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*Heart* 2009 95: e2
doi: 10.1136/heart.2009.178137

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Poster presentations

**001 URINARY 11-DEHYDRO-THROMBOXANE B2 AS A MARKER OF THE ANTI-PLATELET EFFECTS OF CLOPIDOGREL OR ASPIRIN THERAPY IN HEALTHY MALE VOLUNTEERS**

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Background Activated platelets release large amounts of the cyclooxygenase-dependent product, thromboxane A2 (TxA2), and increased urinary levels of the TxA2 metabolite 11-dehydro-thromboxane B2 (Tx-M) appear positively associated with atherosclerotic disease. Anti-thrombotic doses of aspirin significantly lower these levels, and such measurements have been linked to risk determination of stroke, myocardial infarction and cardiovascular death. Consequently, Tx-M is considered a marker of both platelet activation and aspirin anti-platelet effectiveness. Clopidogrel is another important anti-platelet therapy, but no study has been conducted to determine its effects upon Tx-M levels.

Methods A small non-blinded trial of 16 healthy male volunteers assigned to seven days of standard anti-thrombotic aspirin (75 mg/day) or clopidogrel (75 mg/day) therapy. Blood and urine was collected before and on day 7 of treatment. Platelets were incubated with arachidonic acid (AA) (1 mM) to stimulate the acute production of TxA2 and serum TxB2 levels determined by radioimmunoassay. Urinary 11-dehydro-TxA2 metabolites were measured using a commercial ELISA.

Results Aspirin abolished AA-induced serum TxB2 production ([n = 8, p < 0.05]) and caused a significant reduction in Tx-M levels ([58 ± 9%, n = 8, p < 0.05]). Clopidogrel inhibited AA-induced TxB2 production by 45 ± 9% ([n = 8, p < 0.05]) and urinary Tx-M levels by 59 ± 11% ([n = 8, p < 0.05]).

Conclusion Standard anti-thrombotic doses of clopidogrel and aspirin inhibit Tx-M to similar extents. This suggests that basal platelet activation and TxA2 production in vivo in healthy volunteers is associated with activation of ADP-dependent pathways. The strong effects of clopidogrel upon urinary Tx-M may also explain why addition of aspirin to clopidogrel is not always associated with increased anti-thrombotic efficacy.

**002 MELAGATRAN PREVENTS PLAQUE RUPTURE IN APOLIPOPROTEIN E KNOCKOUT MICE THROUGH A NON-THROMBIN-RELATED MECHANISM**

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Background Thrombin, a serine proteinase, activates platelets, inflammatory cells and smooth muscle cells and has been suggested to play a role in atherosclerotic plaque rupture. Here, we present data indicating that the effects of the thrombin inhibitor, melagatran, in the apolipoprotein E knockout (apoE—/—) mouse model are not the consequence of thrombin inhibition.

Methods Melagatran was administered in the diet to fat-fed male apoE—/—mice at a dose of 250 or 500 µmol/kg of bodyweight/day for 8 weeks.

Results Both doses of melagatran produced plasma levels of melagatran sufficient to fully inhibit thrombin but only the higher dose had any significant effect on brachiocephalic artery atherosclerosis and plaque stability, showing that mere inhibition of thrombin is insufficient to influence atherosclerosis. The effects of melagatran on arterial smooth muscle cell proliferation and migration were studied in vitro using tissue obtained from wild-type, tPA—/— or uPA—/—mice. The behaviour of uPA—/—cells was indistinguishable from wild-type, but tPA—/—cells showed reduced proliferative and migratory responses. There was a dose-dependent inhibition of both proliferation and migration of wild-type and uPA—/— smooth muscle cells by melagatran. The concentration range over which this inhibition was observed was identical to that produced by melagatran treatment in vivo. In contrast, in tPA—/— smooth muscle cells melagatran had no effect, suggesting that it acts by inhibiting tPA and that thiszymogen is an important mediator of apoE—/—mouse atherosclerosis.

Conclusions The thrombin inhibitory actions of melagatran are probably not responsible for its beneficial effects on atherosclerosis and plaque stability. We suggest that these actions are exerted through inhibition of tPA and that the role of thrombin in murine atherosclerosis is still unknown.

**003 MATRIX-BOUND AGGREGATED IGM DRIVES MACROPHAGE CYTOTOXICITY VIA MACROPHAGE SCAVENGER RECEPTOR A AND IS INCREASED IN RUPTURED PLAQUES**

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Objective Humoral (antibody-mediated) immunity is an important regulator of vascular inflammation and atherosclerosis. Although IgM in solution (sIgM) protects arteries, we tested the hypothesis that insoluble aggregated IgM (IA-IgM) is selectively increased in ruptured plaques and can activate macrophages.

Methods and Results Immunohistochemistry revealed that ruptured plaques contained a granular matrix of IA-IgM with little IgC. To determine the contribution of IgM to destabilisation, we tested whether IA-IgM would activate cultured macrophages to kill vascular smooth muscle cells (VSMCs). IA-IgM, but not sIgM, activated macrophage cytotoxicity, which was mediated by hydrogen peroxide (H2O2)-induced apoptosis. Thus, catalase, which degrades H2O2, was strongly protective and prevented Annexin-V binding. Macrophage activation by IA-IgM was linked to its insoluble physical format through frustrated phagocytosis, since presentation of IA-IgM on beads reversed the activation of macrophage oxidative burst. Finally, our data suggest that macrophage activation was initiated by IA-IgM binding to macrophage scavenger receptor A (MSRA), since IA-IgM and MSRA co-immunoprecipitated in lysates of activated cells and macrophage cytotoxicity was blocked by MSRA siRNA knockdown.

Conclusions Ruptured atherosclerotic plaques are characterised by immobilised deposits of IA-IgM that may promote plaque destabilisation by stimulating macrophages via MSRA to become lethal to VSMCs.
MONOCYTE DIFFERENTIATION TO AN ATHEROPROTECTIVE PHENOTYPE EXPRESSING HEME OXYGENASE-1 PI VOTALLY REQUIRES A HEME-INDUCIBLE TRANS-ACTIVATING FACTOR AND IS COUNTERACTED BY THE NUCLEAR RECEPTOR REVERB

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doi:10.1136/hrt.2009.178137d

We have previously shown that macrophages associated with intraplaque haemorrhage (HA-mac) have a putatively atheroprotective CD163highHLADRlow phenotype with evidence of reduced oxidative stress (low 8-oxo-guanosine), high heme oxygenase (HO-1), low myeloperoxidase (MPO) and high interleukin-10 (IL-10) (Amer J Pathol. in press). This phenotype was reproduced by culturing monocytes with haemoglobin (Hb)-haptoglobin (Hp) complexes, and required HbHp internalisation via CD163, lysosomal processing and autocrine IL-10. We have now found that heme-mediated induction of HO-1 is a key step in HA-mac differentiation. HbHp treated cell contained free heme detectable by spectrophotometry. Oxidatively-modified RBCs, whose phagocytosis in CD204-dependent, and free purified heme, also led to monocyte differentiation to a CD163highHLADRlow phenotype which suppressed highly reactive oxygen species (ROS) measured by aminophenyl-fluorescein, and increased survival. Blocking HO-1 with ZnPPIX prevented these effects. Conversely, inducing HO-1 with cobalt protoporphyrin IX induced IL-10, reduced ROS, increased cell survival, and induced CD163highHLADRlow differentiation. Depleting HO-1 with siRNA knockdown reversed the protective effects of HbHp. Finally, we found that heme induction of HO-1 mRNA was blocked by cycloheximide, implicating a transactivating factor, but was superinduced by siRNA to RevErb-α (NR1D1) or RevErb-β (NR1D2) suggesting that these heme-binding nuclear receptors are repressive. Taken together, these data indicate that release of free heme and consequent HO-1 induction, is a key signalling event that integrates multiple forms of macrophage heme loading and converts these into a coordinated protective phenotypic response. We are currently identifying the heme-inducible factor by genome-wide methods.

MRI OF ENDOTHELIAL ADHESION MOLECULES IN CAROTID ATHEROSCLEROSIS USING TARGETED ULTRASMALL SUPERPARAMAGNETIC PARTICLES OF IRON OXIDE

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doi:10.1136/hrt.2009.178137e

Introduction There is currently no clinical imaging techniques available to assess the degree of inflammation associated with atherosclerotic plaques. This study aims at visualising and characterising atherosclerosis using targeted ultrasmall superparamagnetic particles of iron oxide (USPIO) as a magnetic resonance imaging (MRI) probe for detecting infamed endothelial cells.

Method The in vitro study consists of detection and characterisation of inflammatory markers on activated endothelial cells by immunocytochemistry and anti-E-selectin antibody conjugated USPIO. The ex vivo stage involves characterisation of inflammatory markers on atherosclerotic plaques, and finally the in vivo stage consists of development of a rat model with focal lesions in carotid arteries to allow targeted molecular imaging by MRI.

Results We have established an in vitro cellular model of endothelial inflammation induced with tumour necrosis factor-alpha (TNF-α). We have confirmed the inflammation of endothelial cells with both immunocytochemistry and MRI. These preliminary results revealed a temporal expression of the inflammatory markers, such as, E-selectin and vascular cell adhesion molecules-1 (VCAM-1), and the expression of these markers was dose dependent on exposure to TNF-alpha. Furthermore, we imaged rat carotid arteries in vivo by MRI.

Conclusion We successfully developed an in vitro model to detect and characterise inflamed endothelial cells by immunocytchemistry and MRI. This will allow us to develop agents and protocols for imaging vascular inflammation in atherosclerosis in the future. We have also successfully imaged the carotid arteries in a live rat by in vivo MRI. This pilot study will form the basis for a translational study to provide clinicians with a novel tool for in vivo assessment of atherosclerosis.

ENDOTHELIAL CELLS AT ATHEROSUSCEPTIBLE SITES EXPRESS ACTIVATED C-JUN N-TERMINAL KINASE WHICH INDUCES PRO-APOPTOTIC MOLECULES

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doi:10.1136/hrt.2009.178137f

Atherosclerosis is a focal disease which occurs predominantly at branches and bends of the arterial tree. We previously demonstrated that c-Jun N-terminal kinase (JNK) is activated in EC at atherosusceptible sites but is inactivated at atheroprotected sites. Here we examined the physiological role of JNK in cultured EC in the presence or absence of a specific pharmacological inhibitor (CT536706) using Affymetrix® microarrays followed by functional annotation. This approach and subsequent validation by gene silencing revealed that JNK positively regulates the expression of 35 transcripts that control apoptosis including Caspase 3 (a downstream effector of apoptosis) and RIP1 (a component of the TNF receptor signalling complex). We examined whether activation of JNK was associated with expression of pro-apoptotic proteins and apoptosis in murine aortic EC by en face immunostaining and TUNEL. We observed in untreated animals that RIP1 and inactive pro-Caspase-3 were expressed at significantly higher levels in EC at the atherosusceptible site compared to the atheroprotected site (p<0.01), however apoptotic EC were not identified at either site. Lipopolysaccharide treatment (4 mg/kg for 6 h) induced caspase-3 activation and apoptosis in EC at the susceptible site, whereas EC at the protected site were resistant. Thus we suggest that JNK1 primes EC in the susceptible region for apoptosis in response to noxious stimuli by elevating expression of pro-Caspase 3 and other pro-apoptotic molecules. In summary, differential activation of JNK1 may delineate the spatial variation in pro-apoptotic gene expression and apoptosis in arterial EC and could influence the spatial distribution of atherosclerotic plaques.

Acknowledgement: Funded by the British Heart Foundation and NHLI Trustees Foundation.

IMPAIRED EXERCISE INDUCED ENDOTHELIAL PROGENITOR CELL MOBILISATION IN SOUTH ASIAN MEN IS NITRIC OXIDE DEPENDENT

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doi:10.1136/hrt.2009.178137g

Background South Asian ethnicity is associated with endothelial dysfunction and a propensity to atherosclerosis. Endothelial
Pro-inflammatory mediators trigger nuclear localisation and activation of NF-kappaB transcription factors which influence atherogenesis by activating endothelial cells (EC). The outer curvature of the aorta is exposed to high unidirectional shear and is protected from EC activation, inflammation and atherosclerosis, whereas the inner curvature is exposed to low/oscillatory shear and is susceptible to atherosclerosis. Here we assessed the effects of shear stress on NF-kappaB transcriptional activity in aortic EC using transgenic NF-kappaB-luciferase reporter mice treated with lipopolysaccharide (4 mg/kg, 5 h) and observed that luciferase activity was significantly reduced in the atheroprotected region compared to the atherosusceptible region. Analysis of wild-type mice (C57BL/6) by confocal microscopy, providing a feasible protocol for 3-dimensional (3D) ex vivo imaging of abdominal atherosclerotic plaques from ApoE−/− mice at a cellular and molecular resolution.

**Rationale for the Study**-In the present study, we compared multiphoton laser scanning microscopy (MPLSM) with conventional confocal microscopy, providing a feasible protocol for 3-dimensional (3D) ex vivo imaging of abdominal atherosclerotic plaques from ApoE−/− mice at a cellular and molecular resolution. MPLSM could be used to evaluate vascular and plaque collagen content using second harmonic generation. Staining with ORO allowed visualisation of lipid accumulation directly below the fibrous cap. Immunohistochemistry permitted the acquisition of high resolution, multi-colour 3D images. Staining with ORO allowed visualisation of lipid accumulation directly below the fibrous cap. Confocal microscopy permitted the acquisition of high resolution, multi-colour 3D images. Staining with ORO allowed visualisation of lipid accumulation directly below the fibrous cap. Confocal microscopy permitted the acquisition of high resolution, multi-colour 3D images.

## Acknowledgement

Funded by the British Heart Foundation and NHLI Trustees Foundation.
function, however, showed a stronger influence on SDMA than on ADMA. Both ADMA and SDMA were predictive of cardiovascular disease in multivariate analysis and the associated hazard ratios over the 5-year observation period were of similar strength: 3.86 (1.36–10.9) and 7.91 (1.94–32.3) for ADMA and SDMA, respectively (p = 0.011 and 0.004). Separate analyses focused on quintile groups of SDMA revealed that the increase in cardiovascular risk was mainly confined to the top category (>0.80 mumol/L).

**Conclusion** This study argues against an exclusive ADMA effect in mediating cardiovascular risk. Instead, SDMA, its supposedly inactive counterpart, has similar diagnostic value in this large prospective cohort.

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**THE ROLE OF TRIBBLES 2 IN PI3K CASCADE REGULATION AND AKT/PKB ANTI-APOPTOTIC SIGNALLING CASCADE**

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doi:10.1136/hrt.2009.178137k

Apoptosis is a key event in atherosclerotic plaque formation. The phosphoinositide-3-kinase (PI3K) cascade is involved in many cellular activities in plaques, such as migration and cell-survival. Our previous data have identified an interaction between Tribbles 2 (Trb2) and signalling adaptor molecule Grb2 associated binder protein (Gab1). The functional consequences of this interaction are unknown. Gab1 interacts with the p85 domain of PI3K to mediate downstream activation of the Akt/PKB anti-apoptotic signalling pathway. We examined whether tribbles, a new family of scaffold proteins link with PI3K to activate Akt and potentially inhibit apoptosis. HEK293 cells were transfected with trb2 and mutant or wt Gab and PI3K binding to trb2/Gab1 complexes was quantified using a GFP protein fragment complementation assay. We show that the SH2 and PI3K domains on Gab1 are not involved in trb2/Gab1 complex formation. However, stimulation of the PI3K cascade enhanced the interaction between trb2/Gab1 by almost 2 fold (P<0.05, n = 5). In addition, over-expression of the PI3Kδ (catalytic) and the PI3Kɑ (regulatory) subunit also had the same effect (binding intensity 2.48 SEM vs 3.9 ± SEM, P<0.05, n = 6 and 2.52 ± SEM vs 5.3 ± SEM, n = 6). These data suggest that Gab1/Trb2 binding plays a role in PI3K cascade catalysis and that trb2 may act as a co-regulator. This may control cell survival and, potentially, plaque development or rupture.

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**FTY720, A SPHINGOSINE-1-PHOSPHATE ANALOGUE, PREVENTS ISCHAEMIC/REPERFUSION-INDUCED CARDIAC ARRHYTHMIAS IN AN EX-VIVO RAT HEART MODEL VIA ACTIVATION OF P21 ACTIVATED KINASE/PROTEIN KINASE AKT SIGNALLING**

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doi:10.1136/hrt.2008.178137l

Organ dysfunction followed by single-organ, or even multi-organ failure, due to ischaemia–reperfusion injury (I/RI) is a common complication in transplant, liver, trauma, and heart surgery. Here we report that FTY720, an analogue of sphingosine-1-phosphate, prevents the arrhythmias induced by I/RI in an ex vivo rat ischaemic heart via activation of p21 activated kinase (Pak1)/protein kinase-Akt signalling. The Langendorff ex vivo heart model was prepared according to established methods. The ex vivo hearts were equilibrated for 50 min and then exposed, in the presence and absence of FTY720, to global ischaemia for 20 mins followed by 30 min of reperfusion. The effect of FTY720 on rhythm disturbance during I/RI were examined by an ECG.

Secondly, we investigated the activation of Pak1 and Akt during I/RI by Western blot analysis. In 15 hearts examined, FTY720 significantly prevents the occurrence of premature ventricular beats, ventricular tachycardia, sinus bradycardia as well as Atrio-Ventricular conduction block caused by I/RI. From our Western blot analysis, the levels of phospho-Pak1 and phospho-Akt were decreased by 49% and 31% at ischaemia condition and by 20% and 15.5% at reperfusion condition compared to baseline level under the control condition. In the presence of FTY720, phospho-Pak1 and phospho-Akt levels were increased by 88% and 19% at ischaemia condition and by 15% and 47% at reperfusion condition.

Our results demonstrate, for the first time, that FTY720 prevents the ischaemia/reperfusion-induced arrhythmia via activation of Pak1/Akt signalling and can be a potentially important and new agent protecting against such a clinical condition.

Acknowledgement: The work was supported by the Wellcome Trust (ML).

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**SHEAR STRESS AND NITRIC OXIDE TRANSPORT AFFECT NF-KB DYNAMICS IN ENDOTHELIAL CELLS**

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doi:10.1136/hrt.2009.178137m

Introduction While shear stress is involved in the regulation of pro-inflammatory transcription factors such as NF-kB and vasoregulators such as nitric oxide (NO), the role of NO transport and its relation to endothelial cell activation is presently unknown. We investigated the hypothesis of NO as a mediator of the NF-kB shear dependant activation via a negative feedback mechanism.

Methods Pig aortic endothelial cells were studied in parallel flow chambers at two shear stress levels (2 and 10 dyne/cm²), at 12 different time points (0–330 minutes) and at different locations along the length of the flow chamber. The conditions were determined by extensive CFD-simulations of shear stress and mass transport in the chambers coupled to a mathematical model of NO interaction with the NF-kB feedback mechanism.

Results NF-kB oscillated in and out of the nucleus with an amplitude and a frequency dependent on the shear stress level and nitric oxide concentration in the flow chamber (period at 2 dyn/cm²>10 dyn/cm²). Analysis by two-way ANOVA, P<0.01. Such dynamic behaviour was predicted from our simulations of the NF-kB–NO interaction.

Conclusion The dependence of NF-kB dynamics on shear stress level and location in the flow chamber may be explained with a nitric oxide transport dependent mechanism interacting with a direct effect of shear stress.

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**3D STRESS ANALYSIS ON CAROTID ARTERIAL PLAQUES BASED ON MRI DATA: A COMPARISON BETWEEN SYMPTOMATIC AND ASYMPTOMATIC PATIENTS**

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doi:10.1136/hrt.2009.178137n

Plaque rupture has been extensively considered as the leading cause of death, it is believed that high stress within plaque can be an important factor triggering plaque rupture.1 In this study, two groups of patients with carotid plaque were studied by stress analysis with fluid structure interaction (FSI).
14 patients in which 5 were asymptomatic patients as G1 and 9 were symptomatic patients as G2, were selected for the study. The patients were subjected to multi-spectral MR scan to provide multi-component plaque images which were then used for 3D model reconstruction. FSI stress analyses were performed on each subject. Parameters such as maximum wall tensile stress (WTS\textsubscript{max} representing first principle stress) in the plaque, minimum Fibrous Cap Thickness (FCT\textsubscript{min}), stenosis degree and lipid size were obtained for comparisons between the two groups. The fibrous cap thickness (FCT) is much thicker for the patients in G1 comparing with G2 (G1: FCT\textsubscript{min} = 0.42 ± 0.28 mm vs G2: FCT\textsubscript{min} = 0.20 ± 0.11 mm). As a consequence, the stress value in symptomatic patient is significantly higher (G2: WTS\textsubscript{max} = 198.8 ± 47.4 kPa vs G1: WTS\textsubscript{max} = 128.6 ± 41.2 kPa, P<0.05). It is also interesting to notice that the mean FCT\textsubscript{min} value of 0.2 mm found for the symptomatic patients agreed very well with the critical FCT proposed by Jessica et al on assessing plaque vulnerability.

In summary, symptomatic patients generally have a thinner fibrous cap and larger lipid region. They also experience a higher plaque stress than asymptomatic patients. Stress analysis combining with morphological analysis will help advance the understanding of plaque rupture.

Acknowledgement: This work is supported by British Heart Foundation (FS/06/048).

REFERENCES

THE EFFECT OF ALTERED MACROPHAGE BEHAVIOUR ON ZEBRAFISH COLLATERAL VESSEL DEVELOPMENT

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doi:10.1136/hrt.2009.178137b

The ability of blood vessels to remodel and enlarge into collateral vessels is of great therapeutic relevance. Macrophages play a role in collateral formation, but the mechanisms behind this are unclear. Using a zebrafish model of aortic occlusion (gridlock mutants) we investigated the effects of genetic manipulation of macrophage associated genes on macrophage distribution, number, migration to a site of injury and collateral formation.

Methodology Macrophages were visualised in vivo by neutral red uptake. Knockdown of the actin binding genes WASP and WIP or the chemokine receptor CXCR4a in was achieved by injection of morpholino oligonucleotide antisense. Macrophage migration to injury was assessed by quantifying macrophage number in the tailfin following surgical removal of a small portion. Collateral formation was assessed by quantifying recovery of blood flow to the occluded aorta in gridlock mutants.

Results Total macrophage number and distribution was unaffected by WASP, WIP or CXCR4a knockdown, but each significantly inhibited macrophage migration to tailfin injury. As anticipated from mammalian studies, CXCR4a knockdown significantly impaired the ability of gridlock mutants to restore aortic blood flow via collateral vessels. However, both WASP and WIP knockdown significantly enhanced the percentage of gridlock embryos that recover aortic blood flow.

Conclusions CXCR4a knockdown impairs, whilst WASP or WIP knockdown promotes, collateral vessel remodelling. This effect is not mediated by effects on macrophage number, distribution or migration. We have now generated a novel fms:Gal4 transgenic to elucidate the role of the macrophage in collateral development.

CHEMERICIN STIMULATES THE RAPID ADHESION OF LEUKOCYTES TO FIBRONECTIN AND VCAM-1 VIA ACTIVATION OF VLA-4 AND VLA-5

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doi:10.1136/hrt.2009.178137p

Chemerin is a potent macrophage chemoattractant protein that is activated at sites of inflammation; other potential inflammatory properties of chemerin have not been investigated. Integrin-mediated adhesion plays a key role in the migration of leukocytes from the bloodstream into atherosclerotic lesions. To explore the possibility that chemerin can stimulate leukocyte adhesion, an assay was developed in which the number of cells bound to different immobilised integrin ligands was quantified following chemerin stimulation. We show for the first time that chemerin stimulates the rapid adhesion of leukocytes to fibronectin and VCAM-1 (optimal concentration = 10 nM), an effect mediated primarily through ChemR23, a G-protein coupled receptor. Chemerin-stimulated adhesion of leukocytes to fibronectin is mediated via activation of the integrin VLA-4, whilst chemerin’s effect on adhesion to VCAM-1 is mediated via the integrin VLA-5. Pharmacological inhibition studies showed that phosphatidylinositol-3-kinase, Akt and p38 play a role in chemerin-stimulated adhesion to fibronectin and VCAM-1. Future work will investigate chemerin’s effects on cell adhesion under flow and in an in vivo setting. Chemerin stimulation of leukocyte adhesion to VCAM-1 and fibronectin may play an important role in leukocyte recruitment and retention within atherosclerotic plaques.

A NOVEL CHICK EMBRYO MODEL REVEALS THAT ENDOTHELIN RECEPTOR B IS ESSENTIAL FOR COLLATERAL VESSEL DEVELOPMENT

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doi:10.1136/hrt.2009.178137q

After arterial occlusion, collateral vessels develop by remodelling of endothelial communications between occluded and neighbouring arteries to restore blood flow. Collateral vessel formation is difficult to visualise in mammalian models in vivo. The extra-embryonic vasculature of the developing chick embryo is easily accessible and manipulated. We therefore established a novel model of collateral development in the chick, and used this to assess the contribution of endothelin receptor B to collateral formation.

Unilateral artery ligation was performed in 2.5 d old chick embryos. Collateral vessels arise from the unligated artery and cross the midline to perfuse the occluded territory, following arterial ligation. The diameter of persisting collaterals and their flow carrying capacity steadily increases over 48 h. Endothelin receptor B contributes to this process. Blocking the receptor with a selective antagonist greatly inhibits development of collateral vessels.

Endothelin receptor B antagonist BO788 (or DMSO) treated filter paper discs were placed over the midline of the chick at T0 post-ligation. After 24 h the discs were removed. Collateral vessels were quantified by number, diameter and total cross sectional area (as a surrogate of flow carrying capacity). BO788 significantly reduced collateral number (DMSO: 3 ± 0.5. BO788: 0.9 ± 0.3. n = 8, p<0.01), and vessel diameter (DMSO: 76 ± 6 mm. BO788 16 ± 4 mm. n = 18, P<0.01). Further experiments revealed that endothelin receptor B is most active at T0–6 h post-ligation. Treatment after 6 h causes no significant difference to collateral vessel formation.
The effects of endothelin receptor B inhibition suggest that this receptor is required for collateral formation in response to arterial occlusion in the early chick embryo.

**018** MMP-13: A NOVEL PLATELET PROTEIN AND A POTENT INHIBITOR OF PLATELET AGGREGATION

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doi:10.1136/hrt.2009.178137r

**Introduction** Thrombus formation following atherosclerotic plaque rupture underpins acute coronary syndromes. Matrix metalloproteinases (MMPs) are proteolytic enzymes which are up-regulated in inflamed plaques, and enhanced collagenolytic activity of MMP-13 has been implicated in vulnerable plaque destabilisation and increased likelihood of plaque rupture. Atherosclerotic plaque rupture exposes thrombotic plaque components including collagen rich surfaces which trigger platelet activation and aggregation; however plaque rupture also exposes platelets to collagen-associated MMP-13.

**Rationale** Although the role of MMP-13 in plaque rupture is becoming increasingly clear, its function in thrombus formation has not yet been determined.

**Results** We have identified MMP-13 as a potent inhibitor of platelet aggregation stimulated by numerous agonists including U46619, thrombin, calcium ionophore and crosslinked collagen related protein (CRP); the inhibitory effect of which is attenuated by aspirin. Moreover we have identified MMP-13 as a novel platelet protein. Western blotting and immunofluorescence have confirmed the presence of MMP-13 within and on the platelet surface. Enzyme linked immunosorbant assays using antibodies to individual platelet activatory receptors indicate that the inhibitory effect of MMP-13 on platelet aggregation is primarily through its interaction with alpha IIb beta 3 integrin and glycoprotein (GP)VI, though our experiments to date have not identified MMP-13 as a GPVI (sheddase).

**Conclusions** Our data suggest that MMP-13 plays a dual role in atherothrombosis by degrading collagen in the unstable plaque and by limiting the platelet thrombus that forms as a result. We are currently investigating the molecular mechanism(s) by which MMP-13 interacts with platelet surface receptors.

**019** MT1-MMP DEFINES A POPULATION OF PRO-INFLAMMATORY FOAM-CELL MACROPHAGES ASSOCIATED WITH UNSTABLE ATHEROSCLEROSIS

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doi:10.1136/hrt.2009.178137s

Accumulation of foam-cell macrophages (FCMs) and expression of metalloproteinases (MMPs) are associated with atherosclerosis. We recently showed that membrane-type1 (MT1)-MMP expression defines a sub-population of FCMs. We now investigate whether macrophages expressing MT1-MMP (MT1-MMP-FCMs) associate with features of plaque instability and markers of inflammation in rabbit and human plaques.

New Zealand White rabbits were studied immediately after being fed a cholesterol-rich diet for 8 weeks to generate aortic atherosclerotic plaques with a “mature”, lipid-rich, morphology, or after returning to a normal diet for 8 weeks to yield “healing” plaques with pronounced fibrous caps. Immunohistochemistry revealed that the percentage of MT1-MMP-FCMs in “mature” plaques (74±5%) declined to (49±4%) in “healing” plaques (n= 8 each, p<0.001). We also compared sections of human carotid plaques from lipid-rich and fibrous plaques (n = 20 each) obtained from the AtheroExpress biobank. The percentage of MT1-MMP-FCMs observed (mainly in the shoulder region) of lipid-rich plaques (59.1±8%) was significantly reduced in fibrous plaques (5.47±2%) (p=0.001). A trend towards correlation between the percentage of MT1-MMP-FCMs and the measurable MT1-MMP activity (by ELISA) was observed in lipid rich plaques (r² = 0.16, p = 0.07), but not in fibrous lesions. Moreover, MT1-MMP-FCMs also tended to express MHCIIm, a pro-inflammatory marker, but not CD163, an anti-inflammatory marker.

We conclude that expression of MT1-MMP in FCMs in both rabbit and human atherosclerotic plaques associates with morphological characteristics of unstable plaques. Furthermore, MT1-MMP-FCMs are a pro-inflammatory subpopulation, which may be appropriate targets for plaque stabilising therapy.

**020** RNA EDITED ORAI1 CHANNEL IN VASCULAR SMOOTH MUSCLE CELL PROLIFERATION AND MIGRATION

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doi:10.1136/hrt.2009.178137t

We recently reported a non-selective cationic current in vascular smooth muscle cells that is activated by store-depletion but only partially contributed by TRPC1-containing channels (Li et al 2008 Circulation Research 103, e97-). Here we investigated the role of Orai1 (Feske et al 2006 Nature 441, 179-). Experiments were performed on human vascular smooth muscle cells, in part using robotic planar patch-clamp to measure ionic current (Milligan et al 2009 Nature Protocols In Press). Orai1 protein was detected by western blotting and suppressed after knock-down of Orai1 expression by RNAi. Knock-down of Orai1 also suppressed the non-selective cationic current, generating an inconsistency because Orai1 is suggested to form highly calcium-selective ion channels in lymphocytes. Sequencing of Orai1 mRNA revealed RNA editing at a specific residue, providing the potential means to explain altered ionic selectivity. Strikingly, knock-down of the RNA editing enzyme ADAR1 returned the Orai1 sequence to that associated with calcium-selective channels, greatly reducing the non-selective cationic current. The Orai1 was found to have pivotal roles in cell migration and proliferation. The data reveal a previously unrecognized ion channel subunit and importance of RNA editing in vascular smooth muscle cells.

Acknowledgement: Supported by the British Heart Foundation and the Wellcome Trust.

**021** 3-DIMENSIONAL EX VIVO ANALYSIS BY OPTICAL PROJECTION TOMOGRAPHY DEMONSTRATES THAT EXOGENOUS GLUCOCORTICOIDS AUGMENT ATHEROGENESIS IN APOE–/– MICE

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doi:10.1136/hrt.2009.178137u

Despite evidence that glucocorticoid excess (eg in Cushing’s syndrome or chronic therapy) increases cardiovascular disease, their influence on atherogenesis is not well understood. Indeed, large exogenous doses inhibit lesion development in animal models. We
addressed the hypothesis that exogenous glucocorticoids inhibit atherosclerosis by developing the technique of optical projection tomography (OPT) to allow 3D analysis of atherosclerotic lesions.

Male ApoE−/− mice (5 weeks) underwent either adenectomy or sham surgery (n = 8/group). Adrenalectomised mice received oral dexamethasone (0.1 mg/kg/day) in 0.9% saline or saline alone and allowed to recover (1 week). All mice then received high (0.2%) cholesterol Western diet (12 weeks) after which the perfusion fixed aortic arch was isolated for analysis of lesion volume and acellular clefts within the lesion (which may represent extracellular lipid and cholesterol accumulation) using OPT.

Adrenalectomy had no effect on body weight, whereas dexamethasone reduced both body (p<0.005) and thymus (p<0.001) weight. Analysis of the brachiocephalic trunk revealed dexamethasone treatment significantly increased lesion volume (2.70±0.20×10^6 μm^3), whereas adenectomy did not (1.92±0.20×10^6 μm^3). In addition, dexamethasone-treated mice (3.59±0.54×10^6 μm^3) were significantly smaller (p<0.01), but not adenectomised mice (1.20±0.51×10^6 μm^3), had increased acellular clefts within the lesions compared with sham (1.70±0.31×10^6 μm^3).

This study demonstrates successful application of OPT to allow 3D analysis of atherosclerotic lesions and indicates glucocorticoid administration augments atherogenesis in ApoE−/− mice, perhaps by increasing lipid incorporation. Removal of endogenous glucocorticoids by adrenalectomy did not reduce lesion development suggesting a limited role for physiological levels in regulation of atherosclerosis.

**022 TELOMERES ARE SHORTER IN MYOCARDIAL INFARCTION PATIENTS COMPARED TO HEALTHY SUBJECTS; CORRELATION WITH ENVIRONMENTAL AND GENETIC RISK FACTORS**

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doi:10.1136/hrt.2009.178137v

**Objectives** Shorter telomeres have been reported in premature myocardial infarction (MI) patients. Our work aims to confirm this association in two case-control studies and in familial hypercholesterolemia (FH) patients. The association of telomere length with risk factors was evaluated.

**Methods** The HIFMECH study compares 598 white male patients (<60 years) who survived a First MI and 653 age-matched controls from North and South Europe. Additionally, 413 Coronary Artery Bypass Graft (CABG) patients and 367 FH patients of whom 145 had premature CHD were recruited in the UK. Leukocyte telomere length (LTL) was measured using real-time PCR-based method.

**Results** HIFMECH study: LTL was significantly shorter in subjects from the North (1.19, SD 0.43) compared to the South (1.25, SD 0.35) (p = 0.02) and in cases (1.16, SD 0.32) compared to controls (1.20, SD 0.42) (p = 0.04). CABG study: LTL was significantly shorter (0.95, SD 0.35) compared to the HIFMECH UK controls (1.09, SD 0.60) (p = 0.007). IL6 level pre-operation positively correlated with LTL (r = 0.23, p<0.0001). Interestingly, LTL was also associated with the IL6 genotype of SNP -174G>C, with the C allele being protective (p = 0.09). FH study: Telomeres were shorter in those with CHD (1.51, SD 0.48) compared to the non CHD subjects (1.41, SD 0.48) (p = 0.09).

**Conclusions** These data confirm that telomeres are shorter in MI cases and show, for the first time, shorter telomeres in FH patients with CHD compared to FH patients without CHD.

**023 TOLL-LIKE RECEPTOR-2 MEDIATES INFLAMMATION AND MATRIX DEGRADATION IN HUMAN ATHEROSCLEROSIS**


**References**


**024 NUCLEAR FACTOR ERYTHROID 2-RELATED FACTOR 3 MEDIATES STEM CELL DIFFERENTIATION INTO SMOOTH MUSCLE CELLS THROUGH INCREASED REACTIVE OXYGEN SPECIES GENERATION AND PLASMA PHOSPHOLIPASE A2 PRODUCTION**

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doi:10.1136/hrt.2009.178137x

**Rationale for the Study** Nuclear factor erythroid 2-related factor 3 (Nrf3), a member of the cap’n’collar family of transcription factors that binds to the DNA-antioxidant responsive elements, have been reported to be involved in the early embryo development and
There are no specific markers for EPCs and advice caution in demonstrating the advantage of using an unbiased proteomic decade of research on EPCs, this has not been reported so far, from an uptake of platelet antigens by mononuclear cells. Despite a consistent with current definitions of an EPC phenotype arises. Conclusions Our findings provide novel information on the role of Nr3f and Pla2g7 in EPC differentiation towards SMC, and indicate that they could be potential new targets for influencing SMC differentiation.

**PROTEOMIC ANALYSIS REVEALS PRESENCE OF PLATELET MICROPARTICLES IN ENDOTHELIAL PROGENITOR CELL CULTURES**


doi:10.1136/hrt.2008.178137y

**Background** The concept of progenitor cells has attracted considerable interest in cardiovascular research, but results from clinical trials remain controversial. Microparticles (MP) are small membrane vesicles originating from the cell surface that retain membrane antigens specific for the parent cell they originate from. Thus, they represent an ideal subproteome to clarify the progeny of cells in endothelial progenitor cultures (EPC).

**Methods** Using liquid chromatography tandem mass spectrometry, we provide the first repository of membrane proteins in MPs originating from EPCs. Results: Our proteomic data revealed that conventional methods for isolating mononuclear cells lead to a contamination with platelet proteins. Notably, platelets readily disintegrate into platelet MPs, which transfer “endothelial” marker proteins to the mononuclear cell population. Platelet MPs also determine the pro-angiogenic effects of the conditioned medium and endothelial tube formation in the Matrigel assay was significantly inhibited by antibodies targeting the platelet-specific integrin GPIbβ. Finally, platelets emerged as positive predictor for the number of colony-forming units and “early-outgrowth” EPCs in a large population-based study (n = 526).

**Conclusions** Our study provides evidence that the cell type consistent with current definitions of an EPC phenotype arises from an uptake of platelet antigens by mononuclear cells. Despite a decade of research on EPCs, this has not been reported so far, demonstrating the advantage of using an unbiased proteomic approach to assess cellular phenotypes. Our findings explain why there are no specific markers for EPCs and advises caution in attributing clinical benefits in trials using unpurified bone marrow mononuclear cells to “stem cell”-mediated repair.

**INSULIN LIKE GROWTH FACTOR BINDING PROTEIN-1 PROTECTS AGAINST ENDOTHELIAL DYSFUNCTION**


doi:10.1136/hrt.2009.178137z

**Background** Insulin-like growth factor binding proteins (IGFBP) are key regulators of IGF-I bioavailability and may also exert IGF-independent effects. Cross-sectional studies inversely correlate circulating IGFBP-1 with insulin-resistance and vascular disease. In transgenic mice over-expressing IGFBP-1 (but with intact fast/ feed regulation of this axis), we previously demonstrated favourable effects on endothelial function and blood pressure. We thus sought to further explore the influence of IGFBP-1 on vascular and metabolic homeostasis.

**Methods** Male C57BL/6 transgenic mice and wildtype littermates were fed an obesogenic diet or crossed with heterozygous insulin-receptor knockout mice (IRKO). Metabolic and non-invasive haemodynamic testing were conducted in conscious mice. Endothelial function was assessed by testing aortic vasomotor responses to insulin ex vivo in the organ-bath, and aortic ser1177-eNOS phosphorylation at Western blot after in vivo insulin bolus. Complementary in vitro studies with exogenous IGFBP-1 were also conducted, using western blot to assess NO signalling pathways in cultured endothelial cells and the organ-bath to assess vasomotor responses in intact aorta.

**Results** Wildtype mice with obesity or IRKO developed hyper-tension and endothelial dysfunction; obese mice were also hyperglycaemic. In contrast, transgenic mice with obesity or IRKO had preserved endothelial function, remained normotensive, and were relatively gluco-competent despite similar weight gain. In vitro, exogenous IGFBP-1 upregulates ser1177-eNOS phosphorylation and blunts aortic vasoconstriction via increased NO generation.

**Implications** IGFBP-1 over-expression rescues endothelial function in both a normoglycaemic and a hyperglycaemic model of insulin-resistance, through preserved endothelial insulin-sensitivity and increased NO production. Attenuated IGFBP-1 levels in insulin-resistance may thus be implicated in pro-atherogenesis.

**HUMAN CD34+ BUT NOT CD34-VE CELLS SURVIVE IN THE ZEBRAFISH VASCULATURE**

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doi:10.1136/hrt.2009.181668a

**Introduction** Endothelial progenitor cells (EPCs) home to sites of vascular damage and play a significant role in the reendothelialisation and neovascularisation of injured endothelium. Since then there has been growing interest in the use of EPCs to repair endothelial damage in pathological processes. Zebrafish embryos possess many advantages for the in vivo visualisation of cell behaviour. We therefore evaluated whether human CD34+ve EPCs can be visualised when injected into the zebrafish vasculature, and whether they attach and survive.

**Methods** CD34+ve or –ve cells were isolated by FACS from human umbilical cord blood and labelled with Dil. Cells were resuspended and a microinjector was used to inject cells into the circulation of 2 d old Fl:1-GFP transgenic zebrafish embryos, in which endothelium expresses GFP. Embryos were imaged using a spinning disc confocal microscope immediately after injection and 24 and 42 hours post injection.
Results Immediately after injection, CD34+ve and –ve cells could be observed both circulating in the blood and attached to zebrafish endothelium throughout the vasculature, but particularly in the cardinal vein. Mean number of adherent labelled cells did not change over 42 h in CD34+ injected fish whereas the number of adherent cells in CD34- injected fish significantly decreased.

Conclusion Both CD34+ve and CD34-ve human cells adhere to zebrafish endothelium, but whilst CD34-ve cells numbers rapidly decline, CD34+ve cells appear to survive and remain adherent. This ability to remain adherent and survive in the vasculature may enhance their reparative capacity.

NOTCH SIGNALLING IN VASCULAR SMOOTH MUSCLE CELL SURVIVAL

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doi:10.1136/hrt.2009.181669b

Vascular smooth muscle cells (VSMCs) in the fibrous cap of atherosclerotic plaques play an essential role in maintenance of the plaque stability. Apoptosis of VSMCs leads to plaque rupture resulting in myocardial infarction and sudden death. A number of signalling pathways contribute to the regulation of VSMC growth and survival, and Notch signalling is a new member on the list. We have previously reported the anti-apoptotic role of Notch signalling by stable transfection of Notch3 in Human Embryonic Kidney (HEK) 293 cells. Here we use VSMCs to explore further the molecular mechanisms of Notch signalling in VSMC survival. Endogenous Notch signalling was stimulated in vitro by immobilised Notch ligand Jagged1/PC chimera in a WKY rat smooth muscle cell line; and exogenous Notch signalling was introduced by stable transfection of Notch3 or the constitutive active form of Notch3 (N3IC) in these cells. Western blotting of cells following either Jagged1 stimulation or overexpression of Notch3 or N3IC showed up-regulations of total Akt and phosphorilated-Akt, a key component of the cell survival pathway. The activation of Akt induced by Jagged1 is associated with decreased expression of PTEN, a negative regulator of the Akt pathway. We conclude that activation of the Akt pathway contributes at least in part to Notch signalling-mediated VSMC survival and one of the possible mechanisms is suppression of PTEN expression. Further studies aimed at elucidating the signalling network that relates to VSMC survival by Notch receptors.

PAPP-A FROM HUMAN ATHEROSCLEROTIC PLAQUES IS AN ACTIVE ENZYME THAT CLEAVES IGFBP-4

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doi:10.1136/hrt.2009.181669d

Pregnancy-associated plasma protein A is a member of metalloproteinase metzincin superfamily and exists in two isoforms: heterotetrameric 2:2 complex PAPP-A/procMBF (htPAPP-A) found in blood of pregnant women and homodimeric form (dPAPP-A) that was found recently in atherosclerotic plaques. PAPP-A does not display protease activity in htPAPP-A complex. It is still unknown whether atherosclerotic dPAPP-A is active protease, but it was shown that recombinant dPAPP-A is an active enzyme and cleaves IGFBP-4 and IGFBP-5 in vitro. It was hypothesised that dPAPP-A in atherosclerotic plaques is an active enzyme cleaving IGFBPs complexed with IGF-1, thus releasing free IGF-1. Local growth of IGF-1 concentration serves plaque destabilisation.

The aim of the current study was to investigate biochemical and enzymatic properties of dPAPP-A from human atherosclerotic plaques. We have elaborated a method of PAPP-A isolation from human atherosclerotic tissue using affinity chromatography utilising MAb 4G11 (HyTest, Finland).

Comparing this endogenous protein with recombinant dPAPP-A and htPAPP-A, we have shown that in all cases PAPP-A subunit had equal apparent molecular masses about 200 kDa being analysed by SDS–PAGE.

Identity of proteins was confirmed by mass spectrometry analysis. Using Western blotting analysis and sandwich immunnoassay method we have demonstrated the absence of htPAPP-A form in the atherosclerotic dPAPP preparation. For the first time we have shown that endogenous dPAPP-A is an active protease and cleaves IGFBP-4 in the presence of IGF-1 in vitro.

Our findings support the hypothesis that enzymatically active dPAPP-A can participate in atherosclerotic plaque destabilisation and rupture.
Healthy EC maintain homeostasis through a balance between protective and pro-inflammatory genes.

Atherogenic stimuli up-regulate the expression of adhesion molecules such as ICAM-1, which support the adhesion and extravasation of leukocytes into the sub-endothelial space, which drive plaque progression and contribute to its complications. Erg is a transcription factor constitutively expressed in EC and involved in the regulation of EC homeostasis. Erg expression is down-regulated in EC by the pro-inflammatory cytokine tumour necrosis factor-alpha (TNF-alpha). To investigate the role of Erg in vascular inflammation, we performed a microarray study on human umbilical vein endothelial cells (HUVEC) treated with oligonucleotides against Erg. Erg inhibition resulted in the up-regulation of several pro-inflammatory genes, including ICAM-1. This was confirmed by RT-PCR and Western blotting. Conversely, Erg over-expression in HUVEC led to a decrease in ICAM-1 expression, both in basal conditions and following TNF-alpha treatment. These data suggest that Erg represses ICAM-1 expression in resting and activated EC. The NF-kB pathway is known to lead to ICAM-1 up-regulation by TNF-alpha. We tested whether Erg affects NF-kB activation by TNF-alpha. In HeLa cells, TNF-alpha-induced NF-kB luciferase reporter activity was inhibited by Erg over-expression.

Thus Erg may repress pro-inflammatory pathways, and its down-regulation by TNF-alpha may be required for a complete inflammatory response. Interestingly, human coronary artery staining showed that Erg is absent from endothelium over the atherosclerotic plaque area, which is ICAM-1 positive and activated. In conclusion, Erg may exert a protective role in the endothelium by modulating the balance between pro- and anti-inflammatory genes.

Treatment of vasculature with CA4P reduced blood flow in tumour vessels but not control vessels. The advantages to this model are manifold and include the fact that zebrafish embryos are: (a) inexpensive to maintain and generate in large numbers, (b) require very few (~250) tumour cells for xenografting into each embryo, (c) permit the simultaneous visualisation of the vasculature associated with primary and metastatic lesions in the same animal.
phospho-Akt expression was reduced in human plaque (intimal) VSMCs compared to medial VSMCs suggesting that Akt-mediated survival signalling regulates VSMC apoptosis in atherosclerosis. Importantly, we identified the transcription factor FOXO3a as a target of Akt. Phosphorylation of FOXO3a by Akt impairs its ability to transcriptionally activate targets that promote apoptosis such as Bim. The activity of FOXO3a is also regulated by acetylation at lysine residues and we show that phosphorylation and acetylation of FOXO3a may be co-regulated in VSMCs and contribute to their survival in vitro. These studies demonstrate that post translational modifications of FOXO3a are a major mechanism by which VSMC survival is controlled in atherosclerosis.

I am interested in the development and rupture of atherosclerotic plaques and, in particular, the factors that govern the proliferation, migration and survival of vascular smooth muscle cells (VSMC). There is growing evidence that the loss of VSMC from the atherosclerotic plaque is associated with plaque rupture. Consistent with this, increased apoptosis of VSMC and macrophages has been detected in unstable compared with stable angina lesions. Death of vascular cells is also the basis for the generation of microparticles within the circulation, which act as potent procoagulant substrates both locally and systemically. However, the atherosclerotic plaque is complex and composed of several cell types. Using a mouse model of induced VSMC death we have demonstrated that loss of VSMC in vivo recapitulate features of human vulnerable plaques. However, the factors that govern the survival of VSMC in vivo are not clear. Therefore, we have begun to elucidate the signalling pathways that regulate the survival of VSMC.

**PRELAMIN-A DISRUPTS NESPRIN-2 FUNCTION AND IMPAIRS DNA DAMAGE REPAIR PROCESSES DURING NORMAL AND PREMATURE VASCULAR AGING**

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doi:10.1136/hrt.2008.181669

DNA damage is a key mediator of both normal and premature vascular ageing. Accumulating evidence suggests that disruption of nuclear lamina organisation leads to accelerated DNA damage in premature ageing syndromes such as Hutchinson Gilford Progeria Syndrome (HGPS), where affected individuals develop premature atherosclerosis characterised by vascular smooth muscle cell (VSMC) depletion leading to myocardial infarction or stroke before the second decade. In this study we show that prelamin-A accumulation is a novel biomarker of normal VSMC ageing that triggers disruption of the nuclear lamina, impinges upon DNA damage responses (DDR) and induces VSMC senescence and death. In addition, we describe a novel role for prelamin-2, a lamin-A binding protein, in mediating DNA damage induced cell cycle arrest. We demonstrate that in VSMCs, prelamin-2 associates with both promyelocytic leukaemia protein (PML) and extracellular regulated kinases 1/2 (ERK1/2) within the nucleus. Depletion of prelamin-2 via siRNA mediated knockdown or overexpression of a dominant negative fragment resulted in the accumulation of DNA damage, activation of the ATM/ATR signalling pathways and S-phase delay. In addition, VSMCs depleted of prelamin-2 displayed mitotic defects indicative of G2/M cell cycle checkpoint failure and ultimately underwent cell death by mitotic catastrophe. Importantly, prelamin-A accumulation abolished prelamin-2 function within the DDR reiterating the phenotypes of increased DNA damage and mitotic catastrophe. Our study is the first to describe a mechanism driving both normal and premature vascular ageing and highlights an essential role for prelamin-2 in DNA damage repair.

**THE CHEMOKINE FRACKTALINE (CX3CL1) HAS ANTI-APOPTOTIC AND PROLIFERATIVE EFFECTS ON PRIMARY HUMAN SMOOTH MUSCLE CELLS VIA EPIDERMAL GROWTH FACTOR RECEPTOR SIGNALLING**

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doi:10.1136/hrt.2009.181669

Chemokines are a family of low molecular weight proteins which mediate the directed migration of leukocytes during tissue homeostasis and inflammation. Many chemokines have been implicated in the development and progression of atherosclerosis. CX3CL1 (fractalkine) is an unusual membrane-bound chemokine which signals through the G protein-coupled receptor CX3CR1 expressed on monocytes, T cells and smooth muscle cells. We have shown that CX3CL1 induces smooth muscle cell (SMC) chemotaxis in vitro and we sought to examine further roles for CX3CL1 in primary human SMC biology.

In primary human coronary artery SMCs, CX3CL1 (50 nM) significantly reduces apoptosis induced by staurosporine treatment, as quantified by caspase 3/7 activity assays and cleaved caspase 3 staining. Furthermore, CX3CL1 is a potent mitogen for primary human SMCs as measured using tritiated thymidine incorporation assays and Ki67 staining. With the use of pharmacological inhibitors and western blotting, CX3CL1 signalling was shown to be mediated via ERK, Akt, and epidermal growth factor receptor (EGF-R) signalling via release of a soluble EGF-R ligand.

Specifically, CX3CL1 induces transcription of the potent EGF-R ligand, epiregulin.

In conclusion, we have demonstrated two novel and important functions of CX3CL1 on primary human SMCs: anti-apoptosis and proliferation. We have demonstrated a previously undocumented role of the EGF-R in CX3CL1 signalling in SMCs. This may have important implications in vascular pathologies including atherosclerosis, restenosis and transplant accelerated arteriosclerosis, where the balance of SMC proliferation and apoptosis critically determines both plaque stability and vessel stenosis.

**GLYCOSAMINOGLYCAN CHAIN SYNTHESIS ON DECORIN CONTRIBUTES TO OXIDATIVE STRESS-INDUCED CALCIFICATION OF HUMAN VASCULAR SMOOTH MUSCLE CELLS**

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doi:10.1136/hrt.2009.181669

Background Oxidised low density lipoprotein (ox-LDL) has been shown to induce osteogenic differentiation of vascular smooth muscle cells (VSMCs) and over-expression of the small proteoglycan decorin (DCN), has also been shown to induce calcification of VSMCs, but links between these two events and their mechanisms of action are unknown. Decorin is composed of a core protein and a single glycosaminoglycan (GAG) chain. Oxidative stress plays an important role in the modification of the extracellular matrix. This study aimed to investigate whether oxidative stress modulated the GAG chain on decorin during calcification of VSMCs in vitro.

Methods and Results Human VSMCs treated with ox-LDL showed increased alkaline phosphatase activity and increased mineralisation. Western blot analysis showed that less decorin core protein was detected in ox-LDL-treated cells compared to untreated controls. However, removing GAG chains with chondroitinase ABC digestion, yielded similar levels of core protein in ox-LDL-treated cells compared to untreated controls. Oxidative stress had...
no effect on decorin mRNA, but increased mRNA expression of Xylosyltransferase 1 (XT-1), the key enzyme responsible for the biosynthesis of GAG chains. Furthermore, usage of XT-1 siRNA blocked the effects of oxidative stress on calcification of VSMCs. Adenoviral-mediated over-expression of decorin (Ad/DCN) also accelerated mineralisation in VSMCs compared to cells infected with a mutated form of decorin, free of the GAG chain, (Ad/DCN-S34A), suggesting GAG chain addition on decorin is crucial to the process of calcification.

**Conclusions** We conclude that oxidative-stress mediated calcification of VSMCs in vitro involves the addition of the GAG chain on decorin.

### 038 NADPH OXIDASE PRODUCED HYDROGEN PEROXIDE-MEDIATED SMOOTH MUSCLE CELL DIFFERENTIATION FROM STEM CELL

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doi:10.1136/hrt.2009.181669

NADPH oxidase (Nox4) produces reactive oxygen species (ROS) that are important for vascular smooth muscle cell (SMC) behaviour, but the potential impact of Nox4 in stem cell differentiation is unknown. When Mouse ES cells were plated on collagen IV-coated dishes/flasks, a panel of SMC-specific genes was significantly and consistently upregulated. Nox4 expression was markedly correlated with such a gene induction as confirmed by real-time PCR, immunofluorescence and Western blot analysis. Overexpression of Nox4 specifically resulted in increased SMC marker production, while knockdown of Nox4 induced a decrease. Furthermore, SMC-specific transcription factors, including serum response factor (SRF) and myocardin were activated by Nox4 gene expression. Moreover, Nox4 was demonstrated to drive SMC differentiation through generation of hydrogen peroxide (H₂O₂). Confocal microscopy analysis indicates that SRF was translocated into the nucleus during SMC differentiation, in which SRF was phosphorylated. Additionally, auto-secreted TGF-beta1 activated Nox4 and promoted SMC differentiation. Interestingly, cell lines generated from stem cells by Nox4 transfection and G418 selection displayed a characteristic of mature SMCs, including expression of SMC markers and cells with contractile function. Thus, we demonstrate for the first time that Nox4 is crucial for SMC differentiation from embryonic stem cells, and enforced Nox4 expression can maintain differentiation status and functional features of stem cell-derived SMCs, highlighting its impact on vessel formation in vivo and vascular tissue engineering in the future.

### 039 ACTIVATION OF NF-E2 RELATED FACTOR-2 (NRF2) PROTECTS ARTERIES FROM EXHIBITING A PRO-INFLAMMATORY STATE

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doi:10.1136/hrt.2008.181669m

Pro-inflammatory mediators play an important role in atherosclerosis by inducing adhesion molecules (eg VCAM-1) on endothelial cells (EC) via activation of signalling intermediaries including p38 MAP kinase. Blood flow influences atherosclerosis by generating shear stress, which alters endothelial physiology. Regions of the arterial tree exposed to high laminar shear stress (LSS) are protected from EC activation, inflammation and atherosclerosis, whereas low-shear regions are susceptible. Previous studies demonstrated that high LSS activates the transcription factor Nrf2 in EC. The interaction between shear stress and Nrf2 was investigated by en face staining which showed that phosphorylation of p38, and expression of VCAM-1 in EC occurs constitutively and can be enhanced by lipopolysaccharide treatment at a low-shear, atherosusceptible site in the murine aorta (C57BL/6), whereas EC in the high-LSS, atheroprotected site were resistant to pro-inflammatory stimuli (p<0.001). We concluded that Nrf2 negatively regulates p38–VCAM-1 signalling at the protected site, because local p38 activation and VCAM-1 expression was enhanced in Nrf2 gene-targeted mice (p<0.001). Furthermore, we examined whether EC activation at the susceptible site can be reduced by pharmacological activation of Nrf2 using the dietary antioxidant sulforaphane. Treatment with sulforaphane activated Nrf2 and reduced p38 activation and VCAM-1 expression at the susceptible sites in wild-type mice but not in Nrf2+/- animals, indicating that sulforaphane suppresses p38–VCAM-1 signalling in EC via Nrf2. Our findings demonstrate a central role for shear- and antioxidant-induced Nrf2 in protecting arteries from exhibiting a pro-inflammatory state and may inform novel therapeutic or dietary strategies to reduce inflammation at atherosusceptible sites.

### YIA awards

#### 001 GLOBAL HETEROZYGOUS KNOCKOUT OF THE INSULIN-LIKE GROWTH FACTOR 1 RECEPTOR IN MICE RESULTS IN ENHANCED METABOLIC AND VASCULAR INSULIN SENSITIVITY AND INCREASED ENDOTHELIAL NITRIC OXIDE PRODUCTION

doi:10.1136/hrt.2009.178129a

Insulin-like growth factor 1 (IGF-1) enhances glucose uptake and nitric oxide (NO) production, acting via similar signalling pathways to insulin. Observational studies suggest a role for IGF-1 in insulin resistance and cardiovascular disease.

To investigate this further we used a murine model with global heterozygous knockout of the IGF-1 receptor.

Data are expressed as mean (SEM). Glucose tolerance tests demonstrated impaired glucose handling in IGF1RKO mice compared to WT (AUC IGF1RKO mice = 1086 (26.82) (mmol/L)minutes, n = 5, p = 0.005). Insulin tolerance tests revealed that IGF1RKO mice were more insulin sensitive than controls (AUC IGF1RKO = 552.2 (25.18) (mmol/L)minutes, n = 10; AUC for WT = 651.4 (22.26) (mmol/L)minutes, n = 11; p = 0.008).

Dose–response curves from ex vivo aortic rings of IGF1RKO mice were hyporesponsive to phenylephrine compared to those from WT (Emax IGF1RKO mice = 0.60 (0.05) g, n = 11; Emax for WT mice = 0.79 (0.06) g, n = 10, p = 0.03). Addition of the NO synthase inhibitor, NG-monomethyl-l-arginine (l-NMMA), led to a 65.40% (n = 9) increase in Emax in IGF1RKO mice compared to 15.78% (12.00)% (n = 9) increase in WT mice (p = 0.01).

Western blot analysis showed the basal protein level of phosphorylated endothelial NO synthase (phNOS) in the aortae of IGF1RKO mice was 35.3% greater than in WT mice (n = 5 in each group).

Upon insulin stimulation, protein levels of phNOS increased by 198.6% in WT mice compared to an increase of 198.8% WT mice (n = 5 in each group).

These data raise the possibility that reduced IGF-1 receptor number in the endothelium has a favourable effect on NO bioavailability, possibly through upregulation of insulin signalling.
Smooth muscle cell (SMC) migration occurs in many arterial diseases, including aneurysm, angioplasty restenosis and atherosclerosis. Eph receptor tyrosine kinases and ephrin ligands signalling play an important role in SMC migration. It has been reported that expression of one member of Eph family, EphB2 receptor, is upregulated in injured vessels. To get more insight into the regulation of vascular integrity by EphB2 in pathological contexts, we interbred Ephb2−/− mice with atherosclerosis-prone Apoe−/− mice and thereby generated Ephb2+/− Apoe−/− offspring. Animals were fed a normal diet and analysed at the age of 25 weeks. We detected a 40% increase in lipid staining in Ephb2+/− Apoe−/− thoracic aortas relative to controls (6.58±2.5% and 3.9±1.1%, respectively; n = 10). Atherosclerotic plaques of Ephb2−/− Apoe−/− mice exhibit a marked thinning of the tunica media. Loss of medial SMC and elastic fibres was observed without any significant increase in intimal macrophage content. Interestingly, we detected the formation of sacular aneurysms in high haemodynamic stress regions within the arterial tree, such as aortic root and collateral arteries of the abdominal aorta. These aneurysms are composed of only intima and adventitia. Transgenic mice expressing a fusion protein, in which kinase domain of EphB2 have been replaced by beta-galactosidase (beta-Gal), showed similar sacular aneurysms in Apoe−/− genetic background, suggesting that EphB2 kinase activity is indispensable in this setting Ephb2-beta-Gal staining is enhanced in atherosclerotic plaques and aneurysms. In conclusion, Ephb2 receptor regulation could play a pivotal role in the maintenance of vascular integrity in atherosclerosis.

**ENDOTHELIAL SHIP2 PLAYS A CRITICAL ROLE IN GLUCOSE REGULATION AND INSULIN SENSITIVITY**

Insulin resistance is thought to promote atherosclerosis through a closely coupled association with endothelial dysfunction. Accumulating evidence suggests that the relationship between insulin resistance and endothelial function is reciprocal; however, the role of the endothelium in whole body glucose regulation remains controversial. The lipid phosphatase SHIP2 is as a negative regulator of insulin signalling. In this study we used Cre-lox technology to generate endothelial-specific SHIP2 haploinsufficient mice to determine whether enhanced insulin signalling in endothelium modulates vascular function and whole body glucose regulation. Male EC-SHIP2+/− offspring were compared with control sex-matched littermates at 12–15 weeks of age. EC-SHIP+/− mice were morphologically indistinguishable from control littermates, exhibited normal development and body and organ masses were similar in both groups. No significant differences were observed in heart rate or blood pressure. Glucose tolerance after glucose challenge was significantly better in EC-SHIP+/− than controls (p < 0.05), with no differences in plasma insulin SHIP+ concentrations; fasted or after glucose challenge. Improved insulin sensitivity was confirmed in EC-SHIP+/− mice in insulin tolerance tests (p < 0.05). ex vivo vasomotor studies in aorta revealed no significant differences between EC-SHIP+/− and controls in contractile responses to phenylephrine or relaxation to acetylcholine or sodium nitroprusside. In conclusion; endothelial partial deletion of SHIP2 improves glucose tolerance and insulin sensitivity in mice which supports a critical role for endothelial cells in whole body glucose regulation and suggests that endothelial insulin signalling is an appropriate target to improve insulin sensitivity.
(MT1-MMP) in monocyte/macrophage invasion through matrigel in vitro and recruitment into subcutaneous sponges in vivo. We also investigated whether MT1-MMP-mediated activation of MMP-2, which requires tissue inhibitor of metalloproteinases-2 (TIMP-2) as a cofactor, participates in monocyte migration.

Total MT1-MMP and MMP-2 protein and surface labelling were detected in freshly-isolated monocytes, demonstrating that MT1-MMP is available to mediate migration, either directly or by activating MMP-2. Monocyte migration utilising an in vitro, transwell, matrigel invasion assay, was significantly reduced by 80 ± 10% by addition of recombinant TIMP-2 (an endogenous inhibitor of MT1-MMP and MMP-2) or 85 ± 5% by an MT1-MMP blocking antibody (both n = 3; p < 0.01 against no addition).

Migration was significantly increased 194 ± 21% in TIMP-2/−/− (but not TIMP-1/−/−) monocytes vs wild-type control (n = 3, p < 0.05), supporting the hypothesis that MT1-MMP itself drives monocyte migration through matrigel in vitro.

Conversely, when subcutaneous polyurethane sponges were implanted into TIMP-2/−/− mice, which do not support MT1-MMP-mediated MMP-2 activation, we observed a significant reduction in the accumulation of MT1-MMP positive macrophages compared with wild-type controls (42 ± 2%; n = 3, p < 0.05). These data suggest that MT1-MMP mediates mouse monocyte migration in vivo in part through activating MMP-2.

Taken together, our results suggest that targeting MT1-MMPs may have therapeutic potential for stabilising atherosclerotic plaques.

**PARATHYROID HORMONE RECEPTOR 1 GENE KNOCKDOWN INDUCES AORTIC OCCLUSION IN ZEBRAFISH EMBRYOS**

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doi:10.1136/hrt.2009.178129f

Mutations in parathyroid hormone receptor 1 (PTH1R) are responsible for Blomstrand’s chondrodysplasia, a fatal bone disorder associated in some cases with aortic coarctation. Mouse PTH1R knockout mice die due to cardiovascular defects but do not display aortic coarctation. We therefore determined the effect of morpholino antisense knockdown of PTH1R on embryonic vascular development in transgenic zebrafish embryos which allow detailed serial imaging.

Morpholino antisense oligonucleotides (MO) were designed to either totally prevent PTH1R mRNA transcription or induce aberrant splicing. These were then injected into one-cell transgenic zebrafish embryos which expressed GFP in the endothelial cytoplasm or localised to the endothelial nuclei.

We observed a total occlusion of the mid-distal aorta in 36 ± 16% of PTH1R morphants (of 155 embryos) at 2 days post fertilisation (dpf). Other morphants exhibited a variable degree of reduced blood flow in the distal aorta suggestive of partial aortic occlusion. Aortic occlusion was accompanied by diversion of blood flow into the intersegmental vessels proximal to the occlusion. This phenotype diminished over time; by 5 dpf this phenotype was seen in only 2 ± 2% of embryos, suggesting that as PTH1R mRNA splicing recovered from the MO, the embryo can repair the occlusion through innate mechanisms.

We conclude that reducing translation of PTH1R mRNA is associated with localised defects in aortic patterning analogous to aortic coarctation. This supports the suggestion that aortic coarctation associated with Blomstrand’s chondrodysplasia is directly attributable to PTH1R mutation. The zebrafish is therefore a useful model with which to examine the genetic contribution to vascular formation.
were inhibited by co-incubation with IL-4 or LAP-IL-4). LAP-IL-4 was also activated by human atherosclerotic plaque extracts.

These data suggest that LAP-OM14-IL-4 could be an excellent latent anti-inflammatory cytokine that could be targeted for release at sites of atheromatous lesions in the later stages of disease, when MMP-13 is over-expressed. This strategy could improve the effectiveness of site-specific targeted anti-inflammatory cytokine delivery without exposure of the entire vascular bed.

REFERENCES

009 HDAC3 EXPRESSION AT BIFURCATION AREAS PLAYS A CRITICAL ROLE IN PRESERVING THE ENDOTHELIAL MONOLAYER AND MAINTAINING THE VASCULAR INTEGRITY

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doi:10.1136/hrt.2009.178129i

Background Histone deacetylase 3 (HDAC3) is known to play a crucial role in differentiation of endothelial progenitors. The role of HDAC3 in mature endothelial cells (EC) however, is not well understood.

Methods and Results Here, we investigated the function of HDAC3 in preserving the endothelial integrity in bifurcation areas prone to atherosclerosis development. En face staining of aortas from ApoE<sup>−/−</sup> mice revealed increased expression of HDAC3, specifically in these branching areas in vivo, while rapid upregulation of HDAC3 protein was observed in EC exposed to disturbed flow in vitro. Co-immunoprecipitation experiments showed that HDAC3 and Akt form a complex. Enforced expression of HDAC3 resulted in increased phosphorylation of Akt and higher viability was also observed when EC were treated with H<sub>2</sub>O<sub>2</sub>, indicating that elevation of HDAC3 expression acts as a prosurvival signal in conditions of oxidative stress. In line with these findings knockdown of HDAC3 using lentiviral vectors (Lenti-shHDAC3) led to a dramatic decrease in cell survival accompanied by apoptosis in EC. In aortic isografts of ApoE<sup>−/−</sup> mice treated with Lenti-shHDAC3, a robust atherosclerotic lesion was formed. Surprisingly, 3 out of the 8 mice that received a Lenti-shHDAC3 infected grafts died within 2 days postoperationally. Miller’s staining of the isografts revealed a disruption of the basement membrane and rupture of the vessel.

Conclusions Our findings demonstrated that HDAC3 serves as an essential prosurvival signal with a critical role in maintaining the endothelial integrity via Akt activation, and that severe atherosclerosis and vessel rupture occur when HDAC3 is knockdown.

Provenance and peer review: Not commissioned; not externally peer reviewed.