

## ABSTRACTS OF THE 61<sup>st</sup> MEETING OF THE BRITISH MICROCIRCULATION SOCIETY 18<sup>th</sup>–19<sup>th</sup> April, 2011, William Harvey Research Institute, Barts & the London School of Medicine and Dentistry, London, UK Editor : Neena Kalia, PhD Birmingham, UK

### ORAL COMMUNICATIONS

#### OC1

##### **Cd49d, Cd44 and Chemokine SDF1 $\alpha$ Govern HSC Adhesion Within Ischaemically Injured Kidney**

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**Introduction:** Many studies have demonstrated that haematopoietic stem cells (HSCs) migrate to injured kidney and aid in tissue repair. However, clinical success remains poor and is partially due to limited HSC recruitment to sites of injury. Identifying the adhesive mechanisms that retain HSCs within injured tissue may aid in the development of strategies that enhance stem cell recruitment. Therefore, this study determined the role of the integrins CD49d and CD18 and the non-integrin CD44 in mediating HSC adhesion to ischaemia-reperfusion (I/R) injured kidney *in vivo*. The role of the chemokine SDF-1 $\alpha$  in recruiting HSCs was also determined.

**Methods:** Anaesthetised (ketamine/xylazine) C57BL6 mice were subjected to 45 minutes renal ischaemia followed by 1hr reperfusion. HSCs were incubated with SDF1 $\alpha$ , IgG, anti-CD18, anti-CD49d or anti-CD44 function blocking antibodies prior to administration. Numbers of adherent CFSE-labeled HSCs within peritubular capillaries was monitored intravitaly.

**Results:** I/R injury led to a significant ( $p < 0.01$ ) increase in HSC adhesion. This was significantly reduced by pre-treating HSCs with an anti-CD49d ( $p < 0.01$ ) or anti-CD44 ( $p < 0.05$ ) antibody. SDF-1 $\alpha$  pre-treatment significantly ( $p < 0.01$ ) enhanced HSC adhesion within I/R injured kidney.

**Conclusion:** Increasing HSC surface expression of CD49d or CD44 (or their affinity for their counterligand), may be used as a therapeutic strategy to enhance HSC adhesion within the kidney. Alternatively, renal homing may be increased by pre-treating HSCs with SDF-1 $\alpha$ . Such thera-

peutic strategies may improve the clinical outcome of cellular therapies.

Acknowledgment: This work was supported by the MRC.

#### OC2

##### **Neutrophils Exhibit Multiple Forms of Transendothelial Cell Migration (TEM) *In Vivo*: An Investigation Using High Resolution Confocal Intravital Microscopy**

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A high resolution real-time 3D imaging technique was established for analyzing the characteristics and dynamics of neutrophil transmigration *in vivo*. The model involves rapid confocal microscopy imaging of the cremaster muscle of *lys*-EGFP mice (which exhibit EGFP<sup>+</sup>-neutrophils) in conjunction with fluorescently labelled endothelial cell (EC) junctions using an anti-PECAM-1 mAb. With this method we have examined the profile and dynamics of neutrophil TEM as elicited by several stimuli, namely IL-1 $\beta$ , FMLP and ischemia/reperfusion injury. In all reactions the majority (~90%) of the TEM events occurred via the paracellular route where the formation of EC junctional pores at bi- and tri-cellular junctions could be clearly imaged and analyzed in terms of their frequency (no significant difference noted between bi- and tri-cellular junctions) and duration (eg  $6 \pm 0.5$  minutes in IL-1 $\beta$ -stimulated tissues). Clear evidence for the formation of transcellular pores was also obtained ( $9.3 \pm 2.1$  % for all reactions). Additionally we report that neutrophils can exhibit disrupted polarised

TEM (“hesitant” and “reverse”) *in vivo*. Collectively the high spatial and temporal resolution of our approach has led to novel qualitative and quantitative analysis of neutrophil TEM *in vivo*.

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### OC3

#### Platelet Bridges Selectively Recruit Monocytes to Endothelial Cells in Human and Mouse Models of Vascular Inflammation

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Cells of the monocyte lineage are major effectors of the inflammatory cascade and in some chronic inflammatory diseases they dominate the inflammatory infiltrate, indicating the presence of disease driven mechanisms supporting their selective recruitment. Using primary human cells in an *in vitro* flow based adhesion assay, we found that secretory arterial smooth muscle cells (SMC), cocultured with EC, promote preferential recruitment of monocytes from blood in a TGF- $\beta$ 1 dependent manner. Approximately 85% of leukocytes recruited to the endothelium were CD14+. Formation of adhesive platelet bridges on EC was essential for monocyte recruitment, as removal of platelets from whole blood, or inhibition of their adhesion to EC, abolished monocyte recruitment. Monocytes were recruited from flow by platelet P-selectin and activated by EC derived CCL2, although the presentation of CCL2 to adherent monocytes was dependent upon platelet activation and release of CXCL4 from alpha granules. In an intravital model of TGF- $\beta$ 1 driven vascular inflammation in mice, platelets were also necessary for efficient leukocyte recruitment to vessels of the microcirculation in the cremaster muscle. Thus, we demonstrate that stromal cells found at sites of inflammation may promote the preferential recruitment of monocytes and this is achieved by establishing a cascade of interactions between EC, platelets and monocytes.

### OC4

#### Hematopoietic Stem Cell Recruitment to Injured Gut is Enhanced Following HSC Treatment with Hydrogen Peroxide

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Introduction: Hematopoietic stem cells (HSCs) migrate to injury sites and aid in tissue repair. However, clinical suc-

cess is poor and is partially due to limited HSC recruitment. We hypothesised that HSC pre-treatment with H<sub>2</sub>O<sub>2</sub>, known to be released within ischemic sites, would enhance their recruitment to injured gut.

Methods: Anaesthetised (ketamine/xylazine; i.p.,) mice were subjected to intestinal ischemia-reperfusion (I/R) injury and HSC recruitment was examined intravitaly. HSC adhesion to endothelial cells (ECs) and frozen injured gut sections was also quantitated *in vitro*.

Results: Significantly ( $p < 0.05$ ) increased HSC adhesion was observed in injured gut *in vivo*. Pre-treating HSCs with H<sub>2</sub>O<sub>2</sub> (100  $\mu$ M; one hour) significantly ( $p < 0.01$ ) enhanced this recruitment threefold. A concomitant reduction ( $p < 0.05$ ) in pulmonary adhesion was also observed. Pre-treatment also significantly ( $p < 0.05$ ) enhanced adhesion to ECs and injured gut sections and significantly ( $p < 0.05$ ) increased HSC surface expression of CD49d and CD18. Significant ( $p < 0.01$ ) F-actin polymerisation was observed in pre-treated HSCs. Importantly, H<sub>2</sub>O<sub>2</sub> did not reduce HSC viability or proliferative ability.

Conclusion: HSC recruitment to injured gut can be modulated by H<sub>2</sub>O<sub>2</sub>. This may be through increasing expression of integrins previously demonstrated by us to mediate HSC homing to injured sites, or through stimulating the migratory apparatus rendering HSCs more likely to migrate towards chemotactic stimuli. Strategies that enhance HSC recruitment may affect their therapeutic efficacy.

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### OC5

#### Regulation of the Platelet Collagen Receptor GPVI by the Tetraspanin Tspan9

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Tspan9 is a member of the tetraspanin superfamily of transmembrane proteins that is preferentially expressed on platelets and endothelial cells. A major characteristic of tetraspanin proteins is their self-association into clusters called microdomains. These microdomains contain not only tetraspanins but also other surface membrane proteins. On platelets these include the collagen receptor GPVI, the laminin-binding integrin  $\alpha$ 6 $\beta$ 1, the scavenger receptor CD36 and the ectodomain sheddase ADAM10. We hypothesise that tetraspanins fine-tune cell function by helping to organise such cell surface proteins. Tetraspanins cover a substantial proportion of the platelet surface and to date five tetraspanins have been found on platelets using antibodies: CD9, CD151, CD63, Tspan9 and Tspan32. Platelets deficient in CD151 or Tspan32

exhibit impaired *in vivo* thrombus formation. In contrast, CD9-deficient platelets generate larger thrombi while CD63 appears to play no detectable role in this process. In the present study we report the generation of Tspan9-deficient mice. These mice are viable, healthy and have no major bleeding phenotype as measured using the tail bleeding assay. However, we have identified a mild but specific defect in platelet aggregation and spreading in response to GPVI activation. Future experiments will investigate the mechanism by which Tspan9 regulates GPVI and will evaluate this tetraspanin as a potential anti-platelet drug target.

Acknowledgment: This work is funded by the BBSRC and British Heart Foundation.

## OC6

### Mapping Megakaryocytes in Bone Marrow Microenvironment

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Bone marrow (BM) provides specialized microenvironments, composed of matrix proteins, stromal cells and chemokines to regulate self-renewal of stem cells and their differentiation along all blood cell lineages. How differentiation along a single lineage is orchestrated with different maturation stages co-existing while migrating from the stem cell niche to the vascular niche is poorly understood. Megakaryocytes (MK) of every maturation status are distributed all over the BM, whereas proplatelet and platelet shedding of mature MK takes place across the endothelial barrier at the sinusoids. We used multi-colour immunohistochemistry to map BM components in mouse femurs. Beneath endoglin, PECAM-1, laminin and integrins we detected a tight association of MK with fibronectin and collagen IV known to support proplatelet formation [JTH, 6, 1900]. However, premature platelet release was absent in the vicinity of collagen IV-associated MK, suggesting a mechanism to prevent premature MK fragmentation. We disrupted the MK equilibrium using a model of immune-mediated platelet depletion to study the effect on BM homeostasis. The depletion antibody was detected upon MK from day 3 to 7 while total MK numbers peaked on day 3 and went down to normal within 14 days. Our data show that the antibody does not lead to substantial MK apoptosis. When mice were fed with EdU after depletion, MK did not stain positive. We conclude that MK are extending from a pre-existing pool rather than proliferating *de novo*.

## OC7

### Elucidating the Mechanism of Platelet Mediation in Physiological Angiogenesis

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We have previously reported endothelial sprouting by muscle overload is dependent on platelets, but longitudinal splitting by hyperaemia is not [1]. Recent work confirms platelet mediation in these divergent forms of physiological angiogenesis using mouse models. We examined the role of platelets by depletion with anti-GPIIb/IIIa antibody treatment. To exclude possible immune regulation, we depleted granulocytes with anti-Ly6G/6C, and saw no change to the effect on capillary:fibre ratio (C:F) either before or after induction of endothelial sprouting. Since clopidogrel hydrogen-sulfate/aspirin are used clinically to inhibit platelet aggregation, we treated mice with a dual regimen of 25 mg/kg each after overload, and found abolition of the normal angiogenic response. To further elucidate the mechanism, mice received single regimens of either drug. We identified the cyclooxygenase inhibitor aspirin as the active agent, while clopidogrel had no effect. Aspirin had no effect on platelet independent longitudinal splitting however. Determination of cellular proliferation by BrdU pulse-labelling showed platelet depletion was not affecting proliferative ability, suggesting an effect further downstream, such as inhibition of matrix metalloproteases that also has no effect on proliferation [2].

Acknowledgment: This work was supported by the BHF.

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## OC8

### Effects of Aggregation on Human Blood Flow in a Microchannel

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Blood can be considered as a two phase fluid consisting of deformable cells suspended in a continuous fluid phase. Microvascular blood viscosity is velocity and geometry dependent, which has been attributed to the deformability and aggregation tendency of the erythrocytes. Erythrocyte aggregation occurs in the vasculature under sufficiently low shear conditions in both physiological and pathological states. There is as yet no consensus on the influence of aggregation on the resistance to flow. Microscopic PIV has

been previously used to measure blood flow both *in vitro* and *in vivo* using either images of erythrocytes or fluorescent particles seeded in the flow. Dusting *et al* [1] described a method for simultaneously measuring velocity fields and erythrocyte aggregation in an optical shearing microscope. In the present study this technique is further developed and used to analyse the flow of aggregating blood through a microchannel. Dextran 2000 was added to a suspension of erythrocytes in phosphate buffered saline (PBS) to induce aggregation. For aggregating cases, local deviations from the expected flow profile have been observed in straight portions of the channel. Further experiments are being carried out at a T-junction in order to provide detailed information on the effect of aggregation on blood flow behaviour in bifurcating and converging microvessels.

Reference:

1. Dusting *et al.*, *J Biomechanics* 42:1438–1443, 2009.

#### OC9

### Human Islet Mesenchymal Cells Interact with Endothelial Cells and Promote Loss of Vegfr2 via TGFBR1 Signalling

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Cultured human endothelial cells (ECs) have mesenchymal potential [1], suggesting that mesenchymal stem cells (MSCs) and ECs may have overlapping functions. Since MSCs exhibit apparent angiogenic potential, co-transplantation with islets may offer a new approach for optimizing post-transplantation islet vascularisation in type 1 diabetes. We used a heterotypic co-culture model to investigate the mechanisms mediating MSC:EC communication. Islet-derived MSCs express CD73, CD90, CD44 and CD105 and exhibit multilineage potential but do not express CD34, CD31 or VEGFR2. These cells form tube-like structures (<two hours) on Matrigel, express VEGF 121/165/189 mRNA, and medium conditioned by islet MSCs increases EC viability ( $p < 0.01$ ). Direct contact co-culture of MSCs and labeled ECs highlights rapid EC loss (<eight hours) correlated with reduced VEGFR2 protein expression. TGF- $\beta$  signalling is known to inhibit EC proliferation via Smad 2/3 and we found that Smad 2 phosphorylation increased during co-culture. SB431542 (SB), a selective pharmacological inhibitor of TGF $\beta$ RI activity enhanced EC viability ( $p < 0.0001$ ) and treatment of MSC:EC co-cultures with SB prevented the loss of EC populations. These data identify a role for TGF $\beta$  signalling in paracrine control of EC function by islet MSCs. Positive modulation of these interactions could improve islet transplantation success by maintaining islet viability.

Reference:

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#### OC10

### Endothelial Progenitor Cell Responses to Hypoxia: Time-Course Dependent Changes in Signalling Pathways and Gene Expression

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Current treatments for diabetic retinopathy (DR) do not address the early stages of this disease. The aim of this work is to examine endothelial progenitor cells (EPCs) as a cell based autologous therapy to revascularise the ischaemic tissue of the diabetic retina. To mimic low O<sub>2</sub> concentrations in the diabetic retina, we have exposed EPCs to 1% O<sub>2</sub> for varying times. Changes in Akt phosphorylation were noted after 30 minutes exposure to hypoxia which then returned to basal levels by one hour and remained so up to 48 hours exposure to 1% O<sub>2</sub>. Considerable alterations in the phosphorylation status of several proteins involved in mTOR signalling were also observed with acute exposure to hypoxia provoking a significant induction in phosphorylation of p70S6K. In contrast chronic hypoxia resulted in a considerable decrease in p70S6K which correlated closely with an increase in both AMPK and raptor phosphorylation. Global gene array analysis identified distinct cohorts of early and late response genes in EPCs to hypoxia. As expected we saw induction of several hypoxia related genes. We also revealed alteration in expression of a number of novel hypoxia genes including several involved in the BMP/Smad signalling pathway. We are currently determining the significance of these gene expression patterns in EPCs. These data will provide useful tools to allow defects in OECs from diabetic patients to be rescued *ex vivo* for future treatment of DR.

#### OC11

### Involvement of Semaphorin 3B in Regulation of Human Dermal Wound Healing

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Angiogenesis is up-regulated early in wound healing then following inhibition vascular regression occurs, the mechanisms of which are largely uncharacterised. Class 3 semaphorins, including Sema3B, are soluble ligands that inhibit

vascular endothelial growth factor (VEGF) activity by competing with VEGF for binding neuropilins (Np1 & Np2) and are thought to be anti-angiogenic. This study aimed to test the hypothesis that *Sema3B* is involved in regulation of angiogenesis and vascular regression during wound repair. Immunohistochemistry of human scar biopsies taken from patients between three days and two years post surgery ( $n = 94$ ) demonstrated up-regulation of *Sema3B* expression from 53–78 weeks post-surgery, i.e., during the vascular regression phase. Moreover, recombinant *Sema3B* significantly ( $p < 0.03$ ) inhibited HuDMEC migration by 45% and significantly ( $p < 0.05$ ) decreased the number of tubules formed on Matrigel in the presence and absence of VEGF (control =  $51 \pm 0.6$ ; *Sema3B* =  $26 \pm 0.5$ ; VEGF =  $60 \pm 0.2$ ; VEGF + *Sema3B* =  $32.8 \pm 1.1$ ). Moreover, when  $1 \mu\text{g}/\text{kg}$  *Sema3B* was injected into mice bearing a subcutaneous wound, wound angiogenesis (microvessel density; MVD) was significantly ( $p < 0.005$ ) inhibited (median MVD: control = 25; *Sema3B* treated = 13). Work is currently underway to establish whether *Sema3B* enhances vascular regression *in vivo*. These data suggest that *Sema3B* is an anti-angiogenic agent that may be involved in vascular regression in human dermal wound healing.

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#### OC12

### Unravelling the Placental Maternal Circulation in Diabetes Using a Novel Standardized Approach to 3D Power Doppler Angiography.

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In the human placenta, chorionic villi lie bathed in maternal blood flowing from endometrial spiral arteries that pierce the basal plate. Altered flow dynamics here may lead to placental changes and adverse fetal outcome in maternal diabetes. Non-invasive imaging techniques to assess spiral arteries may be useful in describing the maternal circulation. Using 3D power Doppler angiography (3D PDA), which allows computation of flow and vascular indices, we designed a novel standardized procedure which further manipulated the 3D renders to produce 2D cross-sections of spiral arteries suitable counting. Using the basal plate as a reference point, sampling position and volumes of renders were standardised; a final tilting of  $90^\circ$  produced the 2D cross-sections. Numbers and flow signal diameters of spiral arteries in normal ( $n = 9$ ; N) and Type 1 diabetic ( $n = 9$ ; T1D) women were measured at 20 weeks gestation. Normal placenta had an average of  $0.6$  spiral arteries/ $\text{cm}^2$ ; comparable to histological studies. This distribution was similar in diabetic placenta. Flow signal diameters showed

an increase in T1D ( $p < 0.05$ ). This is a first study showing that the number of maternal arteries supplying the placenta does not change in T1D but their cross-sectional flow signal does. 3D PDA is a new and evolving technique and may allow a more detailed description of the placental maternal circulation in pregnancy in future studies.

#### OC13

### The Plasma Membrane Calcium ATPase Negatively Regulates Enos Activity in Endothelial Cells

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The ability of the endothelium to synthesise and release nitric oxide (NO) plays a major role in the regulation of cardiovascular pathophysiology. Agonist stimulation triggers NO synthesis in endothelial cells *via* activation of the endothelial nitric oxide synthase (eNOS). eNOS activity is tightly regulated by multiple interdependent mechanisms including its functional association with partner proteins. In this sense, we have detected a novel inhibitory interaction between eNOS and the plasma membrane calcium ATPase (PMCA) in human endothelial cells. Ectopic expression of PMCA leads to a decrease in eNOS activity and subsequent NO synthesis (30% reduction). In contrast, disruption of the PMCA/eNOS interaction (by expression of the PMCA interaction domain) reverses the PMCA-dependent inhibition of eNOS activity. An initial insight into the molecular mechanisms implicated in the PMCA-mediated inhibition of eNOS, has revealed that PMCA/eNOS interaction promotes a significant increase in the inhibitory phosphorylation of eNOS at Thr-495. In conclusion, our results suggest a novel role for PMCA as a negative regulator of eNOS activity and therefore, NO production by endothelial cells. Considering the relevant role of NO in cardiovascular pathophysiology, our findings might have an important significance to design new therapeutic approaches to treat cardiovascular patients.

#### OC14

### A Unique Morphology of WPB in Human Heart Microvascular Endothelial Cells

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Endothelial cells (EC) secrete a large coagulation protein the von Willebrand factor (vWF), which is stored in EC-exclusive storage organelles the Weibel Palade bodies

(WPB). Storage or secretion of vWF may be an important factor for the differences between the macro and microvasculature in vascular pathology. We found that the vWF immunoreactivity in cultured primary human heart microvascular EC (HHMEC) is composed of primarily small diffraction-limited punctate organelles, rather than the distinctive rod-shaped WPB found in human umbilical vein EC (HUVEC). Electron microscope images of HHMEC interestingly show a disordered vWF in PB. Secretagogue-evoked WPB exocytosis was investigated using sub-second resolution live cell imaging of GFP-labelled WPB in Fura-2 loaded HHMEC or HUVEC, allowing simultaneous visualisation of WPB exocytosis and  $\text{Ca}^{2+}$  signalling. The kinetics of WPB exocytosis do not differ between the HHMEC and HUVEC. Furthermore we studied the intragranular pH ( $\text{pH}_g$ ) using GFP as a pH indicator ( $\text{pH}_g 5.7$  and  $\text{pH}_g 5.5$  for the HHMEC and the HUVEC WPB respectively) and the life time of the organelles using cycloheximide (CHX) treatment. After CHX treatment the WPB in HHMEC decreased in numbers whereas in HUVEC they were hardly affected. The platelet binding efficiency of vWF secreted from HHMEC is being investigated using a parallel flow chamber. In summary we are exploring why HHMEC have a unique WPB morphology and the physiological consequences, specifically in coagulation.

#### OC15

### Compartmentalisation of Endothelial Actinomyosin Activity, *In Situ*, Induced by Ischaemia

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We have demonstrated in a rat heart Langendorff perfusion that Rho kinase-dependent capillary constriction can occur against continuous flow when stimulated with histamine, known to induce contractility of cultured endothelial cells. Here we present evidence for compartmentalisation of endothelial actomyosin activity induced by ischaemia. Langendorff perfused rat hearts were subjected to ischaemia, reperfusion and selective agents which affect the endothelial actomyosin contractile system; Rho-kinase inhibitor (Y-27632) ( $1 \mu\text{M}$ ) and myosin light chain kinase inhibitor, ML7 ( $1 \mu\text{M}$ ). Morphometry from electron micrographs showed that perfusion with Y-27632 (n6) prevented changes in cross-sectional capillary and luminal areas, as well as capillary and luminal perimeters induced by ischaemia ( $p < 0.001$ ). While not as effective as Y-27632, ML7 modulated luminal ( $p = 0.01$ ) and abluminal ( $p = 0.017$ )

perimeter length reduction in ischaemic capillaries ( $n = 6$ ). In similarity with Y-27632, ML7 did not affect endothelial cell shape parameters in oxygenated capillaries ( $p > 0.05$ ). ML7 increased microvillous protrusions on the luminal surface of capillaries in oxygenated and ischaemic capillaries by 28% and 54% respectively. Y-27632 decreased their numbers by 21.5% in oxygenated and 91% in ischaemic capillaries.

**Conclusion:** These findings provide strong evidence, in intact vessels, for the existence of distinct functional compartments of actomyosin in endothelial cells. This is consistent with the compartmentalisation of actomyosin observed in studies of cultured cells.

#### OC16

### Transmission Electron Microscopy Tomography of Endothelial Glycocalyx

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Endothelial cell glycocalyx (ECG) is thought to determine the permeability of microvascular walls to macromolecules. Determining the pore size and fibre structure from standard transmission electron microscopy (TEM) can be problematic due to sample and staining thickness. In this study we have used dual axis TEM tomography to reconstruct 300nm sections in order that the ECG can be visualised from the capillary lumen rather than in cross-section. Two distinct methods of tissue staining have been used. The Rostgaard technique using tannic acid [1] and a novel perfusion technique using ionic lanthanum and dysprosium glycoaminoglycan adhesion (LaDy GAGA). The LaDy GAGA technique stains the ECG selectively and by observation has excellent coverage. Upon reconstruction of the tomograms the xyz stack can be corrected for section thinning (z-shrinkage) and then oriented to view from the desired direction. Autocorrelation (AC) of these planar images gives intensity peaks at common spacings. Our initial findings indicate well defined fibre to fibre spacings of ( $24 \pm 3$ ) nm, agreeing with previous 2D analysis of conventional TEMs by ourselves [1] and by Squire *et al* [2]. Advantages of this approach include (i) improved signal to noise (ii) better fibre diameter estimates and (iii) investigations of directional and longer range periodicities ( $>300$  nm).

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OC17

### Intervention with a Novel, Erythropoetin-Derived Peptide Protects Against Neuroglial and Vascular Degeneration during Diabetic Retinopathy

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Erythropoietin (EPO) shows anti-inflammatory and anti-apoptotic properties although there are concerns that the protein could cause thrombosis and neovascularisation. A novel peptide based on the EPO helix-B domain (pHBSP) is non-erythrogenic but retains tissue protective properties. Diabetic retinopathy is characterised by early-phase neurovascular degenerative pathology and late-stage ischaemia-driven neovascularisation. This study has evaluated the potential of pHBSP to protect the retina from diabetes-related damage. Streptozotocin (STZ)-induced diabetes was maintained in rats for six months. Diabetic (n = 12) and age-matched non-diabetic control (n = 12) rats were then given pHBSP or scrambled peptide and injected daily (10 µg/kg; i.p.) for one month. In parallel, a murine model of oxygen induced retinopathy (OIR) was used to evaluate the effects of pHBSP on neovascularisation. Mice received pHBSP or scrambled peptide (ip). In the retina microglia were increased in diabetic retina and showed active morphology and this was attenuated by pHBSP. Diabetic retina showed many TUNEL positive cells and pHBSP significantly reduced this apoptotic response and also prevented acellular capillary formation. In OIR, pHBSP had no effect on pre-retinal neovascularisation. Systemic delivery of an EPO-derived peptide after diabetes is fully established can protect against neuroglial and vascular degenerative pathology, without exacerbating neovascularisation.

OC18

### Vegf<sub>165</sub>b significantly Reduces Intravitreal Neovascularisation (IVNV) in an Oxygen Induced Retinopathy (OIR) Model of Retinopathy of Prematurity (ROP)

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VEGF, a key regulator of angiogenesis, is linked to abnormal growth of retinal blood vessels in retinopathy of prematurity (ROP). The VEGF gene is alternatively spliced into two sister families of isoforms, angiogenic VEGF<sub>xxx</sub> and anti-angiogenic, VEGF<sub>xxx</sub>b. VEGF<sub>165</sub>b has previously demonstrated inhibition of retinal angiogenesis and laser induced choroidal neovascularisation in mice. We investi-

gated whether VEGF<sub>165</sub>b could prevent intravitreal neovascularisation (IVNV), stimulated by repeated fluctuations between hyperoxia and hypoxia. We used the 50/10 oxygen-induced retinopathy (50/10 OIR) model that exposes newborn rat pups to repeated cycles of 24 hours of 50% oxygen alternating with 24 hours of 10% oxygen to cause a condition similar to human ROP. At P14 pups were given an intraocular (IO) injection of 10 ng VEGF<sub>165</sub>b, 1 µg anti-VEGF (G6-31) or saline, removed from the oxygen chamber and left in normoxia until P20. Retinas were isolectin stained, and IVNV was determined by clock hour analysis. Both G6-31 ( $p < 0.04$ ) and VEGF<sub>165</sub>b ( $p < 0.001$ ) significantly reduced the number of clock hours containing IVNV compared to control eyes ( $p > 0.05$ ). This is consistent with previous results demonstrating that VEGF<sub>165</sub>b inhibits hypoxia driven pathological vessel growth. Cytoprotective VEGF<sub>165</sub>b has the potential to be an effective alternative to cytotoxic anti-VEGF agents in the treatment of hypoxia driven eye disease and tumour growth *in vivo*.

Acknowledgment: Supported by Fight for Sight Hans and Gertrude Hirsch Award and the Skin Cancer Research Fund.

OC19

### VEGF may act as a Survival Factor for Astrocytes during Remodeling of the Immature Post-Natal Murine Retinal Vascular Plexus

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The superficial retinal vascular plexus (RVP) in C57BL/6J mice forms on an astrocytic network which migrates in advance of endothelial cells. We investigated the poorly characterized process of superficial RVP remodeling in wild-type and transgenic mice over expressing lens specific VEGF<sub>188</sub> [1]. In wild-type mice, a ten-fold reduction in astrocyte density is observed between P3 and P20 ( $p \leq 0.0001$ ), in parallel to superficial RVP remodeling events. Immunohistochemistry with activated caspase-3, Pa × 2 or GFAP and isolectin B4 revealed that endothelial capillary segments and astrocytes undergo apoptosis in a progressive sequence from the optic chiasm towards the retinal periphery. Up to post-natal day (P) 8, astrocyte density in wild-type and VEGF<sub>188</sub> mice is similar; however, in VEGF<sub>188</sub> mice decreased apoptosis results in a significantly higher ( $p \leq 0.0001$ ) astrocyte density in P20 and adult mice. When examined using GFAP, the astrocytes in VEGF<sub>188</sub> mice exhibit typical stellate morphology but extend larger and more numerous processes than wild-type mice at P20. Increased astrocyte density in VEGF<sub>188</sub> retinas is not linked to significant differences in vessel density at

either P8 or P20 compared to wild-type mice, though mean superficial RVP capillary segment length is shorter in VEGF<sub>188</sub> mice at P20 ( $p = 0.0022$ ). These results indicate VEGF<sub>188</sub> directly or indirectly acts as an astrocyte survival factor. We are currently investigating the molecular pathway responsible for the mechanism by which VEGF affects astrocyte survival during RVP remodeling.

Acknowledgment: Funding was provided by BBSRC grant BB/F002807/1.

Reference:

- Mitchell *et al.*, *Angiogenesis*, 9: 209–224, 2006.

## OC20

### Tumour Growth Inhibition by Over-expression of TIA-1 is VEGF dependent

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The TIA-1 RNA binding protein is alternatively spliced in colonic adenocarcinoma cells to form a short isoform (shTIA-1) lacking the last two zinc finger domains. This isoform is expressed in 66% of colon cancer samples tested, and in colon carcinoma cells in culture (e.g., LS174t). Over-expression of the full length TIA-1 isoform (flTIA-1) resulted in VEGF<sub>165b</sub> expression. RNA immunoprecipitation and RNA pull down assay using the MS2 system demonstrated flTIA-1 binding to VEGF mRNA, but not in cells expressing shTIA-1 (e.g., LS174t). Over-expression of flTIA-1 also suppressed the ability of carcinoma cells to form colonies in soft agar ( $p < 0.05$ ). Carcinoma cell growth was enhanced by treating cells with rhVEGF<sub>165</sub> (59%) or with neutralizing antibodies to VEGF<sub>165b</sub> (85%), while untransfected cells were unaffected by either treatment. In tumour studies, flTIA-1 over-expressing carcinoma cells showed significantly reduced growth and vascular density in nude mice compared to shTIA1 expressing cells (both  $p < 0.05$ , ANOVA). To test whether this was due to the VEGF splicing switch, a VEGF<sub>165</sub> expression plasmid was co-expressed with flTIA-1 in LS174t cells, and these, vector transfected and flTIA-1 transfected cells were implanted into nude mice. The VEGF<sub>165</sub> expressing tumours grew significantly faster than the flTIA-1 parental line ( $p < 0.01$ ). These results suggest that TIA-1 splicing to shTIA-1 induces an angiogenic switch by preventing distal splicing of VEGF to anti-angiogenic isoforms, and that over-expression of flTIA-1 is anti-angiogenic.

Acknowledgment: This work was supported by AICR.

## POSTER COMMUNICATIONS

### PC1

#### Up-Regulation of Orphan Chemokine Receptor GPR15/BOB on Leukocytes in Rheumatoid Arthritis- Hunting the Ligand

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Rheumatoid arthritis (RA) is a chronic inflammatory condition of the joints in which migration of leukocytes from the blood across microvessels and into the synovial membrane and fluid results in development of inflammation, joint destruction and pain. Chemokines and their receptors play a central role in this process. GPR15/BOB is a member of the chemokine receptor family whose ligand is unknown. We previously found expression of GPR15/BOB mRNA to be up-regulated in RA compared to non-RA synovial tissue. In this study we used flow cytometry to investigate GPR15/BOB expression on peripheral blood (PB) leukocytes from RA patients and healthy donors. To identify the ligand for GPR15/BOB a calcium flux assay was developed to study GPR15/BOB receptor activation when a GHOST cell line transfected with GPR15/BOB was stimulated with a panel of known chemokines. Up-regulation of GPR15/BOB expression was observed on PB monocytes and neutrophils from RA patients compared to healthy donors. The chemokine panel produced differential levels of flux in the calcium flux assay with GPR15/BOB transfected cells. Identification of the ligand for GPR15/BOB would allow investigation of its expression in the sub-compartments of the joint and determination of the functionality of GPR15/BOB on leukocytes in the context of the rheumatoid joint. Up-regulation of expression of GPR15/BOB in RA may be linked to migration of leukocytes into affected joints.

### PC2

#### Role of Syndecan-3 in Inflammation

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Syndecans are a family of transmembrane proteoglycans expressed in the glycocalyx and mediate cell adhesion, growth and migration. We used the antigen-induced arthritis model and the neutrophil-driven model of inflammation

in syndecan-3 null (*sdc-3<sup>-/-</sup>*) and wild-type (WT) mice to test whether syndecan-3 is important in leukocyte extravasation and pathophysiology of arthritis. The magnitude of the inflammatory response (assessed by joint swelling) was significantly greater in WT compared to *sdc-3<sup>-/-</sup>* mice 24 hours after arthritis induction ( $p < 0.0001$ ). Four hours after KC injection into the skin a significant increase ( $p < 0.03$ ) of myeloperoxidase activity over WT was observed in *sdc-3<sup>-/-</sup>* mice, histological staining revealed increased influx of leukocytes and there were significantly more blood vessels with a luminal endothelial distribution of E selectin compared to WT ( $p < 0.001$ ). In skin, syndecan-3 deletion provokes an increase in neutrophil recruitment possibly due to increased luminal distribution of E selectin. This anti-inflammatory role may be explained by the loss of syndecan-3 from the endothelial glycocalyx in *sdc-3<sup>-/-</sup>* mice resulting in adhesion molecules becoming more accessible. In contrast the severity of arthritis assessed by joint swelling is less pronounced in *sdc-3<sup>-/-</sup>* mice suggesting that syndecan-3 play a pro-inflammatory role in arthritis.

### PC3

#### Male & Female Rats Exhibit Differential Regulation of Leukocyte Integrins, Tissue Adhesion Molecules & Chemokines in Response to Ischaemia/Reperfusion Injury

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Incidence and severity of ischaemia/reperfusion (I/R) injury is lower in women than men. I/R injury is characterised by leukocyte recruitment that has a causative role in tissue damage. We investigated whether sex-differences in I/R are due to differences in leukocyte and/or tissue phenotype. Male & female Wistar rats were anaesthetised (Na pentobarbitone; 60 mg/kg). The superior mesenteric artery was occluded for 30 minutes followed by two hours reperfusion and leukocyte recruitment was quantified by intravital microscopy. Leukocyte L-selectin & integrin ( $\beta 1$  &  $\beta 2$ ) expression (FACS), tissue chemokine and adhesion molecule mRNA (qPCR), gut necrosis (nitroblue tetrazolium) and lung neutrophil (PMN) accumulation (myeloperoxidase) were quantified. Mesenteric I/R caused PMN recruitment in both sexes but leukocyte rolling flux, adhesion and emigration as well as gut necrosis and lung MPO was significantly less in females. In line with these observations, I/R stimulated neutrophilia in males but not females and whilst no difference in PMN L-selectin expression was noted, PMN integrin  $\beta 1$  &  $\beta 2$  levels and tissue PECAM-1, JAM-A, VCAM-1, ICAM-1 and chemokines CXCL1,

CXCL5, CCL2 were elevated in males but not females. In summary, mesenteric I/R-induced tissue injury is significantly less in female versus male rats, a response that appears to be associated with reduced activation state of neutrophils and the local vasculature in females.

### PC4

#### The Role of the Scaffold Protein Cybr in Leukocyte Recruitment During Tumour Development

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Cytohesin Binder and Regulator (Cybr) is a scaffold protein highly expressed in the hematopoietic/immune system whose physiological role is poorly understood. Here we show leukocyte recruitment and angiogenesis in tumours of Cybr-null (*Cybr<sup>-/-</sup>*) mice in comparison to wild-type following intraderma inoculation with murine colon adenocarcinoma (MCA-38) cells or implanting tumour fragments in dorsal skin window chambers. *Cybr<sup>-/-</sup>* does not limit or increase tumour parenchyma ( $0.73 \pm 0.07$  versus WT  $0.84 \pm 0.09 \text{ cm}^3$ ,  $p > 0.05$ ) or necrosis ( $0.22 \pm 0.07$  WT  $0.27 \pm 0.08 \text{ cm}^3$ ,  $p > 0.05$ ). Average vessel length, vessel diameter and vessel density in *Cybr<sup>-/-</sup>* and WT mice are similar at day 7, 10, 13 and 16 post tumour-implanted as assessed by longitudinal intravital microscopy ( $p > 0.05$ ). *Cybr<sup>-/-</sup>* mice have significantly lower tumour leukocyte velocity than wild-type littermates ( $p < 0.05$ ) at each time point examined. We also found that *Cybr<sup>-/-</sup>* mice have a lower number of extravasating leukocytes at the edge of tumours at 7, 10, 13 and 16 day ( $P < 0.05$ ). This data shows that loss of Cybr reduces leukocyte velocity through tumour vasculature *in vivo* and affect leukocyte recruitment at the advancing tumour periphery.

### PC5

#### Inflammatory Cytokines Induce Pericyte Shape Change *In Vivo*: Role in Facilitating Leukocyte Transmigration?

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Despite our increased understanding of the mechanisms associated with leukocyte migration through endothelial cells, little is known about the mechanisms mediating the subsequent migration through the pericyte layer, the second cellular barrier of venular walls. Here, we have analysed the

effect of TNF- $\alpha$  and IL-1 $\beta$  on pericyte shape change *in vivo* and *in vitro* using the mouse cremaster muscle model and the murine pericyte-like cells (C3H/10T1/2), respectively. Our results showed that in TNF- $\alpha$ - and IL-1 $\beta$ -stimulated cremasters, post-capillary pericytes exhibited shape change resulting in a significant increase in mean gap size between adjacent cells (e.g., TNF- $\alpha$  induced a 97% increase after two hours;  $p < 0.001$ ). Time-course studies indicated that TNF- $\alpha$ -induced shape change preceded neutrophil transmigration. This response was PMN-independent as it was also noted in PMN depleted mice. C3H/10T1/2 cells also exhibited significant shape change in response to direct TNF- $\alpha$ - and IL-1 $\beta$ -stimulation *in vitro*. The results indicate that pericytes can respond to pro-inflammatory cytokines by active shape change, a response that may regulate the process of leukocyte transmigration at sites of inflammation. Funding sources: Funded by the Barts & The London RAB and the Wellcome Trust.

## PC6

### Neutrophil Elastase (Ne) Plays a Non-Redundant Role in Mediating Neutrophil Transmigration in Response to Ischemia/Reperfusion (I/R) Injury

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Neutrophils are the first leukocyte subtype to be rapidly recruited into tissues following I/R injury where they contribute to the pathology of the disease. Here we have investigated the role of neutrophil elastase (NE) in leukocyte transmigration in four murine models of I/R injury, namely cardiac, renal, mesenteric and cremaster muscle by using NE deficient (NE<sup>-/-</sup>) mice. In all models studied, both neutrophil transmigration and tissue injury were significantly reduced in NE<sup>-/-</sup> animals as compared with wild-type littermates. Specifically, neutrophils appeared to be arrested in the NE<sup>-/-</sup> mice at the level of the venular basement membrane (BM). Immunofluorescent staining of inflamed tissues for NE indicated reduced neutrophil-associated and enhanced released NE within the venular wall, a response that appeared to correlate with remodelling of the venular BM (i.e., enhancing the size of leukocyte permissive regions termed matrix protein low expression regions; LERs) in WT but not in NE<sup>-/-</sup> mice. Collectively, the findings suggest a non-redundant role of NE during neutrophil transmigration as induced by I/R injury.

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## PC7

### Platelets Enhance Superoxide Generation by Equine Neutrophils

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Platelets can potentially influence bacterial clearance by modifying neutrophil phagocytosis and free radical generation. This study has looked at how resting and activated equine platelets affect neutrophil superoxide (O<sub>2</sub><sup>-</sup>) production. Neutrophils were co-incubated with unstimulated or thrombin (0.1 U/ml)-stimulated platelets (50:1) and O<sub>2</sub><sup>-</sup> production measured 30min after addition of PMA (0.1  $\mu$ M) or opsonised zymosan (OPZ; 1 mg/ml), as described [1]. The effect of increasing the platelet:neutrophil ratio was then examined. Results are expressed as mean  $\pm$  SEM nmol reduced cyt C/10<sup>6</sup> neutrophils, n = 6. \* =  $p < 0.05$ ; repeated-measures ANOVA and Bonferroni's test. Unstimulated platelets increased neutrophil O<sub>2</sub><sup>-</sup> production in response to OPZ (\*57  $\pm$  9 vs. 41  $\pm$  8) and PMA (\*87  $\pm$  15 vs. 62  $\pm$  13). Using OPZ, it was shown that more O<sub>2</sub><sup>-</sup> production occurred with unstimulated platelets at a 100:1 ratio but no further increase was seen at higher ratios. Activating platelets did not affect the magnitude of the increased response. O<sub>2</sub><sup>-</sup> generation was increased in the presence of fixed platelets (100:1), but not platelet supernatant, although the increase was not significant (\*33  $\pm$  10 (platelets); 26  $\pm$  10 (fixed platelets); 17  $\pm$  9 (supernatant) vs. 17  $\pm$  7)). Blocking P-selectin did not affect the increase in O<sub>2</sub><sup>-</sup> production. Equine platelets enhance neutrophil O<sub>2</sub><sup>-</sup> production in a manner that is not dependent on activation but does require cell-cell contact which could take place after platelet extravasation into infected tissue.

Reference:

1. Foster & Cunningham, *Vet Immunol Immunopathol* 59: 225–237, 1997.

## PC8

### Platelet Based CLEC-2 Signalling Contributes to Formation of the Lymphatic Vasculature Through Effects on Lymphatic Endothelial Cells

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Historically, platelet function has been considered in haemostasis and thrombosis, but recently a critical role for platelet receptors in development, focusing on the C-type lectin-like

protein type 2 (CLEC-2) has been shown. CLEC-2 is a receptor for podoplanin, which is expressed by lymphatic endothelial cells. CLEC-2 activation by podoplanin initiates signalling that involves Syk, SLP-76 and PLC $\gamma$ 2. Mice deficient in these proteins have blood-filled lymphatics with varying levels of morbidity at birth. What is unclear is whether this phenotype is mediated solely at the platelet level. In the present study, we have systematically studied embryos from mice deficient in CLEC-2 alongside that of mice in which CLEC-2 has been deleted in the megakaryocyte/platelet lineage. These investigations provide evidence for a critical role of platelet based CLEC-2 in the separation of the blood and lymphatic vessels, which contributes to lymphatic function. *In vitro* experiments show that platelets can decrease the migratory and tube forming ability of lymphatic endothelial cells. We speculate that these findings reflect a critical role for CLEC-2 signalling in platelets for regulating migration of lymphatic endothelial cells to form lymphatic vessels during development.

Acknowledgment: This work was supported by the Wellcome Trust.

#### PC9

##### **Overexpression of Nitric Oxide Synthase and Soluble Guanylate Cyclase Associated With an Inhibition of Urea Cycle in Platelets from Patients Under Haemodialysis**

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Chronic renal failure (CRF) is characterized by the presence of platelet dysfunction associated with increased levels of nitric oxide (NO). Arginase competes with NO synthase (NOS) for the substrate L-arginine, modulating its activity. We have previously reported an activation of L-arginine-NO pathway in blood cells in CRF. The aim of this study was to investigate the effects of end stage CRF in the activity and expression of arginase, NOS isoforms and soluble guanylate cyclase (sGC) in human platelets. In the present study were included 13 CRF patients under hemodialysis and 12 healthy controls. Inducible NOS (iNOS), endothelial NOS (eNOS), sGC and arginase I and II expressions were accessed by Western blotting. It was detected an overexpression of iNOS, eNOS and sGC in CRF with no alterations of arginase expression. However, arginase activity, analyzed through the conversion of L-[C14]-arginine into [C14]-urea, was decreased in CRF patients. We demonstrated that the increment of NO synthesis previously reported in CRF may be explained in part by an overexpression of NOS in platelets. Moreover, the increased expression of sGC could lead to higher levels of cGMP,

which could impair platelet function. Nevertheless, low arginase activity may provide more substrate for NO production. In this context, the rise of NO bioavailability may contribute to bleeding tendency presented in CRF.

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#### PC10

##### **Platelet-Mediated Adhesion of Endothelial Progenitor Cells during *In Vivo* Transplantation**

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The involvement of endothelial progenitor cells (EPCs) in the angiogenic response to vascular damage has been widely studied, including the release of pro-angiogenic factors and the incorporation of EPCs into repairing vessels [1]. EPCs may be aided, at least in part, by their interaction with platelets: by tethering circulating EPCs to the endothelium, a 'platelet bridge' may support recruitment during angiogenesis [2]. We have demonstrated this interaction by transplantation of EPCs into isofluorane-anaesthetised C57BL/6 mice with angiogenic stimulation, following systemic platelet depletion. Acute (30 minutes) or chronic (two and seven days) hindlimb ischaemia was induced by femoral artery occlusion, with platelet depletion by anti-GPIIb $\alpha$  antibody prior to transplantation. Synergistic muscle overload (7d) of *m. extensor digitorum longus* and *m. soleus* was also induced by extirpation of *m. tibialis anterior*. In each model,  $2 \times 10^6$  fluorescently labelled EPCs were introduced *via* a carotid artery cannula and recruited EPCs localised by flow cytometry of hindlimb muscle and viscera digests. Preferential localisation of EPCs to the contralateral hindlimb was observed in all models except chronic (7d) ischaemia, suggesting significant EPC homing to sites affected by the angiogenic stimuli. Systemic platelet depletion ablated the significant EPC recruitment observed following acute ischaemia, illustrating the importance of platelet presence on adhesion of circulating EPCs.

References:

1. Ding *et al.*, Cell Transplantation 16: 273–284, 2007.
2. Langer & Gawaz. *Basic Res Cardiol* 103: 299–307, 2008.

#### PC11

##### **The Predictive Role of Chronic Periodontal Disease for all Cause Mortality in NHANES III**

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Periodontal disease is a bacterial disease causing destruction of the tooth supporting structure. It is associated with

systemic disease by the ability of bacteria and cytokines to access the vasculature via the fenestrated capillaries of the periodontal microcirculation. This study explored the influence of periodontal disease on mortality in the NHANES III dataset and determined which clinical parameter carried the most prognostic information. A dental assessment was made in 9502 subjects. In subjects with teeth, two sites per tooth of a randomly determined half mouth were assessed. Survival data was collected for 18 years. Multivariate Cox-proportional hazard analyses were performed to determine the independent predictive role of periodontal health. After adjustment for age, sex and ethnicity, the 1272 subjects without teeth had higher mortality than the 4476 with tooth loss or 3754 with all their teeth. Clinical attachment loss (CAL: measures destruction of the periodontal ligament) of  $\geq 4$  mm at any site of at least one tooth predicted a three-fold increase in mortality (99% CI 2.58–3.66;  $p < 0.001$ ). Adjusting for cardiovascular risk factors (predominantly microalbuminuria and proteinuria) accounted for the increased mortality associated with no teeth. However such adjustment didn't alter the risk associated with CAL in those with all their teeth. Ongoing periodontal disease, measured by CAL  $\geq 4$  mm at any site of any tooth, has the same risk of mortality to a similar degree as diabetes or smoking, independent of conventional risk factors.

#### PC12

##### Is Differential Regulation of Arginine Status Related to Ethnicity?

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The aetiology for an increasing incidence of hypertensive cardiovascular disease amongst Africans in Southern Africa is unclear. Hypertension may be induced by inadequate release of L-arginine (L-arg) derived nitric oxide impairing vascular tone regulation. In addition, asymmetric dimethylarginine (ADMA) is associated with cardiovascular disease. We compared the profiles of L-arg with cardiovascular risk factors in African and Caucasian men of similar age to determine whether the association of these risk factors with serum L-arg differentiated between these groups. We studied 183 Caucasian and 162 African men respectively (20 to 70 years) measuring serum L-arg, ADMA, creatinine, urea, symmetric dimethylarginine (SDMA) and blood pressure. L-arg levels were significantly greater in Caucasians. Although serum ADMA and SDMA were similar in both groups of men ( $p > 0.05$ ), linear regression showed ADMA more strongly associated with L-arg in Caucasians ( $r = 0.59$  vs.  $0.249$ ). SDMA's association was significant only in Caucasians. Diastolic blood pressure was lowest in

Caucasians but correlated negatively with L-arg in Africans ( $r = -0.18$ ,  $p = 0.013$ ).

Conclusions: Our findings show that the relationship of cardiovascular risk factors with serum L-arg and some of its catabolites is different in African and Caucasian men and that this may be associated with a relatively greater incidence of hypertension in African men.

#### PC13

##### Relationship Between Capillary Density and Exchange Capacity in Skeletal Muscle in Older men

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Our aim was to investigate the relationship between muscle morphology, microvascularisation and exchange capacity in older men. We studied 22 men (68–77 years) who were previous participants of the Hertfordshire Sarcopenia Study (HSS) [1]. The study was approved by the Hertfordshire Research Ethics Committee. We quantified (i) muscle morphology and microvascularisation (capillary density: CD and capillary to fibre ratio: C:F) from *vastus lateralis* muscle biopsy (ii) microvascular exchange capacity ( $K_f$ ) and blood flow ( $Q_a$ ) using venous congestion plethysmography; and (iii) strength using hand held dynamometry.  $K_f$  was  $3.76(0.25) \times 10^{-3}$  ml/min/100 ml/mmHg, CD 158(18) per  $\text{mm}^2$  and C:F 1.14(0.13) (mean(SEM)). We found no correlation between these measures and age. Our preliminary results do not support a relationship between age,  $K_f$  and CD in this cohort of older men.

Acknowledgment: Supported by the MRC, BBSRC and British Geriatrics Society. We gratefully acknowledge the HSS team.

Reference:

1. Patel *et al.*, Geriatrics, *BMC*, 10: 43–50, 2010.

#### PC14

##### Fluorescence Microlymphography to Study Alterations in Interstitial Fluid Uptake by the Lymphatics in Mouse

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Understanding alterations in interstitial fluid flow towards the lymphatics represents a key step in the comprehension of several lymphatic diseases, such as lymphoedema and lymphatic metastasis. To study the lymphatic clearance in mice, we performed fluorescence microlymphography by measuring the decay in intradermal fluorescence. 0.16%

FITC-Dextran 150 KDa solution was ultracentrifuged with a 30 KDa cut-off filter to remove unbound FITC. 50  $\mu$ l was injected intradermally in nude mice. Mice were anaesthetised with 4% isoflurane in 1% oxygen and fluorescence recorded every hour, up to six hours after the initial injection, with an IVIS Lumina imaging system (Caliper Life Sciences), using an exposure of 0.5 seconds and specific GFP filters. FITC-Dextran was removed by lymphatic clearance with a half-life of  $85 \pm 2.7$  min ( $n=6$ ). 1  $\mu$ M adrenaline significantly enhanced FITC-Dextran clearance ( $t_{1/2} = 71 \pm 4.9$  minutes,  $n = 6$ ), significantly less than control ( $p < 0.05$ ,  $t$  test). 100  $\mu$ M histamine, which did result in visible oedema, did not affect the half-life ( $84 \pm 8.5$  minutes,  $n = 6$ ), but when histamine was combined with 10 nM of recombinant VEGF-C,  $t_{1/2}$  was increased to  $103 \pm 8.0$  minutes ( $n = 6$ ). These results indicate that adrenaline increases clearance through the lymphatics, whereas VEGF-C acutely hinders lymphatic clearance in presence of an inflammatory mediator (histamine).

Acknowledgment: This work was supported by the Wellcome Trust.

#### PC15

### VEGF<sub>165b</sub> Over-expression Alters Female Estrous Cycle Length in mice

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VEGF and its receptors are expressed in the female reproductive system and their expression levels are shown to change during the estrous cycle. VEGF pre-mRNA is differentially spliced to form two families, VEGF<sub>xxx</sub> and VEGF<sub>xxx</sub>b, where xxx denotes the amino acid number. VEGF<sub>165b</sub> inhibits VEGF<sub>165</sub>-induced angiogenesis. To identify the function of VEGF<sub>165b</sub> on female reproductive biology, we generated transgenic mice (TG) overexpressing human VEGF<sub>165b</sub> under the control of the MMTV promoter. RT-PCR indicated expression of hVEGF<sub>165b</sub> was strong in the ovary and weak in the uterus of TG females. TG females and paired WT female littermates were subjected to vaginal smear screening for at least two consecutive estrous cycles. The TG mice had a significantly greater cycle length than the littermate controls ( $6.0 \pm 0.25$  vs.  $4.85 \pm 0.26$  days per cycle,  $p < 0.05$ ). Determination of the lengths of the phases in each mice showed that estrus was longer by a day in the TG ( $2.5 \pm 0.19$  days) compared with WT mice ( $1.6 \pm 0.24$  days  $p < 0.05$ ). This suggests VEGF involved in the determination of progression of the estrous cycle and overexpression of VEGF<sub>165b</sub> alters the estrus phase of the cycle in mice. This suggests that the process of

follicle rupture, which initiates the end of estrus, is extended by inhibiting angiogenesis.

Acknowledgment: This work was supported by the Wellcome Trust & BHF.

#### PC16

### Characterisation of an *In Vivo* Model to Study Prostate Metastasis to Bone

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Cancer metastasis to the skeleton is the cause of significant mortality in prostate cancer. Despite its prevalence, the mechanisms involved in the development of bone metastasis remain unclear. We have recently developed an *in vivo* model using the dorsal skinfold chamber (DSC) with implanted metatarsals, to study single tumour cells homing to bone using *in vivo* microscopy. The aim of this study is to evaluate prostate cancer colonisation of the bone micro-environment *in vivo* using different clinically relevant bones implanted into the DSC. Bones (tibia, calvaria) from newborn mice (1- to 3-day-old) were grafted into a DSC implanted on a SCID mouse (5- to 6-weeks-old). Tibia fragments were re-vascularised by inosculation with the host vasculature by day 7 after engraftment but calvaria fragments do not re-vascularise. Prostate (PC3-GFP) cancer cells ( $1 \times 10^5$ ) were then injected via the heart (i.c) [of animals implanted with the DSC containing tibia fragments]. Recordings of the chamber tissue and tibia fragment were made at 48 hours intervals for the duration of the experiment for the presence of PC3-GFP cells. At the end of the study, tissue was harvested and processed for microCT, multi-photon analysis and histology. PC3 cells are identified within the tibial bone marrow four days after injection ( $9.7 \pm 3$  cells) and are maintained for the duration of the experiment (26 days). We now wish to develop this model further to determine the mechanisms of adhesion and whether the tibia supports tumour cell growth.

Funding sources: Project is funded by Yorkshire Cancer Research (YCR).

#### PC17

### Increased Coagulability in Orthopaedic Patients Assessed via Thromboelastography

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Introduction: Patients undergoing major joint replacement surgery are at increased risk of deep vein thrombosis (DVT). Low molecular weight heparin (LMWH) reduces but does not abolish DVT risk. This observational study

explores changes in clotting activity after orthopaedic surgery, and the effect of LMWH, via thromboelastography.

Methods: Ethics approved study recruiting patients undergoing elective hip or knee replacement surgery. Control thromboelastograph (TEG<sup>®</sup>) was obtained before surgery. TEG curves were obtained on days 1, 2 and 3 post-operatively, in the morning (am) and evening (pm). LMWH activity was assessed in the presence of heparinase. Percent change from preoperative R time (time to clot initiation, minutes), maximum amplitude (MA, final clot strength, millimetres), and maximum rate of thrombin generation (MRTG, millimetres/minute) were measured. Values: mean (+/- s.e.m.).

Results: A total of 17 patients were examined. R times did not change postoperatively, and there was no change in the presence of heparinase. There was a progressive rise in MA (percent change from control: day 1 pm 8.6 (5.3), day 2 pm 17.9 (5.9), day 3 pm 20.2 (5.1). MRTG also rose progressively though with wide variability (percent change from control: day 1 pm 199.5 (100.6), day 2 pm 206.1 (117.5), day 3 pm 291.8 (163.3)).

Conclusions: Post-operative coagulation assessed via TEG<sup>®</sup> showed a progressive rise in coagulability not altered by LMWH. R time did not change, but MA and MRTG both rose progressively, suggesting an increase in platelet activity. LMWH may not adequately protect against DVT.

## PC18

### Resuscitation Improves Perfusion More in Septic Capillaries than Other Shock States

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Purpose: 1. To investigate the pre and post effects of Early Goal Directed Therapy (EGDT) on sublingual microcirculatory density and flow in patients with baseline unresuscitated septic shock compared with non-septic, age and shock matched. 2. To study true capillaries (<10  $\mu\text{m}$ ) and compare behaviour with larger diameter microvessels (11–20  $\mu\text{m}$ ).

Methods: Prospective observational study in emergency and Intensive care departments.

Subjects: A total of 19 septic and 10 critically ill control patients with similar levels of macrohaemodynamic shock.

Measurements: Sidestream Dark field sublingual videomicroscopy to measure microvessel flow and density, at EGDT initiation and on attainment of consensus cardiovascular goals.

Results: In 1–10  $\mu\text{m}$  vessels, flow was significantly worse in shocked septic patients (64% [25.7%]) compared to con-

trols (88.2% [12.0%]  $p < 0.05$ ) with similar baseline shock. Protocolised resuscitation has a larger effect on the flow in septic capillaries, but the flows achieved are lower than control patients (79% [15.6] vs 92.7% [3.9]  $p < 0.009$ ). 2. In 11–20  $\mu\text{m}$  vessels, although flow increases (67.5% [26.6] to 78.7% [24.9]  $p < 0.14$  in sepsis and 93.8% [13.8] to 95.4% [4.7]  $p < 0.46$  in controls), total length  $\mu\text{m}/\text{mm}^2$  decreases with resuscitation in all patients (5589.7 [1717.3] to 4660.8 [1691.5]  $p < 0.018$  in sepsis and 5583.6 [2075] to 3902 [1341.4]  $p < 0.02$  in controls).

Conclusions: Septic capillaries respond to EGDT better than controls. With resuscitation, blood from medium vessels appears to divert into other vessels.

## PC19

### Validation of Haemoglobin Saturation Measurements by Optical Reflectance Spectroscopy in the Microcirculation Using a Skin Phantom

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Mean blood oxygen saturation can be measured non-invasively in the cutaneous microcirculation using optical techniques. However, quantitative measurements are difficult to obtain due to the optical complexity of the skin. This *in-vitro* study evaluates the accuracy of two commercial instruments the O2C (Lea Medizintechnik GmbH) and the moorVMS-OXY (Moor Instruments Ltd) against a Clark-type  $\text{pO}_2$  and the TCM<sup>TM</sup>400 Transcutaneous  $\text{pO}_2$  Monitoring System (Radiometer). The phantom, designed to simulate the optical properties of skin, consisted of whole blood in a scattering solution of 0.5% Intralipid in saline ( $\text{pH} = 7.4$ ). The high scattering coefficient and low absorption coefficient of Intralipid provides a robust model to replicate the reduced scattering coefficient of tissue. The solution was maintained at a temperature of  $37 \pm 2$  °C on a hot plate stirrer and bubbled with nitrogen to deoxygenate the blood. A direct comparison is made between oxygen saturation calculated from the absorption spectra from the blood and  $\text{pO}_2$  measurements using the two Clark-type electrodes over an oxygen saturation range from 0% to 100%. The blood oxygen saturation calculated from the phantom reflectance spectra of the O2C and moorVMS-OXY showed good correlation between the devices and with the Clark electrodes (Pearson's correlation coefficient,  $r = 0.99$ ,  $p < 0.01$ ).

PC20

### Effects of High Dietary Sodium Intake on Microvascular Function During Hyperinsulinemic Euglycaemic Clamp in Overweight / Obese Middle-Aged and Older Adults

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Obesity is associated with insulin resistance, hypertension and impaired endothelial function; however the mechanisms involved are largely unknown. We are testing the hypothesis that dietary sodium intake modulates microvascular endothelial function and insulin sensitivity in prehypertensive/hypertensive overweight middle-aged and older adults. Here we report an interim analysis (mean  $\pm$  SD). Twelve (eight male) untreated adults (body mass index  $30 \pm 3$  kg/m<sup>2</sup>) aged  $60 \pm 7$  years, systolic blood pressure 130–159 mmHg consumed a 7-day high and low sodium diet with a 2-week washout (randomized crossover design). Urinary sodium excretion was  $211 \pm 41$  mmol/day and  $108 \pm 48$  mmol/day during high versus low sodium diet ( $p < 0.01$ ). Microvascular skin blood flow response to iontophoresis of the endothelium dependent vasodilator acetylcholine was increased (area under the curve (AUC):  $p < 0.02$ , peak response:  $p < 0.03$ ) in the high versus low sodium condition during a hyperinsulinaemic euglycaemic clamp. This interim analysis suggests that high dietary sodium intake is associated with increased endothelium dependent vasodilatation in the presence of hyperinsulinaemia.

PC21

### Oxygen Saturation and Perfusion Changes during Dermatological Methyl-Aminolevulinat Photodynamic Therapy

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Methyl-aminolevulinat photodynamic therapy (MAL-PDT) is a successful topical treatment for a number of (pre)cancerous dermatological conditions. In combination, light of the appropriate wavelength, the photosensitiser, protoporphyrin IX (PpIX) and tissue oxygen result in the production of singlet oxygen and reactive oxygen species inducing cell death. This study investigates real-time changes in localised tissue blood oxygen saturation and perfusion in conjunction with PpIX fluorescence monitoring during dermatological MAL-PDT using a range of non-invasive monitoring techniques. Significant reductions in mean blood oxygen saturation ( $p < 0.005$ ) and PpIX fluorescence ( $p < 0.001$ ) were observed within the first minute of irradiation commencing ( $4.75$  J cm<sup>-2</sup>). The changes in oxygen saturation and PpIX

fluorescence were positively correlated during the initial phase of treatment ( $r^2 = 0.766$ ). In contrast the perfusion within the lesions was observed to significantly increase ( $p < 0.01$ ) during treatment. Rapid reductions in the localised blood oxygen saturation of the tissue have been observed to occur for the first time within the initial minutes of light irradiation during clinical dermatological MAL-PDT and positively correlate with the concurrent PpIX photobleaching that is also recorded in this time period. Furthermore perfusion increases suggesting that the microvasculature compensates for the PDT induced oxygen depletion.

PC22

### Identification of Diverse Functional Domains in FPR2/ALX Receptor Activation by Selective Agonists

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Inflammation is a protective response of the organism against injurious stimuli, affording their containment and removal to then initiate the healing process. When run uncontrolled, however, it also drives tissue damage and injury. This project focuses on FPR2/ALX, a GPCR belonging to FPRs family. It conveys the homeostatic signals of annexin A1 (AnxA1) and the short-lived lipid lipoxin A<sub>4</sub>. A variety of ligands binds to FPR2, including the pro-inflammatory mediator serum amyloid A (SAA) and the synthetic compound 43 (C43). We aimed, here, to identify the differential functional domains of some ligands and define their impact on AnxA1 receptor-mediated responses. In order to do so, HEK293 cells transfected with FPR2 or chimaeric receptor (formed by distinct domains of FPR1 and FPR2) were used to determine calcium flux, as read out for receptor activation and desensitization. ERK1/2 phosphorylation was also evaluated. HEK293-FPR2 and different clones were stimulated with AnxA1, SAA and C43. AnxA1 (10nM) elicited ~40% calcium mobilization (expressed as % of ionomycin response) in HEK293-FPR2 and 40%, 60% and 60% for clones C, G and H, respectively. SAA (0.1  $\mu$ M) afforded ~50% of response for FPR2 cells and for clones E, F, G, H. No responses were observed in other clones. C43 (1  $\mu$ M), which led to ~50% of response in HEK293-FPR2, was only active in clones E and F (~85%,  $p < 0.001$ ). In addition, AnxA1 and SAA induced ERK1/2 phosphorylation in clones C, G and H, while C43 activated ERK1/2 in clones E and F. In desensitization experiments, FPR2 activation by AnxA1 was significantly reduced after five minutes pre-treatment with AnxA1, SAA and C43, i.e., either in homologous or heterologous ways ( $p < 0.001$ ). AnxA1 administration only induced desensitization in clones G and H, but not in clone C. In conclusion we showed that different ligands bind different loops to activate the FPR2 receptor in different fashion.

PC23

**A Novel Cross Talk in Resolution: H<sub>2</sub>S Activates the Annexin A1 Pathway****V. BRANCALEONE<sup>1,2</sup>, A. L. F. SAMPAIO<sup>1</sup>, G. CIRINO<sup>2</sup>, R. J. FLOWER<sup>1</sup>, M. PERRETTI<sup>1</sup>**<sup>1</sup>WHRI, Barts and The London SMD, QMUL Charterhouse Square, EC1M 6BQ London, UK; <sup>2</sup>Department Exp Pharmacology, University of Naples, Italy E-mail v.brancaleone@qmul.ac.uk

Hydrogen sulphide (H<sub>2</sub>S), a gaseous mediator synthesized in several mammalian tissues by two main enzymes CBS and CSE, increases under inflammatory conditions or sepsis. Since H<sub>2</sub>S and H<sub>2</sub>S-releasing molecules afford potent inhibitory properties on the process of leukocyte trafficking, we tested whether endogenous Annexin A1 (AnxA1) could display intermediary functions. Treatment of human PMNs with H<sub>2</sub>S donor NaHS (10–100 μM) provoked prompted and intense mobilization (>50%) of AnxA1 from the cytosolic pool to the cell surface, supporting the inhibitory effects of the gas in the flow chamber assay. Such *in vitro* actions could be translated in analyses of the inflamed microcirculation, where NaHS (100 μmol/kg s.c., - 30 minutes) afforded marked inhibition of IL-1-induced cell adhesion and emigration in the mesenteric vessels of wild type, but not AnxA1<sup>-/-</sup> mice. Next, we investigated whether endogenous AnxA1 could modulate H<sub>2</sub>S synthesis, indicating existence of a positive circuit. A marked increase in CBS and/or CSE expression in a variety of tissues (aorta, kidney, spleen) tested from AnxA1<sup>-/-</sup> mice, as compared to wild type, was quantified by qPCR. Moreover, NaHS counteracted the increase in expression in iNOS and COX2 (4-fold and 7-fold reduction, respectively) upon LPS-stimulation of bone marrow derived macrophages (BMDM), though it was totally inactive in cells prepared from AnxA1<sup>-/-</sup> mice. Taken together, these data strongly suggest the existence of a positive interlink between AnxA1 and the H<sub>2</sub>S pathway, providing a novel mechanistic explanation for the exquisite properties of H<sub>2</sub>S in the control of experimental inflammation. These finding may be relevant to innovative discovery programmes aiming at harnessing the biological properties of H<sub>2</sub>S.

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PC24

**Angiopietin-2 in Cerebrospinal Fluid and Serum: A Predictive Biomarker for Brain Injury?****C. V. GANTA, P. CHITABOINA, P. MCCARTHY, L. K. SCOTT, W. E. CROMER, J. M. MATHIS, J. S. ALEXANDER**

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As roles of angiopietins in brain injury become better understood, clinical uses for Ang-2 as a vascular stress

biomarker are increasingly being explored. Several studies have established serum Ang-2 as a marker of systemic illness severity, reflecting vascular injury. Less is known about how angiopietins, e.g., pro-inflammatory Ang-2, change in other compartments in brain injury. Since Ang-2 is stable for 24 hours and resists freeze-thaw cycles it may represent a reliable and durable clinical marker for brain vascular injury. We compared Ang-2 in cerebrospinal fluid (CSF) collected from clinically defined subarachnoid hemorrhage (SAH, n = 7) vs. controls (n = 4) by immunoblotting. We found a large (2.66-fold) and significant (*p* = 0.043) increase in Ang-2 (range = 8–370% of control). We also evaluated Ang-2 in serum from mice undergoing middle cerebral artery occlusion for two hours / 24 hours reperfusion, ('I/R'). We observed a significant decrease (*p* = 0.001) in serum Ang-2 levels after I/R. Despite differences in mouse and human samples, these results suggest contrasting changes in serum and CSF Ang-2, with ischemic injury decreasing serum Ang-2 with a concomitant increase in CSF Ang-2. The results suggests that serum/CSF Ang-2 ratios may be a useful biomarker of vascular stress and temporal changes in serum/CSF ratios may predict disease severity and prognosis. Further experiments to confirm serum Ang-2 in SAH and traumatic brain injury TBI are underway to validate these markers in SAH/TBI.

PC25

**Role of Smooth Muscle Cell Hyperpolarization in Modulation of Endothelial Cell Ca<sup>2+</sup> Events and Conducted Dilatation****T. BELEZANI, Y. KANSUI, K. A. DORA**

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Any agonist that stimulates smooth muscle cell hyperpolarization has the potential to be associated with homo- and heterocellular intercellular electrical coupling and conducted dilatation. Both vascular smooth muscle cells and endothelial cells have been reported to produce spontaneous Ca<sup>2+</sup> events. To hyperpolarize the smooth muscle cells and stimulate dilatation of the vessels, we used levocromakalim (LVK) acting at ATP-sensitive K<sup>+</sup> channels. Rat cremaster arteries were isolated, triple-cannulated and tied onto glass pipettes (70 mmHg). With a confocal microscope and 10× objective we simultaneously imaged the feed artery and the bifurcation vessels. LVK with tetramethylrhodamine-labelled dextran was lumenally perfused into one branch of the bifurcation (Branch1) and continuous flow through the feed vessel prevented upstream diffusion of agonists and dye. Endothelial cell Ca<sup>2+</sup> imaging was performed following luminal perfusion of Oregon Green BAPTA-1 AM. LVK stimulated local

dilatation in Branch 1, and conducted dilatation into the feed artery. Furthermore, LVK resulted in a marked increase in spontaneous endothelial  $\text{Ca}^{2+}$  events, which was observed both at the local and upstream sites. These  $\text{Ca}^{2+}$  events were significantly decreased following removal of extracellular  $\text{Ca}^{2+}$ .

These data suggest that LVK evokes robust local and conducted dilatation with simultaneous increases in endothelial cell  $\text{Ca}^{2+}$  events. The  $\text{Ca}^{2+}$  events at all sites may be driven by hyperpolarization per se increasing the driving force for  $\text{Ca}^{2+}$  entry.

#### PC26

### Reactive Oxygen Species (ROS) from Cytochrome P450 2C9 Mediate the Endothelial $[\text{Ca}^{2+}]_i$ Increase during Endothelial Derived Hyperpolarising Factor (EDHF) Responses

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EDHF is the main relaxation pathway in the microcirculation and ROS have been implicated in this response, but their role has yet to be elucidated. The cremaster muscle circulation of freshly killed Wistar rats was perfused with a Krebs buffer solution containing albumin and the  $\text{Ca}^{2+}$  indicator Fura-PE3 AM. The preparation was placed on the stage of an intravital microscope to measure vessel diameter, and endothelial  $[\text{Ca}^{2+}]_i$  was estimated from the 360/380 nm excitation ratio. The preparation was superfused with phenylephrine (30  $\mu\text{M}$ ), L-NAME (300  $\mu\text{M}$ ) and indomethacin (3  $\mu\text{M}$ ). Addition of carbachol (10  $\mu\text{M}$ ) resulted in 72.6%  $\pm$  2.4 relaxation and the 360/380 nm ratio increased by 24.7%  $\pm$  1.5. The EDHF mediated relaxation and endothelial  $[\text{Ca}^{2+}]_i$  increase in response to carbachol was substantially reduced by including a ROS scavenging combination of superoxide dismutase and catalase (100 U  $\text{ml}^{-1}$  each; relaxation 42.3%  $\pm$  15.4, ratio 5.5%  $\pm$  2.1). The specific CYP 2C9 antagonist sulfaphenazole (10  $\mu\text{M}$ ) reduced relaxation (to 23.7%  $\pm$  2.6) and the endothelial  $[\text{Ca}^{2+}]_i$  increase (to 8.6%  $\pm$  1.2). The epoxyeicosatrienoic acids antagonist 14,15-epoxyeicosa-5(Z)-enoic acid (10  $\mu\text{M}$ ) did not significantly affect the EDHF response (relaxation 72.7%  $\pm$  6.1, ratio 21.7%  $\pm$  3.2). These data suggest that ROS produced by arachidonic acid metabolism via CYP2C9 play an important role in EDHF mediated relaxation by increasing endothelial  $[\text{Ca}^{2+}]_i$ .

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#### PC27

### Role of Schwann Cell Junctional Adhesion Molecule (JAM)-C in Inflammation

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JAM-C is an adhesion molecule expressed at junctions between adjacent endothelial and epithelial cells that has been implicated in multiple inflammatory and vascular responses. We recently reported on the expression of JAM-C in Schwann cells (SC) and its role in regulating the integrity and function of peripheral nerves. As peripheral neural activity can trigger and modulate inflammatory responses via sensory and cholinergic pathways, we investigated the potential role of SC JAM-C in regulating inflammatory and vascular events. For this purpose we generated mice with specific deletion of JAM-C in SCs and examined their response to different models of sensory nerve-mediated inflammation i.e., carrageenan-induced paw oedema and mustard oil-induced skin plasma extravasation. Our results demonstrate expression of JAM-C in myelinated A $\delta$ - but not C-sensory fibers. JAM-C SC KO mice exhibited mild neural functional and structural abnormalities but showed a normal inflammatory response. The data suggest that SC JAM-C does not play a major role in regulating neurogenic inflammation.

#### PC28

### Hyperglycaemia Downregulates TRPV4 Channels in Retinal Endothelial Cells

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Presently, the molecular mechanisms underlying endothelial dysfunction in diabetes are not fully understood. TRPV4 channels have been shown to play an essential role in endothelial-dependent vasodilatation in various vascular beds. In the present study, we have characterised the molecular and functional expression of TRPV4 channels in bovine retinal endothelial cells (BRECs) under both normal (5 mM) and high glucose (25 mM) conditions (72-hour incubations). In cells cultured in normal glucose medium,

the TRPV4 agonist, 4 $\alpha$ -PDD (1  $\mu$ M) induced a rise in [Ca<sup>2+</sup>]<sub>i</sub> (fura-2 microfluorimetry) which could be blocked by the non-selective TRPV4 antagonist, ruthenium red (10  $\mu$ M), and siRNA-mediated knockdown of TRPV4 channel expression. We subsequently examined the effects of high glucose on TRPV4 mRNA expression in BRECs. TRPV4 transcripts were approximately 50% less abundant in BRECs after exposure to high glucose compared with normal glucose controls. Using Western blotting, we also confirmed that TRPV4 expression was appreciably lower in cells treated with high glucose. Functional downregulation of TRPV4 channels was evaluated by Ca<sup>2+</sup> microfluorimetry. High glucose was associated with a substantial decrease in the percentage of cells responding to 4- $\alpha$ PDD. These results show that the expression and function of TRPV4 channels is downregulated in retinal endothelial cells under hyperglycaemic conditions. Changes in TRPV4 expression may contribute to endothelial cell dysfunction during diabetes.

#### PC29

##### **Insulin Resistance and Circulating Endothelial Cells in Preeclampsia**

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**Introduction:** Preeclampsia, a hypertensive disorder of pregnancy, is characterised by generalised endothelial cell dysfunction. There is evidence of increased circulating endothelial cells (CECs) and increased insulin resistance (IR) in preeclampsia, which is related to endothelial dysfunction. The aim of this study was to investigate whether CEC count correlates with IR in preeclampsia.

**Method:** Fasting blood samples were obtained from 10 women with pre-eclampsia and 10 women with normal pregnancy matched for age, BMI, ethnicity and gestational age. Free Insulin was estimated by enzyme linked immunosorbent assay (ELISA), and glucose by the glucose oxidase method. IR was calculated using Homeostasis model Assessment (HOMA). CECs were determined using immunomagnetic bead separation using CD146. They were double stained using UEA-1 (an endothelial stain) attached to Fluorescein isothiocyanate, an isochrome.

**Results:** The CEC count in preeclamptic women was significantly higher compared to normal pregnant controls (mean 24  $\pm$  8.27 versus 6.1  $\pm$  1.82,  $p < 0.003$  respectively) IR in pre-eclampsia was also significantly greater in pre-eclampsia compared to normal pregnant controls (6.1  $\pm$  1.82 versus 1.83  $\pm$  0.85,  $p < 0.0001$  respectively). There is a positive correlation between the IR and CEC in the pre-eclamptic patients ( $r = 0.563$ ,  $p = 0.09$ ). However

this was not statistically significant. There was no significant correlation between the IR and CEC in the normotensive patients.

**Conclusion:** There is no correlation between insulin resistance and plasma Circulating Endothelial Cells levels in pre-eclampsia.

#### PC30

##### **Microparticle Formation after Co-Culture of Human Whole Blood and Umbilical Artery Segments in a Novel *In Vitro* Model of Flow**

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Cardiovascular disease (CVD) is the largest killer in western society, and the importance of interactions between the vascular endothelium and different circulating blood components in disease pathogenesis is well established. Microparticles (MP) are heterogeneous blood borne particles arising from blebbing or shedding of cell membranes. Increased numbers of procoagulant MP have been described in plasma from people with CVD. We have developed a novel co-culture method using segments of human umbilical artery perfused at arterial flow rate with anticoagulated diluted human blood to investigate the contribution of endothelial inflammation to MP production. Using flow cytometry to identify MP we observed that when blood was perfused through umbilical arteries which had been pre-stimulated with TNF $\alpha$  for 18 hours, production of MP from platelet and non-platelet sources, in particular from erythrocytes significantly increased. MP produced after co-culture with inflamed endothelium were isolated by centrifugation and found to induce significantly enhanced levels of reactive oxygen species in third party endothelial cells. We conclude that presence of an inflamed endothelium causes release of pro-coagulant and pro-inflammatory MP from circulating blood cells, which could contribute to prolonged endothelial activation and subsequent atherosclerotic changes in blood vessels subjected to inflammatory insult.

#### PC31

##### **Hydrogen Sulfide Donors Protect Human Microvascular Endothelial Cells from Oxidative Stress-Induced Cell Death**

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**Background:** Type 2 diabetes mellitus (T2DM) is associated with microvascular endothelial dysfunction, although the

underlying mechanisms are unknown. Recently we showed that lower levels of the gaseous mediator hydrogen sulfide (H<sub>2</sub>S) were observed in overweight and T2DM subjects compared to age-matched controls and H<sub>2</sub>S levels strongly negatively correlated with impaired microvascular function *in vivo*. These data suggested that impaired H<sub>2</sub>S synthesis could mediate microvascular dysfunction.

**Aim:** To examine whether H<sub>2</sub>S is protective for microvascular endothelial cells.

**Methods:** Human microvascular endothelial cells (HMEc) were exposed to oxidative stress induced by SIN-1 in the presence and absence of endogenous and pharmacological levels of H<sub>2</sub>S, generated *via* novel slow release donor compounds (e.g., GYY4137, MC05, MC06, AP39). Cytotoxicity was assessed by standard metabolic assays and microscopy.

**Results:** SIN-1 induced significant time (6–48 hours) and concentration-dependent (50–2000 μM) cell death. Pre-treatment of HMEc with H<sub>2</sub>S donors (100 nm–500 μM) significantly inhibited ( $p < 0.01$ ; ANOVA) SIN-1 induced cell death. Significant ( $p < 0.01$ ; ANOVA) inhibition of SIN-1 mediated toxicity was also observed when H<sub>2</sub>S donors were added to cells six hours after SIN-1 treatment.

**Conclusions:** H<sub>2</sub>S donors can inhibit and reverse oxidative stress mediated cell injury. These results suggest that H<sub>2</sub>S may represent a novel potential therapeutic opportunity to prevent, treat or reverse endothelial dysfunction in T2DM.

### PC32

#### Lung Tumour Secreted Factors Induce E-Selectin Expression on Human Brain Microvascular Endothelial Cells (HBMECs)

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**Background:** Endothelial adhesion molecules may mediate adhesion of lung tumour cells to the cerebral endothelium during metastasis. Indeed, lung tumour conditioned medium (TCM) increased expression of E-selectin on HBMECs. We have now analysed the TCM for potential pro-metastatic proteins and identified/quantified cystatin C, cathepsin L, insulin-like growth factor binding protein-7 (IGFBP-7), vascular endothelial growth factor (VEGF) and tumour necrosis factor- $\alpha$  (TNF $\alpha$ ).

**Aim:** To examine whether relevant concentrations of lung tumour secreted factors increase E-selectin expression on HBMECs.

**Methods:** HBMECs were treated with increasing concentrations of each identified factor for four hours and surface expression of E-selectin was quantified by ELISA.

**Results:** E-selectin expression was increased by 8, 80 and 800 ng/ml of cystatin C to  $155.3 \pm 62.3\%$ ,  $199.6 \pm 69.5\%$

and  $306.1 \pm 94.1\%$  respectively vs control (100%) (all  $p = 0.037$ ,  $n = 3$ ) and by cathepsin L at 1, 10 and 100 ng/ml to  $253.4 \pm 122.5\%$ ,  $258.4 \pm 101.9\%$  and  $247.5 \pm 40.2\%$  respectively vs control (100%) ( $p = 0.037$ ,  $n = 3$  for all). IGFBP-7 (900 ng/ml) and VEGF (10 and 20 ng/ml) also increased E-selectin expression to  $189.4 \pm 40.3\%$ ,  $221.6 \pm 120.2\%$  and  $224.5 \pm 144.8\%$  respectively vs control (100%) ( $p = 0.037$ ,  $n = 3$  for all). Finally, 100, 500 and 2500 pg/ml of TNF- $\alpha$  elevated E-selectin to  $243.2 \pm 101.2\%$ ,  $260.6 \pm 49.6\%$  and  $260.6 \pm 65.8\%$  respectively vs control (100%) ( $p = 0.037$ ,  $n = 3$  for all).

**Conclusions:** Factors released from lung tumour cells increase expression of E-selectin on HBMECs. This may mediate tumour cell adhesion to cerebral endothelial cells.

### PC33

#### Microvascular and Sensory Dysfunction in Individuals with Non-Alcoholic Fatty Liver Disease (NAFLD) at Risk of Cardiovascular Disease

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Non alcoholic fatty liver disease (NAFLD) is strongly associated with insulin resistance, type 2 diabetes and cardiovascular disease (CVD) but associations between NAFLD and microvascular disease are less certain. In  $n = 50$  people with NAFLD, we have examined relationships between microvascular function, sensory nerve function and CVD risk over 10 years (QRISK score). We assessed microvascular function in the forearm (peak post occlusive reactive hyperaemic response as % resting flux; PF%RF) using laser Doppler fluximetry (Moor Instruments, UK) and functional parameters of small fibres (warm thresholds) and large fibres (vibratory threshold at 125 Hz) at the foot (HVLab, UK). PF%RF was correlated with temperature ( $r = -0.410$ ,  $p = 0.004$ ) and vibratory ( $r = -0.355$ ,  $p = 0.014$ ) thresholds. QRISK score was associated with vibratory threshold ( $r = 0.308$ ,  $p = 0.04$ ) but not with PF%RF ( $r = -0.247$ ,  $p = 0.098$ ). These data show for the first time that in people without evidence of clinical microvascular disease or neuropathy, microvascular and sensory function are related. Furthermore, they show that there is a relationship between sensory nerve function and CVD risk in individuals with NAFLD.

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PC34

### Mesenchymal Stem Cell Interactions with Endothelial Cells

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Mesenchymal stem cells (MSCs) have anti-inflammatory and immunosuppressive properties and may be useful in the therapy of arteriosclerosis. MSCs have some ability to traffic into inflamed tissues, however to exploit this therapeutically their migratory mechanisms need to be elucidated. This study examines the interaction of murine MSCs (mMSCs) with, and their migration across, murine aortic endothelial cells (MAECs), and the effects of chemokines and shear stress. The interaction of mMSCs with MAECs was examined under physiological flow conditions. mMSCs showed lack of interaction with MAECs under continuous flow. However, when the flow was stopped (for 10 minutes) and then started, mMSCs adhered and crawled on the endothelial surface, extending fine microvillous processes (filopodia). They then spread extending pseudopodia in multiple directions. CXCL9 significantly enhanced the percentage of mMSCs adhering, crawling and spreading on MAECs and shear forces markedly stimulated crawling and spreading. CXCL9, CXCL16, CCL20 and CCL25 significantly enhanced transendothelial migration across MAECs. The transmigrated mMSCs were smaller in size with down-regulated receptors CXCR3, CXCR6, CCR6 and CCR9. This study furthers the knowledge of MSC interaction with ECs, their transmigration and the effects of chemokines and shear stress.

PC35

### A Novel Vascular Chamber Discovered in the Human Renal Glomerulus

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Mouse and human renal glomerular diameter (70  $\mu\text{m}$  and 200  $\mu\text{m}$ ) mark the approximate 27 ( $3^3$ ) fold difference in volume between these glomeruli. The perfusion pressures in both glomeruli remain at a high fraction (45–60 mm) of the mean arterial pressure. We have investigated human glomerular structure to find any compensatory mechanism for the different haemodynamics. The vasculature of unused transplant kidneys (3) was pre-perfused with Marshall's transport solution and the kidneys transported on ice. Renal arteries were cannulated and perfused

(100 mmHg hydrostatic pressure) with a Mammalian HE-PES Ringer containing Ficoll 400 as an osmotic balance (25 mmHg Colloid Osmotic pressure). This was replaced with a glutaraldehyde solution at the same pressures. After fixation some of the wet tissues were washed in Ringer and observed using a confocal microscope, others were dehydrated, embedded in resin and serial section reconstructions made of glomeruli. Both techniques showed vascular chambers (VCs) near the vascular pole of perfusion fixed human glomeruli (105  $\pm$  10  $\mu\text{m}$  long (vessel axis) and 33  $\pm$  4  $\mu\text{m}$  wide, n = 5, mean  $\pm$  sem) some of which appeared connected to the afferent arteriole. Many smaller blood vessels (12  $\pm$  1  $\mu\text{m}$ ) lead away from VCs to smaller vessels in the glomeruli. VCs have not been observed in mouse or rat glomeruli. VCs may be a means of distributing flow among a larger population of filtration capillaries in human glomeruli and may have huge implications in glomerular physiology and disease.

PC36

### Conditional Podocyte-Specific Over-Expression of VEGF in Adult Mice Induces A Transient Increase in Proteinuria and Permeability to Water, Which is Prevented by Podocyte-Specific Over-Expression of VEGF<sub>165B</sub>

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VEGF is a family of molecules produced by glomerular podocytes important for maintaining permselectivity. We aimed to determine the roles of the traditional, pro-permeability VEGF<sub>164</sub> isoform, and the novel VEGF<sub>165b</sub> isoform, both alone and in combination, in mature, adult glomeruli *in vivo* using conditional, inducible transgenic overexpression systems. VEGF<sub>164</sub> overexpression was induced in a podocyte-specific manner by the addition of doxycycline to the drinking water of adult podocin-rtTA / TetO-VEGF<sub>164</sub> double transgenic mice, for periods up to 100 days. Simultaneous overexpression of VEGF<sub>164</sub> and VEGF<sub>165b</sub> was assessed in triple-transgenic podocin-rtTA / TetO-VEGF<sub>164</sub> / nephrin-VEGF<sub>165b</sub> mice. VEGF<sub>164</sub> overexpression induced increased glomerular water permeability by day 3, which was accompanied by proteinuria at day 7. Despite persistently increased VEGF levels, both proteinuria and increased glomerular water permeability resolved by day 14, and remained normal for up to 100 days of doxycycline administration. Simultaneous overexpression of VEGF<sub>165b</sub> blocked the changes in glomerular function induced by seven days overexpression of VEGF<sub>164</sub>. VEGF<sub>164</sub> overexpression-induced proteinuria is less marked and elicits only transient proteinuria in mature,

adult animals. VEGF<sub>165b</sub> is capable of preventing VEGF<sub>164</sub>-induced changes in glomerular permeability *in vivo*.

## PC37

### Hyperglycaemia-Induced Release of Active Heparanase 1 from Retinal Endothelial Cells is Associated with Increased Leukocyte Adhesion

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**Background:** Increased leukostasis in retinal capillaries is an early event in diabetic retinopathy (DR) associated with capillary non-perfusion. Growing evidence suggests that leukocyte-endothelial cell adhesion can be influenced by the endothelial glycocalyx. The glycocalyx can be modified by enzymes including heparanase1 (HPSE) which regulates degradation of heparan sulfate (HS) side chains. Our previous results showed that HPSE release from retinal endothelial cells was significantly increased in hyperglycaemia and that this was accompanied by a significant decrease in endothelial cell glycocalyx.

**Aim:** To investigate whether the HPSE released in high glucose is enzymatically active and whether release is associated with increased leukostasis.

**Methods:** Bovine retinal endothelial cells (BREC) were exposed to normal (NG, 5.6 mM), high glucose (HG, 25 mM) or mannitol (5.6 mM glucose + 19.4 mM mannitol) for 24 hour. Activity of secreted HPSE was assessed by heparan degrading enzyme assay kit. Adhesion of leukocytes to BREC was measured at shear stress of 1 dyn/cm<sup>2</sup>.

**Results:** HPSE activity was significantly increased in HG [NG 2.05 ± 0.38 U/ml vs HG 2.62 ± 0.42 U/ml  $p = 0.0262$ ,  $n = 7$ ], mannitol had no significant effect. Leukocyte adhesion was increased significantly in HG treated BREC [136 ± 16% vs NG (100%,  $p < 0.001$ ,  $n = 10$ ).

**Conclusion:** High glucose-induced secretion of enzymatically active HPSE may play a role in increased leukostasis by altering retinal endothelial glycocalyx structure and function.

## PC38

### Modulating Haematopoietic Stem Cell Adhesion to Intestinal Microvasculature

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**Introduction:** The clinical success of hematopoietic stem cell (HSC) therapy is poor partly due to their limited recruitment to sites of injury. If SC therapy is to be realised, strategies that enhance their recruitment need to be identified. We hypothesised that HSC pre-treatment with factors released at injury sites would enhance their adhesion to frozen tissue sections.

**Methods:** HSCs were pre-treated with hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) or an injury conditioned media (ICM) generated from homogenising small intestine (SI) undergoing ischemia-reperfusion (I/R) injury or colon undergoing DSS-induced colitis injury. HSC adhesion to frozen sections of SI and colon, obtained from separate I/R injured or colitis mice, was determined.

**Results:** HSC adhesion to I/R jejunum ( $p < 0.001$ ) and ileum ( $p < 0.01$ ) was significantly increased. Pre-treating HSCs with an I/R ICM significantly increased adhesion further on jejunum ( $p < 0.001$ ) and ileum ( $p < 0.01$ ). HSC adhesion to colitis colon ( $p < 0.01$ ) was also increased. Again, colitis ICM significantly increased adhesion further on colon ( $p < 0.01$ ). H<sub>2</sub>O<sub>2</sub> pre-treatment had similar effects.

**Discussion:** Acute and chronic gut injuries lead to HSC adhesion. This is a modulatable event since adhesion can be significantly enhanced by pre-treating HSCs with ICM obtained from I/R injured or colitis gut. We also demonstrated that H<sub>2</sub>O<sub>2</sub>, known to be released at sites of ischemic injury, was effective at enhancing adhesion. Identification of additional factors within ICM may lead to development of strategies that can be used clinically to increase the efficiency of stem cell recruitment.

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## PC39

### Calcium Dependent Calmodulin Kinase II (CAMKII) Plays an Essential Role in VEGF-Induced Retinal Angiogenic Signalling

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Retinal angiogenesis, the formation of new blood vessels in the eye, is a critical process in the development of many ocular diseases. Vascular endothelial growth factor (VEGF) has been implicated in the pathogenesis of retinal neovascularisation. In the present study, we have investigated the role of Ca<sup>2+</sup>/calmodulin-dependent kinase II (CaMKII) in VEGF-induced retinal angiogenic signalling. Bovine retinal endothelial cells (BRECs) were stimulated with VEGF and intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) estimated using Fura-2 Ca<sup>2+</sup> microfluorimetry. Stimulation of BRECs with 25 ng/ml of VEGF induced a biphasic increase in [Ca<sup>2+</sup>]<sub>i</sub>, with an initial transient peak followed by a sustained plateau phase. Inhibition of CaMKII activity using KN-93 suppressed the VEGF-induced retinal endothelial cell migration, proliferation, tube formation and sprouting angiogenesis. On the other hand, an inactive analogue, KN92, had no impact on VEGF-induced angiogenic responses. KN-93, but not KN92, blocked the VEGF-induced phosphorylation of Akt. **Conclusion:** VEGF-induced phosphorylation of the pro-angiogenic

protein, Akt, is critically dependent upon the activation of CaMKII. Pharmacological inhibition CaMKII in BRECs blocks retinal angiogenic responses. These results suggest that the CAMKII-dependent signalling pathway may provide a rational target for the treatment of angiogenesis-related disorders of the eye.

#### PC40

### SR Protein Overexpression Differentially Affects VEGF Splicing in Primary and Immortalised RPE Cells

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VEGF is alternatively spliced to two distinct families of isoforms (VEGF<sub>xxx</sub> and VEGF<sub>xxx</sub>b). The aim of this study was to assess a panel of splice factors for changes in VEGF expression in both primary Retinal Pigment Epithelial (RPE) cells and an immortalised RPE cell line (ARPE19). Plasmids for 8 SR proteins (ASF/SF2, SC35, 9G8, SAM68, SRp20 & SRp75) plus an empty vector (pCG) control were transfected into primary RPE or ARPE19 cells. Protein and mRNA was assessed for changes in splice factor expression by RT-PCR and changes in VEGF protein expression by ELISA. Analysis of cDNA confirmed an increase in SR protein mRNA expression compared to pCG controls. *In vitro* only ASF/SF2 over-expression showed a significant change in VEGF protein expression; the proportion of VEGF<sub>xxx</sub>b:VEGF<sub>total</sub> was reduced to 70.1% ± 3.5% vs. pCG control ( $p < 0.05$ ). However in ARPE19 cells, over-expression of ASF/SF2 appears to have the opposite effect, increasing VEGF<sub>xxx</sub>b:VEGF<sub>total</sub> ratio to 155% ± 20.4% vs. pCG control ( $p < 0.05$ ). Assessment of TIA1 isoform expression confirmed that ASF/SF2 splicing effects differed between primary and immortalised RPE cells. Alteration in VEGF isoform selection through the manipulation of splice factors is of both physiological and therapeutic importance. However the differences in functional effect between primary and immortalised cells suggests that the cellular environment also contributes to these mechanisms, and the effect of manipulating these factors may differ between tissue types.

#### PC41

### The Receptor for AGEs (RAGE) Plays A Key Role in Choroidal Neovascularisation (CNV)

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CNV is the primary lesion leading to vision loss in wet age-related macular degeneration (AMD). This study investigates

how RAGE and its ligands modulate key pro-inflammatory processes that lead to CNV. C57Bl/6 wild-type (WT) and RAGE<sup>-/-</sup> (n = 12/group) mice were subjected to laser-induced CNV as a model of wet AMD. *In vivo*, fundus photography and cSLO angiography evaluated lesion development. Post-mortem retinal flatmounts were analysed for CNV morphology, and a panel of microglial expression markers. Parallel *in vitro* studies investigated the angiogenic response to the RAGE ligand S100B and also determined RAGE signal transduction and cytokine expression in human endothelial cells (HMEC-1). Retina from RAGE<sup>-/-</sup> mice exhibited significantly reduced CNV lesion size and S100B staining compared to WT controls ( $p < 0.05$ ) (12/group). Abundant active microglia were evident in CNV induced mice compared to control ( $p < 0.001$ ). RAGE<sup>-/-</sup> mice showed significantly less amoeboid activated microglia ( $p < 0.001$ ). S100B-treated HMEC-1 cells showed increased migration and tube formation ( $p < 0.001$ ), Akt phosphorylation and NFκB transcriptional activation. siRNA mediated knockdown of RAGE significantly prevented all these S100B responses. Pro-inflammatory and angiogenic responses appear to be mediated by S100B-RAGE interactions. This study highlights the role of RAGE in angiogenesis, especially in the context of CNV.

#### PC42

### Longitudinal Sprouting in Murine Retinal Vascular Plexus Development is Guided by Müller Glial Cells

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The murine retinal vascular plexi (RVP) develop in a distinct spatial and temporal pattern in order to meet the metabolic needs of developing retina. The primary RVP develops by the process of angiogenesis along the surface of the retinal ganglion cell layer from 0–7 days postnatally (P0–P7) and is guided by cues from underlying retinal astrocytes. The formation of two further plexi, which supply the deeper retinal tissue, begins at P7, with perpendicular angiogenic sprouting, which penetrates the neural retina. The deepest layer is fully formed by P12; on the outer surface of the inner nuclear layer. An intermediate plexus is fully formed by P14 on the inner surface of the inner nuclear layer. The topology of the plexi is determined by specific spatio-temporal expression of R-Cadherin at their borders [1]. Using immunohistochemistry we have shown that longitudinal angiogenic sprouting occurs coincidentally with Müller glial cell differentiation and is guided by processes from these cells. Evidence of the chemotactic cues and haptotactic determinants of RVP formation in the neonatal mouse eye will be presented.

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## PC43

### Angiogenesis and Osseointegration of Bioactive Glass Scaffolds for Bone Regeneration

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A biomaterials type approach is a promising tissue regeneration strategy for facilitating functional regrowth of damaged tissue. We aim to optimise the biocompatibility and integration of bioactive glass scaffolds of the 70S30C (70%SiO<sub>2</sub> + 30%CaO) composition for critical size bone defects. Microcomputed tomography ( $\mu$ CT) and scanning electron (SEM) reveal that 70S30C bioglass has a porous, interconnected structure resembling trabecular bone. The effect of bioglass dissolution ions on endothelial cell (EC), Osteoblast (Ob) and osteoclast (Oc) cultures is being studied using several *in vitro* assays prior to implantation of scaffolds in bone defects. SEM indicates that EC, Ob and Oc cell lines cultured on 70S30C bioglass can adhere, proliferate and exhibit typical behaviour such as mineralisation and tubule formation. Histological examination of cranial and tibial subcritical defects in mice (anaesthetized intraperitoneally with fentanyl citrate/ midazolam) indicates neutrophil infiltration, vascular ingrowth of the defect site and fibrotic encapsulation of the bioglass implant, with no evidence of bone ingrowth after 2 weeks. Analysis of critical size defects using  $\mu$ CT, SEM and vascular casting techniques is in progress. At the meeting we will present evidence of the angiogenic and osteogenic potential of bioglass seeded with differentiated cell types and examining their ability to heal subcritical and critical size bone defects *in vivo*.

## PC44

### Release of Interleukin 8 (IL8) by Circulating Angiogenic Cells (CACs) Promotes Retinal Angiogenesis.

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Endothelial Progenitor Cells (EPCs) have been shown to facilitate revascularisation of ischaemic tissues and hold therapeutic promise for promoting repair and reperfusion in conditions such as stroke and diabetic microvasculopathies. However clinical outcomes have been contradictory due to the lack of a uniform EPC definition, resulting in a diverse range of EPC types being utilised for therapeutic

applications. We recently characterised two distinct EPC cell populations *in vitro*, early EPCs (circulating angiogenic cells; CACs) and Outgrowth endothelial cells (OECs). Despite being widely described as "EPCs", CACs were shown to be monocytic cells without endothelial characteristics. This study investigates the CAC phenotype and potential role in retinal angiogenesis. We demonstrate that CACs can significantly induce endothelial tube formation *in vitro* and vascular repair *in vivo*, without differentiating into endothelial cells or directly incorporate into a microvascular network, suggestive of a paracrine mechanism. IL8 was identified in CAC conditioned media as a key paracrine factor. Blockade of IL8 but not VEGF prevented CAC-induced angiogenesis. Transcriptomic and immunophenotypic analysis indicates that CACs represent alternative activated M2 macrophages. Our findings demonstrate that CACs act as alternative M2 macrophages with pro-angiogenic, anti-inflammatory and pro-tissue repair properties in the ischaemic retina, and that their role in retinal angiogenesis is linked to paracrine release of cytokines such as IL8.

## PC45

### Paracrine Stimulation of Angiogenesis by Platelet-Derived Deoxyribose-1-Phosphate

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**Objectives:** In this study we investigated the mechanism of action and the physiological role of platelet-derived deoxyribose-1-phosphate (dRP) in the regulation of endothelial cell motility and angiogenesis.

**Methods and Results:** Protein-free supernatants from thrombin-stimulated mouse and human platelets were found to increase human umbilical vein endothelial cell (HUVEC) migratory activity in transmigration and monolayer repair assays. The stimulation of endothelial cell motility was abolished by genetic silencing of dRP-generating uridine phosphorylase (UP) and thymidine phosphorylase (TP) in mouse platelets or the pharmacological inhibition of UP in human platelets. The stimulation of endothelial cell migration by platelet-derived dRP correlated with upregulation of integrin  $\beta_3$ , which was induced in a reactive oxygen species (ROS)-dependent manner, and was mediated by the activity of the integrin heterodimer  $\alpha_v\beta_3$ . The physiological relevance of dRP release by platelets was confirmed in a chick chorioallantoic membrane (CAM) assay, where the presence of this metabolite in platelet supernatants strongly induced capillary formation. **Conclusions:** Platelet-derived dRP stimulates endothelial cell migration by upregulating integrin  $\beta_3$  in a

ROS-dependent manner. As demonstrated by our *in vivo* experiments, this novel paracrine regulatory pathway is likely to play an important role in the stimulation of endothelial cell motility and angiogenesis by platelets in physiological conditions.

#### PC46

### Modelling the Developing Murine Retinal Vasculature: A Combined Experimental and Theoretical Approach

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Tumour angiogenesis has been extensively explored by mathematical modellers with few attempts to model angiogenesis during development; despite the availability of animal models with ordered vascular topologies, such as the retina. We aim to create mathematical models of the developing retinal vasculature in neonatal mice, using a coupled experimental biological and mathematical approach. Formation of the superficial retinal vascular plexus (SVP) occurs in a spatio-temporally defined pattern. At E15.5 PDGF-A expression induces astrocyte migration over the retina, and endothelial cell sprouting begins around birth in response to astrocytic VEGF and fibronectin. We have previously developed a 1D continuum model [1] using partial differential equations to describe the migratory response of astrocytes and endothelial cells to growth factor gradients and haptotactic cues. We have expanded the model to 2D by discretisation of the partial differential equations to enable tracking of individual astrocytes and endothelial cells, incorporated basic flow parameters and included upstream and downstream signalling factors within the expanding endothelium. The simulations of the discrete model provide an excellent correlation with the extent and pattern of astrocyte migration and vascular network formation observed *in vivo*. In the future we hope to expand this model to elucidate the impact of molecular cues on growth and remodelling of the retinal vasculature and the implications of perturbations to these signalling pathways in ocular conditions characterised by aberrant angiogenesis.

Reference:

1. Aubert *et al*, Bulletin of Mathematical Biology, in press.

#### PC47

### Effects of High Insulin on Angiogenesis in Human Umbilical Vein Endothelial Cells from Normal and Type 1 Diabetic Pregnancies

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Fetal hyperinsulinemia is a chronic event in diabetic pregnancies. Whether insulin can affect angiogenesis in the offspring of diabetic mothers is not known and was the aim of this study. Human umbilical vein cells (HUVEC) were isolated from umbilical cords from normal and type 1 diabetic pregnancies. A well established *in vitro* model of angiogenesis was used where cells from normal (nHUVEC) and diabetic pregnancies (dHUVEC) were grown to confluence and overlaid with collagen 1. Cells were incubated in Medium 199 with 5% serum. Hourly observations were made for 27 hours. Insulin (25 mU/ml) was introduced at time zero (T0), three hours (T3) and 24 hours after overlay (T24). Control cells did not contain any insulin. Within an hour of overlay, monolayer remodelling was seen with cord formation and branching observed from two hours onwards reaching a peak at 22 hours and then reducing in number. Increased branching was seen in nHUVEC exposed to insulin from T0 at all time points. Introduction of insulin at later stages of angiogenesis, T3 or T24 did not affect cord branching. In dHUVEC, insulin given at initiation of angiogenesis had very little affect, however at T24, introduction of insulin resulted in a further increase in branching. These studies show that HUVEC cells are vulnerable to insulin with initiation of angiogenesis being affected by insulin in normal pregnancies, whilst in the offspring of diabetic mothers, insulin increased angiogenesis in HUVEC once an established plexus had formed.

#### PC48

### Inhibiting Neuropilin to Enhance Anti-VEGF Therapy

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Vascular endothelial growth factor (VEGF) correlates with disease progression and poor prognosis in breast cancer. Bevacizumab (Bz), an anti-VEGF antibody, inhibits VEGF binding to VEGF-R1/2 and increases progression free survival in breast cancer patients. Eventually tumours escape treatment control and we hypothesize that this is because Bz does not prevent VEGF from binding to its alternative receptors, the neuropilins (Np1/2). Using two Np1 binding peptides (p7b & p10) we found significant inhibition of

endothelial cell (EC) tubule formation (47 & 58% respectively, compared to control,  $p < 0.005$ ), but this was unaffected by combined treatment with Bz. p7b and p10 significantly inhibited MDA-MB-436 breast cancer cell proliferation (20% compared to cells alone,  $p < 0.001$ ) but not EC or MDA-MB-231 proliferation. p7b and p10 had no effect on EC or breast cancer migration. The Boyden chamber is currently being used to confirm these results. These data show that the two Np1 binding peptides inhibit EC differentiation and breast cancer proliferation *in vitro* to varying extents, suggesting that one of these peptides may have therapeutic potential for breast cancer treatment. We are currently evaluating the response to Np1 inhibition, using p10 and p7b, alone and in combination with Bz, in an *in vivo* model of breast cancer.

Acknowledgment: This work was supported by the Breast Cancer Campaign.

#### PC49

### **A2780 Ovarian Cancer Cells and Insulin Like Growth Factor Binding Protein 7 (IGFBP-7) Stimulate Activation, Proliferation and Angiogenesis in Human Omental Microvascular Endothelial Cells (HOMECS)**

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Background: During ovarian cancer metastasis to the omentum secondary tumours implant onto the omentum and induce angiogenesis in the omental microvasculature. However, the molecular mechanisms involved are unknown.

Aim: To investigate whether A2870 ovarian cancer cell conditioned medium (CM) and IGFBP-7 (secreted from ovarian cancer cells) alter activation, proliferation and angiogenic tubule formation of HOMECS.

Methods: HOMECS proliferation was assessed by WST-1 assay and activation by surface expression of CD105 measured by cell based fluorescence assay. Angiogenic tubule formation was assessed using an *in vitro* assay.

Results: A2780 CM significantly increased membrane expression of CD105 on HOMECS ( $176.3 \pm 56.2\%$  vs control (100%);  $p < 0.001$ ,  $n = 12$ ) and also proliferation to  $259.6 \pm 16.5\%$ ,  $336.5 \pm 16.8\%$  and  $379.3 \pm 19.5\%$  vs. control (100%) ( $p < 0.001$ ,  $n = 6$ ) at 24, 48 and 72 hours respectively. In co-culture A2780 cells induced tube structure formation in HOMECS ( $26.88 \pm 9$  vs control ( $10.7 \pm 5.2$ ),  $p = 0.0016$ ,  $n = 8$ ). IGFBP-7 (100 ng/ml) significantly stimulated proliferation to  $112.7 \pm 8.3\%$  vs control (100%) ( $p = 0.028$ ,  $n = 4$ ) and tubule formation  $31.5 \pm 3.7$  vs control ( $7.6 \pm 1.5$ ) ( $p \leq 0.0087$ ,  $n = 6$ ). SU5416 (VEGF receptor kinase inhibitor) did not inhibit A2780-induced tubule formation.

Conclusion: A2780 cells secrete potent factors that stimulate activation, proliferation and angiogenic tube structure formation in HOMECS. A2780-induced angiogenesis may not be solely VEGF dependent.

#### PC50

### **Inhibition of Hydrogen Sulfide Synthesising Enzymes Reduces Human Microvascular Endothelial Cell (HMEC) Viability**

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Background: Hydrogen sulfide ( $H_2S$ ) is a novel gaseous mediator produced endogenously by cystathionine  $\beta$ -synthase (CBS) and cystathionine  $\gamma$ -lyase (CSE).  $H_2S$  may be pro-angiogenic since inhibition of  $H_2S$  synthesis reduced endogenous  $H_2S$  production and neovascularisation in a chick chorioallantoic membrane model. In addition, in human umbilical vein endothelial cells  $H_2S$  increased cell number, capillary-like structure formation and motility. However, the role of  $H_2S$  in these processes in HMECs is unknown.

Aim: To examine whether inhibition of  $H_2S$  synthesising enzymes reduces cell growth in human microvascular endothelial cells.

Methods: Cerebral HMECs were treated with propargylglycine (PAG; CSE inhibitor) and aminooxyacetic acid (AOAA; CBS inhibitor) for either 18 or 48 hours. The number of viable cells was estimated by alamar blue assay.

Results: PAG reduced viable cells to  $89.7 \pm 14.4$ ,  $80.0 \pm 11.4$ ,  $77.9 \pm 11.0$  at 100, 300 and 1000  $\mu M$  respectively vs controls (100%),  $p \leq 0.05$  for all,  $n = 3$  at 18 hours, and  $57.2 \pm 5.1$ ,  $52.9 \pm 15.9$ ,  $43.1 \pm 19.3$  at 48 hours respectively, vs controls (100%),  $p \leq 0.05$  for all,  $n = 3$ . Similarly, AOAA reduced viable cells to  $63.2 \pm 11.5$ ,  $65.0 \pm 14.1$ ,  $61.5 \pm 19.5$  at 100, 300 and 1000  $\mu M$  respectively vs controls (100%),  $p \leq 0.05$  for all,  $n = 3$ , at 18 hours and  $51.2 \pm 2.6$ ,  $47.1 \pm 4.6$ ,  $46.5 \pm 4.7$  at 100, 300 and 1000  $\mu M$  respectively vs controls (100%),  $p \leq 0.05$  for all,  $n = 3$ , at 48 hours.

Conclusions: Inhibition of  $H_2S$  in HMECs reduced viable cell number, suggesting an essential role for  $H_2S$  in endothelial cell proliferation.

#### PC51

### **A Role for the Endothelial Tetraspanin Tspan18 in Calcium Signalling**

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Tetraspanins organise the cell surface through the creation of microdomains and have recently emerged as essential

regulators of endothelial cell function. For example, tetraspanins CD9 and CD151 cluster the adhesion molecules ICAM-1 and VCAM-1 in adhesive platforms which are important for leukocyte recruitment during inflammation. CD151 is also necessary for angiogenesis, while Tspan12 is essential for vascularisation of the retina, with mutations leading to familial exudative vitreoretinopathy. Despite these advances, most other tetraspanins have not been studied in endothelial cells. In this study, we have found endothelial cells to express at least 22 of the 33 human tetraspanins. Of these, the previously unstudied Tspan18 was highly expressed in endothelial cells versus other cell types. Moreover, Tspan18 was unique amongst several tetraspanins tested in its capacity to activate a calcium-responsive NFAT transcriptional reporter. In primary endothelial cells, Tspan18 induced a striking dendritic morphology. Finally, *in situ* hybridisation showed Tspan18 localisation to the vasculature of developing zebrafish embryos, consistent with an endothelial expression profile. In conclusion, the present study suggests that Tspan18 might regulate calcium signalling in endothelial cells, possibly through an interaction with a calcium channel.

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## PC52

### **A Call for More Research into Diseases of the Lymphatic System**

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As a founder member of the British Microcirculation Society, I look at the development of research into the microvascular system with satisfaction. Research into the more neglected lymphatic system is less satisfactory. In a recent tour of Ethiopia, I saw a few hundred of possibly two and half million persons who had swollen feet due to lymphatic failure. Recently labelled as one of the neglected diseases (podoconiosis) it is in need of basic science research into its causation which is assumed to be due to exposure to silica. My main preoccupation is with 20 million causes of lymphoedema in India. The huge hypertrophy of tissues (elephantiasis) responds to integrated biomedical and Indian systems of medicine approaches, but the mechanism is unknown. The points for discussion, after viewing of the problem, will be the effects of exchange failure and the growth factors and cytokines involved. The role of movement of tissues and of tissue temperature will be referred to as well as parallels with wound healing and the fibrosis of scarring.

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## PC53

### **CD31 is Not a Pan Marker for Endothelial Microparticles**

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Endothelial microparticles (EMPs) are generated upon stimulation of endothelial cells. EMPs may have important roles in thrombosis, inflammation, cell survival, cell activation, and angiogenesis. To date there is no universal method for identifying EMPs but many papers identify EMPs by gating on CD31 as a pan endothelial marker in flow cytometry. From our own observations in human plasma, CD31 did not seem to associate with other markers we would expect to see on EMPs. Therefore we have used a purified endothelial cell culture system to test the hypothesis that not all EMPs produced by purified endothelial cells express CD31. Purified human umbilical vein endothelial cells (HUVECs) were used to generate EMPs using TNF $\alpha$ , and serum starvation to initiate apoptosis versus untreated cells in a pilot study. Flow cytometry was used to determine whether all EMPs expressed CD31 when gating with CD105, CD62e and CD54. The pilot data (Table 1) suggests that if you select EMPs based on CD105, CD62e or CD54 then CD31 expression may be low and stimulus-dependent. These results suggest that CD31 is not present on all EMPs. We are currently replicating and validating these results using additional endothelial cell models.

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**Table 1.** Expression of CD31 on endothelial microparticles is dependent upon expression of CD105, CD62e or CD54 and is also stimulus-dependent.

Treatment of HUVECs	CD31 expression in		
	CD105+ EMPs	CD62e+ EMPs	CD54+ EMPs
Untreated	18.8% / 5.4	0% / 11.9	28.95% / 20.2
TNF $\alpha$	16.7% / 8.4	0.8% / 3.7	46.61% / 27.6
Serum-starved	1.8% / 1.4	9.61% / 2.2	28.42% / 10.7

Values expressed; % expression/mean fluorescence.

\* $p < 0.05$  vs control

PC54

**Pilot Study to Assess Optical Coherence Tomography as a Marker of Disease for Systemic Sclerosis**

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Introduction: Systemic sclerosis (SSc) is a connective tissue disease characterised by fibrosis (e.g., skin thickening

[scleroderma]) and ischaemia. Our aim was to validate optical coherence tomography (OCT) as a technique to measure skin thickness, and relate to perfusion. Methods:

A total of 20 patients with SSc and 20 healthy controls were recruited. The thickness of the epidermis (representative of disease severity) was assessed at three sites using OCT and high-frequency ultrasound (HFUS, a previously validated technique for measuring skin thickness). Laser Doppler imaging (LDI, a measure of microvascular function) and nailfold capillaroscopy (NC, a measure of microvascular structure) were measures of vascular involvement of disease.

Results: Epidermal thickness could be measured by both OCT and HFUS. LDI and NC confirmed concomitant functional and structural microvascular abnormality (Table 1).

Conclusion: OCT, a measurement tool for skin thickening may be complementary to measures of structural and functional microvascular abnormality in the assessment of SSc disease severity.

**Table 1.** Epidermal thickness ( $\mu\text{m}$ ), distal dorsal perfusion difference (perfusion units) and capillary density (number/mm) shown as mean (SD)

	Controls (N = 20)				Patients (N = 20)			
	HFUS	OCT	LDI	NC	HFUS	OCT	LDI	NC
Distal digit	240 (33)	273 (41)			264 (41)*	270 (69)		
Proximal digit	244 (35)	255 (37)			265 (41)*	290 (47)*		
Hand dorsum	184 (37)	213 (26)			206 (37)*	252 (35)*		
Distal-dorsal difference			211 (101)				117 (102)*	
Capillary density				31 (5)				25 (12)*

\* $p < 0.05$  vs control.

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