# Annual Scientific Meeting 2023

## Novel therapeutics & emerging technology in haemostasis & thrombosis

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# Scientist in Training abstracts

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## 01

**A microfluidic model to study the initiating events in venous thrombosis**

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**Background**
A primary pathogenic factor of venous thromboembolism is alteration to normal blood flow, particularly within venous valve pockets where thrombi initiate. Despite the association of venous thrombi as 'red thrombi', von Willebrand factor (VWF), platelets and neutrophils are increasingly recognised as contributing to thrombus initiation and development.

**Aims**
To investigate the influence of alterations to venous flow patterns upon interactions between VWF and platelets, as well as capture and activation of neutrophils.

**Methods**
HUVECs were cultured in both straight channels and those approximating the shape of pathogenic venous valves, designed and produced in-house. Plasma-free blood or isolated platelets and neutrophils were perfused over stimulated endothelial cells (ECs) to assess interactions between endothelial-derived VWF, platelets and neutrophils under venous shear conditions.

**Results**
Under venous shear stress, platelets bound to endothelial-released VWF via GPIbα. Under linear flow, this led to characteristic VWF-platelet strings however, when oscillatory flow was applied, tangling of VWF-platelet cables occurred. Platelet binding to VWF strings under low shear stress induced intracellular $Ca^{2+}$ signalling. Subsequently, neutrophils interacted with these platelets, leading to $Ca^{2+}$ spiking within neutrophils, a pre-cursor for neutrophil activation and NET formation. Blockade of both interactions between $\alpha_{IIb}\beta_3$-SLC44A2 and P-selectin-PSGL-1 reduced neutrophil interactions on platelet-VWF strings. In comparison, inhibition of SLC44A2 did not impact interactions between neutrophils and ECs. Neutrophil-EC binding was also diminished by blocking P-selectin and the β2 integrins on neutrophils, LFA-1 and Mac-1.

**Conclusions**
Under venous shear conditions, platelets bind VWF strings and facilitate neutrophil capture. This involves $\alpha_{IIb}\beta_3$-SLC44A2 and P-selectin-PSGL-1. Fluidic models will undoubtedly aid the investigation of important early stages of neutrophil capture on platelets and/or ECs and may highlight novel targets for therapeutic intervention.

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## 02

**Antibodies are a key component in *Salmonella* induced platelet aggregation**

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**Background**
Invasive non-typhoidal *Salmonella* is responsible for over 75,000 deaths/year in Sub-Saharan Africa. An increasing number of the >500,000 cases/year found in Sub-Saharan Africa are from multi-drug resistant *Salmonella* strains. It is therefore of vital importance that the mechanisms involved in this bloodstream infection are elucidated, as antibiotic treatments are becoming increasingly limited.

**Aims**
To assess platelet aggregation to different strains of *Salmonella*, and to identify which components of platelet rich plasma (PRP) are key in this response.

**Methods**
Light transmission aggregometry was used to assess platelet aggregation in PRP in response to 3 strains of *Salmonella* Typhimurium: SL3261 (a lab attenuated strain), SL1344 (a wild-type virulent strain) and D23580 (an invasive strain from Sub-Saharan Africa), in 19 healthy donors.

**Results**
Platelet aggregation varied both between donors and strains. SL1344 was the strain that gave the strongest aggregation in terms of magnitude and speed, with 58% of donors classed as strong responders (maximum aggregation >50%) and a lag time of 7.3 ± 2.3 minutes (mean ± SD), followed by D23580, with 32% strong responders and an average lag time of 10.5 (± 2.8) minutes. Aggregation to all three strains was blocked by the αIIbβ3 antagonist eptifibatide and the FcγRIIa-blocking monoclonal antibody IV.3. To identify whether platelets or plasma were responsible for variation between donors, 'donor-swap' experiments were carried out, swapping platelets and plasma from a responder with a non-responder. This revealed plasma to be a key component involved in causing platelet aggregation. Anti-*Salmonella* IgG levels positively correlated with the degree of platelet aggregation to all 3 strains, and negatively correlated with lag time to SL1344 and D23580. To confirm the role of IgG, plasma was depleted of anti-*Salmonella* IgG, which abolished the platelet aggregation response.

**Conclusions**
IgG antibodies are a key component in platelet aggregation responses induced by *Salmonella*.

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The authors report no conflict of interest

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## 03

**Inflamed Endothelial Cells Reduce Clot Surface Fibrin Film Coverage**

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**Background**
Thrombi are heterogeneous, consisting of fibrin, platelets and red blood cells. Recent studies showed that fibrin films develop on the surface of thrombi. Inflammatory response is known to be involved in thrombus formation; however, little is known about the role of fibrin films in thrombosis under inflammatory conditions.

**Aims**
Investigate the effect of inflamed endothelial cells on fibrin film formation.

**Methods**
Human umbilical vein endothelial cells (HUVECs) were stimulated with tumour necrosis factor-α (TNF-α), lipopolysaccharide (LPS) or interleukin-1β (IL-1β). Inflammation markers, including monocyte chemoattractant protein-1 (MCP-1), tissue factor, von Willebrand factor (vWF) and thrombomodulin (TM) were analysed by enzyme-linked immunoassay. Plasma clots were formed on the top of the cells. Effects of HUVEC inflammation on fibrin fibre density was analysed by laser scanning confocal microscopy, and fibrin film coverage was investigated by scanning electron microscopy. Clotting lag time was measured by turbidity assay.

**Results**
MCP-1 levels were high and TM levels were low in all cells treated with inflammatory mediators. Tissue factor level was high in TNF-α treated cells, while there was no significant difference in vWF secretion in these cells. TNF-α-treated cells showed denser clot and less fibrin film coverage than control cells. There was no significant different in fibrin fibre density and film coverage of clots on top of LPS and IL-1β treated cells. Lag time was shorter in clots on top of all inflamed cells compared to control cells, but this was most significant for TNF-α treated cells.

**Conclusion**
Plasma clots on top of TNF-α treated cells showed less fibrin film coverage and were characterised by higher fibre density and shorter lag time, compared to control cells. These findings are consistent with increased thrombin generation on TNFα inflamed cells leading to reduced film coverage. Reduced film formation under thromboinflammatory conditions may support continued clot growth.

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## 04

### Reprogramming of glucose metabolism in murine platelets in type 1 diabetes

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**Background**
Cardiovascular diseases are the leading cause of death in patients with diabetes. Patients with diabetes exhibit increased platelet activation and thrombosis. Platelet aggregation and activation have been previously studied in these patients. However, the glucose metabolism in diabetic platelets is poorly understood.

**Aims**
In the present study, we assessed the effect of diabetes on platelet metabolic functions and mitochondrial integrity in the murine model Streptozotocin (STZ)-induced diabetes.

**Methods**
Diabetes was induced in male C57BL/6 mice using STZ (50mg/kg body weight) for three days. Three months after STZ induction, glucose uptake was measured in resting and convulxin-stimulated platelets using flow cytometry. Glycolysis was measured using Extra Cellular glycolysis (ECAR) by a Seahorse bioanalyser. Finally, correlative analysis was carried out between fasting blood glucose (FBG) and other parameters.

**Results**
STZ induced a significant increase in fasting blood glucose levels and decreased weight of the mice. When we compared the glucose uptake by platelet from STZ-induced diabetic mice relative to those of control mice we found significant increase in platelet glucose accumulation after STZ treatment. The increase in glucose uptake was coupled elevated glycolysis, including glycolytic capacity and glycolytic reserve. When we examined oxygen consumption as a marker of mitochondrial activity we found that this was unaffected, suggesting glucose oxidation was not increased. However, we did observe a significant increase in mitochondrial membrane potential in STZ-treated mice compared to control mice. Interestingly this increased glucose metabolism was not associated with overt platelet activation since both resting P-selectin expression and annexin binding was not altered by STZ

**Conclusions**
These data suggest that hyperglycaemia promotes a glycolytic phenotype in platelets without causing overt platelet activation

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## 05

**Cytokines stimulate expression and release of plasminogen activator inhibitor-1 (PAI-1) from endothelial cells**

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**Background**
PAI-1 is the primary inhibitor of tissue-plasminogen activator (tPA) and urokinase. PAI-1 is expressed in hepatocytes, endothelial cells, adipocytes, and megakaryocytes, subsequently giving rise to the major circulating pool within platelets. Elevated levels of PAI-1 induce a hypofibrinolytic state and are a known cardiovascular risk-factor. Thromboinflammation is now a well-recognised phenomena, whereby activation of the inflammatory cascade invokes thrombotic complications. These complications arise due to pathogenic infection or in sterile inflammatory conditions, such as diabetes, and autoimmune diseases.

**Aim**
To examine the expression and release of PAI-1 from endothelial cells in response to inflammatory stimuli.

**Methods**
EA.hy926 cells were stimulated with several inflammatory cytokines, including IL-6, TNFα, TGF-β1, IL-8 or thrombin, to assess PAI-1 expression. Secreted cellular PAI-1 was quantified by simple-plex (ELLA), and mRNA measured by qPCR. Cellular localisation was visualised by fluorescence microscopy. Plasma clot formation and tPA-mediated clot lysis on the surface of cells were observed by turbidimetric analysis ± neutralising PAI-1 antibodies.

**Results**
Stimulation with IL-6, TGF-β1, or thrombin for 24 h significantly elevated PAI-1 gene expression (1.7-fold, 2-fold, and 2.8-fold respectively) compared to unstimulated controls. IL-8 and TNFα stimulation did not significantly increase over basal expression. PAI-1 protein in media was significantly elevated (approximately 4-fold) in response to TNFα, TGF-β1, or thrombin when compared to the unstimulated cells. Clot lysis time was significantly delayed on the surface of endothelial cells and inclusion of neutralising PAI-1 antibodies enhanced rates of clot lysis.

**Summary**
The inflammatory cytokines, specifically IL-6 and TGF-β1, as well as thrombin, stimulated the expression of PAI-1 from EA.hy926 cells. Subsequent release of PAI-1 into the media was observed from cells stimulated with TNFα, TGF-β1, or thrombin. Functional assays, with neutralising antibodies, reveal that endothelial-derived PAI-1 dictates the fibrinolytic response to tPA and may contribute to the thrombo-inflammatory state.