For this purpose, fluorimetry, isotopic labeling and Seahorse experiments were used to study the metabolism of a human microvascular endothelial cell line (HMEC). The expression levels of transcripts and proteins of different enzymes and transporters related to lactate metabolism were estimated by qPCR and Western blotting. The results obtained indicate that these cells rely on glycolysis for their metabolism, while the oxidation of glucose

and glutamine seems to be considerably low. On the other hand, no lactate oxidation could be detected. We then checked the mRNA expression of the two isoenzymes of lactate dehydrogenase (LDH) and the two main lactate transporters, MCT1 and MCT4, and found that levels of LDH-B and MCT1 were undetectable. We failed to measure any MCT1 mRNA or protein expression either in normoxia or hypoxia. Hence, we can conclude that at least this microvascular endothelial cell line cannot use extracellular lactate as a metabolic fuel.

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### Characterization of a Novel CADASIL-Like Mutation in the NOTCH3 Receptor

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Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is the most common familial cerebral small vessel disease. To date, the prevalence is estimated to affect approximately 5–15/100 000 individuals, which is most likely an underestimation based on analysis of data from large-scale genomic analyses. CADASIL patients suffer increasingly severe strokes and altered cerebral blood supply, resulting in cognitive deficits. The condition is linked to missense mutations in the NOTCH3 gene and exhibits accumulation of the misfolded extracellular domain of NOTCH3. Misfolded Notch3 is thought to recruit proteins from surrounding tissue, leading to disruption of vascular function. While the conventional cysteine-altering mutations give rise to CADASIL, data from Rutten and co-workers, point to the possibility that there may also exist “lighter” forms of CADASIL, with symptomatic but preclinical individuals carrying mutations. It is also of note that some cysteine-sparing mutations have been proposed to result in CADASIL, although not all of these have been fully molecularly analysed. The A1604T mutation is however different because it is in a non-canonical cysteine-rich region, but it was founded in patients that have some CADASIL-like features. This raises the interesting possibility that non-conventional NOTCH3 mutations may give rise to a somewhat distinct disease spectrum.

As proposed by Joutel and co-workers, the increased amount of protein in cells is due to an impairment in protein clearance rather than an elevated production of NOTCH3. We investigated the A1604T mutation on a molecular level by analysing the cellular expression and the ability to respond to canonical Notch activation or inhibition compared to the wild type NOTCH3 receptor. Lysosomal degradation has been proven to be the principal route of degradation of NOTCH3 receptor. To investigate whether the A1604T mutation affects Notch3 degradation route, we also analysed the intracellular localization of the A1604T mutation compared to the wild-type. Preliminary results show that the mutated form of NOTCH3 accumulates in the late endosomes as opposed to the wild-type NOTCH3 that accumulates in the lysosomes.

In sum, the A1604T mutation in the NOTCH3 receptor seems to hinder the endosomal-lysosomal trafficking of NOTCH3 receptor.

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### 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 gene expression. The mechanisms governing the recruitment of these complexes 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 Kcnq1ot12. Our group previously identified the lncRNA MANTIS as a crucial component of the endothelial SWI/SNF complex. MANTIS stabilised the interaction between the core ATP-ase, BRG1, and BRG1-associated factor 155 (BAF155) and thereby 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 treatment against BRG1 clearly confirmed 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 have so far identified lncRNAs including EPHA1-AS1, CACNA1G-AS1, MALAT1 and NEAT1 that may have implications in the endothelium. Importantly, there was a higher degree of lncRNA profile overlap between the SWI/SNF complex members BAF170, BRM and BRG1 than with the PRC2 member, EZH2.

Initial screens with an siRNA library have highlighted those lncRNAs which may have functional relevance within the endothelium. 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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### Metabolic Control of Vascular Function: The Role of Gastrointestinal Hormones

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**Background and Rationale:** The global rise in obesity and type 2 diabetes mellitus (T2D) is associated with increased cardiovascular mortality. The dysfunction of endothelial cells is a key player in cardiovascular and metabolic disease. However, the molecular mechanisms that disrupt endothelial homeostasis remain unclear. Recent evidence indicates that circulating bile acids (BA) may act as crucial signalling molecules controlling lipid and glucose metabolism in a variety of tissues and cells, including the endothelium. BA activate the G-protein coupled membrane receptor TGR5 and the nuclear farnesoid X receptor (FXR), as well as other receptors and intracellular signalling pathways. Whether TGR5 and FXR are involved in endothelial cell metabolism and function is poorly investigated. BA can, however, act as endogenous vasodilators, suggesting that they may function as cardiovascular endocrine regulators. A portion of BA in the circulation is carried by high density lipoprotein (HDL). Hypothesis. Thus, HDL may be a crucial shuttle, delivering BA directly to endothelial cells and, in turn, BA may contribute to HDL's endothelial-protective effects.

**Results and Conclusions:** Our study conducted in diet induced obese mice and in obese patients suggests that TGR5 and FXR mediated mechanisms underline the improvement of obesity-associated endothelial dysfunction after bariatric surgery, likely through the action of GLP-1 and insulin. Moreover, we observe a post-operative remodeling of BA content of HDL in patients with increase of BA species which are either agonists of the FXR, e.g. chenodeoxy-CA (CDCA), cholic acid (CA) or for the membrane TGR5 receptor, e.g. deoxy-CA (DCA). The composition-function analysis revealed that among all BA subclasses, the specific enrichment in CA and in CDCA bound to HDL correlated with an improved endothelial anti-apoptotic capacity of HDL ( $R = -0.52$ ,  $p = 0.006$  for CA-HDL and  $R = -0.35$ ,  $p = 0.07$  for CDCA-HDL). Further, the exogenous loading of CA onto healthy native HDL isolated from human serum significantly enhanced their endothelial anti-apoptotic function. In the case of obese, dysfunctional, pro-apoptotic HDL, exogenous CA loading was able to restore HDL anti-apoptotic function thus suggesting a crucial interaction between BA and the endothelium for the improvement of HDL's endothelial-protective properties. The molecular mechanisms are under further investigation.

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### Integrated Assessment of Cerebral Blood Flow Regulation in Humans: Dynamic Cerebral Autoregulation, CO<sub>2</sub> Reactivity and Neurovascular Coupling

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**Introduction:** Neural stimulation increases cerebral blood flow (CBF), due to the mechanism of neurovascular coupling (NVC), but simultaneous changes in arterial blood pressure (BP) and arterial carbon dioxide (PaCO<sub>2</sub>) also occur, stimulating vasomotor activity due to the mechanisms of dynamic cerebral autoregulation (dCA) and CO<sub>2</sub> reactivity (CVR). This presentation describes approaches to obtain simultaneous estimates of dCA, CVR and NVC, from a single recording during cognitive or sensorimotor tasks.

**Methods:** Healthy subjects and patients with acute ischaemic stroke (AIS) have been studied following neural stimulation with cognitive paradigms or repetitive elbow flexion, at a frequency of 1 Hz. CBF velocity (CBFV) was recorded with transcranial Doppler in both middle cerebral arteries, continuous non-invasive BP was recorded with the Finapres device, and end-tidal CO<sub>2</sub> was measured by capnography. An electrical signal marked the duration of stimulation, normally lasting 60 s, represented as St. A multivariate, autoregressive moving-average (ARMA) model was adopted with BP, EtCO<sub>2</sub> and St as inputs and CBFV as output. The model orders were [2,4,1,1] (Panerai et al AJP-HCP 2012). Model coefficients were derived by least squares and used to obtain estimates of the CBFV step responses for each of the inputs (BP, EtCO<sub>2</sub>, St). Single parameters derived to express the integrity of dCA, CVR and NVC, were the autoregulation index (ARI) (Tiecks et al Stroke 1995), or the plateau value of the CBFV-EtCO<sub>2</sub> or CBFV-St step responses.

**Results:** Model predicted CBFV was in excellent agreement with measured values ( $r^2 > 0.9$ ). The fraction of CBFV variance explained by the three inputs ranged from 15–40% (BP), 10–25% (EtCO<sub>2</sub>) and 30–50% (St) demonstrating the relevance of including BP and EtCO<sub>2</sub> as relevant contributors in studies of NVC. In healthy subjects and AIS patients, the three step responses were in excellent agreement with experimental values or alternative approaches. Breathing 5% CO<sub>2</sub> in air reduced extracted values of ARI from  $6.1 \pm 0.7$  to  $5.1 \pm 1.6$  ( $p < 0.005$ ) and shifted the CBFV-St step response downwards, without affecting the CBFV-EtCO<sub>2</sub> step response. AIS patients were hypocapnic compared to controls and ARI was not different during repetitive elbow flexion. However, both CBFV step responses to EtCO<sub>2</sub> and St were shifted downwards significantly in patients compared to controls ( $p = 0.01$ ;  $p = 0.009$ ).

**Conclusions:** Integrated modelling of dCA, CVR and NVC responses to neural stimulation provide a powerful tool for assessment of CBF regulation in humans. Further work is needed to validate this approach in different physiological settings and clinical conditions.

## Interaction of Moesin with $\alpha\beta 3$ Integrin Regulates Endothelial Cell Migration

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Moesin, a member of the ERM protein family, is an actin-binding protein that plays role in cell motility by linking the actin cytoskeleton to a variety of membrane-anchoring proteins. In the present work, by using MALDI-TOF analysis we identified moesin as an  $\alpha\beta 3$ -interacting molecule in human endothelial cells and studied its role in vascular endothelial growth factor-A165 (VEGF-A165) and pleiotrophin (PTN)-induced endothelial cell migration. Moesin- $\alpha\beta 3$  interaction was verified by immunoprecipitation, immunofluorescence and proximity ligation assays in human endothelial cells and was decreased by both VEGF-A165 and PTN at concentrations that stimulate cell migration. Down-regulation of moesin expression in human endothelial cells resulted in significant enhancement of cell migration. Collectively, these observations suggest that  $\alpha\beta 3$ -moesin interaction may have an inhibitory effect on cell migration and is decreased by angiogenic growth factors. PTN induced moesin Thr558 phosphorylation through its receptor protein tyrosine phosphatase beta/zeta (RPTP $\beta/\zeta$ ), PI3K, CDK5 and Rac1 but phosphorylation is not linked to stimulation of cell migration, since PTN also induced moesin Thr558 phosphorylation in cells in which it inhibits cell migration. These data uncover a novel partner of  $\alpha\beta 3$  that regulates its effect on endothelial cell migration; they may thus help explain the multifaceted role of  $\alpha\beta 3$  on angiogenesis and highlight a novel important player in the PTN and VEGF-A165 signaling cascades.

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## A Potential Role for Extracellular Vesicle-Mediated Communication from Smooth Muscle Cells to Endothelial Cells in Development of Human Pulmonary Arterial Hypertension

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**Introduction:** Pulmonary Arterial Hypertension (PAH) is a pathology in which the TGF $\beta$  superfamily signalling pathway is impaired in Human Pulmonary Arterial Smooth Muscle Cells (HPASMCs) due to BMP2 receptor alterations. TGF $\beta 1$  treatment of HPASMC is a well-known stimulus to mimic in vitro mechanisms associated with PAH. Although extracellular vesicles (EVs) have been shown to influence the vascular environment, their role remains obscure for many pathologies. Recent work in our laboratory demonstrated that HPASMCs derived EVs are involved in this pathology.

**Objective:** The main aim of this project was to assess whether EVs from HPASMCs treated with an excess of TGF $\beta 1$  are involved in transporting functional cargo to HPAECs.

**Methods:** We first used the PKH67 fluorescent membrane labelling to analyse take up of HPASMC-EVs by HPAECs. To demonstrate the transfer of mRNA and its translation into protein, we optimised the Cre-loxP method for use in primary cell cultures. Donor cells (HPASMC) enclose Cre protein on EVs while recipient cells (HPAECs) show whether Cre+ EVs have been taken up by switching from red to green fluorescence. Single-stranded low-input RNAseq was used in order to characterise HPASMC-EV cargoes.

**Results:** PKH67 staining showed that EVs from TGF $\beta 1$  treated and control HPASMCs are able to bind HPAECs membranes and be taken up. The Cre-loxP method applied to this system proved transfer and translation of Cre mRNA into protein from HPASMC-EVs to HPAECs which switched into green cells with a % eGFP/DsRed ratio of  $1.53 \pm 0.26$  measured by FACS. The Cre-loxP method also showed the level of HPASMC-HPAEC communication is not altered under TGF $\beta 1$  stimulation (10 ng/ml). For this reason, we analysed the cargoes present in EVs from control and TGF $\beta 1$  treated HPASMC. A total amount of 2417 transcripts were detected in HPASMC-EVs. Among these, a subset of 759 RNAs was found significantly enriched in Control EVs compared to their donor cells. Furthermore, EVs from TGF $\beta 1$  treated cells showed 90 differential transcripts when compared to Control EVs. Gene Ontology Enrichment Analysis related these transcripts to cell differentiation, migration and response to wounding, all well-known characteristics of cells' behaviour during PAH.

**Conclusion:** We demonstrate that HPASMC-EVs can bind HPAEC membrane in an in vitro model of PAH, and release functional cargo that can be translated into protein. Additionally, we found several differential transcripts in EVs derived by TGF $\beta 1$  treated cells, compared to Control EVs, which may suggest a potential role for these in PAH development.

### Agent-Based Model Simulates Effect of Dose-Dependent Inhibition of Angiogenesis by Increasing Secretion of Extracellular Soluble PDGF Receptor-Beta by Pericytes

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**Introduction:** Crosstalk between vascular endothelial growth factor-A (VEGF-A) and the Notch pathway coordinates sprouting of endothelial “tip” cells from existing vessels, thus facilitating angiogenesis. Tip cells secrete platelet-derived growth factor-BB (PDGF-BB) to bind PDGF Receptor- $\beta$  (PDGFR- $\beta$ ) on pericytes, thereby recruiting these specialized cells to stabilize nascent vessel branches. Recent studies from our lab (Darden et al. *Angiogenesis* 2018) and others (Sagare et al. *Neurosci Lett* 2015, Hutter-Schmid and Humpel *Curr Neurovasc Res* 2016) have suggested that pericytes also produce a soluble PDGFR $\beta$  (sPDGFR $\beta$ ) isoform that may further modulate pericyte recruitment and investment. We therefore expanded our current agent-based model (ABM) of angiogenesis in the developing mouse retina to identify potential functional roles for pericyte-derived sPDGFR $\beta$ . By varying sPDGFR $\beta$  production by pericytes in silico, this model will complement and inform experimental approaches for determining the effect of this PDGFR $\beta$  isoform on pericyte investment along nascent tip cells.

**Methods:** Our prior ABM was designed in NetLogo, an open source software, to simulate the interaction between pericytes and endothelial cells at the angiogenic front of the developing postnatal mouse retina by implementing a rule set derived from current literature. In our extension of this original model, we incorporated rules simulating extracellular secretion of sPDGFR $\beta$ , which in turn modifies extracellular gradients of PDGF-BB ligand that coordinate pericyte recruitment and investment. In addition to monitoring output levels of each molecular species, endpoints in this in silico study included quantifying the degree of stabilization of nascent tip cells by recruited pericytes.

**Results:** By considering extracellular secretion of sPDGFR $\beta$  by associated pericytes, we were able to examine interactions between pericytes and endothelial cells. We hypothesized that decreased production of sPDGFR $\beta$  from pericytes led to an accumulation of PDGF-BB in the system which in turn facilitate pericyte stabilization of endothelial tip cells.

**Discussion:** Our data suggest that pericyte-derived sPDGFR $\beta$  may represent an important means for pericytes to regulate local levels of PDGF-BB and thereby “fine-tune” their investment in developing vessels. A similar paradigm exists for the VEGF-A pathway, in which the soluble VEGF receptor Flt-1 provides key feedback regulation of VEGF-A levels. Loss of this Flt-1-mediated feedback mechanism leads to vascular overgrowth and dysmorphogenesis. Results from our computational modeling suggest that this sPDGFR $\beta$  isoform may also keep PDGF-BB signaling in pericytes within a physiological range to promote and maintain adequate pericyte coverage during angiogenic remodeling.

### Coupled Multi-Scale Models of the Microcirculation in the Cerebral Cortex

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In our previous work, we have developed a number of different models of the cerebral circulation, from 0D lumped compartment models to highly detailed 3D models of the microcirculation. Through the use of homogenisation techniques we have been able to scale up the microcirculation to a single compartment porous medium model that couples with models of individual penetrating cortical vessels. In the first part of the talk, I will present our current work that expand this through the use of a dual-porosity porous medium model in order to model the cerebral cortex more accurately. This work also directly links to multiple-compartment poro-elastic models, developed by a number of groups, which I will discuss. In the second part of the talk, I will present our current work on bridging the length-scale gap between large vessels (i.e. those that can be individually imaged) and the microcirculation, over which length scales surprisingly little anatomical information is known. I will present our proposed modelling approach and discuss how such whole-brain blood flow models can be validated and used within a patient-specific model, in particular in response to ischaemic stroke through considering the effect of thrombi on the circulation.

### Association of Skin Microvascular Function with Pulsatile and Resistive Hemodynamics

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**Introduction/Objective:** Pulsatile and resistive components of hemodynamics are in a complex relationship with each other in both sexes. Remodelling in resistive arterioles due to impaired endothelial function over the life span is hypothesized to play role in arterial stiffening process during vascular aging. Data on the association of the microvascular function with pulsatile and resistive hemodynamics are scarce. In this study, we aimed to investigate the relationship of the skin microvascular function, as a proxy of systemic microvascular function, with pulsatile and resistive components of hemodynamics at population level and whether this potential association is modified by sex.

**Method:** In this cross-sectional analysis we included participants of the Rhineland Study. The Rhineland Study is a population-based cohort study in Bonn, Germany, which recruits participants aged 30 years and older. Participant recruitment started in the first half of 2016. The skin microvascular function was measured with skin laser Doppler flowmetry as response to local ther-

mal heating on ventral surface of forearm and reported as percentage change in cutaneous skin conductance (CVC, %). Hemodynamic parameters were measured with impedance cardiography. Pulsatile hemodynamics was assessed as total arterial compliance (TAC, ml/mm Hg), and resistive hemodynamics was assessed as systemic vascular resistance (SVR, dyn-s-cm<sup>-5</sup>). We investigated the association of skin microvascular function to hemodynamic parameters with linear regression analysis. Regression models were adjusted for age and sex. Additionally, we examined these associations in groups stratified by sex. CVC was log-transformed due to skewed distribution. Results were reported as regression coefficients per standard deviation increase in CVC.

**Results:** 1629 participants were included in the present analyses (Age; 30–95 years old, mean±sd: 53.7 ± 13.7, Women; n = 902, 55.4%, CVC; median [IQR]: 627.8 [306.5–793.0], SVR; mean±sd: 1128.0 ± 253.1, TAC; mean±sd: 1.96 ± 0.48). Higher CVC was associated with lower SVR (beta, 95% CI; -0.06, -0.11–-0.02) and higher TAC (beta, 95% CI; 0.04, -0.01–0.08). However, the positive association between CVC and TAC did not reach statistical significance. In the stratified analyses, there was no sex-specific effect on these associations.

**Conclusion:** In this study, greater CVC was associated with lower SVR and higher TAC, independent of sex. These results support the hypothesis of remodelling of the resistive arterioles due to impaired microvascular function during arterial stiffening process across the lifespan. However, the cross-sectional nature of our data limits the interpretation of causal pathways.

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### Evidence of the TLR4 Participation on the Impact of Metabolic Syndrome on Brain Microcirculation and Cognitive Function

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**Background:** Metabolic syndrome (MS) is defined as a chronic low-grade pro-inflammatory state in which abnormal metabolic and cardiovascular factors increase the risk of developing cardiovascular disease and neuroinflammation. A consistent activation of innate immunity through Toll-like receptors (TLR) appears to be related as a chronic immune stress to the body. In this study, we investigated the role of TLR4 receptors in the brain microcirculation and cognitive performance of MS mice induced by diet.

**Methods:** C3H/He (WT) and C3H/HeJ (TLR4-mut) mice were fed with high-fat diet (HFD) or normolipid diet (ND) for 24 weeks. All experiments were conducted in accordance with internationally accepted principles for the Care And Use of Laboratory Animals and approved by the Oswaldo Cruz Foundation Animal Welfare Committee (license L038/15). After long-term ingestion of HFD, the animals had systolic blood pressure (SBP) evaluated by photo-plethysmography, cerebral microcirculation flow evaluated by Laser Speckle contrast Imaging and the brain functional capillary density, endothelial function and endothelial-leukocyte interactions were evaluated by intravital microscopy. The Morris water

maze test was performed to evaluate learning and the spatial memory, the inhibitory avoidance task was performed to evaluate amygdala dependent aversive memory. The ICAM-1, TLR4 and HMGB1 protein levels have been determined by western blotting analysis.

**Results:** The WT-HFD animals presented alterations on the metabolic and hemodynamic parameters when compared to the WT-ND: fasting plasma glucose 179 ± 11 vs 116 ± 14 mg/dl p < 0.01; HOMA-IR 7.8 ± 3.7 vs 1.6 ± 0.7 p < 0.05; abdominal fat 1.95 ± 0.07 vs 1.63 ± 0.07 g p < 0.05 and SBP 151 ± 25 vs 94 ± 7 mm Hg p < 0.05, respectively). The TLR4-mut-HFD group did not show difference on the above-mentioned parameters when compared to the TLR4-mut-ND group. Intravital microscopy analysis of the brain vessels of WT-HFD group revealed decreased functional capillary density and increased rolling and adhesion of leukocytes, impaired brain microvascular blood flow and abolished vasodilator response to acetylcholine which were not observed on the WT-ND and TLR4-mut-HFD groups. The learning and memory tests demonstrated cognitive decline in WT-HFD group, when compared to WT-ND and TLR4-HFD groups. Brain protein levels of HMGB1 and TLR4 were two-fold increased and ICAM-1 were three-fold increased on the WT-HFD group, but not on WT-ND and TLR4-HFD groups.

**Conclusions:** Our results demonstrate that TLR4 are involved on the microvascular dysfunction and neuroinflammation associated to HFD diet-induced MS and possibly play a causative role on the development of cognitive decline.

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### Metabolite Signaling in the Vascular Endothelium

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Angiogenesis – the growth of new blood vessels from pre-existing vasculature – is traditionally viewed from the perspective of how endothelial cells coordinate migration and proliferation in response to growth factor stimulation. However, endothelial cells must also coordinate their metabolism and adapt metabolic fluxes to the rising energy and biomass demands of sprouting vessels. Recent studies have highlighted the importance of such metabolic regulation in the endothelium and uncovered core metabolic pathways and mechanisms of regulation that drive the angiogenic process. In this presentation, current principles of endothelial metabolic regulation will be discussed. A particular focus will be given to the role of metabolite signaling and its role in controlling endothelial growth state.

## In Vivo Imaging of the Outflow of Cerebrospinal Fluid Through Lymphatic Vessels in Mice

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Cerebrospinal fluid (CSF) is produced by choroid plexuses within the ventricles of the brain and circulates throughout the subarachnoid space surrounding the brain and spinal cord. From the subarachnoid space, CSF has long been considered to clear through arachnoid villi directly to venous sinuses within the dura mater meningeal lining of the brain. However, evidence for lymphatic vessels draining at least some component of this fluid has been presented in studies dating back 150 years. Previous studies have relied on cannulation of lymphatic and/or blood vessels to study the outflow of CSF injected tracers. We have developed fluorescence imaging methods that can non-invasively assess the dynamics of near-infrared tracer outflow to lymphatic vessels or to the systemic blood circulation after injections into the lateral ventricle or cisterna magna of mice. Surprisingly, we found that tracers were cleared predominantly by lymphatic vessels with no evidence suggesting a direct blood vascular uptake within the ventricles or subarachnoid space. Tracer outflow from the skull was detected at the exits of the cranial nerves, including at the cribriform plate, optic canal and jugular foramina, to drain to deep cervical and mandibular lymph nodes.

We have extended these studies to examine the relationship between tracer efflux to lymphatics and influx to paravascular spaces of the brain. Recent studies have suggested that a large proportion of CSF, including macromolecular solutes, enters the brain parenchyma through a convective flow along penetrating arteries and transport through aquaporin-4 channels of astrocytes lining the vessels. This process, deemed “glymphatic flow”, was proposed to occur with greater efficiency during sleeping or anesthetized conditions, as more tracers were found to enter the brain under these conditions. However, our studies have indicated that lymphatic efflux of tracers from the CSF to systemic blood is inhibited under anesthetized conditions. This led to more tracer being present in the paravascular spaces on the surface of the cortex. Furthermore, using near-infrared imaging through the skull of the mouse, we were unable to visualize tracer influx along the penetrating arteries under in vivo conditions. Indeed, tracer influx was only detected immediately after death through overdose of injection anesthesia. These findings have indicated that convective influx of CSF into the brain likely does not occur under physiological conditions. More work is necessary to elucidate the intercommunication of brain interstitial fluid and CSF.

## Crosstalk of VEGF-, BMP- and Hippo-Signaling in Angiogenesis

Heidi Pulkkinen, Miika Kiema, Jari Lappalainen, Anu Toropainen, Annakaisa Tirronen, Lari Holappa, Henri Niskanen, Minna Kaikkonen-Määttä, Seppo Ylä-Herttua, Johanna Laakkonen

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**Introduction and Objectives:** Vascular endothelial growth factors (:VEGFs) are known to be involved in angiogenesis both in physiological and pathological conditions. Furthermore, VEGF utilizing therapies have been under research focus for both pro- and anti-angiogenic purposes. Especially VEGF-mediated pro-angiogenic therapies for ischemic tissues such as coronary artery disease have been challenging due to e.g. aberrant neo-vessel structure leading to hyperpermeability. Recently, bone morphogenetic proteins (:BMPs) have been linked to have a role in vascular pathologies, to have synergic effects with VEGF and even to modulate angiogenic phenotypes in endothelial cells. The main objective in our study was to identify novel targets for VEGF-mediated angiogenesis by defining the interaction mechanisms of VEGF and BMPs. In addition, their mechanistical connection to a cancer-related signaling pathway, Hippo-signaling, was explored.

**Methods:** In this study intravenous adenovirus-mediated VEGF gene transfer was performed for C57/Bl6 mice. The effects of VEGF overexpression were examined by RNA-sequencing from mice liver. To further reveal the interaction mechanisms of VEGF and BMPs various primary endothelial cell models were utilized. The effects were investigated on transcriptome (qPCR), proteome (WB) and functional (3D angiogenesis assay) levels.

**Results:** VEGF gene transfer led to increased vascular area in mice liver and induced expression of genes linked to transforming growth factor  $\beta$  (:TGF $\beta$ )-, BMP-, NOTCH- and Hippo-signaling pathways. VEGF was shown to alter the expression of BMP2 and BMP4 in endothelium-specific manner. Furthermore, BMPs were shown to have a regulative role in endothelial cell sprouting in a 3D-angiogenesis assay. Interestingly, BMPs were revealed to modulate Hippo-signaling activation.

**Conclusions:** Our data unveils the complex interaction mechanisms of VEGF-, BMP- and Hippo-signaling pathways. BMPs were shown to regulate VEGF-mediated sprouting angiogenesis and to modulate Hippo-signaling activity. As a conclusion, BMP-signaling can be targeted to finetune VEGF-induced neovessel formation, thus it can be used in the development of VEGF-therapy for ischemic tissues.

### Abnormal K<sup>+</sup>-Signaling in Cerebral Arteries Disturbs Neurovascular Coupling in Mouse Model for Migraine

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Familial hemiplegic migraine type 2 (FHM2) is a sub-type of migraine with aura. Patients with FHM2 are bearing single-point mutations in the gene of G301R resulting in reduced expression of the Na,K-ATPase  $\alpha$ 2 isoform. A mouse model with the corresponding mutation phenocopies several FHM2-relevant disease traits.

We hypothesize that an altered expression in the Na,K-ATPase  $\alpha$ 2 isoform changes function of the K<sup>+</sup>-inward-rectifying (Kir) channels and this results in an abnormal neurovascular coupling (NVC) in FHM2 mice compared to wild type (WT) controls.

Neurovascular coupling was investigated in vitro in isometric myograph-setup, in situ in brain slices and in vivo using Laser Speckle imaging. Relative expression of the Kir channels was quantified using immunohistochemistry.

Pre-contracted middle cerebral arteries (MCA) from FHM2 mice had stronger relaxation than WT in response to elevation of [K<sup>+</sup>]<sub>out</sub>. Inhibition of the Na,K-ATPase  $\alpha$ 2 isoform by 10  $\mu$ M ouabain had no significant effect on the K<sup>+</sup>-induced relaxation in both groups. BaCl<sub>2</sub> (30  $\mu$ M), a Kir-channel inhibitor, almost completely suppressed the K<sup>+</sup>-induced relaxation in both groups indicating major dependence on the Kir channels for this relaxation.

Electrical field stimulation in brain slices elicited stronger dilation of parenchymal arteries from FHM2 mice compared to WT. The results suggest an increased neurovascular signaling between excited neurons and the arterial wall mediated by astrocytic end-feet.

Whisker stimulation induced an increased blood flow to the corresponding sensory cortex, which was stronger in FHM2 mice compared to WT.

The expression of Kir channels was significantly increased in MCA endothelial cells from FHM2 mice compared to WT. There was no difference in smooth muscle cell Kir expression between the two groups.

FHM2-associated mutation in the Na,K-ATPase  $\alpha$ 2 isoform leads to an abnormal NVC in the FHM2 mouse model which might be due to an increased contribution of endothelial Kir channels. This might be the mechanism underlying hyperperfusion associated with the headache stage of migraine attack seen in FHM2 patients.

### High-Throughput Permeability Assay on Perfused 3D Microvessels In Vitro

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In current state of the art, the majority of in vitro models to test barrier function of vasculature are based on 2D monolayers. These monolayers lack the 3D architecture of bloodvessels found in vivo as well as exposure to flow and interaction with an extracellular matrix (ECM). Here, we demonstrate a high throughput microfluidic platform, called OrganoPlate<sup>®</sup>, that allows to culture endothelial cells into perfusable 3D micro vessels. The platform harbours 96 perfusable blood vessels.

Endothelial cells were seeded against an ECM and formed a blood vessel in 4 to 7 days. The micro vessels were continuously perfused using a rocker platform and remained viable for over 60 days. The barrier integrity was quantified by the diffusion of 20 kDa and 150 kDa fluorescent dextrans from the lumen into the ECM using high content imaging techniques. The vessels exhibited a selective permeability of 20 kDa dextran over 150 kDa. In addition, we show a dose dependent response to VEGF, TNF $\alpha$  and cytokine exposure, resulting in a change of permeability of the micro vessels. The results are consistent with known in vivo responses.

The compatibility and throughput of the platform allows researchers in vascular biology to transition to 3D culture methods. By incorporating flow and ECM interaction, the culture conditions of micro vessels mimic in vivo conditions.

### BBB On-a-Chip: A 3D In Vitro Model of the Human Blood Brain Barrier (BBB)

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The BBB ensures a homeostatic environment for the brain and is made up of specialized endothelial cells and supporting astrocytes and pericytes. The BBB protects the brain from harmful substances. It also prevents large lipophilic compounds, including most therapeutic drugs, from entering the brain. This makes it difficult to treat brain diseases<sup>1</sup>.

While recent developments in microfluidic engineering have resulted in promising in vitro models of the BBB, the throughput and ease of use of these systems is low. This makes these models not suited for regular academic research and drug development. Here we present a novel BBB model using the 3-lane OrganoPlate<sup>®</sup>. This platform is based on a 384-well microtiter plate and

allows for parallel culture of 40 perfused miniaturized tissues, making it fully compatible with standard lab procedures and equipment.

The BBB-on-a-chip model comprises a perfused 3D microvessel of human brain microvascular endothelial cells. Perfusion through the lumen of the vessel is induced without pumps and can be controlled to model mechanical cues. In addition, the microvessel is supported by human astrocytes and pericytes that interact and support the endothelial vessel. The phenotype of the BBB-on-a-chip was characterized using immunofluorescent staining and showed presence of junctional markers VE-cadherin, PECAM-1, Claudin-5, and ZO-1. In addition, we have confirmed barrier function and adopted transporter assays to show functionality of two major BBB transporters, Pgp and GLUT1.

In conclusion, we present a novel human BBB model in an easy to use microfluidic platform. This model can be used for fundamental BBB research, drug development or studying neurological disorders.

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### Perfusable 3D Angiogenesis in High Throughput Microfluidic Culture Platform

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The transition from 2D to 3D cell culture is a first step towards more physiological relevant models in vitro. Microfluidic techniques enable to add more physiologically relevant cues, such as long term gradient stability and continuous perfusion. Furthermore, microfluidic techniques allow patterning of cell layers as stratified co-cultures, in order to capture complex tissue architectures found in vivo. We use a standardized microfluidic 3D tissue culture platform, called OrganoPlate<sup>®</sup> to generate precisely controlled gradients, pump-free and in high throughput (n = 40) for growing blood vessels and inducing controlled 3D angiogenic sprouting thereof.

The blood vessel is grown against an extracellular matrix gel and subsequently exposed to (anti-) angiogenic compounds to direct sprouting into an ECM gel. The exposed vasculature shows many of the important hallmarks of angiogenesis found in vivo, including tip cells induction and migration and stalk cells formation. Importantly, the stalk cells develop perfusable lumen that are connected to the parental vessel.

This model will be used as an in vitro screening platform to unravel the important drivers in angiogenesis and vasculogenesis and the mechanism of action of anti-angiogenic compounds. By combining this culture platform with mural cells, cell-cell interactions can be studied. In parallel we will combine the platform with our current Organ-on-a-Chip models to create tissue models with integrated vasculature.

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### Microvascular Dysfunction Is Associated with Worse Cognitive Performance: The Maastricht Study

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**Introduction:** Microvascular dysfunction may be associated with worse cognitive performance. Most previous studies evaluated individual measures of microvascular dysfunction only, did not adjust for important potential confounders, and showed inconsistent results. We therefore evaluated the association between a comprehensive set of indirect and direct measures of microvascular dysfunction and cognitive performance in the population-based Maastricht Study.

**Methods:** We used cross-sectional data including 3,011 participants (age 59.5 ± 8.2; 48.9% women, 26.5% type 2 diabetes mellitus [oversampled by design]). Measures of microvascular dysfunction included MRI features of cerebral small vessel disease, plasma biomarkers of microvascular dysfunction, albuminuria, flicker light-induced retinal arteriolar and venular dilation response and heat-induced skin hyperemia. These measures were summarized into a total microvascular dysfunction composite score. Cognitive domains assessed were memory, processing speed and executive functions. Global cognitive function was calculated as the standardized sum of the scores on these three cognitive domains.

**Results:** The microvascular dysfunction score was statistically significantly associated with worse global cognitive function (standardized  $\beta$ , -0.087; 95% CI -0.127; -0.047), independent of age, sex, education level, type 2 diabetes mellitus, smoking, alcohol use, hypertension, total/HDL cholesterol ratio, triglycerides, lipid-modifying medication, prior cardiovascular disease, depression and plasma biomarkers of low-grade inflammation. The fully adjusted beta-coefficient of the association between microvascular dysfunction and the global cognitive function score was equivalent to two (range: one to three) years of aging for each standard deviation higher total microvascular dysfunction score. The microvascular dysfunction score was statistically significantly associated with worse memory and processing speed, but not with worse executive function.

**Conclusion:** The present study shows that microvascular dysfunction is associated with worse cognitive performance. This suggests that microvascular dysfunction may contribute to the development of cognitive impairment.

## The Myocardial Infarction Milieu Favours the Development of Protective T-Cell Auto-Reactivity

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T-cell autoreactivity is a hallmark of autoimmune diseases, but it can also benefit self-maintenance and foster tissue repair. Herein, we investigated whether heart-specific T cells exert salutary or detrimental effects in the context myocardial infarction (MI). Recent studies revealed that MI triggers the activation of CD4 + T-cells in mice, which in turn modulate myocardial inflammation, healing and remodeling. In the present study, we sought to assess how T-cell responses are fine-tuned in the injured myocardium milieu. Thus, cardiac-myosin-specific Thy1.1 + CD4 + T-cells (TCR-M) were adoptively transferred into Thy1.2 + WT recipients prior to MI induction. Flow cytometry and light-sheet microscopy analyses showed that TCR-M cells accumulate in the heart and mediastinal lymph nodes of infarcted, but not of sham-operated animals, at the peak of healing process (day 7), whereas no TCR-M accumulation was observed in other irrelevant sites (spleen, subiliac lymphnode). Notably, these cells vanished at later chronic remodeling phase, indicating that post-MI T-cell auto-reactivity is self-limiting. Most strikingly, TCR-M cells, which are pathogenic and drive lethal myocarditis in their donors, differentiate into Foxp3 + cells, acquire a unique pro-healing gene expression profile and promote cardioprotection when transferred into MI-recipients, leading to preserved functional parameters. Next-generation sequencing of T-cell receptors (TCR) further revealed that T-cells infiltrating the infarcted myocardium display a unique repertoire signature, showing signs of antigen-specific expansion. Tregs were also detected in myocardial autopsies from patients who suffered MI, peaking in the healing phase. Noninvasive PET/CT imaging using a CXCR4 radioligand revealed enlarged med-LNs and increased T-cellularity in MI-patients, and these alterations corre-

lated with infarct size and cardiac function. Our study provides evidence that the MI-context induces antigen-specific, protective T-cell autoimmunity in mice, and confirms the existence of an analogous physiological heart/med-LN/T cell axis in MI patients.

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## The Effect of Two Weeks of Leg Immobilisation and Intense Aerobic Exercise Training on Endothelial Function

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**Introduction:** The vasculature is an extremely plastic tissue that rapidly adapts to the habits of an individual. For instance, endothelial dysfunction which is a strong predictor of cardiovascular disease is a common cause of a sedentary lifestyle. However, most studies have focused on physical inactivity over a longer period whereas the consequences of short-term immobilisation as seen with hospitalisation are not fully understood. In the present study, we hypothesised that a short period of leg immobilisation would lead to endothelial dysfunction and that a four-week period of intense aerobic exercise training would reverse the dysfunction.

**Methods:** Twelve healthy young male subjects (20–24 years) completed two weeks of leg immobilisation accomplished by a full-leg cast followed by four weeks of intense aerobic exercise training. Endothelial function was examined in the leg by femoral arterial infusion of acetylcholine (ACH) and sodium nitroprusside (SNP) and measurement where collected at baseline, after two weeks of immobilisation and after four weeks of aerobic exercise training. In addition, skeletal muscle biopsies were obtained from the thigh and analysed for protein expression of endothelial nitric oxide synthase (eNOS) and superoxide dismutase 2 (SOD2).

**Results:** The vasodilatory response to femoral arterial infusion of ACH was 25% lower ( $P < 0.05$ ) after the two weeks of leg immobilisation compared to baseline. Furthermore, after aerobic exercise training the vasodilatory response was normalised to baseline values. There was a tendency ( $P < 0.1$ ) towards a lower vasodilatory response to femoral arterial infusion of SNP after leg immobilisation compared to baseline whereas exercise training had no effect on the response.

The protein expression of eNOS and SOD2 were unchanged after the two weeks of leg immobilisation compared to baseline, but aerobic exercise training increased both eNOS and SOD2 expression ( $P < 0.05$ ) compared to levels after leg immobilisation ( $n = 7$ ).

**Conclusion:** These findings clearly demonstrate that a short period of immobilisation is sufficient to induce endothelial dysfunction in the vasculature, but also that this detrimental effect can be reversed by a four-week period of intense aerobic exercise training.

## PDGFR $\beta$ is Required in Perivascular Cells to Generate Aortic Hematopoietic Stem Cells In Vivo

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The first adult-type hematopoietic stem cells (HSCs) are generated in the aorta-gonads-mesonephros (AGM) region in the mid-gestation mouse embryo. Signals from the surrounding microenvironment are required for HSC generation. However, the current impossibility of specifying HSCs in vitro for clinical use suggests that key signals and the identity of cells releasing those signals in vivo remain unknown. The embryonic microenvironment includes perivascular cells. Pericytes support HSC maintenance in the adult bone marrow but whether they are also involved in the HSC birth has not been studied so far. Pericytes are recruited to the developing blood vessel wall through PDGFB/PDGFR $\beta$  signalling which was recently shown to mediate HSC specification in zebrafish. We here hypothesize that PDGFR $\beta$  signalling is required to generate the first HSCs in vivo. To answer this question, we used the PDGFR $\beta$ -knock out (KO) mice which lack pericytes and die prenatally. Results from our hematopoietic progenitor assays and transplantations show that the germline deletion of PDGFR $\beta$  affects both hematopoietic progenitor numbers and hematopoietic stem cell activity in the embryonic aorta in vivo. This is not due to changes in the blood vessel structure. Using sophisticated technology to visualise the full aorta by confocal imaging and quantification of vascular cells by flow cytometry, we demonstrate that the dorsal aorta is properly formed in the PDGFR $\beta$  KO mid-gestation mouse embryos. Interestingly, hematopoietic progenitor numbers in other highly vascularised hematopoietic organs at the same developmental stage such as the placenta, yolk sac, liver and head are not affected, although PDGFR $\beta$  is expressed, suggesting an organ-specific role for PDGFR $\beta$  signalling to control hematopoiesis. We next aimed to identify and characterise the PDGFR $\beta$ +cells that control hematopoiesis in the AGM. By immunohistochemistry, flow cytometry and RNA sequencing, we here show for the first time that the dorsal aorta in the wild-type mid-gestation mouse embryo is surrounded by three phenotypically and transcriptionally distinct perivascular cell layers that include pericytes (PDGFR $\beta$ +NG2+) and PDGFR $\beta$ +NG2- adjacent cells. PCs are highly enriched in HSC niche genes described in the adult microenvironment, suggesting their involvement in the AGM mid-gestation hematopoiesis. In conclusion, our results define PDGFR $\beta$  signalling as key component of the HSC generating niche in the mouse embryo in vivo

that could be tested in vitro to derive HSCs from hematopoietic and non-hematopoietic cell sources for cell therapy to treat patients with blood diseases.

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### S100A4, a Key Player in Plaque Stabilization

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During atherosclerosis, intimal smooth muscle cells (SMCs) acquire a synthetic phenotype. We have previously isolated spindle-shaped (S) and rhomboid (R) SMCs from porcine coronary artery. R-SMCs display the features of synthetic/dedifferentiated SMCs. S100A4 was identified as being a marker of R-SMCs in vitro and of intimal SMCs, both in pig and man.

S-SMCs were treated with multimeric recombinant S100A4, which resulted in partial transition from S- to R-phenotype and NF $\kappa$ B activation. Remarkably, treatment of S-SMCs with multimeric S100A4 and platelet-derived growth factor-BB (PDGF-BB) together induced a complete SMC transition toward a R-phenotype associated with NF $\kappa$ B activation compared with multimeric S100A4 or PDGF-BB alone, likely through toll-like receptor-4 (TLR-4). RNA sequencing showed strong upregulation of pro-inflammatory genes when cells were treated with multimeric S100A4 and PDGF-BB together compared with multimeric S100A4 or PDGF-BB alone (e.g. granulocyte-macrophage colony-stimulating factor: 229 fold increase in S100A4 alone, no increase in PDGF-BB alone and 558 fold increase in S100A4+PDGF-BB vs controls or PDGF-BB alone).

In vivo, ApoE<sup>-/-</sup> mice were fed with high-cholesterol diet for 9 weeks. The 3 last weeks they were injected intraperitoneally with neutralizing monoclonal S100A4 antibody (clone 6B12, n = 11) or with control IgG1 (n = 12). Neutralization of extracellular S100A4 induced decreased number and size of atherosclerotic lesions, decreased necrotic core and cholesterol cleft, decreased number of CD68 positive cells and increased number of  $\alpha$ -smooth muscle actin and smooth muscle myosin heavy chains-positive cells when compared to control groups.

Our results indicate that extracellular S100A4 could be a new target to influence the evolution of atherosclerotic plaque, leading to plaque stabilization.

## Involvement of Fibrinogen in the Microcirculation

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The majority of plasma protein fibrinogen (Fib) is synthesized in the liver and in small amounts by the platelets. In addition to be a biomarker of inflammation the Fib participates in blood hemostasis, gliomas, tumor metastasis and erythrocyte aggregation (EA). The aim of this presentation is to summarize the knowledge obtained from in vivo, ex vivo and in vitro studies regarding the effects of human soluble fibrinogen molecule on the properties of blood components and the resulting on microcirculation.

In vivo studies conducted on microvessels of the experimental animal models with or deprived of Fib will be discussed. In vivo human microcirculatory parameters have been shown to be associated with ex vivo red blood cell (RBC) deformability, aggregation and nitric oxide efflux from red blood cell demonstrated the participation of plasma fibrinogen.

In vitro studies of fibrinogen binding to the membrane targets on neutrophils and erythrocytes showed its influence on activation on leukocytes and on the biorheology properties of RBCs, such as deformability and the NO availability. Those effects were observed under the absence and the presence of endogenous or exogenous human molecules. In vivo studies showed the soluble fibrinogen and RBC aggregation as partners in dysfunctional microcirculation.

## Role of Arginase II in Hypoxia-Induced Endothelial Inflammation

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**Objective:** Hypoxia induces endothelial inflammation and tissue remodeling in cardiovascular diseases like atherosclerosis, pulmonary arterial hypertension, and myocardial infarction by deregulating many cellular processes. Arginase II plays an important role in maintaining the homeostasis in the endothelium which is disturbed in hypoxia. However, the transcriptional regulation and signaling cascade involved in upregulation of arginase II in response to hypoxia is not characterized well, therefore our primary objective is to understand the molecular mechanisms involved in hypoxic regulation of Arginase II.

**Methods:** Human Umbilical Vein Endothelial cells were freshly isolated and used as model system for cell culture-based assays. Arginase II mRNA levels were measured using semi-qPCR and protein levels were measured using immunoblotting. Dual luciferase reporter assays were performed in EA.hy926 endothelial cell line to characterize promoter activity of Arginase II.

**Results:** Hypoxia enhanced arginase II mRNA and protein levels in HUVECs. Hypoxia enhanced total protein levels as well as surface expression of inflammatory markers like E-selectin. Also,

naive Peripheral Blood Mononuclear Cells (PBMCs) showed increased adherence to hypoxia treated HUVECs, showing an inflammatory phenotype of the endothelium. In-silico tools like Consite, Jasper, and MatInspector were employed to analyze the promoter of arginase II to find putative transcription factors. The transcription factors thus found include GATA 2, HIF 1 $\alpha$ , c-FOS, SNAI1 and SOX – 5. Based on the results, five Arginase II promoter fragments of sizes 1.5 kb, 1.1 kb, 0.5 kb, 0.3 kb and 0.2 kb were cloned in pGL3 basic luciferase vectors deleting different clusters of transcription factor binding sites. Basal promoter activity characterization of the deletion constructs through dual luciferase reporter assay showed the highest activity for 1.1 kb fragment suggesting a repressor site in the deleted region between 1.5 kb and 1.1 kb. Further, hypoxia enhanced the promoter activity of the deletion constructs, giving insights to critical transcriptional regulators of Arginase II.

**Conclusion:** Hypoxia induces endothelial inflammation by up-regulating Arginase II protein levels. Repressor elements and hypoxia responsive elements were discerned in the promoter of Arginase II and putative transcription factors corresponding to these regions were identified.

## TSP-1:CD47 Orthosteric Antagonist TAX2 Peptide has Platelet Anti-Aggregating and Antithrombotic Activities

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**Introduction:** Thrombospondin-1 (TSP-1), one of the most expressed proteins in platelet  $\alpha$ -granules, is a homotrimeric glycoprotein that plays a major role in haemostasis and thrombosis. Platelet-released TSP-1 interacts with various membrane receptors, including CD47. This interaction enhances platelet adhesion and aggregation and contributes to stabilization of platelet aggregates (Isenberg et al., 2008). Moreover, TSP-1 and CD47 are over-expressed within the vascular wall of atherosclerotic patients (Rogers et al., 2014). Thus, specific targeting of TSP-1/CD47 axis offers interesting therapeutic perspectives. Previous works from our lab (Jeanne et al., 2015) allowed engineering of a cyclic dodecapeptide, named TAX2, acting as an orthosteric antagonist for TSP-1:CD47 interaction.

**Results:** In this study, we evaluated the effect of TAX2 peptide (100–800  $\mu$ M) on collagen-induced aggregation of human platelets. Aggregation was significantly decreased by  $38.5 \pm 15.2\%$  ( $n = 3$ ) in whole blood,  $21.4 \pm 9.6\%$  ( $n = 8$ ) in platelet-rich plasma, and  $80.3 \pm 11.4\%$  ( $n = 3$ ) in washed platelets, compared to a scrambled

peptide. Comparable results were obtained when using other agonists i.e. ADP and TRAP. In washed platelets, inhibition of collagen-induced aggregation was associated with a significant decrease in protein phosphorylation. Moreover, co-immunoprecipitation experiments from human and murine washed platelets show a significant decrease of the amount of TSP-1 co-immunoprecipitated with CD47 when platelets were incubated with TAX2 (800  $\mu$ M versus scrambled peptide) of  $84.8 \pm 13.1\%$  ( $n = 4$ ) and of  $43.0 \pm 12.8\%$  ( $n = 3$ ), respectively. In a microfluidic collagen-coated perfusion chamber model, we found that TAX2 (100  $\mu$ M) reduces by  $38.1 \pm 11.0\%$  ( $n = 10$ ) the area covered by platelets after 5 minutes of whole blood perfusion at arterial shear rates. At last, we studied TAX2 effect in two murine models of FeCl<sub>3</sub>-induced arterial thrombosis (mesenteric arteriole and carotid artery). In both models, systemic administration of TAX2 (10 mg/kg i.v. versus scrambled peptide) significantly delays time to complete thrombotic occlusion by  $236.2 \pm 36.0\%$  for mesenteric arteriole ( $n = 15$ ) and  $174.5 \pm 16.4\%$  for carotid ( $n = 16$ ), respectively. Importantly, similar results were obtained with mice being deficient for TSP-1-encoding gene (Thbs1<sup>-/-</sup>).

**Conclusion:** Overall, this study sheds light on the major contribution of TSP-1:CD47 interaction in platelet activation and thrombus formation, while putting forward TAX2 as an innovative antithrombotic agent with high-added value.

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### Nox4 Promotes Angiogenesis by Oxidation of HDAC4 and Abrogated MEF2A Repression

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NADPH oxidases (Noxes) produce reactive oxygen species (ROS). Noxes differ in localization as well as in type and concentration of the produced ROS. Only Nox4 directly produces H<sub>2</sub>O<sub>2</sub> at the endoplasmatic reticulum while the others such as Nox5 produce superoxide anions ( $\bullet\text{O}_2^-$ ) at the plasma membrane. In contrast to  $\bullet\text{O}_2^-$ , 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 reduces angiogenesis after hindlimb ischemia in mice. Histone deacetylases (HDACs) regulate expression of several proteins. They deacetylate histones and other proteins at lysine and arginine residues. Interestingly, HDACs are also involved in regulation of angiogenesis.

We hypothesize that Nox derived ROS and HDACs both act in concert, when regulating endothelial function. Here we aim to analyze if HDACs can be differentially oxidized by Nox4.

Nox4 and Nox5 overexpressing HEK293 cells were generated. Nox4 expression and activity was achieved by tetracycline induced gene expression, while Nox5 was stably overexpressed and acutely activated by treating the cells with PMA. BIAM switch redox assays were used to verify class Iia HDACs 4 and 5 as oxidation targets. We observed an enhanced oxidation of HDAC4 and 5 upon Nox4 upregulation, but not another class Iia member, HDAC7, or the class I member HDAC3. Accordingly, treatment of HUVECs with

H<sub>2</sub>O<sub>2</sub>, also selectively enhanced HDAC4 oxidation. In contrast, Nox5 activation had no effect on HDAC4 and 5 oxidation.

HDAC4 overexpression in HUVECs reduced their ability to form tubes, while overexpression of a redox dead HDAC4 mutant had no effect. MEF2 proteins, which are known targets of class Iia HDACs, induce pro-angiogenic processes. Luciferase reporter assays revealed a reduced repression of MEF2A activity, when cells express the redox dead HDAC4 mutant compared to overexpressed wild type HDAC4.

We conclude that Nox4 oxidizes HDAC4, which potentially reduces the complex formation of HDAC4 and MEF2A. Accordingly, we discovered a new mechanism, of how Nox4 indirectly promotes angiogenesis.

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### Circadian Rhythms in Leukocyte-Endothelial Cell Interactions

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The number of leukocytes circulating in blood is under circadian, i.e. ~24 h, control. This talk will summarize latest findings on the mechanisms governing leukocyte migration from the blood into various organs, focusing on the distinct leukocyte subtype- and organ vascular-specific molecules involved. A focus will be on the oscillatory expression patterns of adhesion molecules, chemokines and their receptors, expressed on endothelial cells and leukocytes, which are critical regulators of rhythmic leukocyte recruitment. Furthermore, the relevance of clock genes in endothelial cells and leukocytes for leukocyte function and migration will be discussed.

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### Brachial and Retinal Endothelial Dysfunction – Methodology and Effect of Exercise

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The endothelial dysfunction of the brachial artery is measured by flow-mediated dilatation. As usually the blood flow is interrupted for a period of five minutes with a cuff. The brachial artery diameters are measured before, at the end of the occlusion period and after releasing the cuff. The dilatation mediated by the flow is the percentage difference between the diameters measured before and after opening the cuff. The endothelial function of the retinal artery is measured by flicker-induced vasodilation. The flicker light is usually applied to the retina at a frequency of 12.5 Hz for up to one minute duration with the pupil in mydriasis. Dilatation of both arteries, the macrovascular brachial artery and the microvascular retinal artery, is at least partially dependent on nitric oxide

(NO), one of the strongest anti-atherosclerotic substances in the human body. The higher the NO production or NO bioavailability, the higher the vasodilation.

Aerobic exercise with large muscle groups result in an increase in cardiac output, as well as general and regional arterial blood flow. This is associated with increased shear stress on the arterial wall and thus endothelial NO production. The flow-mediated dilatation of the microvascular and macrovascular site thus reflects a better arterial function. Endurance exercise with small muscle groups or strength training above a certain intensity has no positive effect on endothelial function. The talk presents the current methodology for assessing endothelial function in research and the effects of movement on the arterial microvascular and macrovascular system.

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### Tight Junction Adapter Protein Cingulin Plays a Role in Vascular Barrier Function In Vivo

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Barrier function in the vascular network is critical for organ function in every part of the human body. Beside adherens junctions, the vascular barrier is safeguarded by a specialized tight junction complex, made up of transmembrane components, cytoplasmic adapter proteins and signaling molecules. Cytoplasmic adapter proteins, like ZO-proteins and cingulin, are the components that connect transmembrane proteins with the actin cytoskeleton and microtubules, as well as signaling molecules.

To investigate whether cingulin plays a role in vascular barrier function, human primary endothelial cells, endothelial cells overexpressing cingulin and histological tissue sections from different vascular beds were analyzed by IHC, WB and qPCR. Differences in barrier function were analyzed by transwell assays and electrical cell impedance sensing. Furthermore cingulin knockout mice were subjected to 1 mm<sup>2</sup> burn wounds to the ear skin. Edema formation and nonperfused area were monitored for 2 weeks after wounding.

Histological sections and isolated human primary endothelial cells show differential expression of cingulin in blood vessels of human lung, skin and brain. In vitro the presence of cingulin in endothelial cells strengthens vascular barrier function for different molecular weight tracers and increased measurements of transendothelial electrical cell impedance. Consequently, cingulin is involved in maintaining vascular barrier function.

In vivo, the knockout of cingulin in C57Bl6 mice leads to a decrease of vascular barrier function in brain endothelial cells. In addition, differences in the response to burn injury in cingulin knockout mice and wild type littermates were seen. In sum-

mary, results indicate that the tight junction adapter protein cingulin regulates vascular barrier function but not angiogenesis in vivo.

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### A Novel Approach of Platelet Function Test to Identify Non-Matured Platelets Having Increased Aggregatory Effect

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**Introduction:** Elevated mean platelet volume (MPV) is predictive for vascular risk. MPV is related to the immature platelet fraction (IPF). Both can be associated with 'on treatment' residual platelet reactivity. Inhibitors of platelet aggregation such as clopidogrel, are used to prevent or treat strokes and cardiac infarction. However, there are cases when antiplatelet function is less effective, yet the underlying mechanism is unclear. Thus we aimed to determine which platelet related parameters (MPV, IPF) assist to reveal reduced response to clopidogrel in stroke patients utilizing a novel method.

**Methods:** Blood was taken from 46 patients and 15 healthy subjects (age: 66 ± 8 vs 40 ± 13) and were analyzed for platelet count, MPV, IPF, large cell platelet ratio (LCR) and high fluorescent immature platelet fraction (H-IPF). As a novelty, not only whole blood, but upper (ascending) and lower (sedimentating) half blood samples after one hour gravity sedimentation were also analyzed. Adenosine diphosphate (ADP) and platelet aggregometry was used for the whole blood and gravity separated samples to explore the area under the curve (AUC) of aggregation in both, patients and healthy controls.

**Results:** The AUC of the whole blood showed significant differences ( $p < 0.05$ ) compared to the upper and lower samples separated after 1-hour gravity sedimentation both in patients and healthy controls. Remarkably, AUC measured in the upper samples in 59% of patients on clopidogrel were exceeding the therapeutic range (AUC: 0–53) suggesting that ascending platelets still aggregate in the presence of ADP. This observation was associated with increased MPV and LCR in the upper samples (both,  $p < 0.05$ ). Thus patients on clopidogrel were characterized as responders ( $n = 34$ ) and non-responders ( $n = 12$ ) and the percentage of H-IPF was significantly ( $p < 0.05$ ) higher among non-responders compared to healthy subjects in the upper samples. The percentage of H-IPF was similar in both samples in the healthy subjects.

**Conclusions:** The modified platelet function test developed by us may help to stratify patients with high residual platelet aggregation despite antiplatelet treatment. Ascending platelets in patients contained significantly more non-matured platelets compared to healthy subjects, which may have pathophysiological consequences.

### Protective Effects of Exercise on Vascular Function are Mediated by NADPH Oxidase 4

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**Introduction/Objective:** Physical activity is one of the most potent strategies to prevent endothelial dysfunction. Recent evidence indicates vaso-protective properties of H<sub>2</sub>O<sub>2</sub> produced by main endothelial NADPH oxidase isoform 4 (Nox4) in the vasculature. Therefore, we hypothesized that Nox4 connects physical activity with vaso-protective effects.

**Methods and Results:** Analysis of endothelial function by Mulvany myograph showed endothelial dysfunction in wild-type as well as in Nox4<sup>-/-</sup> mice after 20 weeks on high-fat diet. Access to voluntary running wheels during high-fat diet prevented endothelial dysfunction in wild-type but not in Nox4<sup>-/-</sup> mice. Mechanistically, exercise led to increased H<sub>2</sub>O<sub>2</sub> release in the aorta of wild-type mice with increased phosphorylation of eNOS pathway member AKT serine/threonine kinase 1 (Akt1), subsequently. Both effects were diminished in aortas of Nox4<sup>-/-</sup> mice. Deletion of Nox4 also led to decreased capacity for intracellular calcium release and reduced phenylephrine-mediated contraction, whereas potassium-induced contraction was unaffected. H<sub>2</sub>O<sub>2</sub> scavenger catalase reduced phenylephrine-contraction in wild-type mice. Supplementation of H<sub>2</sub>O<sub>2</sub> increased phenylephrine-induced contraction in Nox4<sup>-/-</sup> mice. Exercise induced key regulator of mitochondria biogenesis peroxisome proliferative activated receptor gamma, coactivator 1 alpha (Ppargc1a) in wild-type but not Nox4<sup>-/-</sup> mice. Furthermore, exercise induced citrate synthase activity and reduced mitochondria mass in the absence of Nox4. Thus, Nox4<sup>-/-</sup> mice became less active and ran less compared with wild-type mice.

**Conclusions:** Nox4 derived H<sub>2</sub>O<sub>2</sub> plays a key role in exercise-induced adaptations of eNOS and Ppargc1a pathway and intracellular calcium release. Hence, loss of Nox4 diminished physical activity performance and vascular protective effects of exercise.

### Flow-Mediated Dilatation (FMD) and Peripheral Endothelial Function Assessment

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Prediction of cardiovascular adverse events is challenging and specifically the individual at risk. It has become apparent that traditional coronary artery disease (CAD) risk factors are the cornerstones of the European 10-year CAD risk SCORE and the Framingham score. However, despite their importance, the predictive value of general assessment tools such as the SCORE and Framingham in an individual subject is limited, especially in young adults

and women. The trend toward personalized medicine and individualized risk assessment in recent time has gained strength and various functional and imaging screening tests including endothelial function studies have been suggested to improve efficacy and provide the functional implications of these risk factors. Moreover, the ability to assess the disease process rather than the risk factors may lead to better therapy. Endothelial dysfunction is regarded as the early stages of atherosclerosis and has been associated with adverse cardiovascular outcome events, including myocardial infarction, revascularization, stroke and death. The purpose of this presentation is to review the scientific background, available assessment methods of vascular endothelial function [flow-mediated dilation (FMD), and peripheral methods) and interpretation of tests results, the two most common non-invasive methods to assess endothelial function: Brachial artery FMD and peripheral arterial tonometry (PAT) using the EndoPAT technology.

### Loss of the Long Non-Coding RNA MIR503HG Promotes Endothelial-to-Mesenchymal Transition

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Endothelial-to-mesenchymal transition (EndMT) is a process in which endothelial cells lose their properties and transform into fibroblast-like cells. This transition was shown to play a role in biological and pathological conditions in cardiovascular system including valve formation during heart development and diseases such as myocardial infarction, cardiac fibrosis, valve calcification, endocardial elastofibrosis, atherosclerosis, and pulmonary arterial hypertension (PAH).

Despite the important role played by EndMT in pathological conditions, molecular mechanisms governing the transition are not fully understood. Recent emergence of non-coding RNAs regulation of pathological processes in vascular remodelling as well as their therapeutic potential lead us to investigate the role of long non coding RNAs (lncRNAs) in EndMT.

To examine the functions of lncRNAs in EndMT, we reproduced this process in vitro by treating human primary endothelial cells (EC) with a combination of transforming growth factor-β2 (TGF-β2) and interleukin-1β (IL-1β) and identified transcriptional changes, including lncRNAs, by performing deep RNA sequencing. In particular, the loss of the lncRNA MIR503HG was identified as a common signature across multiple human primary EC types undergoing EndMT in vitro. Additionally, we observed decreased expression of MIR503HG in blood outgrowth endothelial cells (BOECs) isolated from PAH patients along with enhanced expression of mesenchymal markers and reduced expression of endothelial markers.

Furthermore, targeted depletion of MIR503HG alone was able to induce a spontaneous EndMT phenotype in HUVEC, while its overexpression repressed hallmark EndMT changes despite TGF-β2 and IL-1β co-stimulation. RNA sequencing analysis of cells overexpressing MIR503 identified that more than 25% of the

EndMT-transcriptome signature was inhibited and showed the role of MIR503 in regulation of genes associated with cell adhesion and migration.

In order to identify potential mechanisms behind MIR503HG effect on EndMT, we investigated the roles of miR-503 and miR-424 genes, which overlapped the MIR503HG locus and found MIR503HG acting independently from the miRNAs. Further, we are currently carrying out pull down experiment with biotinylated antisense probe combined with Mass Spectrometry to identify direct protein binding partners of MIR503 and understand miR503HG mechanism of action.

Overall, our results show MIR503HG as a novel regulator of EndMT and suggest potential mechanism of its action.

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### Effects of Diabetes on Microcirculation in Non-Ocular Tissues: Insights into Hemodynamic Alterations in Diabetic Retinopathy

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**Objective:** To investigate whether diabetes-induced changes in microcirculation in non-ocular tissues are correlated to retinal vascular lesions associated with diabetic retinopathy.

**Methods:** To identify whether changes that develop in microcirculation in different tissues in diabetes correspond to vascular changes in diabetic retinas, intravital microscopy was performed in post-capillar venules of cremaster muscle and ear lobe of mice with diabetes and compared to non-diabetic control mice. Specifically, number and velocity of rolling leukocytes, number of adherent leukocytes, and areas of leukostasis were quantified. In parallel, retinal capillary networks isolated from these animals were examined for retinal vascular lesions, acellular capillaries and pericyte loss, two histological hallmarks of diabetic retinopathy.

**Results:** Intravital microscopy imaging data revealed that cremaster muscle and ear lobe capillaries in the diabetic rats showed a significant increase in the number of rolling leukocytes and a decrease in their rolling velocities when compared to those of non-diabetic control rats. Additionally, mice with severe diabetes (>350 mg/dl) exhibited greater changes compared to those of less severe diabetic rats. Similarly, increased areas of leukostasis in the cremaster and ear lobe capillary beds of diabetic rats compared to those of non-diabetic rats were observed. Interestingly, the presence of acellular capillaries and pericyte loss was most pronounced in those diabetic mice with highest level of changes in the cremaster and ear lobe microcirculation.

**Conclusions:** These results suggest that the extent to which microcirculation changes develops in cremaster and ear lobe capillaries under diabetic conditions could be valuable for predicting vascular changes seen in diabetic retinopathy.

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### Long Term Cardiac and Vascular Changes in Women with a History of Hypertensive Disorders of Pregnancy: A Case-Control Study

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**Background and Aims:** The risk of cardiovascular disease (CVD) increases in women with a history of pre-eclampsia and other hypertensive disorders of pregnancy (HDP). Although mechanisms exploring this association are poorly understood, a reduction in skin capillary density known as capillary rarefaction (CR) and a reduction in tissue perfusion has been proposed in its pathogenesis. Previously, studies have shown CR to occur in individuals with essential hypertension and it has also been shown to accurately predict the onset of pre-eclampsia. Hence, this study aimed to investigate whether persistent capillary rarefaction, as well as raised central haemodynamics and arterial stiffness post HDP, act as a significant risk factors for the development of CVD in the future.

**Methods:** This project was based at a university hospital trust and it received a favourable ethics approval. We recruited 35 women (mean age  $37 \pm 6$  years) using the Foetal Medicine Unit database. Out of these, 30 women had a previous history of HDP (18 with pre-eclampsia and 12 with gestational hypertension) and 5 were non-pregnant healthy controls. We used non-invasive intravital capillaroscopy to quantify skin capillary density on the dorsum of the middle finger. We also used Omron HEM 9000AI for pulse wave analysis (PWA) to measure central systolic blood pressure (cSBP) and arterial stiffness parameters including aortic augmentation index and pulse pressure.

**Results:** PWA showed a higher central systolic ( $122 \pm 22$  mm Hg vs  $103 \pm 13$  mm Hg,  $p = 0.003$ ), systemic systolic ( $119 \pm 14$  mm Hg, vs  $109 \pm 18$  mm Hg  $p = 0.007$ ) and diastolic blood pressures ( $76 \pm 9$  mm Hg vs  $67 \pm 17$  mm Hg,  $p = 0.043$ ) in HDP group, compared to controls. A strong correlation was observed between cSBP and arterial stiffness parameters, including pulse pressure ( $p = 0.033$ ) and heart-rate corrected aortic augmentation index ( $p \leq 0.001$ ). However, no significant changes were observed in capillary density between groups or in relation to the number of years after pregnancy ( $2.5 \pm 2.5$  years).

**Conclusion:** Women with previously hypertensive pregnancies are more likely to have raised systemic arterial stiffness as a result of increased central blood pressure. cSBP's significant association with AIx thereby increases the risk of future CVD, compared to controls. Furthermore, it is important to reproduce CR findings in a larger cohort of patients before it is representative of the HDP population. In the future, we hope our observations can potentially be used as a clinical predictor of future cardiovascular events in high risk individuals post HDP.

## Nitric Oxide Maintains Endothelial Redox Homeostasis Through PKM2 Inhibition

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**Introduction/Objective:** Decreased nitric oxide (NO) bioavailability and oxidative stress are hallmarks of endothelial dysfunction and cardiovascular diseases and although numerous proteins can be potentially S-nitrosated, whether and how changes in their S-nitrosation influence endothelial function under pathophysiological conditions remains unknown. Thus, the aim of this study was to interrogate the endothelial NO synthase (eNOS) interactome for novel binding partners and potential S-nitrosation targets that could contribute to cellular redox regulation and to the NO-mediated protection of the vascular wall against atherogenesis.

**Results:** Pyruvate kinase M2 (PKM2) interacts with active eNOS and is S-nitrosated in vitro and in vivo. As the redox sensitive phosphorylation of eNOS on Y657 was shown to abrogate eNOS activity, Tyr657 mutants and a novel non-phosphorylatable Y656F-eNOS knock-in mouse were used to demonstrate that the extent of PKM2 S-nitrosation is closely linked to the ability of eNOS to generate NO in vitro and in vivo. PKM2 is inhibited by S-nitrosation, thereby supporting the accumulation of reducing equivalents to attenuate nitrosative stress in vitro and in vivo and delaying cardiovascular disease development. In addition, PKM2 pharmacological inhibition exerts significant anti-inflammatory effects in endothelial cells.

**Conclusions:** These findings highlight a novel mechanism linking preserved NO bioavailability to maintained endothelial cell redox homeostasis through S-nitrosation and inhibition of PKM2.

## Epigenetic Regulation of the Glomerular Endothelial Glycocalyx by Enhancer of Zeste Homolog-2 (EZH2) Histone Methyltransferase in Diabetic Nephropathy

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In diabetic nephropathy (DN), the glomerular endothelial glycocalyx is impaired. Injury to the glycocalyx evokes proteinuria and kidney failure. In an epigenetic screen we found the polycomb group methyltransferase Enhancer of Zeste Homolog 2 (EZH2) to be involved in DN. EZH2 inhibits expression of its target genes through methylation of lysine 27 on histone 3 (H3K27Me3). As the glycocalyx is impaired in DN, we hypothesize that EZH2 activity is increased in the glomerular endothelium in DN thereby reducing glycocalyx synthesis.

H3K27me3 was analyzed in glomerular endothelial cells by immunofluorescence in BTBRob/ob mice, a DN mouse model. Endothelial glycocalyx in these mice was analyzed by measuring binding of fluorescently-labeled lycopersicon esculentum agglutinin (LEA), overlapping with CD31 immunofluorescence staining. In glomerular endothelial cells, EZH2 was silenced by RNAi. Gene expression was assessed by Quantitative Real-time PCR. H3K-27me3-enrichment was measured by Chromatin Immunoprecipitation. Thickness of the glomerular endothelial glycocalyx in vitro was measured by binding of fluorescently-labeled LEA.

H3K27me3 in glomerular endothelial cells was increased 1.5-fold compared to non-diabetic mice ( $p = 0.026$ ). Urinary albumin-creatinine ratios of BTBRob/ob mice correlated with the increase in H3K27me3 ( $p = 0.044$ ;  $r_2 = 0.674$ ). A 2-fold loss of glomerular endothelial glycocalyx was observed in BTBRob/ob mice ( $p = 0.002$ ). In vitro, silencing of EZH2 in glomerular endothelial cells led to a decrease in H3K27me3 and an increase in 26 glycocalyx-associated genes. H3K27me3-enrichment analysis showed direct regulation of glycocalyx-associated genes by EZH2 via H3K27me3. Silencing of EZH2 increased the thickness of the glomerular endothelial glycocalyx 1.4-fold ( $p < 0.0001$ ). Preliminary data in human DN biopsies shows an increase in H3K27me3 in glomerular endothelial cells compared to healthy controls. In conclusion, our data suggest that EZH2-mediated epigenetic changes reduce endothelial glycocalyx in DN.

## Single Cell RNA-Sequencing Reveals Plasmalemma Vesicle-Associated Protein (PLVAP) as a Novel Target with a Role in New Blood Vessel Formation in the Ischaemic Heart

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**Introduction:** Ischaemic heart disease remains the leading cause of death worldwide. Restoration of a functional vascular network in the ischaemic border region may enhance myocardial perfusion, limit infarct expansion and promote cardiac regeneration. However, the pathways underpinning neovascularogenesis following myocardial infarction (MI) remain unclear. We aimed to investigate the single cell transcriptional profiles of pro-angiogenic resident endothelial cells (EC) in the adult mouse heart following MI, and to validate the expression of potential novel targets in cardiac tissue from patients with ischemic heart disease.

**Methods:** MI was induced in endothelial-specific lineage-tracing 'Confetti' mice (Pdgfb-iCreERT2-R26R-Brainbow2.1) by permanent ligation of the left anterior descending coronary artery. Ischaemic ventricles (and uninjured controls, n = 4 per group) were dissociated to a single cell suspension and EC were isolated by FACS for single cell RNA-sequencing using the 10X Chromium system. Immunofluorescence staining was used to quantify target expression in healthy and ischaemic mouse and human cardiac tissue.

**Results:** Blood vessel formation via clonal proliferation by Pdgfb-lineage EC was significantly upregulated in the ischaemic border at 7 days post-MI, compared to the healthy heart (Pdgfb-Confetti+ EC per clone =  $4.5 \pm 3.3$  versus  $10.3 \pm 10.6$ ,  $P < 0.0001$ ). Bioinformatics analyses revealed 10 transcriptionally discrete heterogeneous EC clusters in the ischaemic heart at 7 days post-MI and defined the transcriptional hierarchy through which each cluster was likely to mediate neovascularogenesis following MI. Plasmalemma Vesicle-Associated Protein (PLVAP) gene expression was upregulated in MI in a cluster specific manner, indicating its potential relevance to neovascularogenic pathways. Further, pseudotime trajectory analysis revealed that PLVAP-expressing clusters

appeared to initiate a hierarchical fate transition of EC gene expression in response to injury. PLVAP expression was EC-specific and was significantly higher in the infarct border of the post-ischaemic mouse heart compared to the healthy heart (% PLVAP+ EC =  $70.5 \pm 19.9\%$  versus  $38.7 \pm 28.2\%$ ,  $P = 0.002$ ). PLVAP expression was also significantly increased in EC adjacent to regions of fibrosis and scarring in the ischaemic human heart, compared to healthy human hearts (% PLVAP+ EC =  $36.9.8 \pm 10.1\%$  versus  $12.7 \pm 12.1\%$ ,  $P = 0.02$ ).

**Conclusion:** We have generated a single cell gene expression atlas of resident cardiac EC in the healthy and 7-day ischaemic mouse heart, which may guide future therapeutic strategies aimed at enhancing cardiac neovascularogenesis and myocardial regeneration. PLVAP is an exciting new target which may be important to augment endogenous myocardial perfusion following ischaemia.

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## Distinct Atrial Natriuretic Peptide-Induced cGMP Signalling Pathways in Astrocytes and Pericytes Attenuate Retinal Pathological Neovascularization

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**Introduction:** Pathological retinal neovascularization is a main cause of blindness in diabetic patients. Regression of the normal vasculature is followed by hypoxia-driven induction of proangiogenic factors, triggering overgrowth of weak and leaky vessels. These changes result from an altered interplay between endothelial cells, pericytes and astrocytes. Studies with exogenous atrial natriuretic peptide (ANP) suggested a protective therapeutical potential. Whether the endogenous hormone regulates these pathological changes is unknown.

**Methods:** To dissect the cell-specific actions of ANP we studied neonatal mice with global or cell-restricted deletions (KO) of its cGMP-forming guanylyl cyclase (GC)-A receptor in the model of oxygen-induced retinopathy (OIR).

**Results:** Global GC-A KO mice showed stronger vessel regression under hyperoxia (at P12), followed by markedly enhanced formation of vascular tufts (P17), indicating that a local ANP/GC-A pathway attenuates pathological vascular regression and neovascularization. Intriguingly, although ANP regulates peripheral vascular endothelial regeneration, endothelial-specific GC-A inactivation did not alter the vascular changes accompanying OIR. Our in vitro studies revealed ANP/GC-A/cGMP signalling in astrocytes and pericytes. To investigate the role in vivo, we established two novel genetic mouse models with restricted deletion of the GC-A receptor in astrocytes or pericytes.

Astrocyte-restricted GC-A deletion provoked mildly but significantly enhanced neovascularization. Remarkably, astrocyte densities within and around the vascular tufts were unaltered. This

suggested that the loss of GC-A/cGMP signaling did not alter astrocyte proliferation or viability but the communication from astrocytes to endothelial cells. Our studies with cultured astrocytes corroborated this hypothesis showing that synthetic ANP, via GC-A, markedly inhibits hypoxic VEGF induction.

Specific ablation of the GC-A receptor in pericytes induced a very strong phenotype with extensive vascular regression (at P12), followed by markedly enhanced hypoxic neovessel formation (at P17). Of note, the density of pericytes within the zone of neovascularization, especially around and within the neovascular tufts, was diminished in such pericyte GC-A KO mice. This indicates that the loss of GC-A/cGMP signalling decreases pericyte viability and/or enhances their susceptibility to apoptosis. Indeed, our studies in cultured pericytes demonstrated that ANP/GC-A/cGMP stimulation results in activation of a protective pathway that includes Akt/Bcl-2 signalling and prevents TGF- $\beta$ -induced pericyte apoptosis.

**Conclusions:** Our observations demonstrate that endogenous ANP modulates the communication between endothelial cells and surrounding cell types. The endogenous hormone inhibits the excessive hypoxia-driven VEGF-mediated astrocyte-to-endothelial cross-talk and protects pericytes from apoptosis. These cell-specific effects can attenuate endothelial hyperproliferation and neovascularization in vascular retinal diseases.

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### Endothelial-Specific Deletion of Autophagy Protein 5 Attenuates Ischemia-Related Angiogenesis

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Autophagy is an intracellular clearance and recycling process, allowing cells to uphold homeostasis and survival under stressful conditions. Pathological angiogenesis, such as exuberant retinal neovascularization during vision-threatening proliferative retinopathies, is tightly linked to endothelial responses to ischemia/hypoxia and oxidative stress. Here, we addressed the role of the autophagy-related gene *Atg5* in endothelial cells in the context of pathological ischemia-related neovascularization in a mouse model of oxygen dependent neovascularization, recapitulating human retinopathy of prematurity (ROP).

In comparison to WT littermates, mice with endothelium specific deletion of the autophagy essential gene *Atg5* (EC-*Atg5*<sup>-/-</sup>) that were subjected to the ROP model had starkly reduced pathological angiogenesis, reflected in attenuated neovascular tuft formation, lowered EC proliferation and upregulated EC apoptosis. At the same time, EC-*Atg5*<sup>-/-</sup> mice showed no alterations in retinal vascularization when allowed to develop normally, suggesting a specific Role of EC autophagy in angiogenesis under oxidative stress. Consistently, EC specific *Atg5* deletion potently impaired VEGF mediated sprouting activity in an aortic ring assay, when oxidative stress was induced through hypoxia/reoxygenation treatment (H/R), but not in normoxia. In Endothelial cells isolated

from EC-*Atg5*<sup>-/-</sup> mice (EC-*Atg5*<sup>-/-</sup> MLEC) we found increased apoptosis and several mitochondrial alterations, such as disturbed H/R-induced mitochondrial turnover, decreased mitochondrial respiration and reduced mitochondrial reactive oxygen species (mROS) production. Moreover, *Atg5*<sup>-/-</sup> MLEC had decreased phosphorylation of receptor tyrosine kinases (RTKs) involved in endothelial cell survival, such as endothelial growth factor receptor 2 and platelet-derived growth factor receptor. Interestingly, *Atg5*<sup>-/-</sup> MLEC exhibited reduced inactivation of ROS-sensitive protein tyrosine phosphatases (PTPs), a potential cause of reduced RTK signaling in these cells.

Our data suggest that endothelial *Atg5* sustains mitochondrial function and thereby promotes endothelial survival in the context of pathological hypoxia/reoxygenation-related neovascularization. Endothelial *Atg5* therefore represents a potential target for the treatment of pathological neovascularization-associated diseases, such as retinopathies.

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### Microvascular Function and Cardiometabolic Diseases

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Microvascular and metabolic physiology are tightly linked. This Lecture will review evidence 1) that the relationship between hyperglycemia and microvascular dysfunction (MVD) is bidirectional and constitutes a vicious cycle; 2) that MVD in diabetes affects many if not all organs, which may play a role in diabetes-associated comorbidities such as depression and cognitive impairment; and 3) that MVD precedes, and contributes to, hyperglycemia in type 2 diabetes (T2D) through impairment of insulin-mediated glucose disposal and, possibly, insulin secretion. Obesity and adverse early life exposures are important drivers of MVD. MVD can be improved through weight loss (in obesity) and through exercise. Pharmacological interventions to improve MVD are an active area of investigation.

### Exercise Regulates DNA Methylation of p66Shc Gene and Improves Retinal Microvascular Phenotype: A Cross-Sectional and Randomized Controlled Trial (EXAMIN AGE)

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**Background:** Reactive oxygen species (ROS) are major determinants of vascular aging. The mitochondrial adaptor p66Shc is a key driver of aging-induced ROS production and DNA methylation of p66Shc promoter affects its expression. Vascular aging and worse cardiovascular (CV) outcome are associated with narrower retinal arterioles and wider venules. The role of exercise in the epigenetic regulation of oxidative stress remains unclear. This study was designed to investigate whether exercise affects p66Shc expression, oxidative stress and retinal microvascular phenotype in healthy and diseased aging individuals.

**Methods:** Out of 158 subjects (mean age 59.4 ± 7.0 years) included in the study, 38 were healthy active (HA), 36 healthy sedentary (HS) and 84 sedentary at increased CV risk (SR). The SR group was randomized into a 12-week high intensity interval training (HIIT) or standard physical activity recommendations. Retinal arteriolar and venular diameters and the arteriolar-to-venular diameter ratio (AVR) were measured by use of a static retinal vessel analyzer. Plasma 3-nitrotyrosine (3-NT) was measured by ELISA. Gene expression of p66Shc and DNA methylation analysis were assessed in peripheral blood mononuclear cells by RT-qPCR and Methylminer qPCR.

**Results:** HA had wider arteriolar and narrower venular diameters compared to HS and SR. By contrast, SR showed wider venular diameters and a lower AVR. Increased p66Shc expression and subsequent high levels of 3-NT were associated with promoter hypomethylation in HS and SR. Interestingly enough, HIIT improved microvascular phenotype and restored methylation of p66Shc promoter blunting its expression and 3-NT levels.

**Conclusion:** Exercise-induced epigenetic reprogramming of p66Shc gene may protect against aging-related oxidative stress and impaired microvascular phenotype with the potential to postpone the process of vascular aging in the elderly.

### Epigenetic Changes, Oxidative Stress and Endothelial Dysfunction in Diabetes

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Patients with diabetes are at high risk of atherosclerotic cardiovascular disease (CVD). Diabetes-induced hyperglycaemia triggers vascular oxidative stress and inflammation, key hallmarks of endothelial cell dysfunction, leading to a pro-inflammatory and pro-atherogenic environment. Several trials focused on preventing CVD complications by intensive glycaemic control (IGC). So far, targeting HbA1c has not shown clear-cut beneficial effects on reducing cardiovascular events; however, the mechanisms behind these observations are not yet fully understood. Epigenetic modification of chromatin has emerged as a master regulator of transcriptional programs implicated in oxidant and inflammatory pathways and, therefore, may play key regulatory roles in this setting. Whether insulin resistance and glucose fluctuations influence chromatin function remains unknown. We sought to investigate the interplay among glycaemic variability, epigenetic derangement and vascular dysfunction in patients with type 2 diabetes (T2D), characterized by an early development of atherosclerotic vascular lesions, before and after IGC treatment according to guidelines. We found that persistent dysregulation of epigenetic machinery was independently associated to endothelial dysfunction in patients with T2D despite being on target HbA1c levels. Future studies evaluating the epigenetic landscape in patients treated with newer classes of antidiabetic drugs will undoubtedly deserve further exploration.

### Cerebral Hemodynamics and Microcirculatory Function in Patients with Systemic Lupus Erythematosus

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**Background:** Systemic lupus erythematosus (SLE) is a chronic inflammatory disease characterized by multiple organ involvement. Atherosclerosis is the underlying cause for SLE related cardiovascular disease. Reliable non-invasive methods for early detection of vascular involvement including microcirculatory assessment is important. The aim of this study was to detect if macro- and microcirculation is impaired in patients with SLE.

**Methods:** 15 SLE patients (out of 60 investigated SLE-patients), (mean age 51.4 years) with moderate atherosclerotic ultrasound findings in common carotid artery, and 15 individually age and sex matched population controls, (mean age 51.7) were investigated.

Intima-media thickness (IMT) was recorded with high frequency ultrasound (GE Logic E9) in carotid and central arteries.

Microcirculatory oxygen saturation and endothelial function were assessed with EPOS (Enhanced Perfusion and Oxygen Satu-

ration; PeriFlux 6000, Perimed, Järfälla, Sweden) and EndoPAT2000; Itamar Medical, Israel) systems, respectively. The EPOS system measures red blood cell tissue fraction, speed resolved perfusion and oxygen saturation in the skin microcirculation in absolute units. EndoPAT 2000 records changes in finger arterial pulsatile volume, as the reactive hyperemia index, RHI, reflecting microcirculatory endothelial function. A  $\ln(\text{RHI}) \leq 0.51$  indicates impaired endothelial function.

Cerebrovascular reserve capacity was assessed by Transcranial Doppler (TCD) (Sonara TCD; Natus) by mean flow velocities in middle cerebral artery baseline and after 30 seconds of breath holding. A breath-hold-index (BHI) of  $<0.69$  implies impaired cerebrovascular reserve capacity.

**Results:** A significant difference between the patient group and controls was seen in IMT in the aortic arch,  $1.3 \pm 0.3$  vs  $1.1 \pm 0.2$  mm, ( $p = 0.04$ ) whereas no significant difference was seen between the groups in IMT in the common carotid artery,  $0.61 \pm 0.13$  vs  $0.55 \pm 0.10$  mm, ( $p = 0.2$ ).

A significant difference between the groups with lower BHI-values were seen in the SLE-group  $1.29 \pm 0.36$  vs  $1.65 \pm 0.56$ , ( $p = 0.05$ ) whereas both groups had preserved cerebrovascular reserve capacity.

Peak oxygen saturation after ischemia was lower in SLE patients than in controls,  $79.5 \pm 7.8\%$  vs  $86.9 \pm 5.6\%$ , ( $p = 0.006$ ).

Endothelial function using EndoPAT did not differ,  $\ln(\text{RHI}) = 0.72 \pm 0.40$  vs  $0.84 \pm 0.24$ , ( $p = 0.3$ ).

**Conclusion:** This study indicates that microcirculatory vessel disease could be present in patients with SLE with atherosclerotic findings. The impaired microcirculation measured with EPOS and lower values of breath-hold index in the patient group compared to the healthy controls needs further validation in larger patient groups also including non-atherosclerotic SLE-patients.

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### Antagonism of Prostaglandin Signaling Involving FP-Receptors Inhibits the Evolution of Spreading Depolarization in Cerebral Ischemia

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**Introduction:** Spontaneous, recurrent spreading depolarizations (SD) are increasingly more appreciated as the pathomechanism behind delayed ischemic brain injuries. The inhibition of the FP receptor of prostaglandin F<sub>2a</sub> was shown to limit secondary neuronal damage during brain ischemia. Therefore, we set out to test the hypothesis that the neuroprotection by FP receptor blockade is achieved by the inhibition of SD.

**Methods:** Global forebrain ischemia was induced in isoflurane-anesthetized, young, adult, male Sprague-Dawley rats ( $n = 16$ ) by the bilateral occlusion of the common carotid arteries. Two open craniotomies on the right parietal bone served the elicitation of SD with 1M KCl (caudal), and the acquisition of local field potential (rostral). The entire dorsal cranium was thinned to track regional cerebral blood flow (CBF) variations by laser speckle con-

trast imaging. The femoral artery was prepared for the monitoring of mean arterial pressure (MAP) and for sampling for blood gas analysis. The femoral vein was used for the infusion of an FP receptor antagonist (AL-8810; 1 mg/bwkg) or its vehicle (0.1% dimethyl sulfoxide, DMSO).

**Results:** Physiological parameters were similar in the two groups (e.g. MAP:  $82.7 \pm 8$  vs.  $84.5 \pm 9.1$  mm Hg; AL-8810 vs. control). However, AL-8810 markedly reduced the duration of evoked SDs ( $36 \pm 14$  vs.  $56 \pm 15$  s; AL 8810 vs. vehicle). In addition, total depolarization time was reduced by 50% in the AL-8810 group (1339 vs. 2589 s, AL-8810 vs. vehicle). The CBF response to SD involved a more restricted cortical surface in the AL-8810-treated animals. Additionally, the amplitude of the post-SD oligemia was smaller in the AL-8810 group ( $7.8 \pm 3.5$  vs.  $9.8 \pm 4$  pp; AL-8810 vs. control), while the amplitude of reactive hyperemia after reperfusion was substantially higher ( $94.9 \pm 20$  vs.  $79.7 \pm 16\%$ ; AL-8810 vs. control).

**Conclusion:** In summary, the antagonism of FP receptors (located in the vascular wall or neurons) emerges as a promising approach to inhibit the evolution of injurious SDs in cerebral ischemia. Further studies should address, whether the volume of the ischemic infarct is reduced accordingly by this intervention.

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### Identification of Scorpion Venom-Derived Peptides Inhibiting Vascular Endothelial Growth Factor (VEGF)-Mediated Angiogenesis

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**Introduction:** Vascular endothelial growth factor (VEGF) plays an important role in both physiological and pathological angiogenesis through binding to its receptor (VEGFR). Therefore, the disruption of VEGF-VEGFR system is a promising target for anti-angiogenic therapy for treatment of cancer and diabetic nephropathy. Peptides have merged as important therapeutics in anti-angiogenic therapy due to their low toxicity, flexible to modification and high specificity. In the present study, we identified several scorpion-venom derived peptides blocking VEGF-VEGFR interaction could be useful as an alternative antiangiogenic agents.

**Methods and Results:** A set of 34 overlapping peptides spanning the entire sequence of mauriporin (a cationic  $\alpha$ -helical peptide with 48-mer isolated from scorpion venom in *Androctonus mauritanicus*) was identified. Each peptide contains 15-amino acid residues with one residue overlapping with the adjacent peptide. The experimental data from three protein-peptide dock web servers (CABS-dock, HPEPDOCK and GalaxyWEB) suggest that 10 peptides derived from the N-terminal sequence of mauriporin, have a high affinity towards VEGFR2 binding site and thus blocking the VEGF-VEGFR interaction. Among these, the M8 peptide with the most potent antiangiogenicity was chosen for further amino acid modification to have better antiangiogenic activity. With these, 5 new peptides (M8.1-M8.5) are derived from the parental M8 peptide.

The computation studies revealed that all these new peptides (M8.1-M8.5) have shown better affinity towards VEGFR2 binding

site, thus demonstrated their strong antiangiogenic activity than its parental M8 peptide. In addition, these new peptides also found to possess antimicrobial and anticancer activities with low toxicity and hemolytic activity using several computational biology models. The de-novo structural characterization of these peptides reveals these new peptides adopting  $\alpha$ -helical structures which may contribute to its capacity to perforate cell membrane and induce cell death. From our preliminary in-vitro studies, these peptides reduced proliferation of a VEGF-dependent endothelial cell line and expression of metastasis-associated protein such as Slug and Snail in both highly metastatic MDA-MB-231 and PC-3M cell lines.

**Conclusion:** We utilized advanced computational biology simulation approaches to predict, identify and verify potential antiangiogenic, antimicrobial and anticancer activities of peptides derived from scorpion venom. These peptides have high affinity towards VEGFR2 binding site and result in the induction of anti-angiogenesis responses. Future studies should continue to investigate the in-vitro and in-vivo therapeutic potentials and its underlying mechanisms of actions.

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### Acute Aerobic Exercise Increases Systemic Skin Microvascular Functional Responses to Post-Occlusive Reactive Hyperaemia

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**Background:** Chronic exercise causes physiological adaptation throughout the entire vascular tree, including in the cutaneous microvasculature. The acute changes which accompany a single exercise bout, however, are still relatively unknown. Such changes are important to consider, as the accumulation of these episodes may form the basis for chronic adaptations to exercise. Furthermore, there is a paucity of research investigating the local versus systemic effects of exercise on skin microvascular function. Post-occlusive reactive hyperaemia (PORH) provides an insight into combined endothelium-dependent and -independent mechanisms of skin microvessel control, providing a 'global' perspective of skin microvessel functioning.

**Methods:** Participants ( $n = 10$ ,  $26 \pm 6$  years) attended 3 experimental sessions whereby PORH was performed before (PRE), immediately-after (IMM) and 1 hour-after (1HR) a 30-minute bout of low-intensity (50% HRMAX, LOW), high-intensity (75% HRMAX, HIGH), or no-exercise (chair sitting, CON). Exercise was performed on a cycle ergometer and titrated to HRMAX as determined by prior VO<sub>2</sub>max assessment. PORH protocol: Skin microvessel blood flux was measured using laser Doppler flowmetry (LDF, perfusion units, PU) at the volar forearm (ARM) and lateral calf (CALF), and indexed as cutaneous vascular conductance (CVC = PU/mean arterial pressure, PU.mm Hg-1). Suprasystolic occlusion was performed for 5 minutes on the non-dominant arm and thigh simultaneously. LDF monitoring occurred be-

fore, during, and for 10 minutes after cuff release. Peak blood flux per site was defined as the highest second average flow following release (PEAK). PORH was defined as the area-under-the-curve (AUC) for 3 minutes following cuff-release, and can be adjusted to control for baseline AUC (PORH.adj).

**Results:** Regional differences were observed in all measures whereby ARM showed significantly greater responses than CALF ( $P < 0.001$ ).

In ARM, PEAK increased post-exercise (LOW: PRE  $1.14 \pm 0.2$  vs IMM  $1.50 \pm 0.3$  PU.mm Hg-1,  $P = 0.059$ . HIGH: PRE  $1.19 \pm 0.2$  vs IMM  $1.78 \pm 0.3$  PU.mm Hg-1,  $P = 0.022$ ; PRE vs 1 HR  $1.69 \pm 0.2$  PU.mm Hg-1,  $P = 0.016$ ). PORH response significantly increased only with HIGH (PRE  $124.6 \pm 21$  vs IMM  $207.2 \pm 36$  PU.mm Hg-1.s-1,  $P = 0.01$ ; PRE vs 1 HR  $183.7 \pm 26$  PU.mm Hg-1.s-1,  $P = 0.029$ ). PORH.adj showed no significant change at any stage post-exercise.

In CALF, PEAK showed a trend towards an increase after HIGH (PRE  $0.68 \pm 0.1$  vs IMM  $0.88 \pm 0.2$ ,  $P = 0.063$ ; PRE vs 1 HR  $0.97 \pm 0.2$ ,  $P = 0.055$ ). PORH showed significant increases following HIGH (PRE  $77.2 \pm 15$  vs IMM  $102.2 \pm 22$  PU.mm Hg-1.s-1,  $P = 0.1$ ; PRE vs 1 HR  $116.8 \pm 22$  PU.mm Hg-1.s-1,  $P = 0.046$ ). PORH.adj showed no significant change post-exercise.

**Conclusion:** Acute aerobic exercise increases skin microvascular function for up to 1 hour post-exercise at both active and inactive limb sites, with intensity of exercise as a possible dependent factor.

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### Effects of Acute Postprandial Hyperglycemia on Microvascular Endothelial-Dependent Vasodilation

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**Introduction:** High consumption of carbohydrates is known to be associated with the development of cardiovascular diseases (CVD). In addition, epidemiological studies suggest that postprandial hyperglycemia better predicts future CVD morbidity and mortality compared with fasting hyperglycemia in both dysglycemic and normoglycemic individuals. Moreover, macrovascular endothelial dysfunction is known to be transiently induced by sugar consumption in both healthy individuals and patients with metabolic syndrome. The main purpose of the study was to evaluate the acute effects of a single high-carbohydrate (H-CHO) meal on microvascular function of healthy individuals.

**Methods:** Endothelium-dependent microvascular reactivity of healthy volunteers (aged  $27 \pm 5$  years,  $n = 19$ ) was evaluated using cutaneous laser speckle contrast imaging (LSCI) before and 25 min after administration of the H-CHO meal containing 70 g of carbohydrates (408 kcal). LSCI was coupled with the cutaneous iontophoresis of acetylcholine (ACh) using increasing anodal micro currents (30–180  $\mu$ A); post-occlusive reactive hyperemia (PORH) was induced by arterial occlusion for three minutes. The measurements of skin blood flow (arbitrary perfusion units, APU) were divided by values of mean arterial pressure to give the cutaneous

vascular conductance (CVC) in APU/mm Hg. The study protocol was approved by the IRB of our Institution (CAAE 86854318.8.0000.5272) and was registered at ClinicalTrials.gov (NCT03515460). Statistical analyses were performed using two-tailed paired t tests.

**Results:** Volunteers of both sexes (male gender 42%) presented basal values of body mass index of  $24.5 \pm 3.1$  kg/m<sup>2</sup>, fasting glycemia  $84.1 \pm 6.3$  mg/dL, glycosylated hemoglobin  $5.4 \pm 0.4\%$  and plasma insulin  $7.46$  (5.92–11.41)  $\mu$ IU/mL. Plasma glucose levels peaked 25 min after ingestion of the H-CHO meal ( $144.2 \pm 11.4$  vs.  $79.8 \pm 6.6$  mg/dL;  $P = 0.0035$ ). Maximum values of CVC induced by ACh iontophoresis increased after H-CHO (from  $0.49 \pm 0.2$  to  $0.59 \pm 0.2$  APU/mm Hg;  $P = 0.0005$ ). The increase in CVC (peak minus baseline) improved from  $0.23 \pm 0.2$  to  $0.32 \pm 0.2$  APU/mm Hg;  $P = 0.0044$ . The area under the curve of vasodilation induced by ACh also increased from  $16,574 \pm 4,152$  to  $19,806 \pm 6,924$  APU/sec ( $P = 0.0052$ ). Maximum CVC ( $0.71 \pm 0.2$  versus  $0.76 \pm 0.2$  APU/mm Hg;  $P = 0.1844$ ) and increase in CVC ( $0.38 \pm 0.2$  versus  $0.44 \pm 0.2$  APU/mm Hg;  $P = 0.1348$ ) induced by PORH did not differ after H-CHO meal.

**Conclusions:** The ingestion of a high-carbohydrate meal does not induce acute systemic microvascular dysfunction in healthy individuals with no history of familial CVD. The improvement of endothelium-dependent microvascular vasodilation at the postprandial stage during mild increases in plasma glucose appears to be due to the release of insulin that occurs at the postprandial period in health individuals.

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### Penile Microvascular Endothelial Reactivity in Hypertensive Patients with Erectile Dysfunction: Effects of a Chronic Treatment with Sildenafil

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**Introduction:** The pathophysiology of erectile dysfunction (ED) in hypertensive patients is characterized by vascular endothelial dysfunction caused by reduced nitric oxide bioavailability and increased oxidative stress. Sildenafil citrate (SIL) is known to be effective and safe in patients with cardiovascular disease and to restore the penile vasculature vasodilation.

**Methods:** This was a prospective, randomized, double blinded and crossover study, controlled against placebo (PLA), with 30-day washout between treatments. Penile endothelium-dependent microvascular reactivity of hypertensive patients (aged  $57.4 \pm 5.6$  years,  $n = 50$ ) was evaluated using cutaneous laser speckle contrast imaging (LSCI) before and after chronic administration of PLA or SIL (50 mg twice daily during 30 days). LSCI was coupled with the cutaneous iontophoresis of acetylcholine (ACh) using increasing anodal microcurrents (30–180  $\mu$ A). The measurements of skin blood flow (arbitrary perfusion units, APU) were divided by values of mean arterial pressure to give the cutaneous vascular conductance (CVC) in APU/mm Hg. Erectile function was evaluated using the Simplified International Index of Erectile Function (IIEF-

5) score. 24 hour ambulatory blood pressure monitoring (ABPM) was performed using oscillometric devices (Spacelabs). The results were presented as the means  $\pm$  SD or medians (25th – 75th percentile) and analyzed using two-tailed paired Student's t-tests or Wilcoxon matched pairs tests.

**Results:** The basal values of CVC in the penis skin increased significantly after SIL ( $P = 0.004$ ), but not after PLA ( $P = 0.86$ ). Maximum CVC values after cutaneous administration of ACh increased only with SIL treatment ( $P = 0.001$ ; after PLA  $P = 0.63$ ). The area under the curve of penile microvascular vasodilation induced by ACh also significantly increased after SIL ( $P = 0.003$ ) but not after PLA ( $P = 0.98$ ). IIEF-5 scores significantly increased after SIL [18 (16–20) and 21.5 (19.75–23);  $P < 0.0001$ ] but not after PLA [18 (14.75–19.25) and 17.84  $\pm$  3.88;  $P = 0.16$ ]. Finally, 24 hours diastolic arterial pressure significantly decreased after SIL ( $83.5 \pm 8.9$  and  $79.9 \pm 8.2$  mm Hg;  $P = 0.0003$ ) but not after PLA ( $P = 0.27$ ). Systolic blood pressure reduced significantly in the office measurements after treatment with SIL [136 (130–144.3) and 131.5 (119–139.3);  $P = 0.02$ ] but not with PLA [136 (130.8–145) and 134.7  $\pm$  13;  $P = 0.23$ ].

**Conclusions:** Penile endothelium-dependent microvascular reactivity and erectile function improved after chronic treatment with sildenafil. The treatment also reduced blood pressure suggesting that, in addition to the effects on erectile function, chronic treatment with sildenafil could improve blood pressure control. Additionally, laser-based methods may well be valuable non-invasive tools for the evaluation of penile microvascular responses to drug treatment in patients presenting with cardiovascular diseases.

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### Mitochondrial Metabolism Limits ECM Formation in Human iPSC-Derived Endothelium

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Human induced pluripotent stem cells (iPSCs) are widely used for organogenesis, scaffold recellularization, patient specific cell therapy and for disease modelling. iPSCs have been differentiated into many cell types, including endothelium, however maturation and stabilization of iPSC derived endothelial cells (iPSC-ECs) remains challenging.

Here, we investigated functionality of iPSC-EC after exposing these cells to prolonged physiological shear stress and co-culture with pericytes, to provide an instructive environment for iPSC-EC maturation. Prolonged exposure to shear stress alone or in combination with pericyte co-culture, however, failed to induce func-

tional and structural maturation when compared to control microvascular endothelial cells. Furthermore, these cells hardly expressed a luminal glycocalyx critical for vasculature homeostasis, shear stress sensing and signalling. This phenotype was accompanied with a hampered mitochondrial function. As a result, iPSC-ECs produced more reactive oxygen species (ROS), while normal glycolysis and ATP levels were maintained.

Since efficient mitochondrial oxidative phosphorylation is a prerequisite for differentiation of stem cells into a functional endothelial cells, we induced closure of the mitochondrial membrane transition pore by cyclosporine-A. This resulted into increased mitochondrial activity, while lowering the ROS content. Furthermore, we observed an increase in the presence of extracellular matrix and improvement of iPSC-ECs alignment to flow. These findings indicate that mitochondrial maturation is necessary for iPSC-ECs functionality.

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#### **Microcirculation and Red Blood Cell Sensitivity to Nitric Oxide in 2 Type Diabetes Mellitus**

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The vascular effects of gasotransmitters are known (NO, CO and H<sub>2</sub>S), however, significantly less data are published on their regulatory influences on red blood cell (RBC) microrheological properties which are responsible for oxygen supply to tissue in the microvascular network. The aim of this study was to investigate the microcirculation state and red cell microrheology in type 2 diabetes mellitus (DM-2T) under NO alterations.

In patients with DM-2T, capillaroscopy and laser Doppler imaging revealed a decrease in the capillary density by 18% ( $p < 0.05$ ) and microvascular perfusion by 12%, combined with NO metabolism disorders. It was found that the concentration of its stable products of metabolism (NO<sub>x</sub>) was higher (by 56%,  $p < 0.05$ ) under DM-2T. RBCs and their restored ghosts (RGs) in healthy control and in patients with DM-2T were incubated with NO donors: sodium nitroprusside (SNP, 100  $\mu$ M), spermine-NONOate (S-NO, 10  $\mu$ M) and NO-synthase (NOS) substrate, L-arginine (100  $\mu$ M). After that, the deformability (RBCD) and the aggregation of RBCs (RBCA) were registered. It was found that SNP increased RBCD by 12% ( $p < 0.01$ ) in healthy control and only by 5.3% in DM-2T. S-NO showed a similar effect on RBCD, deformability changes were less notable in presence of L-arginine. RBCA was almost unchanged under action of all NO-donors in patients with diabetes. Stimulation of NO production in RBCs by L-arginine was accompanied by a decrease in RBCA. These differences in sensitivity of RBCs to the action of NO donors in norm and in DM-2T were confirmed in experiments with RGs. In response to the action of the SNP and L-arginine, the RG deformability increased up to 8% ( $p < 0.01$ ) in control, and markedly less in DM-2T. Thus, the data obtained indicate the disorders of nitric oxide metabolism in DM-2T.

The decrease in capillary density and microvascular perfusion effectiveness in DM-2T is combined with unfavorable changes of

RBC microrheology regulation. On intact cells and their restored ghosts it was found the decreased sensitivity of RBC to the NO action in diabetes. NO metabolism is disturbed and, probably, its bioavailability to endothelial cells and vascular smooth muscle cells is reduced. Pathological changes in microvascular blood circulation in DM-2T may realize in two ways: 1) by decrease in NO pool available for arterioles and, as a result, a reduction of their dilation potential and 2) by dimension of positive microrheological effects of NO on RBC.

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#### **Effect of Gasotransmitters on Erythrocyte Microrheological Properties in Non-Diabetic Subjects and in Patients with Type 2 Diabetes Mellitus**

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Cardiovascular complications are the leading cause of morbidity and mortality in patients with diabetes mellitus. The blood rheology abnormalities observable in diabetes increase peripheral vascular resistances and ischemia and therefore worsen diabetic vascular disorders. The rheological properties of plasma and blood cells are markedly influenced by the surrounding milieu. It is known that diabetes mellitus is accompanied by dysfunction of the vascular endothelium and the latter loses its ability to adequately synthesize vasodilators. However, little is known about the associations between hemorheological properties and gasotransmitters – endogenous vasodilators – in type 2 diabetes mellitus (T2DM). The aim of this study was to evaluate in vitro potential effect of gasotransmitters (NO and H<sub>2</sub>S) on erythrocyte microrheological properties in 21 diabetic patients compared to 20 non diabetic control subjects. Three times washed erythrocytes were incubated with sodium nitroprusside 50  $\mu$ M (NO donor) and NaHS 50  $\mu$ M (H<sub>2</sub>S donor). To estimate signaling pathways of gasotransmitters action we use pretreatment of RBC by inhibitor of soluble guanylate cyclase (ODQ, 0.5 mM) and blocker of ATP-dependent K-channels (glibenclamide 50  $\mu$ M). RBC aggregation was evaluated by means of Myrenne aggregometer, RBC deformability was determined as elongation index in flow microchamber. In control both gasotransmitters cause improvement of erythrocyte microrheological properties – cell deformability increased (up to 10%,  $p < 0.05$ ) and aggregation decreased – by 28%,  $p < 0.05$ . In T2DM patients effect was also positive but less pronounced – deformability increased by 7%,  $p < 0.05$ , aggregation reduced by 17%,  $p < 0.05$ . Microrheological effect of NO was almost fully neglected in presence of ODQ, while H<sub>2</sub>S influence was more sensitive to treatment by glibenclamide. Gasotransmitters are lipid-soluble, able to diffuse across cell membranes in a receptor-independent manner and activate various cellular targets. Usually gas targets are intracellular enzymes and ion channels, NO and H<sub>2</sub>S, being chem-

ically active, directly modify membrane and intracellular proteins, which leads to a change in their functions. We can propose that optimization of RBC microrheological properties by NO and H<sub>2</sub>S realize by different signaling mechanisms. Both of these gases are vasodilators; the possibility that a deficiency in the biosynthesis of these gases contributes to or predisposes to cardiovascular diseases is appealing. Thus our results indicate that impaired endothelium synthesis of vasodilators in diabetes not only reduces the vascular reserve, but also aggravates hemorheological disorders, causing disturbances in the blood supply to organs and tissues.

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### PDGF-B Retention Motif Deletion Has Mural Cell-Independent Effects Including Leukocytosis and Increased Murine Atherosclerotic Plaque Stability Due to Macrophage Apoptosis and Decreased MMP Activity

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**Introduction:** Hyperpermeability of intraplaque microvessels has been associated with plaque instability and rupture, although causality remains to be addressed. Platelet-derived growth factor B (PDGF-B) is responsible for the recruitment of pericytes towards blood vessels which is facilitated by binding of the PDGF-B retention motif to the extracellular matrix. Therefore, we hypothesized that intraplaque microvessel hyperpermeability could be mimicked with atherogenic PDGF-B retention motif knockout mice (PDGF-Bret/ret) and that this would result in exacerbated atherosclerosis.

**Methods:** PDGF-Bret/ret mice which were crossed with LDLR<sup>-/-</sup> mice received a 0.25% cholesterol diet for 10 weeks. Aortic root plaque size, necrotic core-, macrophage- (MOMA2), smooth muscle cell- ( $\alpha$ -SMA) and collagen content (Sirius red), microvessel density (CD31), intraplaque hemorrhage (Perls) and apoptosis (TUNEL) were assessed. Circulating leukocytes were analyzed by flow cytometry. Bone marrow-derived macrophage susceptibility to apoptosis after incubation with atherosclerosis-relevant stimuli and matrix metalloproteinase (MMP) activity were assessed with OmniMMP Fluorogenic substrate and Annexin-V, respectively. Plasma PDGF-B levels were assessed by ELISA.

**Results:** PDGF-Bret/ret plaque size was increased (+41%,  $p < 0.001$ ) as was collagen content (+25%,  $p < 0.01$ ), whilst necrotic core, microvessel density and intraplaque hemorrhage were unaffected. Unexpectedly, leukocytosis was observed in PDGF-Bret/ret mice as circulating total leukocyte levels and NK cell, monocyte, granulocyte and T cell levels were significantly increased. Leukocytosis could not be explained by increased circulating PDGF-B levels as these were significantly decreased in PDGF-Bret/ret mice (-87%,  $p < 0.0001$ ). Moreover, enhanced leukocytosis was not reflected in the plaque as macrophage content was decreased (-50%,

$p < 0.01$ ). A 2.1-fold increase in TUNEL<sup>+</sup> cells in PDGF-Bret/ret plaques ( $p = 0.0149$ ) and increased macrophage apoptosis upon incubation with the atherosclerosis-relevant stimuli 7-ketocholesterol ( $p < 0.0001$ ) and oxLDL ( $p = 0.031$ ) in vitro could explain reduced macrophage content. Moreover, decreased macrophage MMP activity (-25%,  $p = 0.0286$ ) could explain increased collagen content in PDGF-Bret/ret mice.

**Conclusion:** PDGF-B retention motif deletion results in larger but more stable plaques unrelated to microvessel permeability and differentially affects circulating and plaque-resident macrophage number, viability and function.

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### Traumatic Brain Injury-Induced Cerebrovascular Alterations

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Traumatic brain injury (TBI) impairs autoregulation of cerebral blood flow, which contributes to the development of secondary brain injury increasing mortality of patients. Impairment of pressure-induced myogenic constriction of cerebral arteries plays a critical role in autoregulatory dysfunction; however, the underlying cellular and molecular mechanisms are not well understood. To determine the role of mitochondria-derived H<sub>2</sub>O<sub>2</sub> and large-conductance calcium-activated potassium channels (BKCa) in myogenic autoregulatory dysfunction, middle cerebral arteries (MCAs) were isolated from rats with severe weight drop-impact acceleration brain injury (24 h post-impact). We found that post-TBI MCAs exhibited impaired myogenic constriction, which was restored by treatment with a mitochondria-targeted antioxidant, by scavenging of H<sub>2</sub>O<sub>2</sub> and by blocking BKCa channels and TRPV4 channels. Further, exogenous administration of H<sub>2</sub>O<sub>2</sub> elicited significant dilation of MCAs, which was inhibited by blocking BKCa and TRPV4 channels. In cultured vascular smooth muscle cells H<sub>2</sub>O<sub>2</sub> activated BKCa currents, which were inhibited by blockade of TRPV4 channels. Collectively, our results suggest that after TBI excessive mitochondria-derived H<sub>2</sub>O<sub>2</sub> activates BKCa channels via a TRPV4-dependent pathway in the vascular smooth muscle cells, which impairs pressure-induced constriction of cerebral arteries. Future studies should elucidate the therapeutic potential of pharmacological targeting of this pathway in TBI to restore autoregulatory function in order to prevent secondary brain damage and decrease mortality.

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## Clinical Aspects of Microvessel Dysfunction in Cardiometabolic Disease

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Clinical and epidemiological evidence indicates a strong relationship between obesity and cardiovascular diseases, including hypertension, coronary artery disease and heart failure. Clustering of these obesity-related metabolic disorders is referred to as cardiometabolic disease. Obesity impacts cardiometabolic disease directly through obesity-induced structural and functional adaptations of the heart, vessels and kidneys and through adipokine effects on inflammation and vascular function, and indirectly through co-existing cardiovascular disease risk factors including diabetes, dyslipidaemia, insulin resistance and hypertension. Cardiometabolic disease is characterised by endothelial dysfunction, vascular inflammation and structural remodeling of small vessels, processes that are influenced by surrounding perivascular adipose tissue (PVAT). PVAT produces many factors (adipokines), including hormones, such as Ang II and aldosterone, cytokines and reactive oxygen species (ROS), which actively participate in the regulation of vascular function and local inflammation by endocrine and/or paracrine mechanisms. As a result, the signaling from PVAT to the vasculature is emerging as a potential therapeutic target for obesity and associated vascular dysfunction. We described an important role for adipocyte-derived aldosterone in obesity-related microvessel dysfunction in cardiometabolic disease. Clinical and pre-clinical studies clearly demonstrated a positive relationship between plasma aldosterone levels and adiposity, and hyperaldosteronism is an important cause of hypertension and its cardiovascular complications. We showed that in obesity, PVAT-derived aldosterone induces abnormal production of adipokines, ROS production and systemic inflammation, which in turn contribute to impaired insulin signaling, reduced endothelial-mediated vasorelaxation, and associated cardiovascular abnormalities. Thus, obesity-associated aldosterone excess exerts detrimental metabolic and vascular effects that participate in the development of cardiometabolic disease. Here we will focus on the pathophysiological role of adipocyte-derived aldosterone and mineralocorticoid receptor signaling in the cross-talk between PVAT and the vasculature and implications in vascular dysfunction and remodeling in the context of obesity and cardiometabolic disease. We will also discuss the differential roles of white adipose tissue versus brown adipose tissue in the regulation of vascular function and the potential clinical implications.

## Endothelial Function and Coronary Microcirculation

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Coronary microcirculation is a key regulator of coronary blood flow distribution in the heart. Its role is to match local blood supply to myocardial metabolic demand, which is fulfilled by continuous adaptation of coronary vessel diameter via regulation of smooth muscle tone. Both anatomical and functional integrity are necessary for normal coronary microcirculatory function. Abnormal coronary microvascular function, i.e. coronary microvascular dysfunction is linked with several risk factors for atherosclerosis comprised cardiometabolic disorders. It is also marked as a crucial player in the pathophysiology of the several clinically very important entities with significant morbidity and mortality, including: microvascular angina pectoris and myocardial ischemia with non-obstructive coronary artery disease (INOCA), myocardial Infarction with non-obstructed coronary arteries (MINOCA) and heart failure with preserved ejection fraction (HFpEF).

The mechanisms underlying coronary microvascular dysfunction are complex but endothelial dysfunction appears to play a central role. Endothelial dysfunction can occur in response to increased oxidative stress, when endothelium is changing its phenotype into a state prone to amplify inflammatory responses by secretion of several chemokine and adhesion molecules (endothelin, thromboxane A<sub>2</sub>, prostaglandin H<sub>2</sub>, and superoxide) with subsequent interactions with platelets and leukocytes. Reduced bioavailability of NO shifts the balance between vessel generation and pruning towards microvascular rarefaction and decreased density further contributing to coronary microvascular dysfunction.

Endothelial function in coronary circulation can be tested in humans by intravascular application of the endothelium-dependent vasodilator acetylcholine. In case of coronary microvascular endothelial dysfunction, a reduced flow upon intracoronary infusion of acetylcholine is observed despite normal diameter of epicardial vessels.

There are several newly proposed mechanisms of coronary microvascular dysfunction, including degradation of the endothelial surface layer/glycocalyx, which covers the luminal surface of the endothelium. It consists of a thin layer of membrane bound macromolecules (i.e. the glycocalyx) and an additional thicker layer of adsorbed plasma components. This endothelial surface layer is critically involved in the regulation of blood flow (predominately in transmission of shear stress). Its structural deprivation results in a reduction of shear-dependent NO-mediated arteriolar vasodilation. This endothelial surface layer can be easily degraded by oxygen radicals, ischaemia, inflammation, or altered plasma composition.

Although the importance of coronary microvascular dysfunction is increasingly recognized as a key player in several relevant

pathological conditions, the thorough investigation of complex mechanisms behind coronary microcirculatory dysfunction, including its tight connection with endothelial dysfunction, requires further critical testing in order to elucidate its role in the proposed pathophysiological concepts.

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### **Aging Exacerbates Obesity-Induced Cerebromicrovascular Dysfunction in Preclinical Mouse Models: Novel Targets for Prevention of Cognitive Decline**

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Previous studies demonstrate that obesity promotes cognitive impairment due to, at least in part, its deleterious effects on the cerebral microvasculature. Importantly, the effects of obesity are exacerbated in aging, but the underlying mechanisms remain elusive. Nuclear factor erythroid 2-related factor (Nrf2) is a master transcriptional factor that regulates the expression of anti-oxidant and anti-inflammatory genes. There is increasing evidence that Nrf2/ARE signaling plays a critical role in vascular resilience to obesity and the metabolic syndrome and that aging is associated with progressive Nrf2 dysfunction, promoting vascular oxidative stress. The present study was designed to test the hypothesis that Nrf2 deficiency exacerbates endothelial senescence and cerebro-microvascular dysfunction induced by obesity. To test this hypothesis, we developed a transgenic animal model to investigate senescence induced by Nrf2 deficiency by crossing Nrf2 knock out mice (Nrf2<sup>-/-</sup>) with a senescence reporter mice. In the senescence reporter mice, p16-positive senescent cells can be detected in tissues by red fluorescent protein (mRFP) expression. Nrf2<sup>+/+</sup> and Nrf2<sup>-/-</sup> senescence reporter mice were fed a standard diet or an adipogenic high fat diet (HFD) for 5 months. At the end of the dietary treatment, blood-brain barrier (BBB) integrity was assessed by the permeability of the fluorescent tracer, Cadaverine Alexa-fluor-555 (Cad-555) into the brain parenchyma. Endothelium-dependent increases in cerebral blood flow (CBF) in response to contralateral whisker stimulation were measured by laser speckle contrast imaging. Finally, we assessed senescence in the hippocampus and cortex by quantifying mRFP positive endothelial cells and through gene expression analysis related to senescence pathways. HFD treatment in Nrf2<sup>+/+</sup> mice for 6 months significantly increased the accumulation of Cad-555 in the brain parenchyma, suggesting impaired BBB integrity. The CBF response was significantly decreased in Nrf2<sup>+/+</sup> HFD animals in response to contralateral whisker stimulation compared to standard diet animals. Further, while the CBF responses were decreased by pharmacological inhibition of NO synthesis (L-NAME) in standard diet animals, it remained unaffected by L-NAME treatment in Nrf2<sup>+/+</sup> HFD animals indicating endothelial dysfunction. In addition, HFD treatment significantly increased the expression of senes-

cence related genes and number of RFP positive cells in the hippocampus of Nrf2<sup>+/+</sup> animals. Nrf2 deficiency significantly exacerbated HFD-induced BBB damage, endothelial senescence and worsened endothelial dysfunction, mimicking the aging phenotype. Together, these results provide additional evidence that intact Nrf2 signaling importantly contributes to cerebro-microvascular health, protecting against endothelial senescence and preserving endothelium-dependent regulation of blood flow.

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### **Treatment with a NAD+ Booster Rescues Cerebromicrovascular Function in Aged Mice**

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Adjustment of cerebral blood flow (CBF) to neuronal activity via neurovascular coupling (NVC) has an essential role in maintenance of healthy cognitive function. In aging increased oxidative stress and cerebro-microvascular endothelial dysfunction impair NVC, contributing to cognitive decline. There is increasing evidence showing that a decrease in NAD<sup>+</sup> availability with age plays a critical role in a range of age-related cellular impairments but its role in impaired NVC responses remains unexplored. The present study was designed to test the hypothesis that rescue of NAD<sup>+</sup> biosynthesis may exert beneficial effects on NVC responses in aging. To test this hypothesis 24 month old C57BL/6 mice were treated with nicotinamide mononucleotide (NMN), a key NAD<sup>+</sup> intermediate, for 2 weeks. NVC was assessed by measuring CBF responses (by laser Doppler flowmetry; above the whisker barrel somatosensory cortex) evoked by contralateral whisker stimulation. Vascular relaxation was assessed using wire myography. qPCR was used to analyze mRNA expression. Selected reaction monitoring/tandem mass spectrometry was used for targeted proteomics. Electron microscopy and mtDNA quantitation was used to assess changes in mitochondrial content. Seahorse respirometry and flow cytometry (mitoSox) was used to assess mitochondrial respiration and mitochondrial oxidative stress in cultured cerebro-microvascular endothelial cells (CMVECs) derived from young and aged animals. Radial arm water maze and elevated plus maze tests and computerized gait analysis were used for behavioral characterization. We found that NVC responses were significantly impaired in aged mice. NMN supplementation rescued NVC responses by increasing endothelial NO-mediated vasodilation, which was associated with significantly improved spatial working memory and gait coordination. These findings are paralleled by the sirtuin-dependent protective effects of NMN on mitochondrial production of reactive oxygen species and mitochondrial bioenergetics in cultured cerebro-microvascular endothelial cells derived from aged animals. NMN treatment did not increase cellular mitochondrial content or expression of antioxidant enzymes. Thus, a decrease in NAD<sup>+</sup> availability contributes to age-related cerebro-microvascular endothelial dysfunction and NVC impairment, exacerbating cognitive

decline. The cerebromicrovascular protective effects of NMN highlight the preventive and therapeutic potential of NAD<sup>+</sup> intermediates as effective interventions in patients at risk for vascular cognitive impairment (VCI).

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### **Characterization of the LncRNA miR143HG in The Pathophysiology of Atherosclerosis**

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Long noncoding RNAs (lncRNAs) are emerging as powerful regulators of vascular pathophysiology. However, little is known about their function and regulation during vascular injury. Here we studied miR143HG, a conserved lncRNA whose locus overlaps miR143/145 microRNA loci. Deep sequencing from human atherosclerotic plaque and further qPCR validation in clinical samples, indicated that miR143HG was significantly downregulated (>3-fold decrease,  $P < 0.01$ ) in unstable versus stable region of the plaque. Importantly, the axis was furthermore found downregulated (>2-fold decrease,  $P < 0.05$ ) in advanced plaques versus early lesions isolated from LDLR<sup>-/-</sup> mice by Laser Capture Microdissection technique. Further qPCR validation and in situ hybridisation revealed that miR143HG is widely expressed in vSMCs (vascular smooth muscle cells), monocytes, macrophages and fibroblasts. With the purpose of investigating the molecular mechanism of action of miR143HG and its involvement in disease, we manipulated in vitro the lncRNA expression using antisense oligonucleotides in primary human coronary artery vascular SMCs. We demonstrated that the depletion of miR143HG significantly increased vSMC proliferation in basal (>2-fold increase,  $P < 0.001$ ) and in PDGF-BB (Platelet Derived Growth Factor-BB) treatment (2-fold increase,  $P < 0.01$ ) versus control. Moreover, the knock-down of miR143HG increased the migratory capacity vSMC of more than 1.5-fold ( $P < 0.01$ ) and decreased the production of collagen of 2-fold ( $P < 0.05$ ). Although our understanding of miR143HG regulatory function is early, collectively, our preliminary results suggest that miR143HG, could potentially represents a critical player in the progression of atherosclerotic plaque in vascular disease.

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### **Microvascular Events in Acute Ischemic Stroke**

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While thrombolysis was the only available option for treatment of acute ischemic stroke for years, thrombectomy has revolutionized treatment in the last few years. Yet, still only one out of three treated patients recovers towards independency. A major concern is the lack of maintained microvascular reperfusion in the potentially salvageable penumbra area. Several events in the cerebral microcirculation might be responsible for such impaired microvascular reperfusion. These include spasm of smooth muscle cells or pericytes, compression by local loss of blood brain barrier integrity and oedema, and occlusion by micro-thrombi, activated platelets and leukocytes. Oxidative stress and inflammatory mechanisms appear to be strongly involved. These events do not occur in isolation. Rather, a vicious circle of tissue damage and microvascular events develops during stroke that continues after thrombectomy. The presence of collaterals may delay the initiation of this vicious circle from minutes to hours, buying time for thrombectomy, but a rapid reversal of these microvascular events is required for further improving outcome.

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### **Short-Term Lipopolysaccharide Induces Aortic Valve Thickening and Valve Hemorrhage in ApoE\*3Leiden Mice**

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**Background:** Recently, it was shown that 12 weeks of lipopolysaccharide (LPS) administration to non-atherosclerotic mice induced thickening of the aortic heart valve (AV). Whether such

effects may also occur even earlier is unknown. As most patients with AV stenosis also have atherosclerosis, we studied the short-term effect of LPS on the AVs in an atherosclerotic mouse model.

**Methods:** ApoE\*3Leiden mice, on an atherogenic diet, were injected intra-peritoneally with either LPS or phosphate buffered saline (PBS), and sacrificed 2 or 15 days later. All procedures were approved by the Animal Use and Care Committee at the Amsterdam UMC, Vrije Universiteit. AVs were assessed for size, fibrosis, glycosaminoglycans (GAGs), lipids, calcium deposits, iron deposits and inflammatory cells.

**Results:** LPS-injection caused an increase in maximal leaflet thickness at 2 days (128.4  $\mu\text{m}$ ) compared to PBS-injected mice (67.8  $\mu\text{m}$ ;  $p = 0.007$ ), whereas at 15 days this was not significantly different. LPS-injection caused a non-significant increase in average AV thickness on day 2 (37.8  $\mu\text{m}$ ) and a significant increase at day 15 (41.6  $\mu\text{m}$ ;  $p = 0.038$ ) compared to PBS-injected mice (31.7  $\mu\text{m}$  and 32.3  $\mu\text{m}$  respectively). LPS-injection did not affect AV fibrosis, GAGs and lipid content. Furthermore, no calcium deposits were found. Iron deposits, indicative for valve hemorrhage, were observed in one AV of the PBS-injected group (a day 2 mouse; 9.1%) and in five AVs of the LPS-injected group (both day 2- and 15 mice; 29.4%). No significant differences in inflammatory cell infiltration were observed upon LPS-injection.

**Conclusion:** Short-term LPS apparently has the potential to increase AV thickening and hemorrhage. These results suggest that systemic inflammation can acutely compromise AV structure.

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### Search for the Source of the Retinal Relaxing Factor

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**Purpose:** the retinal relaxing factor (RRF) is an unidentified paracrine factor, which is continuously released from retinal tissue and causes smooth muscle cell relaxation. This study tried to identify the cellular source of the RRF. Furthermore, the possible RRF release by voltage-dependent Na<sup>+</sup> channel activation and the Ca<sup>2+</sup> dependency of the RRF release was investigated.

**Materials and Methods:** mouse femoral arteries were mounted in myograph baths for in vitro isometric tension measurements. The vasorelaxing effect of chicken retinas, which contain no vascular cells, and of solutions incubated with MIO-M1 or primary Müller cell cultures were evaluated. The RRF release of other retinal cells was investigated by using cell type inhibitors. Concentration-response curves of veratridine, a voltage-dependent Na<sup>+</sup> channel activator, were constructed in presence or absence of mouse retinal tissue to evaluate the RRF release. The Ca<sup>2+</sup> dependency of the RRF release was investigated by evaluating the vasorelaxing effect of RRF-containing solutions made out of chicken retinas in absence or presence of Ca<sup>2+</sup>.

**Results:** Chicken retinas induced vasorelaxation, whereas solutions incubated with Müller cell cultures did not. Moreover, the gliotoxin DL- $\alpha$ -aminoadipic acid, the microglia inhibitor minocy-

cline and the tetrodotoxin-resistant voltage-dependent Na<sup>+</sup> channel 1.8 inhibitor A803467 could not reduce the RRF-induced relaxation. Concentration-response curves of veratridine were not enlarged in the presence of retinal tissue, and RRF-containing solutions made in absence of Ca<sup>2+</sup> induced a substantial, but reduced vasorelaxation.

**Conclusions:** the RRF is not released from vascular cells and probably neither from glial cells. The retinal cell type which does release the RRF remains unclear. Veratridine does not stimulate the RRF release in mice, and the RRF release in chickens is Ca<sup>2+</sup> dependent as well as Ca<sup>2+</sup> independent.

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### Shear Stress Regulates Endothelial Glycocalyx Hyaluronan Biosynthesis Through HAS2 and Metabolic Regulation of Its Substrates

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Endothelial cells exposed to laminar shear stress express a thick glycocalyx on their surface that plays an important role in reducing vascular permeability and has anti-inflammatory, anti-thrombotic and anti-angiogenic properties. Production and maintenance of this glycocalyx layer is dependent upon cellular carbohydrate synthesis, but how this is regulated is not known. Here, we studied how synthesis of a major glycocalyx polysaccharide constituent, hyaluronan (HA), is regulated during exposure to shear. Both in vitro as well as in vivo, HA expression on the endothelial surface was increased upon laminar shear and reduced when exposed to oscillatory flow. Laminar shear was accompanied by enhanced synthesis of the HA producing enzyme hyaluronan synthase 2 (HAS2), which also translocated to the endothelial cell membrane in a KLF2 dependent manner, using a CRISPR-CAS9 edited small tetra cysteine tag (TC-tag, Cys-Cys-Pro-Gly-Cys-Cys) to endogenous HAS2. Subsequent HA production by HAS2 was driven by availability of the HA substrates UDP-glucosamine and UDP-glucuronic acid. We show that the known KLF2 regulation of the glycolytic regulator PFKFB3 also allows for glucose intermediates to shuttle into the hexosamine- and glucuronic acid biosynthesis pathways, as measured by <sup>13</sup>C-glucose labelled NMR. Thus increasing the cytosolic availability of the HAS2 substrates. These data demonstrate how endothelial glycocalyx function and functional adaptation to shear is coupled to KLF2 mediated regulation of endothelial glycolysis.

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### Glomerular Function and Structural Integrity Depend Upon Endothelial Hyaluronan Synthesis

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Endothelial cells are covered by a glycocalyx envelop, both luminal and abluminal, which predominantly consists of proteoglycans and adhering proteins. Here we demonstrate that integrity of this layer is critical for glomerular structure and function. Four weeks after tamoxifen induction in endothelial specific has2-cKO mice, glomerular hyaluronan (HA) was reduced by ~80% from the luminal endothelial surface. This resulted in mesangiolysis and capillary ballooning within the glomeruli and micro-albuminuria. Over time this process developed into glomerular capillary rarefaction and glomerulosclerosis, recapitulating the phenotype of progressive human diabetic nephropathy. Recapitulating, we observed loss of glomerular endothelial HA in association with lesion formation in human diabetic nephropathy biopsy samples. Mechanistically, we found that HA harbours a specific binding site for the key regulator of endothelial quiescence and maintenance in glomerular endothelial barrier function, angiopoietin 1 (Ang1), a secreted ligand of the endothelial Tie-2 kinase. We show that, endothelial loss of HA resulted in disturbed Tie-2 kinase dependent glomerular endothelial stabilization. In summary, glomerular endothelial hyaluronan is a hitherto unrecognised key ECM component required for glomerular structure and function, which is lost in diabetic nephropathy.

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### Using a Mouse Model of Vascular Media Calcification to Unravel the Pathophysiology Behind Arterial Stiffness

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Large artery stiffness is a hallmark of arterial aging and a major cause of increased cardiovascular risk. Current treatment options to combat arterial stiffness are rather limited. Therefore, our goal is to unravel the pathophysiological mechanisms of arterial stiffness. This study aims to provide an in vivo model to study the progressive fashion of arterial stiffness.

A mouse model of arterial media calcification (AMC) is used. AMC is closely associated to arterial stiffness. DBA/2 mice were fed a 0.3% warfarin and 0.15% vitamin K1 supplemented diet. Mice were sacrificed after 8, 12 and 16 weeks. In vivo and ex vivo evaluation of stiffness was done using echo-evaluation of the pulse wave velocity (PWV) and by studying cyclic stretch of small aorta segments in our organ bath setup, respectively. Blood pressure was obtained using the tail-cuff method. Calcified tissue was visualized using Von Kossa staining and confirmed by quantification of aortic calcium content, using flame atomic absorption spectrometry. Finally, intima media thickness was histologically evaluated.

Stiffness aggravates over time in warfarin administered mice. PWV was significantly increased after 16 weeks ( $p < 0.001$ ) compared to age-matched controls receiving normal animalarium diet. There was no significant difference in pulse pressure between both groups. Ex vivo evaluation of active cellular components taught us that major vascular smooth muscle cell (VSMC) function was still intact after 16 weeks. Remarkably, endothelial cells (ECs) of warfarin administered animals were hypersensitive to acetylcholine (ACh) ( $p < 0.05$ ). Passive stiffness components were visible after 16 weeks ( $p < 0.05$ ) and a trend towards increased intima media thickness could be observed. Calcifications were clearly present in Von Kossa stained sections of warfarin administered mice. Calcium content in the aorta was significantly increased after 12 weeks ( $p < 0.01$ ) and 16 weeks ( $p < 0.05$ ). No significant correlation between PWV and calcium content could be observed.

Our study illustrates the progressive character of arterial stiffness in a mouse model of AMC. Interestingly, at the time AMC is present, functionality of both VSMCs and ECs is preserved or enhanced, respectively. Further investigation is needed to clarify this hypersensitivity to ACh. Proteomic analysis of the isolated mouse aortic tissue should give more insight on the combined pathophysiological and molecular signatures, which are typical for arterial stiffness. Collectively, our findings contribute to a better understanding of this complex pathology and its treatment options.

## In-Vitro Characterization of Mouse Carotid Artery Mechanics by Dynamic Biaxial Pressure-Myography

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**Objective:** Although in-vitro biomechanical behavior of arteries is commonly studied under quasi-static conditions, dynamic (pulsatile, in-vivo) behavior may differ substantially. We developed a set-up to characterize mouse carotid artery biaxial mechanics under quasi-static and dynamic conditions, using high-frequency ultrasound to track diameter and two-photon laser scanning microscopy (TPLSM) to capture the microstructure. We aimed to (1) quantify reproducibility and (2) compare quasi-static and dynamic elastic behavior.

**Design and Method:** After euthanasia, eight carotid arteries from four male surplus mice were mounted between glass micropipettes. Four carotids were tested directly after euthanasia, four on the day thereafter. Pressure (P) was recorded at the distal pipette; axial force (F) was recorded by a load cell. First, arteries were stretched to in-vivo length and exposed to quasi-static pressure inflation from 0–200 mm Hg. Second, axial stretch ( $\lambda z$ ) was varied for constant pressures of 60/100/140 mm Hg to determine an axial stiffness coefficient. Third, vessels were exposed to pulsatile pressures (systolic pressures of 80/120/160 mm Hg, at 5 Hz) and at frequencies (f) of 2.5/5/10 Hz at 120 mm Hg. Single-point pulse wave velocity (PWV; Bramwell-Hill) was determined and compared with corresponding PWVs calculated from the quasi-static pressure-diameter curve. The axial stiffness coefficient was obtained as the local slope of the F- $\lambda z$  curve. Fourth, information on adventitial collagen-structure deformation was obtained using second-harmonic generation TPLSM. The first three protocol steps were performed in duplicate to determine coefficients of variation (CVs).

**Results:** CVs for PWV were ~9% for low/medium and 27% for high pressure. For f2.5 and f10Hz these were 16% and 26%. Dynamic PWVs were higher than quasi-static PWVs for all test conditions ( $p < 0.012$ ; mean $\pm$ SD, at f5Hz: PWV\_P80  $2.4 \pm 0.2$  vs.  $2.1 \pm 0.1$  m/s, PWV\_P120  $6.3 \pm 0.6$  vs.  $5.0 \pm 0.5$  m/s, PWV\_P160  $13.3 \pm 1.9$  vs.  $10.3 \pm 2.2$  m/s, and at P120 mm Hg: PWV\_f2.5  $3.9 \pm 0.7$  vs.  $3.5 \pm 0.3$  m/s, PWV\_f10  $3.1 \pm 0.9$  vs.  $2.5 \pm 0.2$  m/s). Axial stiffness coefficients increased with pressure 60–140 mm Hg ( $1.3 \pm 0.4$ ,  $2.4 \pm 1.0$ ,  $4.9 \pm 2.8$  grams). Measurements from fresh and non-fresh vessels were not significantly different ( $p > 0.099$ ). From 60–100 mm Hg undulation of adventitial collagen strands disappeared, whereas from 100–140 mm Hg orientation changed.

**Conclusions:** Our innovative set-up shows well-acceptable reproducibility and demonstrates the importance of quasi-static and dynamic conditions when studying arterial mechanics.

## Preferred Exit-Sides for Leukocyte Diapedesis, Identifying Local Signalling Induced by ICAM-1 Clustering Upon Leukocyte Trans Endothelial Migration

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Upon inflammation the endothelial cells of the vasculature become activated and facilitate the extravasation of immune cells into the inflamed tissue. This process involves the upregulation of selectins, cellular adhesion molecules and pathways to enable leukocyte capture from the bloodstream and diapedesis through the vessel wall, a process referred to as leukocyte transendothelial migration. Although several steps during this multistep process have been studied extensively and considerable knowledge is gathered in the recent years, we still do not know why a leukocyte prefers one exit-side over the other. To get a better understanding of the local signalling in the endothelium at the diapedesis site we have used a model system for ICAM-1 clustering. This model induces ICAM-1 clustering in inflammatory endothelial cells similar to that induced by leukocytes prior to the diapedesis step. This clustering is the first step of the diapedesis process and induces a local signalling that primes the endothelium for leukocyte diapedesis. Revealing the nature of the signals that define these primed locations will improve our understanding of what drives leukocyte transendothelial migration under inflammation conditions. ICAM-1 clustering is an active process that induces recruitment of other proteins to the site of transmigration such as filamin a, filamin b and cortactin. To identify other proteins recruited by active clustering we have applied a label-free mass spectrometry approach to this model. The role of the identified proteins in the ICAM-1 clustered complex was then examined in an in vitro trans endothelial migration assay under physiological flow.

Label free mass spectrometry identified 92 proteins that were actively recruited to the ICAM-1 clustering complex upon ICAM-1 clustering in inflammatory endothelial cells. These 92 proteins contained previously identified proteins as well as proteins that have not yet been associated with ICAM-1 clustering or Leukocyte transmigration. We further investigated the role of a selection of these proteins and validated their presence upon clustering using both western blot and immunofluorescent microscopy. The next step in will be investigating the function of these proteins in the leukocyte diapedesis by using shRNA knockdown and the in vitro transmigration model under physiological flow.

We have identified 93 proteins involved in the local complex formed upon ICAM-1 clustering in enflamed endothelial cells among which proteins that have not previously been connected to leukocyte transmigration. Currently we are in the process of further examining the role of a selection of these proteins in the diapedesis process.

### Hybrid Optical and CT Imaging reveals increased Matrix Metalloprotease Activity and Apoptosis Preceding Cardiac Failure in Progeroid *Ercc1* mice

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**Background:** Cardiovascular diseases (CVD) persist as one of the leading causes of morbidity and mortality in the elderly population worldwide. The accumulation of unrepaired DNA damage over time is one of the driving forces of age-related diseases, as exemplified in mice with genetic defects in DNA repair pathways. The *Ercc1* protein is involved in important DNA repair pathways. Hence, *Ercc1* mutations cause accelerated aging phenotypes in humans and mice, including cardiovascular aging. We hypothesized that unrepaired DNA damage contributes to cardiovascular aging and the associated functional decline. To examine this, we used functional MicroCT imaging combined with NIRF probes to report on in vivo activity of key biomarkers for cardiovascular aging with a focus on age-related cardiac failure, using progeroid *Ercc1* mouse models.

**Methods:** *Ercc1Δ/-* mice (15% mutant allele expression), and cardiomyocyte-specific *Ercc1c/-* mice (no *Ercc1* expression in the heart), along with WT controls were imaged with contrast enhanced MicroCT for anatomical reference, to assess cardiac morphology and function. The NIRF probes MMPsense680™ and Annexin-Vivo750™ were used to image matrix metalloprotease (MMP) activity and apoptosis, respectively. Functional microCT analysis was compared to ultrasound imaging and results were validated by histology.

**Results:** *Ercc1Δ/-* deficiency resulted in LV geometry and functional changes at age 24 weeks; increased LV end-diastolic and LV end-systolic volumes were observed, leading to an overall stroke volume decrease and substantial reduction in LV ejection fraction. WT mice showed relative stable volumes over time. *Ercc1Δ/-* hearts had increased myocardial apoptosis at age 12 and 24 weeks, and a gradual increase in MMP activity starting at 6 weeks of age, suggesting these processes precede cardiac failure in progeroid *Ercc1* mice. Cardiomyocyte-specific *Ercc1* inactivation also led to impaired cardiac functioning, increased myocardial apoptosis and MMP activity, indicating association of *Ercc1* deficiency in cardiomyocytes with adverse cardiac remodeling and poor cardiac functioning. In addition, we observed increased apoptosis and MMP activity in *Ercc1Δ/-* aortas, which coincided with changes in elastin and collagen structure and smooth muscle cell loss, as observed with immunohistochemistry.

**Conclusion:** Combined CT and optical imaging allows simultaneous analysis of molecular and functional changes in accelerated aging mouse models and shows increased markers of cardiovascular decline before the disease manifests. In progeroid *Ercc1Δ/-* mice, the age-related cardiac increase in MMP activity was followed by apoptosis and cardiac functional decline. These data show that unrepaired DNA damage contributes to cardiovascular aging.

### Perfused 3D Angiogenic Sprouting Assay of iPSC-Derived Endothelial Microvessels In Vitro

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**Introduction:** It is now well recognized that angiogenesis, the growth of new blood vessels from pre-existing vasculature, plays a fundamental role in both health and disease. For the discovery of new drug targets that target the microvasculature, drug research relies heavily on in vitro models, due their unparalleled level of experimental control. However, it is still common practice to study endothelial cells in culture systems that have limited physiological relevance. To meet the demands of pre-clinal vascular drug research, improved in vitro models of vasculature are required: assays that are amendable to high-throughput screening, with a scalable and robust cell source in a physiological relevant cellular micro-environment.

**Methods:** A standardized microfluidic 3D cell culture platform was used, which consist of 40 individually addressable microfluidic units, integrated underneath a 384-well plate. Within these microfluidic channels, endothelial cells differentiated from human induced pluripotent stem cells (hiPSC-ECs) are cultured against a patterned collagen-1 scaffold and form 3D microvessels under continuous perfusion. Angiogenesis is studied by applying stable biomolecular gradients within the patterned scaffold.

**Results:** We show that iPSC-ECs are able to reproduce important hallmarks of angiogenic sprouting: differentiation into tip cells that display their characteristic filopodia and the trailing stalk cells form lumen. Furthermore, directional and repetitive sprouting is observed, which is an important indication that the cells are able to sense the imposed VEGF-gradient.

Furthermore, we show that Sunitinib, a clinically available anti-angiogenic drug that inhibits the signaling of angiogenic receptors, including VEGFR2 (Flk-1) and PDGFRβ, inhibits the sprouting of hiPSC-ECs at nanomolar levels (IC50 of 21 nM, 95% CI 15–29). This suggests that the angiogenic sprouting of iPSC-ECs in this model is mediated through VEGFR2 signaling.

Prolonged culture of the sprouts resulted in anastomosis with the bottom perfusion channel. This resulted in two interesting phenomena which we have also observed with the primary ECs: retracting and pruning of non-connected angiogenic sprouts. Perfusion with TRITC-labeled albumin before and after angiogenic sprouting showed that the permeability decreases after anastomosis, which is also similar to primary ECs (HUVECs).

**Conclusion:** The method presented here is suitable to study angiogenesis in a physiological relevant 3D cellular microenvironment, including perfusion and gradients. The platform is compatible with automated microscopes, and has the required robustness and scalability to be integrated within the drug screening infrastructure.

## Single Cell Sequencing Reveals Heterogeneity of Adventitial Mesenchymal Cells in Healthy and Diseased Mice

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**Background:** Mesenchymal, fibroblast-like cells were recently detected in atherosclerotic plaques, stemming from the adventitia. Further study of these cells is hampered by lack of specific cell type markers because of cell heterogeneity. Hence, we aimed to identify mesenchymal-specific markers using single cell sequencing, and how mesenchymal transcriptomics and plasticity changed between healthy and diseased vasculature.

**Methods:** The adventitia of the thoracic aorta from 8 healthy, C57Bl/6 mice (chow, 8–12 weeks) and 6 ApoE<sup>-/-</sup> (chow, 35 weeks) was pooled and enzymatically digested. Living, CD45<sup>-</sup>, ICAM2<sup>-</sup> cells were sorted for single cell sequencing (SCS) using 10xGenomics. Mesenchymal subtypes were identified by graph-based clustering (tSNE) – and trajectory analysis (RNA Velocity, PHATE). Flow cytometry of healthy and diseased adventitia quantified PDGFR $\alpha$ <sup>+</sup> and/or PDGFR $\beta$ <sup>+</sup> mesenchymal cells in the CD45<sup>-</sup>/TER119<sup>-</sup>/VE-Cadherin<sup>-</sup>, F4/80<sup>-</sup>/ $\alpha$ SMA<sup>-</sup>, population. Cell type specificity of new and traditional was studied using qPCR, immunohistochemistry, and flow cytometry.

**Results:** Traditional mesenchymal markers (PDGFR $\beta$ , PDGFR $\alpha$ , COL1 $\alpha$ 1, Col1 $\alpha$ 2, FSP1, FAP, lumican and decorin) were not only expressed in murine fibroblast cells (MEF, 3T3) in vitro, but also in primary VSMCs and/or macrophages. Immunohistochemistry and flow cytometry confirmed expression of traditional mesenchymal markers in  $\alpha$ SMA<sup>+</sup> VSMCs and CD68<sup>+</sup> macrophages of human and mouse plaques. Flow cytometry of healthy vs. diseased murine adventitia cells, lacking VSMC, macrophage or endothelial cell markers, showed that PDGFR $\alpha$ <sup>+</sup> cells decreased in diseased adventitia (~73%), while PDGFR $\beta$ <sup>+</sup> and PDGFR $\alpha$ <sup>+</sup>/PDGFR $\beta$ <sup>+</sup> dual positive cells increased in diseased adventitia (+767% and +80% respectively).

To find new markers, analysis of 5000 single cells identified 5 differentiation tracks in murine mesenchymal cells, and three VSMC clusters. Violin plots showed cluster-specific genes for all clusters. Enrichr analysis of all clusters reveals functional difference between central versus outer clusters, e.g. negative regulation of cell cycle progression vs. TGF- $\beta$  signalling or extracellular matrix production. The majority of new murine markers are expressed in human plaques (87%), and ~50% is significantly differentially regulated in human unstable compared to stable plaque segments.

**Conclusion:** Traditional mesenchymal markers lack mesenchymal-specificity among vascular cells. PDGFR $\alpha$ <sup>+</sup> and PDGFR $\beta$ <sup>+</sup> cells, lacking leukocyte, VSMC or EC markers, are however differentially expressed between healthy and diseased adventitia, indicating an adaption of fibroblast-like cells in state of disease. SCS identified 5 mesenchymal differentiation tracks and cluster-specific gene expression in healthy murine adventitia, allowing future study of arterial mesenchymal cells.

## Downregulation of Tie2 Induces Microvascular Leakage in Mice

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**Background:** Critical illness is associated with multiple organ failure and increased mortality. We previously showed microvascular leakage in critically ill rats, which could be restored by pharmacologically targeting of the endothelial angiotensin/Tie2 system. This angiotensin/Tie2 system is involved in regulation of endothelial barrier function by activation of Tie2 receptor via angiotensin-1. During critical illness, angiotensin-2 is released and impairs the endothelial barrier. Angiotensin-2 levels are used as predictor for mortality in critical ill patients. However, it is unclear whether angiotensin-2 needs an additional stimulus to disrupt the endothelial barrier. To further explore the role of angiotensin-2 and Tie2, we studied the effect of angiotensin-2 administration or Tie2 downregulation on microvascular leakage and edema formation.

**Methods:** Seven to nine weeks old heterozygous Tie2 knockout male mice (exon 9 deletion, Tie2<sup>+/-</sup>), and wild type littermate controls (WT) were anesthetized and the carotid artery and jugular vein were cannulated for hemodynamic measurements. Mice received either angiotensin-2 (WT+Ang-2 n = 6) or PBS as control (WT+PBS n = 4, Tie2<sup>+/-</sup>+PBS n = 5) and were monitored for one hour. Microvascular leakage was determined by measuring the extent of Evans Blue dye (EBD) extravasation in kidney and lung tissue. Edema formation was measured using wet/dry ratios of kidney and lung tissue.

**Results:** Angiotensin-2 administration had no effect on hemodynamic values. In healthy animals, administration of angiotensin-2 increased EBD extravasation in lung (0.080  $\pm$  0.022 vs. 0.048  $\pm$  0.010  $\mu$ g/g; p < 0.05) but not in kidney tissue (0.068  $\pm$  0.018 vs. 0.067  $\pm$  0.012  $\mu$ g/g; p > 0.99). Surprisingly, angiotensin-2 administration did increase wet/dry ratios in both lung (7.24  $\pm$  1.47 vs. 4.32  $\pm$  1.45; p < 0.05) and kidney tissue (4.92  $\pm$  0.42 vs. 4.12  $\pm$  0.89; p < 0.05).

Tie2 heterozygosity increased EBD extravasation in lung tissue compared to healthy mice (0.088  $\pm$  0.018 vs. 0.048  $\pm$  0.011  $\mu$ g/g; p < 0.01), but not in kidney tissue (0.065  $\pm$  0.012 vs. 0.067  $\pm$  0.12  $\mu$ g/g; p > 0.99). This result was confirmed by increased wet/dry ratios in lung tissue (8.77  $\pm$  1.26 vs. 4.32  $\pm$  1.45; p < 0.005; kidney 4.75  $\pm$  0.12 vs. 4.12  $\pm$  0.89; p = 0.15).

**Conclusion:** Increasing circulating angiotensin-2 levels alone can induce pulmonary and renal edema formation in healthy animals. Additionally, downregulation of Tie2 resulted in pulmonary microvascular leakage and edema formation. Future studies should focus on reducing angiotensin-2 levels and/or targeting the Tie2 receptor to improve the outcome of critically ill patients.

## The GEF Trio Reinforces Vascular Endothelial Barrier Integrity

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Endothelial cell-cell junctions maintain a restrictive barrier that is tightly regulated to allow dynamic responses to permeability-inducing factors, inflammatory agents and adherent leukocytes. The ability of these stimuli to remodel endothelial adherens junctions (AJs) and tight junctions (TJs) depends on Rho- and Rap-GTPase-controlled cytoskeletal rearrangements. How activity of Rho and Rap-GTPases is spatio-temporally controlled at endothelial AJs by guanine-nucleotide exchange factors (GEFs) is incompletely understood. Here, we identify a crucial role for the Rho-GEF Trio in stabilizing VE-cadherin-based junctions. Trio interacts with VE-cadherin and locally activates Rac1 at AJs during nascent contact formation. The Rac-GEF domain of Trio is responsible for remodeling of junctional actin from radial to cortical actin bundles, a critical step for junction stabilization. Moreover, our data show that this domain of Trio activates the GTPase Rap1. Trio-Rap1 signaling promotes the formation of linear junctions and increases endothelial barrier function, in addition to thick F-actin fibers that colocalize with Myosin-Light Chain phosphorylation. Using advanced imaging techniques such as laser ablation microscopy and super-resolution structured illumination microscopy (SIM), we show that myosin-based tensile forces on F-actin bundles tightly pack endothelial cell junctions into linear arrays. By promoting an optimal junctional surface-tension ratio, Trio in this way improves the stability of the vascular endothelial barrier.

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## Towards a Molecular Atlas of the Mouse Vasculature

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Cerebrovascular disease is the third most common cause of death in developed countries, but our understanding of the cells that compose the cerebral vasculature is limited. In our recent work we provide molecular definitions for the principal types of blood vascular and vessel-associated cells in the adult mouse

brain. The transcriptional basis of the gradual anatomical change (zonation) along the arteriovenous axis was elucidated and we discovered several surprising cell type differences: a seamless continuum for endothelial cells from the arteries, through capillaries into veins, versus a punctuated continuum for mural cells. Indeed, pericytes were found to be very homogeneous throughout the brain while being very similar to venous smooth muscle cells. Arterial smooth muscle cells, on the other hand, had a substantially different transcriptome. We also provide insight into pericyte organotypicity by comparing brain pericytes to lung pericytes, and we define a population of perivascular fibroblast-like cells that are present on all vessel types except capillaries, and lie closely associated with the vessels, outside of the smooth muscle cell layer, but inside of the astrocyte endfeet. Our work illustrates the power of single-cell transcriptomics to decode the higher organizational principles of a tissue and may provide the initial chapter in a molecular encyclopaedia of the mammalian vasculature. Currently, we are expanding this work towards investigation of blood brain barrier heterogeneity during diseases like Primary familial brain calcification (PFBC). Furthermore, we plan to discuss our findings in cellular blood vessel heterogeneity in other organs of the mouse.

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## ROCKs Play a Major Role in the Control of Gene Expression Program by the CCM Complex

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**Background:** Cerebral Cavernous Malformations are vascular lesions composed of clusters of dilated and hemorrhagic capillaries potentially leading to debilitating hemorrhagic strokes. These lesions appear upon the endothelial loss-of-function mutations in *ccm* genes that code for the CCM protein complex. We previously showed that the CCM complex coordinates ROCK1 and ROCK2 activities to dynamically control the architecture and the tensional homeostasis of the endothelium in response to its microenvironment. Exaggerated cell contractility upon CCM2 loss leads to increased integrin-dependent adhesion to the extracellular matrix (ECM) and destabilized VE-cadherin-dependent intercellular junctions. In parallel, others have shown that the loss of the CCM complex leads to endothelial dedifferentiation through an endothelial-to-mesenchymal like transition driven by the overexpression of the transcription factors Klf2 and Klf4. Our driving hypothesis is that the CCM complex controls signaling pathways emerging from adhesives structures to dictate a transcription program preserving endothelial differentiation. The goal of my thesis is to test whether increased ROCK-dependent cell contractility is involved in the cellular identity switch upon CCM loss.

**Methods:** To test the role of ROCKs in the gene expression switch upon CCM loss, we did a comparative transcriptomic analysis of HUVEC silenced for CCM2 or doubly silenced for CCM2 and ROCK1 or ROCK2. We used fluorescent gelatin degradation and Boyden chamber assays to test respectively the ECM degradative skills and invasiveness of CCM2-depleted HUVEC.

**Results:** Our results from transcriptomic analysis confirmed the switch in the gene expression program of HUVEC upon CCM loss and demonstrated the major contribution of dysregulated ROCKs to this switch. Indeed, 50% of the altered genes were dependent on ROCKs. Among them, Matrix Metalloproteinases (MMPs) and ECM remodeling proteins were overexpressed. We showed that their ROCK-dependent overexpression was responsible for a significant increase in the capacities of CCM2-depleted HUVEC to degrade and invade the ECM.

**Conclusion:** Our results confirm the hypothesis that increased ROCK-dependent cell contractility is involved in the cellular identity switch experienced by endothelial cells upon CCM loss. Moreover, we reveal for the first time that CCM-depleted endothelial cells acquire invasive capacities for which dysregulated ROCKs play major structural and transcriptional roles.

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### Regulation of Endothelial Junctions During Leukocyte Extravasation

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Immune defense against pathogens depends on the recruitment of leukocytes to sites of infection. This process is initiated by the induction of leukocyte capturing to the endothelium, which is followed by transmigration through the endothelial barrier mainly, but not exclusively, on a route through junctions between endothelial cells. VE-cadherin is a central adhesion molecule that provides stability of endothelial junctions and that controls junction integrity. Leukocytes seem to be able to modulate endothelial junctions by addressing VE-cadherin function. Recently, we found that tyrosine phosphorylation of certain sites in the cytoplasmic tail of VE-cadherin trigger the destabilization of endothelial junction stability *in vivo*. Unexpectedly, we could show that different tyrosine residues were relevant for the extravasation of leukocytes and for the induction of vascular permeability induced by various pro-inflammatory mediators. Leukocyte-induced modulation of VE-cadherin phosphorylation enhanced its endocytosis. We are presently investigating the signaling process by which the docking of leukocytes to endothelium influences the phosphorylation of VE-cadherin.

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### Retinal Microvascular Biomarker Extraction on Fundus Images from the Maastricht Study Using Supervised Deep Learning

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Retinal fundus imaging enables detailed visualization of the microvascular structure in the retina of the human eye. Geometrical features, related to vessel caliber, tortuosity and bifurcations, have been identified as potential biomarkers for a variety of cardiometabolic diseases, including (pre)diabetes and hypertension. A pipeline of automated unsupervised image analysis methods for extraction of such features from retinal fundus images has previously been developed and evaluated [1]. However, the current computationally expensive pipeline takes 24 minutes to process a single image, which impedes implementation in a screening setting.

In the present work, we approximate the pipeline using a deep neural network that enables processing of a single image in a few seconds. We use a model that contains approximately 23 million trainable parameters and we train it with color fundus images from the Maastricht Study, a population-based cohort study with extensive phenotyping, that focuses on the etiology, complications and comorbidities of Type 2 Diabetes Mellitus. The set comprises 10668 images from 2872 subjects taken from both left and right eyes and are centered either on the fovea or on the optic disc.

We design the model to simultaneously output four global biomarkers that represent key vessel geometries: Central Retinal Arteriolar Equivalent (CRAE), Central Retinal Venular Equivalent (CRVE), global tortuosity and asymmetry ratio of the bifurcations. The outputs from the original pipeline are used as training labels. Eighty percent of the data is used for training, while the remainder is used to evaluate the performance of the model.

We obtain a substantial speed-up, requiring only 5 seconds to process an image. Intraclass correlation coefficient between the predictions of the model and the results of the pipeline showed strong correlation (0.86–0.91) for three of four biomarkers and moderate correlation (0.42) for one biomarker. To visualize what regions in the fundus images contribute to the model predictions, we create class activation maps. The maps show clearly that the local activations overlap with the vascular tree. It is able to differentiate between arterioles and venules around the optic disc when predicting CRAE and CRVE. Moreover, local high and low tortuous regions are clearly identified, verifying that the model is sensitive to key structures in the retina.

In conclusion, key biomarkers can be approximated well using deep learning. The model is fast and can easily be expanded to include other biomarkers.

#### Reference

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### The Influence of Altered Hormonal Pattern on the QTc Dispersion in Hypertensive Diabetic Patients

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**Objective:** To assess the influence of an altered aldosterone and cortisol pattern on the QTc interval in hypertensive diabetic patients.

**Methods:** We have studied non-insulin treated T2DM patients (for more than 5 years), well controlled according to the individual targets chosen by their own physician, all with controlled essential hypertension (for at least 8 years). They didn't have documented coronary disease or heart failure and all were in sinus rhythm (no more than 90 beats/min). For all patients, we obtained the HbA1c, glycemia, plasma cortisol and aldosterone, TSH, lipid profile and a 12-lead standard ECG. The patients were divided in two groups: the study group, with increased cortisol and aldosterone, and the control group, with normal hormone levels. Increased QTc dispersion was defined as >60 ms and a prolonged QTc interval as >440 ms.

**Results:** We included 118 subjects, 52 in the study group and 66 in the control group, with both groups having similar characteristics in terms of age (mean age was 59.3 years in the study group and 58.6 in the control group), sex distribution, prevalence of dyslipidemia and obesity. In the control group the QTc dispersion was 56.2 (±3.4) ms and mean QTc 438 ms. In the study group the QTc dispersion was 68.6 (±3.7) ms and mean QTc 442 ms.

**Conclusion:** In these hypertensive T2DM patients the prevalence of increased QTc and QTc dispersion was relatively high. The patients with an altered cortisol and aldosterone pattern had a significantly higher QTc dispersion and also a numerically higher mean QTc, although the difference was not significant. The main limitation of the study is the small number of patients, so further studies are needed to establish if a relationship exists between these parameters.

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### Downregulation of Endothelial PLXNA4 under Pro-Atherosclerotic Conditions Diminishes Vascular Integrity Enabling Monocyte Transendothelial Migration

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**Introduction:** Atherosclerosis is a systemic inflammatory disease, characterized by the accumulation of macrophages in the vascular wall. Neuroimmune guidance cues (NGCs), originally identified to regulate neuronal and vascular patterning, are significant

players in leukocyte trafficking. We set out to investigate the expression of NGCs and their receptors in human endothelial cells and monocytes under pro-atherogenic conditions and to clarify their function in atherosclerosis-related processes.

**Methods and Results:** Human primary monocytes and endothelial cells cultured in the presence or absence of TNF $\alpha$  or IL1 $\beta$  displayed several significant up- and downregulations in NGC genes. A particularly interesting concurrent downregulation occurred in the receptor PLXNA4 in endothelial cells and its ligand SEMA3A in monocytes under inflammatory conditions. Subsequently, we aimed to investigate the role of endothelial PLXNA4 in atherosclerosis-related processes. Silencing endothelial PLXNA4 markedly induced an inflammatory elongated morphological change. Indeed, endothelial cells with reduced PLXNA4 displayed, already under basal conditions, increased expression of ICAM1, impaired barrier function, and increased RAC1 activity. Importantly, we observed an increase in monocyte migration across endothelial monolayer with reduced PLXNA4 expression.

**Conclusion:** We show that PLXNA4 has important anti-inflammatory properties. Loss of PLXNA4 affects endothelial integrity and monocyte transendothelial migration. These studies provide novel insight into the immune-modulatory roles of semaphorin ligands and receptors in atherosclerosis.

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### Sublingual Microcirculation in Chronic Heart Failure

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**Background:** Microcirculatory changes contribute to clinical symptoms and disease progression in chronic heart failure (CHF). We hypothesized that changes in cardiac microcirculation might also be reflected systemically and visualized by sublingual videomicroscopy. The aim was to study in vivo functional and perfused total capillary density as well as glycocalyx dimensions in patients with CHF vs. healthy controls.

**Methods:** Fifty patients with ischemic and non-ischemic cardiomyopathy and conservative treatment were compared to thirty-five healthy age-matched subjects in a prospective cross-sectional study. Sublingual microcirculation was visualized using a Sidestream Darkfield videomicroscope. Functional and total capillary densities were compared between patients and controls. A reduced glycocalyx thickness was measured by an increased perfused boundary region (PBR).

**Results:** In CHF patients there was a significant rarefaction of median functional and perfused total capillary densities by 30% and 45%, respectively, both  $p < 0.001$ . Dimensions of the glycocalyx were marginally lower in CHF patients than in healthy controls

(<7% difference, n.s.). Loss of glycocalyx was pronounced in patients with overall death after a median follow up time of 31 months (PBR: 2.06  $\mu\text{m}$  (1.98–2.12  $\mu\text{m}$ ) vs. 1.85  $\mu\text{m}$  (1.63–1.97  $\mu\text{m}$ ),  $p = 0.002$ ). In addition, a PBR above 1.95  $\mu\text{m}$  remained significantly associated with all-cause death in a cox regression analysis ( $\beta = 0.148$ ; CI (95%): 0.023–0.973,  $p = 0.047$ ).

**Conclusion:** CHF patients have got markedly lower sublingual functional and perfused total capillary densities when compared to healthy controls. In addition, glycocalyx dimensions might have an impact on patient prognosis.

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### Eph-Ephrin Signalling Patterns Early Venous Valve Formation

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**Introduction:** Venous valves (VVs) ensure unidirectional flow of blood back to the heart. The formation of a competent VV requires organisation of valve forming cells (VFCs) by postnatal day 0 (P0), associated with delineation of restricted connexin expression domains. EphrinB2 (encoded by *Efnb2*) binds the receptor tyrosine kinases EphB2/EphB3/EphB4 and is required for postnatal VV development. However, the roles of Eph-ephrin signalling in prenatal VV development have not been investigated. We aimed to explore the role of Eph-ephrin signalling in patterning the early organisation of VFCs.

**Methods:** An *Efnb2*<sup>GFP</sup> reporter line, and *Efnb2* *in situ* hybridisation, were used to localise *Efnb2* expression in E18-P0 VVs. Conditional deletion of *Efnb2* was performed using floxed alleles and *Prox1CreER*<sup>T2</sup> mice, with administration of 4-hydroxytamoxifen at E15+16. *Efnb2*-deleted and littermate control offspring were analysed at E18-P0. Wholmount immunofluorescence with confocal microscopy was used to localise: EphB2, EphB3, EphB4, integrin- $\alpha$ 9, connexin37, connexin43, as well as the expression of proliferation (Ki67) and apoptosis (cleaved caspase-3) markers. Human VVs were analysed in patients with mutations in *EPHB4* using ultrasonography.

**Results:** *Efnb2* was expressed by the ring of VFCs and in endothelial cells downstream (but not upstream) of the VFCs at P0. Its

receptor EphB4 was immunolocalised throughout the valve region, particularly within clusters of proliferating VFCs at the superior and inferior regions of the valve. EphB2 and EphB3 were not localised to VVs at P0. Deletion of *Efnb2* resulted in disorganised VFCs that failed to form an organised ring (predominantly on the anterior wall, i.e. Stage 1 of development) by P0 ( $p < 0.001$ ), and VFCs exhibited reduced elongation ( $p < 0.001$ ) and reorientation ( $p < 0.05$ ). *Efnb2* deletion also disrupted the normally highly restricted expression patterns of connexin37 and connexin43 at P0. Integrin- $\alpha$ 9 expression was largely localised to the ring of VFCs on the anterior vein wall at P0 in littermate controls, but this was disrupted by *Efnb2* deletion. A reduction in the proportion of proliferating VFCs was seen following *Efnb2* deletion ( $p < 0.001$ ), with no detectable effect seen on apoptosis. In humans, almost no VVs were detected in patients carrying mutated *EPHB4*.

**Conclusion:** The patterning of VFCs to form a VV at P0 requires ephrinB2, likely via its interaction with the receptor tyrosine kinase EphB4. EphrinB2 is critically required to pattern gap junction communication domains, the restricted expression of integrin- $\alpha$ 9, and VFC proliferation, all of which are required for normal VV formation. Patients with *EPHB4* mutations exhibited severe VV disease.

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### Inflammatory Activation of Endothelial Cells Induced by Disturbed Flow is Regulated by Frizzled-4 and $\beta$ -catenin via a Non-Canonical Pathway

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**Objectives:** The development of cardiovascular disease is strongly influenced by local mechanical forces. Endothelial cells are sensitive to haemodynamic stresses, and in particular to perturbations of flow direction. Atherosclerotic plaques develop in regions of arteries where flow is multi-directional or 'disturbed'; such flow is known to promote endothelial dysfunction, although the signalling mechanisms responsible are not fully understood. The Wnt/ $\beta$ -catenin pathway plays an important role in mechanosignalling in non-vascular cells; in this study, its role in mediating the effects of disturbed flow on human aortic endothelial cells was explored.

**Methods:** Cells seeded into 6-well plates were exposed to flow for 72 h using an orbital shaker housed inside an incubator. The swirling of the medium with each rotation of the platform creates two distinct flow environments within each well: an area in the centre exposed to disturbed flow (DF) and a region around the edge exposed to undisturbed flow (UF). Flow metrics within each region were determined by computational fluid dynamics using STAR-CCM+. Following exposure to flow, cells were harvested from each region and either RNA or protein was prepared for qRT-PCR or western blot, respectively. For immunofluorescence studies, cells were fixed directly in glass-bottomed wells and imaged by confocal microscopy.

**Results:** The expression of Frizzled-4 was significantly increased in cells exposed to DF for 72 h, as was the expression and

transcriptional activity of  $\beta$ -catenin. Interestingly, this was not associated with activation (phosphorylation) of Lrp-6, suggesting that  $\beta$ -catenin is activated by a non-canonical pathway. Increased expression of Frizzled-4 protein was associated with increased expression of R-spondin-3 that protects Frizzled receptors from ubiquitin-mediated degradation. Knockdown of either Frizzled-4 or  $\beta$ -catenin significantly reduced the expression of pro-inflammatory transcripts (E-sel, MCP-1, VCAM-1) in cells exposed to DF, and this was associated with reduced activation and nuclear localisation of NF- $\kappa$ B. Similar effects were observed when cells were treated with iCRT5, an inhibitor of  $\beta$ -catenin transcriptional activity. Treatment with iCRT5 also reduced the adhesion of THP-1 monocytes to endothelial cells exposed to DF, confirming the importance of  $\beta$ -catenin activation in mediating inflammatory responses to DF. Treatment with DKK-1, an inhibitor of canonical Wnt signalling, had no effect on the expression of pro-inflammatory genes, NF- $\kappa$ B activity or monocyte adhesion under DF conditions, suggesting that the canonical Wnt-Fzd pathway is not involved in these responses.

**Conclusions:** These data suggest the involvement of a novel Frizzled-4- $\beta$ -catenin mechanosignalling pathway in endothelium exposed to DF that promotes a pro-inflammatory phenotype.

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### Dissecting the Link Between Microcalcification and Macrophage-Led Inflammation in the Vasculature

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**Background and Aims:** Microcalcifications in atherosclerosis hugely impact the inflammatory state of the plaque, and inversely, inflammation drives calcium deposition. We aimed to assess the role that macrophage-led inflammation has in vascular calcification and remodelling, by performing a weighted gene co-expression network analysis (WGCNA) to find functionally associated candidate genes.

**Methods:** A WGCNA was performed on human plaque material obtained via carotid endarterectomy on 36 patients, with stable and unstable regions defined. Calcification was measured by alizarin red staining, and macrophage presence via CD68 immunohistochemistry, which when quantified, was correlated to gene module expression in the same tissue. Genes in correlating modules were ranked by macrophage relevancy and gene cluster centrality, and the top ten chosen. Expression was measured via RT-PCR on a panel of vascular cell types, and on peripheral blood mononuclear cell-derived macrophages exposed for 24, 48 and 72-hours to either hydroxyapatite nanoparticles, high phosphate media or high calcium phosphate media.

**Results:** In stable plaques there was strong correlation between macrophage presence and calcification presence ( $r = 0.869$ ,  $p = 1.2 \times 10^{-5}$ ), whereas in unstable plaques, calcification highly correlated to arginase 1 expression ( $p = 1.2 \times 10^{-4}$ ) and multinucleate giant cell presence ( $p = 0.03$ ). Three gene modules in unstable plaques highly correlated to calcification ( $p$  value 0.02, 0.01,  $0.7 \times 10^{-4}$ ), from which ten candidates were selected. Candidate

gene expression was measured via RT-PCR on VSMC, VEC, and polarised macrophage subsets; seven of the candidates showed significant macrophage relevancy. Expression was then measured in macrophages after calcification stimuli exposure, where six of the genes showed significant changes in expression after incubation with calcifying stimuli; for example, CDK5 was significantly up-regulated after 5 day exposure to both hydroxyapatite and calcium phosphate, and Osteopontin (SPP1) was significantly upregulated after 5 day hydroxyapatite exposure.

**Conclusions and Future Work:** We have here defined seven candidate genes highly associated with macrophages in calcified atherosclerotic plaques. Candidates will now be functionally assessed for roles in macrophage engagement in active calcification through siRNA knockdown studies. Macrophage functional responses to exposure to calcification stimuli will also be evaluated. Our correlation data is in concordance with previous literature suggesting a remodelling phenotype of macrophages engaging with plaque calcification.

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### Lipid Regulation of KIR2.x Channels and the Enabling of Hemodynamic Sensing in Cerebral Arteries

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Inward rectifying (KIR) K<sup>+</sup> channels are expressed in both smooth muscle and endothelial cells of the rat cerebral circulation. This tandem arrangement is unusual for a presumptive background current and suggests a dynamic yet undiscovered role for this channel in tone development. This study defined whether distinct pools of cerebral arterial KIR channels were uniquely modulated by membrane lipids and hemodynamic stimuli. A Ba<sup>2+</sup>-sensitive KIR current was isolated in smooth muscle and endothelial cells of rat cerebral arteries; molecular analyses subsequently confirmed KIR2.1/KIR2.2 mRNA and protein expression in both cells. Patch clamp electrophysiology next demonstrated that each population of KIR channels were sensitive to key membrane lipids and hemodynamic stimuli. In this regard, endothelial KIR was sensitive to phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) content, with depletion impairing the ability of laminar flow to activate this channel pool. In contrast, smooth muscle KIR was sensitive to membrane cholesterol content, with sequestration blocking the ability of pressure to inhibit channel activity. The idea that membrane lipids help confer flow and pressure sensitivity of KIR channels was confirmed in intact arteries using myography. Virtual models integrating structural/electrical observations reconceptualised KIR as a dynamic regulator of membrane potential working

in concert with other currents to set basal tone across a range of intravascular flow and pressure. The data show for the first time that specific membrane lipid-KIR interactions enable unique channel populations to sense hemodynamic stimuli and drive vasomotor responses to set basal perfusion in the cerebral circulation.

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### The Conducted Response as an Electrical Pliant Process: Reimagining Cell Signaling in the Arterial Wall

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Vasomotor responses conduct along and among resistance arteries to coordinate blood flow delivery with energetic demand. The distance these responses conduct is set by the electrical and mechanical properties of vascular cells, the former tied to charge movement via gap junctions and ion channels, elements subject to regulation. Using *in silico* approaches, this study conceptualized the idea of electrical pliancy, illustrating in clear terms how gap junction and ion channel properties impact conduction along a virtual artery or branching arteriolar network. Initial simulations reproduced known conducted behavior, highlighting the endothelium's importance as an electrical conduit and a key charge source. Next, each set of gap junctions was shown to uniquely impact charge distribution, with diminished: 1) endothelial-to-endothelial coupling, impairing longitudinal spread; and 2) myoendothelial coupling modestly enhanced conduction by limiting radial spread to the smooth muscle layer. A final set of simulations noted how subtle change in ionic conductance impact charge loss through membranes and consequently conduction. Of note was detailed work on inward rectifying- or voltage dependent - K<sup>+</sup> channels showing how their voltage dependent properties tune conducted hyperpolarization and depolarization, respectively. This analysis led us to view conduction as an electrically pliant process, with subtle regulatory changes in ion channel activity, most likely to impact charge spread in network structures and consequently blood flow control.

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### Brain-Derived-Neurotrophic-Factor, Dynamic Retinal Vessel Responses During Flicker Light and Stroke Risk: The SABPA study

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**Background:** Assessment of the retinal vessels' structure and function provides an automated and objective approach to non-invasively assess the condition of the cerebral microvasculature and indicates susceptibility for stroke. Neurons, glial cells and the cerebral endothelium exhibit a unique relationship as they function as a cohesive unit namely, neurovascular coupling, similar to that of the retinal microvasculature. Brain-derived neurotrophic factor (BDNF) plays an essential role in the survival, growth and maintenance of neural structures. BDNF is actively released by cerebral endothelial cells, constituting a link between maintaining cerebral and possibly retinal blood flow as well as effective neurovascular coupling.

**Aims:** We investigated associations between retinal vessel structure and functionality during flicker-light-induced-provocation (FLIP), systemic BDNF levels, and potential stroke risk.

**Methods:** Prospective observations were obtained from a bi-ethnic cohort (N = 254), aged 23–68 years. Fasting serum BDNF levels and 24 h blood-pressure (BP) were obtained. At 3-year follow-up, retinal vessel calibres were quantified from mydriatic eye fundus images and dynamic retinal vessel responses were determined during FLIP. The University of California stroke risk score was applied to assess subclinical 10-year stroke risk.

**Results:** Lower BDNF levels were observed in the total cohort compared to reference ranges (average of 1.57 ng/mL vs normal ranges of 6.97–42.6 ng/mL). Overall, Africans exhibited greater 3-year increases in BP and BDNF levels. At follow-up they presented with a greater prevalence of retinopathy, 68% vs 38% in Caucasians; exhibited wider venules and attenuated arteriolar responses during FLIP, compared to Caucasians. Mean BDNF changes over 3-years were inversely associated with arteriolar dilation (p = 0.032) and arteriolar constriction time (p = 0.026), again, in the African group exclusively. A similar trend was observed in Caucasians (borderline significant p = 0.062). Furthermore, a novel BDNF cut-point of 1.5 ng/mL predicted the presence of hypertensive/diabetic retinopathy (95% CI 0.45, 0.71; AUC 0.60; sensitivity/specificity 49%/68%), irrespective of race/gender. Yet, independent of race or gender, lower BDNF levels predicted an increased 10 year Stroke risk with an odds ratio of 1.56 (95% CI, 0.94; 2.06, p = 0.011).

**Conclusions:** The neuro-protective effect of BDNF might be diminished in the SABPA cohort. BDNF may directly act on vascular-smooth-muscle-cells to alter arteriolar vascular resistance and contribute to disturbed neurovascular coupling and increased stroke risk. BDNF up-regulation, specifically in Africans, might

imply an attempt to maintain adequate neurovascular function yet, its protective mechanisms fail and manifests as attenuated retinal arteriolar responses during and following flicker light stimulation.

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### Heart Rate Variability and the Dynamic Nature of the Retinal Microvasculature: Providing Insight into the Brain-Retina-Heart Link: The SABPA Study

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**Introduction:** Controversy exists regarding the contribution of the autonomic nervous system (ANS) to the retinal microvasculature's autoregulatory capacities. It was demonstrated that mean retinal vessel responses to systemic sympathetic stimulation were significantly reduced in sympathectomised eyes. Decreased heart-rate-variability (HRV) indicates increased sympathetic nervous system (SNS) activity and modulation with a shift in the sympatho-vagal balance towards SNS predominance, vagal withdrawal or both. Increased SNS activity may precede volume-loading hypertension, endothelial dysfunction and small vessel disease.

**Aim:** Therefore, we investigated the retinal vasculature, HRV during flicker-light-induced-provocation (FLIP) to provide further evidence in support of the brain-retina-heart link.

**Methods:** Cross-sectional observations were obtained from a bi-ethnic cohort (N = 264), aged 23–68 years. Retinal vascular calibres were quantified from mydriatic eye fundus images taken with a Carl Zeiss FF450Plus camera (Carl Zeiss, Mediatech Jena, Germany), and dynamic retinal vessel calibre responses were determined during FLIP according to the standard flicker protocol developed by IMEDOS Systems. Frequency and time domain parameters of HRV were calculated during FLIP for each participant.

**Results:** Overall, Africans had wider venules and attenuated time domain parameters during FLIP. Again in Africans, inverse associations emerged between arteriolar dilation and both cTnT and root-square-mean-difference of successive RR-intervals (rMSSD) ( $p = 0.030$ ), and between arteriolar constriction and both low-frequency expressed in normalised units (LFnu) ( $p = 0.003$ ) and high-frequency expressed in normalised units ( $p = 0.021$ ). Wider venules were inversely associated with standard-deviation-of-successive-RR-intervals (SDNN) as well as LFnu ( $p = 0.009$ ) in Africans. An opposite profile was observed in Caucasians with both time and frequency domain parameters of HRV indicating physiological SNS variation in relation to retinal vessel structure and function.

**Conclusion:** We conclude that FLIP elicited increased SNS activity and modulation in this bi-ethnic cohort. SNS hyperactivity may cause loss of tone and/or altered haemodynamics of the reti-

nal microvasculature. Further, the attenuated arteriolar and venular responses during FLIP, accompanied by decreased HRV variables, imply that the SNS exerts a significant effect on the smooth muscle tone of the retinal vasculature, either directly or indirectly. Increased SNS modulation, activity and/or vagal withdrawal, related to altered retinal dynamics in Africans. Disrupted retinal autoregulation may be a result of general, sustained ANS dysfunction, exemplifying central control by the brain on all systemic regulatory functions, regardless of the vascular bed. Disrupted retinal autoregulation may imply general autonomic nervous system dysfunction; exemplifying central control by the brain on all systemic regulatory functions, across different vascular beds.

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### Autocrine C-Type Natriuretic Peptide Signalling Improves Macrovascular Endothelial Functions and Prevents Arterial Stiffness

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**Introduction:** Endothelial C-type natriuretic peptide (CNP) participates in the local regulation of vascular tone. Our recent work revealed that CNP-induced guanylyl cyclase (GC)-B/cGMP signalling in microvascular smooth muscle cells and pericytes is essential for the maintenance of normal microvascular resistance and blood pressure (Spiranec et al., *Circulation* 2018). In addition, the GC-B receptor is expressed in endothelial cells with unknown functions. To dissect the (patho)physiological roles of the endothelial CNP/GC-B/cGMP pathway we generated a novel genetic mouse model with endothelial-restricted inactivation of the GC-B receptor (Npr2fl/fl; Tie2Cre: EC GC-B KO (Spiranec et al., *Circulation* 2018)).

**Methods:** The effects of CNP on cGMP levels (by radioimmunoassay) and activity (phosphorylation of the vasodilator-stimulated phosphoprotein (VASP), by western blot analysis) were studied in primary cultured murine lung endothelial cells (MLECs). Arterial blood pressure of awake mice was measured by tail-cuff and telemetry. Aortic stiffness was estimated by non-invasive pulse wave velocity (PWV) measurements. Aortic thickness and collagen/elastin contents were evaluated in sirius red- or elastica van gieson-stained sections. The expression levels of the adhesion molecules E-selectin and VCAM-1 were analyzed by qRT-PCR.

**Results:** In vitro, in MLEC prepared from EC GC-B KO mice, the CNP stimulated cGMP formation and VASP phosphorylation were almost fully abolished indicating successful GC-B deletion. In vivo, EC GC-B KO mice exhibited mild but significant chronic increases in arterial blood pressure. Young KO mice showed isolated systolic hypertension whereas old mice had increases in both systolic and diastolic pressure levels. Notably such changes were

only observed in the female KO mice while their male littermates were normotensive. Consistently only female EC GC-B KO mice showed significantly higher PWV. Histology revealed increased aortic adventitial and medial thickness with augmented collagen content, decreased ratio of elastin to collagen and numerous elastin breaks. Molecular studies provided mechanistical insights showing that endothelial E-selectin and VCAM levels were enhanced in aortae from the EC GC-B KO females, indicating a pro-inflammatory phenotype.

**Conclusions:** We conclude that auto/paracrine endothelial CNP/GC-B/cGMP signalling participates in the maintenance of arterial blood pressure homeostasis and macrovascular elasticity/integrity. The morphological and molecular alterations of aortae from female EC GC-B KO mice indicate that CNP prevents vascular inflammation and remodelling and thereby aortic stiffness. The mechanism(s) of the sex difference in the consequences of endothelial deletion of CNP/GC-B signalling remain an important question for our current studies.

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### **Pomelo Extract Attenuates Cerebral Endothelial Dysfunction Through Activating PI3K-Akt-eNOS Pathway in A Rat Model of Type 2 Diabetes**

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**Introduction/Objective:** Several studies show an association of high Citrus fruit intake with a lower risk of CVD and stroke but the mechanisms involved are not fully understood. Pomelo belongs to the genus Citrus is source of flavonoids. Recently, a number of studies on pomelo extract have reported the antioxidant and antidiabetic properties. Therefore, the present study aimed to examine the effects of pomelo extract (PE) on cerebral endothelial function involvement of PI3K-Akt-eNos pathway in a rat model of type 2 diabetes.

**Methods:** Male Sprague-Dawley rats were divided into three groups (n = 8/group): normal control (Control) and diabetes with (DM2-PE) or without pomelo extract (DM2) administration. Type 2 diabetes was induced by feeding high fat diet for three weeks followed by an I.V. injection of low dose streptozotocin (STZ). Daily gavage feeding of PE (200 mg/kg BW) was performed for 12 weeks. The effect of PE on blood glucose (BG), HbA1C, serum insulin (S.insulin) were examined. To examine the endothelial functions, leukocyte adhesion (LA) to the venular endothelium was evaluated by counting leukocytes labeled with rhodamine 6G. In addition, functional responses of cerebral arterioles to endothelium-dependent (acetylcholine; ACh) and -independent (nitroglycerin; NTG) vasodilators were measured. Cerebral blood flow (CBF) perfusion was evaluated using a laser Doppler flowmeter. Additionally, levels of phosphorylation insulin receptor substrate-1 (pIRS-1) was examined by ELISA analysis. The expression levels of eNOS, PI3K

and phosphorylation of Akt (pAkt) in hippocampus were determined by western blot analysis. The experiments were approved by the ethical committee, Faculty of Medicine, Srinakharinwirot University, Thailand.

**Results:** The elevated BG, HbA1c and S.insulin were observed in DM2 rats. Our results showed that insulin resistance, as evaluated by an increasing in S.insulin was developed in HF fed and low dose of STZ induced DM2 rat model. DM2 rats had significantly decreased CBF, while LA was increased. Endothelial function in DM2-PE rats as evidenced by the increasing CBF and reducing LA was demonstrated, as compared to DM2 rats. The magnitude of vasodilation to ACh was significantly less in DM2 rats and was restored to near normal level in DM-PE rats. Moreover, we found that PE administration promoted PI3K, pAkt, eNOS expression and decreased level of pIRS-1 in the hippocampus of DM2-PE rats.

**Conclusion:** The present study suggested that pomelo extract could attenuate endothelial dysfunction and improve cerebral blood flow probably through its effect by increasing the activating PI3K-Akt-eNOS pathway in type 2 diabetes.

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### **Translational Approaches to Understanding Aging-Induced Cerebrovascular Dysfunction**

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**Background:** Age-related vascular cognitive impairment and dementia (VCID), defined as cognitive decline caused by cerebrovascular pathologies, is a major public health issue that affects >10 million individuals aged 65 and older in the US with annual healthcare costs totaling >\$100 billion. Aging is a major risk factor for the development of VCID and current strategies for VCID treatment are limited to targeting cardiovascular risk factors such as hypertension, diabetes mellitus, etc. Here, we hypothesized that aging, similarly to other risk factors, affects vasculature uniformly and that age-related changes in the peripheral circulation can be used as an important tool to predict cognitive decline.

**Methods:** For our study, we have enrolled 55 healthy 22–92 yo individuals and divided them into two groups of young (<45 yo) and aged (>65 yo). Peripheral macrovascular and microvascular endothelial function was measured using a standard flow-mediated dilation (FMD) and Laser Speckle Contrast Imaging tests (LSCI) in response to increased flow-mediated shear stress after a 5-min of blood flow restriction with arterial cuff. Pulse Wave Analysis was used to evaluate the augmentation index (AIx) as a measure of arterial stiffness. A principal component analysis (PCA) approach was used to create a vascular health index (VHI) that included all vascular measurements for each participant. Cognitive function including domain of learning, memory and executive function was measured via a selection of tests from the Cambridge Cognition CANTAB cognitive panel. A PCA approach was used to generate a cognitive impairment index for each participant.

**Results:** Subjects from the aged group showed significantly impaired macrovascular endothelial function (FMD,  $5.4 \pm 0.7\%$  vs.  $8.5 \pm 0.6\%$  in young,  $p < 0.01$ ) and increased arterial stiffness compared to young (AIx  $28.9 \pm 1.7\%$  vs  $4.5 \pm 2.6\%$  in young,  $p < 0.01$ ). Microvascular endothelial function showed significantly lower skin perfusion in aged subjects ( $2.8 \pm 0.1\%$  vs  $3.4 \pm 0.1\%$  in young,  $p = 0.03$ ) that correlated with FMD measurements ( $r = 0.34$ ,  $p = 0.014$ ), and significantly lower reperfusion rate in aged individuals ( $25.8 \pm 2.2$  PU/s vs  $35.1 \pm 2.7$  PU/s in young,  $p = 0.01$ ). PCA-generated Vascular Health Index (VHI) showed a significant negative correlation with age ( $r = -0.55$ ,  $p < 0.01$ ). VHI correlated with the Cognitive Impairment Index ( $r = -0.44$ ,  $p < 0.01$ ).

**Conclusion:** Our study demonstrates that deterioration of peripheral macro- and microvascular endothelial function is associated with and can predict VCID in healthy aged individuals.

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### Identification of Lipopolysaccharide-Activated Endothelial Subpopulations with Distinct Inflammatory Phenotypes and Regulatory Signaling Mechanisms

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**Introduction:** Endothelial cells (ECs) actively contribute to microvascular dysfunction associated with sepsis. Upon Lipopolysaccharide (LPS) exposure, TLR4 and RIG-I mediate downstream NF- $\kappa$ B and p38 MAPK signalling pathways to mediate E-selectin and VCAM-1 adhesion molecule expression that facilitates leukocyte recruitment into organs. This process is organ and microvascular compartment specific. How these heterogenic responses are molecularly controlled is unknown. Here, we investigated the extent of heterogenic EC responses in vivo and in vitro based on E-selectin and VCAM-1 expression patterns, and determined in vitro the contribution of the molecular signaling pathways to these responses.

**Materials and Methods:** We immunofluorescently double stained E-selectin and VCAM-1 in kidney cryosections from LPS-treated mice. HUVEC were stimulated with  $1 \mu\text{g/mL}$  LPS, flow cytometry was used to determine EC subpopulation formation based on E-selectin and VCAM-1 expression levels, fluorescence activated cell sorting to isolate EC subpopulations. Inflammatory gene expression was determined by RT-qPCR. TLR4 and RIG-I were knocked down using siRNA, BAY11-7082 and LY2228820 were used to inhibit NF- $\kappa$ B and p38 MAPK signaling, respectively.

**Results:** Expression of E-selectin and VCAM-1 in the microvasculature of kidneys of LPS-treated mice differed between microvascular compartments and between adjacent ECs within the same compartment. HUVEC exposed for 4 h to LPS revealed the formation of EC subpopulations that had similarities with the responses observed in the microvasculature in the kidney. A large

subpopulation of HUVEC expressed both E-selectin and VCAM-1, while a significant subset remained 'quiescent' despite exposure to LPS. Moreover, two additional EC subpopulations were only E-selectin or VCAM-1 positive. Sorting of these subpopulations followed by inflammatory cytokine/chemokine gene expression analysis revealed that the quiescent and the VCAM-1+ subpopulations expressed low levels of inflammatory genes, while the Esel+/VCAM-1+ subpopulation exhibited the most pronounced inflammatory phenotype. TLR4 and RIG-I predominantly controlled the formation of the Esel+ and Esel+/VCAM-1+ population, as did the p38 MAPK pathway. The VCAM-1+ population was not affected by any of the pathways investigated.

**Conclusion:** In mouse kidneys, ECs in different as well as in the same microvascular bed exert a heterogenic pattern of E-selectin and VCAM-1 expression following LPS challenge. Such a heterogenic response was recapitulated in vitro when HUVEC were exposed to LPS. The here identified endothelial subpopulations have distinct inflammatory phenotypes and are regulated by different signaling mechanisms downstream of the TLR4/RIG-I receptor system.

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### How to Build a Polarized Endothelial Cell: Non-Centrosomal Microtubules and Not the Centrosome Control Endothelial Cell Polarity and Sprouting Angiogenesis

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Despite extensive use of microtubule targeting compounds in vascular-directed anticancer treatment, knowledge of the cellular mechanisms underlying microtubule regulation during angiogenesis remains elusive. Microtubules have been described as crucial regulator of cell polarity during diverse processes important for angiogenesis such as directional migration. Microtubule organization in interphase animal cells is traditionally believed to be a radial network anchored at the centrosome. Whereas a pivotal role in setting up polarity is generally attributed to the forward positioning of the centrosome, the presence of microtubules that do not originate from the centrosome can potentially generate cell asymmetry. Here, by using sprouting angiogenesis as a paradigm of a polarised physiological process, we deciphered the contribution of centrosomal and non-centrosomal microtubules to controlling cell polarity. Strikingly, we showed that the loss of centrosomes had no effect on the ability of endothelial cells to polarize and move in 2D and 3D environments. In contrast, we uncovered a key function for non-centrosomal microtubules in establishing endothelial cell polarity. We show that, by destabilizing non-centrosomal microtubules silencing of the microtubule minus-end binder CAMSAP2 dramatically perturbs the ability of endothelial cells to establish polarity and leads to defects in Golgi positioning

and trafficking, 2D directional migration and formation of large persistent protrusions in 3D. Importantly CAMSAP2 was also required for persistent endothelial cell sprouting during in vivo zebrafish vessel development. Altogether our results demonstrated a key role for CAMSAP2-protected non-centrosomal microtubules in the guided process of sprouting angiogenesis, pointing towards the inability of a radial and symmetric centrosomal-anchored MT array to support cell polarization.

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### Retinal Vessel Caliber and Vessel Reaction to Light Flicker in True Normotensive Young Healthy Black and White Adults: The African-PREDICT Study

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**Introduction:** A detrimental shift in cardiovascular disease risk factors and a higher mortality level are reported in some black populations. The retinal microvasculature provides insight into the pathogenesis of systemic vascular diseases, but it is unclear

whether retinal vessel calibers and acute retinal vessel functional responses differ between young healthy black and white adults.

**Methods:** We included 115 black and 144 white adults (20–30 years). Participants did not have masked hypertension or white coat hypertension and displayed normal nocturnal blood pressure dipping status as determined by 24 hr ambulatory blood pressure (BP) monitoring and clinic BP, making them true normotensives. The Retinal Vessel Analyzer (IMEDOS Systems, GmbH) was used to determine 1) retinal vessel calibers (central retinal artery and vein equivalent (CRAE and CRVE)) from retinal images and 2) the retinal arterial and venous reaction to flicker light induced provocation (FLIP). Independent of the commercial software we assessed parameters describing vessel dilation and constriction (artery) or minimum reaction (vein). Additionally, anthropometry and blood samples were collected.

**Results:** The groups displayed similar 24 hr ambulatory BP profiles, body mass index and waist circumference (all  $p > 0.24$ ). Black participants demonstrated a smaller CRAE ( $158 \pm 11$  vs.  $164 \pm 11$  MU,  $p < 0.001$ ) compared to the white group, whereas CRVE was similar ( $p = 0.57$ ). In response to FLIP, arterial maximal dilation was greater in the black vs. white group ( $5.6 \pm 2.1$  vs.  $3.3 \pm 1.8\%$ ;  $p < 0.001$ ). The time to the minimum venous reaction value was longer in the black group ( $70.61 \pm 1.87$  vs.  $64.25 \pm 1.71$  s,  $p = 0.024$ ). Time to maximal arterial dilation, arterial constriction and venous dilation in response to FLIP did not differ between groups.

**Conclusion:** Already at a young age, healthy black adults demonstrate narrower retinal arteries relative to the white population. As retinal arterial narrowing may be predictive of future hypertension development, our finding aligns with demographic figures on hypertension prevalence in black populations. The reason for the larger vessel dilation responses to FLIP in the black population is unclear and warrants further investigation.

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