**Introduction and objectives** New methods of high throughput sequencing provide unparalleled access to the human genome and transcriptome. We hypothesised that next generation DNA sequencing technologies would allow us to identify an elusive novel disease gene for a pulmonary vascular disease inherited as an autosomal dominant trait: The *HHT3* interval on chromosome 5 is predicted by linkage studies to contain a mutation causing pulmonary arteriovenous malformations and hereditary haemorrhagic telangiectasia.[1,2]

**Methods** Published expressed sequence tag (EST) databases and tiling array data were used to supplement sequencing analysis of the *HHT3* interval. Sheared, Agilent SureSelect adaptor-ligated genomic DNAs from six related patients and four controls were hybridised to single stranded biotinylated RNA baits. Samples were pooled for multiplexed sequencing on an Illumina HiSeq2000. Sequence data were processed with RTA version 1.7.45, CASAVA Eland pair algorithm, and CASAVA 1.7 demultiplexing algorithms. Validations of sequence variants were performed using conventional PCR and Sanger sequencing.

**Results** Conventional exon-based sequencing strategies did not identify the *HHT3* causative gene mutation. For individual candidate genes, up to 108 alternatively spliced transcripts per gene were predicted from EST databases. For intergenic regions, tiling array data indicated that up to 44 different transcribed fragments were present in the nucleus and/or cytoplasm of different cell types. For each NextGen sequencing DNA sample, ~8 million reads per sample uniquely mapped to the *HHT3* interval which represents ~1/5,000 of the genome. Using a 2:1 threshold, an average of ~4,000 differences to NCBI36/hg18 were identified in each sample. 113 differences to NCBI36/hg18 were present in all six HHT3-affected individuals and absent in all four controls. 60% of novel shared variants were validated by wet lab PCR. Following exclusion in 100 normal chromosomes, and computational predictions of potential function, multiple candidate sequence variants remain.

**Conclusions** Genomic sequencing capturing intronic sequences yields challenging numbers of sequence variants for wet lab validations, even when multiple replicate chromosomal strategies are employed.

## REFERENCES

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## P18 ROBO1/4-SLIT2 EXPRESSION IN PULMONARY VASCULAR CELLS: IMPLICATIONS FOR PAH?

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**Introduction and objectives** Pulmonary artery hypertension (PAH) is associated with inappropriate vascular remodelling and inflammation. Recent studies have shown that vascular cells express the transmembrane roundabout (Robo) proteins, Robo1 and Robo4, and that interaction with a secreted glycoprotein ligand, Slit2, controls cell migratory and inflammatory response. We hypothesise that Robo1, Robo4 and Slit2 are expressed on pulmonary artery (PA) endothelial cells (EC) and smooth muscle cells (SMC). We also hypothesise that Slit2 will modulate PAEC and PASMC migration and inflammatory mediator release.

**Methods** Real-time-PCR determination of Robo1, Robo4 and Slit2 expression and the house-keeping gene, GAPDH, in PAEC, PASMC and for comparison, human umbilical vein endothelial cells (HUVEC); following incubation with TNF$\alpha$ (10ng/ml) or Slit2N (10nM) for 2h. Enzyme-linked immunosorbent assay measurements of granulocyte-macrophage-colony stimulating factor (GM-CSF) in supernatants of HUVEC, PAEC or PASMC pre-treated (1h) with Slit2N, followed by TNF$\alpha$ (17h). Migration assays (PAEC or PASMC) towards serum-containing medium (0.05 and 0.02%, respectively), for 4h with/ without Slit2N.

**Results** Basal mRNA expression of Robo1, Robo4 and Slit2 was detected in PAEC, PASMC and HUVEC (n = 3–4). Slit2N (2h) significantly ($p < 0.05$, n = 3) decreased Robo4 and Slit2 mRNA expression, but not Robo1, by 35% in PAEC; and had no effect on HUVEC or PASMC. TNF$\alpha$ had no significant effects on Robo1, Robo4 or Slit2, regardless of cell type. Despite a small (23%), but significant ($p < 0.05$) reduction of GM-CSF release from TNF$\alpha$-activated HUVEC (n = 7), no similar effects were seen in PAEC or PASMC (n = 3). Moreover, whilst PAEC or PASMC migration to serum-containing medium increased (2.7- and 5.3-fold, respectively), co-incubation with Slit2 had no significant effect.

**Conclusion** The novel discovery of Robo1, Robo4 and Slit2 mRNA in PAEC and PASMC; and that Slit2 down-regulated Robo4 and Slit2 in PAEC, but not PASMC/HUVEC, might suggest negative feedback on the Robo4-Slit2 axis unique to PAEC. That neither PAEC nor PASMC responded to Slit2 in functional assays could reflect limitations in experimental assays. However, down-regulation of Robo4-Slit2 in PAEC might also explain lack of effect on GM-CSF release, when compared with HUVEC. Further studies to better delineate the role of the Robo-Slit2 pathway in PAH are required.

## P19 THE ROLE OF DIFFERENTIAL TNFR SIGNALLING IN MAINTENANCE OF ALVEOLAR EPITHELIAL HOMEOSTASIS

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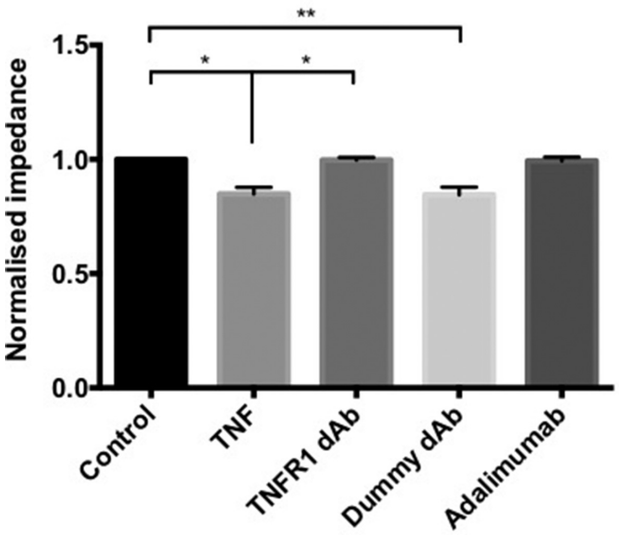
Alveolar epithelial activation and disruption of the alveolar epithelial barrier promote recruitment of neutrophils into the alveolar space and cause alveolar oedema respectively thereby playing key roles in the pathogenesis of Acute Respiratory Distress Syndrome (ARDS). Tumour necrosis factor alpha (TNF) is an early mediator of inflammation in ARDS. TNF signals through two cell surface receptors, TNFR1 and TNFR2 initiating distinct signalling pathways and cellular responses.

Using a novel, highly selective TNFR1 domain antibody (dAb™), a dummy dAb and the dual TNFR antagonist Adalimumab™, we investigated the role of differential TNFR signalling on human pulmonary alveolar epithelial cell (human alveolar type 2 cells and A549 cell line) activation, permeability and repair.

Human alveolar epithelial type 2 cells (haT2) expressed both TNFR, whilst A549 cells only expressed TNFR1. TNFR1 signalling mediated release of the neutrophil chemokines IL-8 and GMCSF as well as IL-6 in hAT2 (pin vitro scratch model of epithelial (haT2) wound repair.

TNFR1 signalling induced pro-inflammatory cytokine expression from alveolar epithelial cells and mediated increased epithelial permeability. TNFR1 induced permeability did not appear to




**Abstract P19 Figure 1** A549 cells plated on an iCelligence 8-well gold electrode coated plate were incubated with TNFR1 dAb™, a dummy dAb or Adalimumab™ for 1 h then exposed to exogenous TNF or vehicle control. Electrical impedance was measured continuously over 50 h. Trough normalised impedance was measured over 50 h post treatment (n = 3–5). Data are presented as mean ±SEM analysed by Kruskal-Wallis (Dunns). *p < 0.05, **p < 0.01

be due to disruption of epithelial junctional proteins; we speculate that this may alternatively be due to TNFR1 induced cell death.

## P20 DELINEATING THE CONTRIBUTION OF FORMYLATED PEPTIDES AND FORMYL PEPTIDE RECEPTOR 1 TO THE PATHOGENESIS OF ACUTE LUNG INJURY

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**Background** Acute respiratory distress syndrome (ARDS) remains an often fatal condition without effective pharmacological therapies. Characteristically, a neutrophil-dominant disorder, it is associated with a dysregulated inflammatory response and tissue injury. Neutrophil migration into inflammatory sites is controlled by a variety of factors; in sterile tissue injury mitochondrial formylated peptides are released following necrotic cell death and bind to formyl peptide receptor 1 (FPR1) on neutrophils to induce migration and activation.

**Hypothesis** That mitochondrial formylated peptides are elevated in ARDS and drive FPR1-mediated neutrophil recruitment. Inhibition of FPR1 in sterile lung injury would therefore attenuate the inflammatory response through multiple FPR1-mediated effects.

**Methods** Mitochondrial DNA and formylated peptides were quantified in plasma of ARDS patients and healthy controls by qPCR, western blot and LC-MS/MS. Healthy volunteer neutrophils were stimulated with mitochondrial formylated peptides and chemotaxis assays and flow cytometry used to assess neutrophil function. Intracellular signalling was assessed by western blotting. Mouse models of infective (E. coli) and sterile (hydrochloric acid) acute lung injury were used.

**Results** Free mitochondrial DNA and formylated peptides were elevated in ARDS patients. Mitochondrial formylated peptides induced FPR1-dependent neutrophil chemotaxis through PI3K- and MAPK-mediated control of the $\beta_2$-integrin heterodimer Mac1. In sterile acid-induced injury FPR1 inhibition resulted in reduced neutrophil migration, pulmonary haemorrhage, protein leak and pro-inflammatory cytokine expression. Furthermore, acid-induced reduction in alveolar macrophage number was inhibited while interstitial macrophages displayed an alternatively activated phenotype. FPR1 was also found to be expressed on mouse type 1 alveolar epithelial cells suggesting further possible mechanisms through which FPR1-mediated alveolar leak occurs. Importantly, delivery of FPR1 antagonists 12 h after injury also reduced acute lung inflammation demonstrating potential therapeutic relevance. In non-sterile E. coli-mediated lung injury partial antagonism of FPR1 resulted in reduced alveolar neutrophil numbers and attenuated vascular leak without altering bacterial clearance.

**Conclusions** Mitochondrial formylated peptides and FPR1 play an important role in the pathogenesis of sterile acute lung injury. This appears to be predominantly through neutrophil-dependent means but their role in macrophage and epithelial cell function could also be important. FPR1 antagonism may therefore represent a multi-cellular therapeutic target in the treatment of ARDS.

## P21 HYPOXIA-INDUCED NEUTROPHIL SURVIVAL IS DEPENDENT ON PHOSPHOINOSITIDE 3-KINASE (PI3-K)-MEDIATED SIGNALLING

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**Introduction and objectives** Neutrophils (PMNs) are a key component of the innate immune response to invading pathogens. They accumulate at sites of inflammation and infection, which are typically characterised by low oxygen tensions (e.g. in the acute respiratory distress syndrome (ARDS)). Human PMNs undergo constitutive apoptosis, their survival contingent upon pro-survival and pro-apoptotic signals derived from their microenvironment. Hypoxia profoundly delays PMN apoptosis, resulting in persistence of PMNs at inflammatory foci and this may perpetuate hypoxia-mediated lung injury. Given the importance of phosphoinositide 3-kinase (PI3-K) signalling in cytokine-mediated neutrophil survival, we hypothesised that hypoxia-induced PMN survival may also involve PI3-K-mediated signalling.

**Methods** Highly pure PMNs isolated from healthy volunteers were incubated for 20 h under normoxic (20 kPa) and physiologically relevant hypoxic (3 kPa) conditions with a pan-PI3-K inhibitor (LY294002 at 10 μM), a novel pan-Class I PI3-K inhibitor (ZSTK474 at 1 μM, 3 μM and 10 μM) or novel PI3-K Class I isoform-selective inhibitors (PI3-Kδ at 1 μM; PI3-Kγ at 3 μM and 10 μM, or PI3-Kδγ at 3 μM). PMNs were also incubated in normoxia and hypoxia in the presence of GM-CSF (1 ng/ml) with the same panel of inhibitors, allowing comparison with GM-CSF mediated survival, which is largely PI3-K dependent. PMN apoptosis was assessed using two complementary techniques – morphology and flow cytometry following annexin V-FITC and propidium iodide staining.