Abstract Number - P01

A potential role of gap junctions in platelet-neutrophil interactions.

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Background:
Platelets are increasingly recognised for playing roles beyond thrombosis and haemostasis and mediating inflammation by direct interactions with innate immune cells. Connexins are a family of membrane proteins forming hexameric hemichannels, which dock to create gap junctions between platelets, allowing adjacent cells to communicate directly through intercellular signalling. The Cx gene family has 21 members in humans and 20 in mice, and their anticipated molecular weights are used to designate them. Platelets express several connexin family members, including Cx37, Cx40 and Cx62. Notably, Cx37 and Cx40 are also expressed in neutrophils. Given the importance of platelet interactions with neutrophils, we explored the impact of selective and non-selective gap junction and hemichannel inhibitors on platelet-neutrophil aggregation.
Methods and Results:
Following stimulation of washed human platelets with TRAP-6 (30μM), they were mixed with neutrophils isolated from human blood using the MACSxpress Whole Blood Neutrophil Isolation Kit (Human) and the interaction between platelets (detected using anti-CD41a) and neutrophils (detected using anti-CD11b) were assessed by flow cytometry. TRAP-6 stimulation resulted in 57.08±2.8% (P<0.0001, n=5) of platelets bound to neutrophils. Cbx (a non-specific gap junction blocker) reduced the interaction by 19.54 ± 5.7% (P=0.009, n=5), whilst the connexin 37 (Cx37) and 40 (Cx40) specific peptide inhibitors 37,43Gap27 and 40Gap27 led to a 21.15±6.6% (P=0.01, n=5) and 22.1±6.2% (P=0.008, n=5) reduction, respectively. Conversely, neutrophils were stimulated with PMA (10ng/ml) before adding platelets, resulting in a 54±2.6% (P=0.0003, n=5) increase in platelet neutrophil aggregates. Aggregation was decreased by 28.79±6% (P=0.001, n=5), 27.60±8% (P=0.01, n=5) and 31.4±8% by Cbx, 37,43Gap27 and 40Gap27, respectively. When fMLP (1μM/ml) was used as an alternative approach to activate neutrophils, the rate of platelet-neutrophil aggregation was 42.76±1.09% (P<0.0001, n=5). The interaction was reduced by Cbx (20±3%, P=0.0002, n=5), 37,43Gap27 (16.15±5%, P=0.01, n=5), and 40Gap27 (21±4%, P=0.003, n=5) blockers.
Conclusion:
These data implicate connexins in mediating platelet-neutrophil interactions. Ongoing work explores whether this involves these proteins' gap junction or adhesive functions and the implications for platelet and neutrophil function.

Abstract Number - P02

Fibrin film limits platelets interactions and is reduced by inflamed endothelial cells

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Background:
Thrombi are heterogeneous, consisting of platelets, fibrin, and red blood cells. Recent studies show that fibrin films develop on the surface of air exposed clots, playing a protective role against infection, and on thrombi. Platelets play a crucial role in clot formation and stability by interacting with fibrin and fibrinogen. Little is known about the role of fibrin film and their interactions with platelets and the vasculature.
Aims:
Investigate the role of fibrin film in platelet adhesion and spreading, and the impact of inflamed endothelial cells on fibrin film.
Methods:
Platelet adhesion and spreading on clot surfaces made from purified fibrinogen, recombinant wild-type (WT) and γ'/γ' fibrinogen was investigated by laser scanning confocal microscopy (LSCM). Eptifibatide was used to study the role of integrin αIIbβ3. Human umbilical vein endothelial cells were stimulated with tumour necrosis factor-α (TNF-α), lipopolysaccharide (LPS) or interleukin-1β (IL-1β). Plasma clots were formed on the top of the cells. Clot density was analysed by LSCM. Fibrin film coverage was investigated by scanning electron microscopy. Clotting parameters were measured by turbidity assay.
Results:
Adhesion and spreading of platelets on purified fibrin clots with film was reduced compared to clots without film. Eptifibatide significantly reduced platelet adhesion and spreading on the surface of clots formed without film, but no difference was observed in clots with film, suggesting that the integrin binding site of fibrin may be hidden in the film. This was supported by studies of clots made from γ'/γ' fibrin, which lacks the AGDV binding site for αIIbβ3 on the γ-chain C-terminus, since there was no difference in platelet spreading on the surface of film from clots made of γ'/γ' fibrin or WT fibrin. TNF-α-treated cells showed denser clot and less fibrin film coverage than control cells, but there was no significant difference in clot density and film coverage of clots on top of LPS and IL-1β treated cells. Lag time was shorter in clots formed on top of all inflamed cells compared to control cells, and this was most significant for TNF-α treated cells.
Conclusion:
Fibrin film impedes platelet adhesion and spreading, likely because the platelet-fibrin binding site (αIIbβ3) on fibrin is not exposed on the surface of the fibrin film. Reduced film formation under thromboinflammatory conditions may support continued clot growth by supporting platelet adhesion.

Abstract Number - P03

Regulation of glucose uptake by platelets

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Background: It is established that the metabolism of glucose is critical for ATP production that supports several key platelet functions. While it is known that platelets express GLUT 1 and GLUT 3 transporters, the mechanisms that drive glucose uptake under different conditions is unclear.

Aims:

In the present study, we wished to determine the mechanisms that facilitate glucose uptake in platelets under quiescent and activated conditions.

Methods:

We used 2-(Nitro-2,1,3-benzoxadiazol-4-yl)-D-glucosamine (2-NBDG), a fluorescent glucose analogue, to monitor glucose uptake into washed platelets by flow cytometry.

Results:

Washed platelets were found to accumulate glucose under resting conditions. Stimulation with thrombin (0.01-0.1U/ml) or the GPVI agonist convulxin (50 – 500ng/ml) led to a concentration dependent increase in glucose uptake. Focusing on GPVI, we found that convulxin induced glucose uptake was ablated by inhibition of the key signalling enzymes, Src, Syk or PI3kinase. We next examined the role of secondary mediators and found that apyrase and indomethacin abolished glucose uptake, while in contrast inhibition of integrin aIIbb3 with tirofiban had no effect. The uptake of glucose requires the maintenance of a concentration gradient created by the conversion of glucose to glucose-6-phosphate by hexokinase. We found that convulxin activated platelet hexokinase and that inhibition of its activity blocked glucose uptake.

Conclusions:

These data demonstrated that GPVI signalling increases the uptake of glucose and PI3K/Akt and hexokinase inhibitors suggest a crosstalk between glucose metabolism and GPVI pathway

Abstract Number - P04

Platelet dependent priming of monocyte inflammatory phenotypes

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Background:
High levels of platelet-monocyte aggregates (PMAs) have been observed in sepsis and major trauma with these levels associated with negative clinical outcomes. It has previously been suggested that platelets can induce changes in monocyte phenotype, but it remains unclear whether this is a patho-physiologically detrimental interaction, or a protective component of the physiological innate immune response.
Aim:
To compare the monocyte phenotypes following stimulation of whole blood with a thrombotic trigger or pathogen associated molecular patterns (PAMPS).
Methods:
Whole blood, obtained from healthy human volunteers, was stimulated with the platelet GPVI specific ligand collagen related peptide (CRP-XL), TLR4-ligand lipopolysaccharide (LPS) or TLR2-ligand PAM3CSK4 at 37C for up 4 hours. PMA formation, proportion of CD14/16 monocyte subtypes (classical/ intermediate), intracellular cytokine content (tumour necrosis factor alpha-TNFa, monocyte chemoattractant protein - MCP1, interleukin 6 - IL6) and tissue factor expression were all measured using flow cytometry.
Results:
Platelet-GPVI stimulated whole blood resulted in a rapid (<10 mins) PMAs formation with greater than 70% CD14+ monocytes decorated with platelet material. This PMA formation was accompanied by a 10-fold expansion in the pro-inflammatory intermediate subtype. No acute changes PMA formation or expansion in intermediate subtype were present in LPS-stimulated whole blood. Similarly, surface tissue factor expression significantly increased on PMAs but not unbound monocytes. Finally, differential intracellular cytokine production patterns were observed. CRP-XL, not LPS or PAM3CSK4, caused MCP1 expression in CD14+ monocytes; CRP-XL and LPS, not PAM3CSK4 in TNFa expression, and LPS and PAM3CSK4, not CRP-XL, in IL6 expression.
Conclusion:
These data strongly support the hypothesis of thrombotic priming of the innate immune response, however further downstream functional characterisation is required to discern protective and detrimental traits.

Abstract Number - P05

Inositol-1,3,4,5-tetrakisphosphate (IP4) is a negative regulator of platelet activation

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Background:
Inositol-1,3,4,5-tetrakisphosphate (IP4) is formed from inositol-1,4,5-trisphosphate (IP3) by IP3 -3 kinase. Its function is not established but has been suggested to be involved in Ca2+ entry, IP3 regulation and PI-3K manipulation. In order to explore its role further studies were carried out using a specific inhibitor of IP3-3kinase (ITPKI) and activation reactions in saponin permeabilised platelets

Methods:
Fresh human platelets isolated using local ethical guidelines, and prepared in plasma (PRP), or labelled with Fura2-AM for Ca2+ studies, or resuspended in a normal Hepes Tyrode buffer or one that has higher K+ and low Na+ for permeabilization studies. The effects of the ITPKI were investigated in PRP, in Fura2 labelled cells and in aggregometry experiments. Saponin permeabilised platelets were used for aggregation, phosphorylation of Akt-Ser473 and Rap1-GTP formation induced by a non-hydrolysable analogue of GTP (GTPγS). Rap1-GTP was estimated using RalGDS-RBD beads followed by Western blotting. Akt phosphorylation was estimated using Western blotting and aggregation was determined using light transmission. Extraction of RASA3 from platelet lysates was determined using PIP3-beads followed by western blotting.

Results:
In intact platelets ITPKI enhanced the aggregation seen by low concentrations of ADP, collagen, thrombin and U46619. In washed Fura2 labelled platelets the ITPKI at micromolar concentrations induced a transient elevation of Ca2+ . The inhibitor enhanced the peak height of Ca2+ elevated by all agonists tested. In permeabilised platelets IP4 inhibited GTPγS induced formation of Akt-Ser473 phosphorylation and platelet aggregation. IP4 also reduced GTPγS stimulated Rap1-GTP levels. IP4 was potently effective at inhibiting extraction of RASA3 by PIP3 beads.

Conclusions:
This study shows that in the presence of an inhibitor of IP3-3K there is an enhancement of Ca2+ elevation and aggregation induced by all agonists tested. Further in permeabilised platelets IP4 negatively regulates GTPγS stimulated Akt phosphorylation, Rap1 activation and platelet aggregation. IP4 potently inhibited PIP3 binding to RASA3 and other PH domain containing proteins. Thus IP4 may serve as a negative regulator of platelet function and this presents an approach to control platelet activation during thrombosis.
This study is supported by the British Heart Foundation grant (PG/19/72/34642).

Abstract Number - P06

pHrodo-labelled dextrans: An assay to study platelet endocytosis

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Background:
Platelets endocytose many molecules from their environment and traffic them into their granules. Key endocytic regulators such as dynamin, clathrin, CDC42 and Arf6 are expressed in platelets but their roles in fluid-phase endocytosis is not known. Although some small molecule inhibitors of these proteins are available, an assay is needed that can be used in platelets to evaluate the efficacy of these inhibitors.
Aim:
The aim of this study was to develop an endocytosis assay in platelets and assess the routes of endocytosis in the different subpopulations of platelets.
Methods:
Washed human platelets were treated with a range of different endocytosis inhibitors and stimulated using different activators. The rate of endocytosis was assessed using pHrodo green, a pH sensitive labelled dextran of 10,000kDa. pHrodo is essentially non-fluorescent in a neutral extracellular environment, fluorescing when internalised via endocytosis and trafficked to acidic intracellular vesicles. Platelets were incubated with 50mg/ml pHrodo green at 37°C for up to 90 minutes. The increase in fluorescence over time was assessed using flow cytometry and confocal microscopy.
Results:
We found that pHrodo-labelled dextrans can be used to assess the rate of endocytosis in platelets under different conditions. In unstimulated platelets, pHrodo fluorescence increased over time and accumulated as intracellular puncta. Stimulated platelets (both pro-aggregatory and pro-coagulant) had an elevated rate of endocytosis compared to unstimulated platelets. Dynamin inhibitors were shown to be effective in blocking endocytosis in unstimulated, pro-aggregatory and pro-coagulant platelets indicating that most platelet fluid-phase endocytosis is dynamin dependent, in all platelet subpopulations. However, although endocytosis was clathrin-independent in unstimulated and pro-coagulant populations, clathrin partially contributed to endocytosis in pro-aggregatory platelets.
Conclusion:
pHrodo green uptake is a simple and effective assay of platelet endocytosis. Using this assay, we have shown that the inhibition of dynamin effectively blocks endocytosis in all platelet subpopulations. This assay will be a useful tool for understanding the function and regulation of endocytosis in platelets.

Abstract Number - P07

Platelet Biogenesis from Mouse Megakaryocytes at Variable Shear Stress Levels by Using Microfluidic Systems

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Background:
An ageing population, haematological malignancies, and a critical shortage of registered donors have contributed to considerable strain and the requirement for readily available platelet stocks. Therefore, donor-independent platelet biogenesis circumvents complications associated with traditional allogeneic platelet donation and transfusions such as a short shelf-life and the risk of bloodborne infections.
Aim:
Two microfluidic systems have been designed for potential use in the generation of large quantities of platelets in a consistent and cost-effective manner. The initial system is primarily based on the structure of the pulmonary microvascular network, as it has been reported that platelet biogenesis can occur in the pulmonary capillary network. The second microfluidic system mimics better the physiological shear stress and pressure of the pulmonary capillary system.
Methods:
We fabricated the microfluidic systems from Polydimethylsiloxane and glass through soft lithography, simulating the performance through Computational Fluid Dynamics. Mouse megakaryocytes, differentiated from mouse bone marrow, were isolated, stained with anti-CD41, and then passaged, at different flow rates through each system a total of six times (inspired by preliminary experiments). The number of generated platelets was quantified using Flow Cytometry and the resultant platelets were examined by Transmission Electron Microscopy.
Results:
We obtained 450 Calcein+ and CD41+ platelets per megakaryocyte which is the highest value through the microfluidic system at 600 µl/min (shear stress between 0.042 Pa and 97 Pa). These platelets have a well-defined cell membrane and organelles including alpha- and dense granules, and open canalicular systems.
Conclusion:
We propose the use of microfluidic systems, particularly those that mimic pulmonary microvasculature, as a viable method for platelet biogenesis. The quantity and quality of the generated platelets demonstrate that they would be viable candidates for in vitro platelet biogenesis platforms and compare favourably with other systems previously reported in the literature.

Abstract Number - P08

Platelet Proteome Study in COVID-19

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Background:
Platelets, which are the smallest cells in human blood and play a crucial role in primary hemostasis, have also been found to interact with pathogens and contribute to the immune response. In severe cases of severe coronavirus disease 2019 (COVID-19), platelets become overactivated, exacerbating inflammation and contributing to a cytokine storm.
Aim:
This study aimed to further investigate the role of platelets in COVID-19 progression and identify predictive biomarkers for disease outcome.
Methods:
With this, we performed a comparative proteome analysis of highly purified platelet from severely diseased COVID-19 patients (survivors and deceased) who were invasively ventilated and, in most cases, also supported with extracorporeal membrane oxygenation (ECMO) as well as age- and sex-matched controls.
Results/Conclusion:
Platelets from the severely ill COVID-19 patients presented a distinctly different proteomic profile compared to the controls, with proteins associated with protein folding being prominent. In addition, a number of proteins with isomerase activity were found to be more abundant or uniquely present in patient samples, apparently affecting platelet activity through nongenomic properties of the glucocorticoid receptor (GR) and nuclear factor κ-light-chain-enhancer of activated B cells (NFkB). In addition, acute phase proteins and erythrocyte-related proteins were found at significantly higher levels in platelets from COVID-19 patients. Moreover, carbonic anhydrase 1 (CA-1) was found to be a candidate biomarker in platelets, showing a significant increase in COVID-19 patients.

Abstract Number - P09

SIRT1-mediated deacetylation regulates integrin αIIbβ3 and actin cytoskeleton dynamics through a decrease in talin-1 cleavage

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Background:
Sirtuin 1 (SIRT1) is an NAD+ dependent deacetylase with vasculoprotective properties. Patients at high risk of atherothrombosis, such as diabetics and obese, display reduced SIRT1 levels, which were associated with enhanced thrombus formation in a murine model of arterial thrombosis. However, the importance of SIRT1 in regulating human platelet function has not been evaluated.
Aim:
The objective of this study was to investigate the role of SIRT1-dependent deacetylation on platelet function.
Methods:
Platelet aggregation was studied through plate-based aggregometry. Flow cytometry was used to assess platelet activation (fibrinogen binding), α-granule secretion (CD62P) and dense granule release (CD63). Platelet adhesion and spreading were evaluated on collagen and fibrinogen-coated surfaces and Western Blotting was performed to determine protein expression and phosphorylation, as well as actin and tubulin polymerization.
Results:
SIRT1 expression was confirmed in human platelets and reduced platelet aggregation was observed in response to collagen following incubation with SRT1720 ($P<0.05$). Further evaluation showed that SIRT1 activation caused a moderate reduction in fibrinogen binding and α-granule secretion ($P<0.05$), but not dense-granule secretion. SIRT1-induced deacetylation inhibited platelet adhesion and spreading on both collagen and fibrinogen ($P<0.05$). Consistent with this, agonist-induced actin polymerization was reduced by SRT1720, but there was no effect on tubulin polymerization or coiling. Clot retraction was also perturbed by SIRT1-mediated deacetylation ($P<0.05$). Signalling studies revealed that SIRT1 activation decreases talin-1 cleavage, which is important for tethering integrin αIIbβ3 to the cytoskeleton.
Conclusion:
SIRT1 mediated deacetylation regulates platelet integrin αIIbβ3 activation and the subsequent cytoskeletal rearrangement through talin-1 cleavage. Reduced SIRT1 expression in diabetic and obese patients may contribute to their enhanced platelet reactivity. Therefore, SIRT1 activation could be a novel therapeutic approach to reduce thrombotic risk in these patients.

Abstract Number - P10

Venlafaxine and surveillance for induced platelet dysfunction. A case report.

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Background:
Venlafaxine is a frequently prescribed antidepressant. It works by inhibiting reuptake of serotonin (5-HT) and noradrenaline (NA) (selective serotonin reuptake inhibitor, SSRI). 5-HT and NA receptors also exist on the surface of platelets, and SSRI are considered to impair platelet aggregation.
Aim:
Herein, we report a case of a 54-year-old patient treated with venlafaxine, who presented with an hematoma in the anterior abdominal muscle.
Methods:
5 months after starting therapy with venlafaxine for moderate anxious depression, the woman went to the emergency department due to pain in the right iliac fossa. Abdominal ultrasound revealed a solid mass along the midline without a blood flow and a subsequent CT scan revealed an hematoma in the right anterior abdominal muscle. Clinical assessment of bleeding history (Bat score) and family history were found normal. Blood tests (including blood count, kidney, liver, and thyroid functions) were normal and no abnormalities of coagulation were detected. The platelet function investigation consisted of the platelet number and size using an automated cell counter, blood film report for platelet size and white cell inclusions, aggregation testing in platelet rich plasma with light transmission aggregometry by the use of 5 agonists (arachidonic acid, ADP, adrenaline, collagen and ristocetin)(Helena Biosciences Europe). Platelet count (236×109/L), MPV=9,4 fL and blood film were normal as well as von Willebrand factor activity and FVIII levels. The only finding was abnormal platelet aggregation in response to adrenaline and arachidonic acid.
Results:
Platelet function dysfunction due to SSRI is reported in the literature. As our patient was not taking any other medication known to alter platelet functions, idiosyncratic hypersensitivity to the platelet inhibitory effects of venlafaxine was suspected.
Conclusions:
In the group of patients prescribed SSRIs, such as venlafaxine, the rare side effect of impairment of platelet aggregation should be considered.

Abstract Number - P11

Systematic prioritisation of GWAS variants for platelet side scatter reveals candidate genes for cytoplasmic complexity.

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Background:
Genome-wide association studies (GWAS) of platelet count (PLT) and size (MPV) have revealed new genes relevant for platelet and megakaryocyte (MK) biology. However, additional platelet flow cytometry parameters measured in the full blood count may also be informative. Here we focus on the platelet side scatter parameter (PLT SSC), a surrogate marker of cytoplasmic complexity.
Aim:
To use statistical genetic approaches to annotate and prioritise GWAS variants associated with PLT SSC.
Methods:
We utilised previously reported GWAS data for PLT SSC adjusted for PLT and MPV captured using Sysmex automated haematology analysers in 39,656 blood donors from the INTERVAL study. Genomic loci independently associated with PLT SSC were linked to genes using Ensembl Variant Effect Predictor (VEP). Variants were annotated against putative regulatory elements in cord blood-derived MKs from the BLUEPRINT Epigenomes Project and colocalised with whole blood expression (eQTL) and plasma protein quantitative trait loci (pQTL). Linked genes were evaluated for biological plausibility by assessing variant impact on gene function and expression in MKs.
Results:
48 variants independently associated with PLT-SSC accounted for 13% of trait variance. 43/48 variants mapped to 45 genes which were enriched with Gene Ontology terms for secretory granule membrane and platelet activation. 32 of these genes were expressed in MK and 27 were reported to be associated with PLT or MPV in previous GWAS. 6 variants occurred in gene coding regions (IQGAP2, SVEP1, TUBB1, GP6, KCKN6). Non-coding variants were enriched in regions of open chromatin with histone modifications indicative of gene promoters. 40% of loci colocalised with cis-eQTLs, and 10% colocalised with plasma pQTLs. A pipeline for prioritising variants with the strongest evidence for a role in platelet/MK biology identified SLC35D3, TRIM58 and ZFPM2 as convincing candidate genes for PLT SSC.
Conclusion:
We present an approach for prioritising variants associated with the PLT SSC trait that utilises orthogonal evidence from open-access population genomics datasets. This pipeline identified genes with established roles in platelets but also novel genes. The later included SLC35D3, previously implicated in dense granule formation in mice, ZFPM2 which encodes a GATA cofactor, and TRIM58, which facilitates erythroblast enucleation. This in-silico approach enables gene prioritisation for functional evaluation in model systems.

Abstract Number - P12

Phosphatidylinositol-4-kinase alpha regulates integrin activation and cytoskeletal organization in human platelets

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Background:
Phosphatidylinositol-4-kinase alpha (PI4KA) converts phosphatidylinositol (PI) to phosphatidylinositol-4-monophosphate (PI4P), which further serves as the major source of phosphatidylinositol-(4,5)-bisphosphate (PIP2) at the plasma membrane (PM). PIP2 is known to be a crucial signalling molecule in mediating cytoskeletal reorganization during platelet adhesion and spreading. PI4KA has previously been shown to be highly expressed in human platelets (hPLTs).
Aim:
To determine the role of PI4KA in hPLTs activation and spreading.
Methods:
PI4KA was pharmacologically inhibited using a GSK-A1 inhibitor (100 nM). PI4KA expression and signalling pathways were analysed using Western blot. Platelets were spread on either immobilized fibrinogen or collagen to monitor: cytoskeletal organization and platelet morphology, PI4P/PIP2 levels at the PM using immunofluorescence (IF) and calcium flux in live-cells using Oregon Green BAPTA-1. Following PI4KA inhibition, hPLTs were also activated with Collagen Related Peptide (CRP) and monitored for activation status (P-selectin exposure, integrin αIIbβ3 activation) using flow cytometry and ATP secretion and aggregation using lumi-aggregometry, with the size and number of aggregates formed observed by light microscopy.
Results:
PI4KA inhibition resulted in a decrease in both PI4P and PIP2 levels at the PM, and decreased lamellipodia formation and spreading area as well as the impairment of F-actin and microtubule organization on both collagen and fibrinogen. Interestingly, PI4KA inhibition was also shown to decrease integrin αIIbβ3 activation but had no effect on alpha (P-selectin) or dense granule (ATP) release in CRP-activated hPLTs. Western blot signalling studies showed an increase in phosphorylation of Syk and PLCγ2, which was associated with shorter Ca^{2+} spike duration and higher amplitude in spread platelets. Finally, the inhibition led to a decrease in total aggregation, with the formation of smaller aggregates, in contrast to the larger aggregates seen in controls.
Conclusion:
The results demonstrate a critical role for PI4P precursor molecule in cytoskeletal rearrangements, with the decrease in its levels leading to impaired GPVI-mediated integrin αIIbβ3 activation and hPLTs spreading. The effect on integrin activation could explain the formation of smaller aggregates, possibly due to abnormality in later stages of platelet aggregation.

Abstract Number - P13

Impaired Platelet Activation Combined with Increased Platelet Leukocyte Aggregate Formation in Murine Biliary Liver Fibrosis

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Background:
Liver fibrosis is characterized by the accumulation of extracellular matrix proteins, including collagen, that occurs in most types of liver disease including chronic cholestatic liver injury. Advanced fibrosis can compromise normal liver function and result in cirrhosis and hepatocellular carcinoma. Mice with a targeted disruption of the Mdr2 (Abcb4) gene develop a cholestatic liver disease resembling human primary sclerosing cholangitis with chronic periportal inflammation and fibrosis progressing to cirrhosis. Liver macrophages are key cells to maintain liver homeostasis that can promote progression or reversal of both acute and chronic liver diseases. Besides their role in hemostasis, platelets are important players in immune responses and have been shown to interact with circulating and tissue resident immune cells. However, the consequences of the interplay between platelets and innate immune cells in the development/resolution of liver fibrosis is incompletely understood.
Aim:
The aim of this project is to investigate the role of platelet leukocyte interactions in the development of liver fibrosis.
Methods:
Platelet activation, platelet receptor expression and platelet-leukocyte aggregate (PLA) formation were measured by flow cytometry using whole blood from Mdr2-/- mice. Liver tissue was analyzed by immunofluorescence staining for liver macrophages (F4/80+ derived from monocytes and CLEC4F+ resident Kupffer cells), and immune cell influx was characterized by flow cytometry.
Results:
Analysis of blood from Mdr2-/- mice revealed significantly increased platelet count and size. At the same time, platelet expression of GPVI was reduced. Moreover, Mdr2-/- platelets exhibited reduced activation and platelet-leukocyte aggregate (PLA) formation upon stimulation with collagen-related peptide. Immunofluorescent staining and flow cytometry showed an infiltration of monocyte-derived macrophages (F4/80hiCD11b+TIM-4neg), monocytes, neutrophils and CD3+CD8+ T cells into the liver of Mdr2-/- mice. Moreover, intrahepatic platelet accumulation was increased in Mdr2-/- compared to control mice. Importantly, there was an enhanced colocalization between platelets and liver infiltrating macrophages and Kupffer cells in Mdr2-/- livers.
Conclusions:
Taken together, we observed significantly altered platelet morphology and functionality in Mdr2-/- mice as well as an increased immune cell infiltration and platelet-macrophage interactions in the liver.

Abstract Number - P14

The Aspergillus fumigatus metabolite gliotoxin disrupts platelet activation and NETosis induction

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Background:
Gliotoxin is an epipolythiodioxopiperazine (ETP) metabolite produced by the pathogenic fungus Aspergillus fumigatus upon invasion from lung into the blood stream. Gliotoxin has attracted attention due to diverse antimicrobial, anticancer, and pro-apoptotic effects. The ETP structure of gliotoxin contains a disulphide bridge that facilitates cellular uptake and induces apoptosis. In addition, gliotoxin can form covalent disulphide bonds that disrupt proteasomal activity and zinc release, which are critical events in the formation of neutrophil extracellular traps (NET). Platelets are increasingly recognised to play roles beyond thrombosis and haemostasis, including direct influences on immune cells via a mechanism known as immunothrombosis. In particular, platelets are known to release several factors that induce 'NETosis', a mechanism whereby activated neutrophils release DNA, histones, and antimicrobial proteins into the extracellular space to defend against infection, while also promoting coagulation and thrombosis.
Aim:
We investigated the effects of A.fumigatus-derived gliotoxin on platelet activation and the ability of platelets to modulate NETosis.
Methods and Results:
Gliotoxin reduced TRAP6- and Collagen-induced platelet aggregation in a concentration-dependent manner, while also limiting TxA2 release (measured by ELISA) and decreasing expression of activated αIIbβ3 and P-selectin on the platelet surface (measured by FACS). When whole blood was stimulated in presence of gliotoxin, the formation of platelet-neutrophil complexes (measured by FACS) was significantly reduced. Pre-incubation of washed platelets with gliotoxin (prior to activation with Collagen/TRAP/ADP) significantly impaired platelet-induced NET formation, as visualised by epifluorescent microscopy and confirmed by reduced MPO-DNA complexes measured by ELISA.
Conclusions:
We show that the A.fumigatus metabolite gliotoxin impairs platelet aggregation, granule release and TXA2 production, and reduces platelet-induced NETosis. Gliotoxin's characteristics could be exploited to synthesise structurally related analogs to be tested in immunothrombotic settings.

Abstract Number - P15

Influence of platelets on monocyte phenotype and function

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Background:
Several studies have suggested that binding of activated platelets to monocytes results in a more pro-inflammatory and migratory phenotype, leading to a potentiated inflammatory response. Despite prognostic indications for higher numbers of platelet-monocyte complexes (PMC) relating to negative clinical outcome in a range of diseases, characterisation of these phenotypic changes remains limited, and the functional implications are yet to be fully elucidated.
Aim:
Here we aim to characterise the impact of platelet and monocyte interactions on monocyte function and phenotype.
Methods and Results:
Platelet-monocyte complexes were formed in vitro by incubating whole blood, or isolated monocytes with washed platelets, with collagen related peptide (CRP; 1µg/ml, 37°C, 1 hour). In whole blood, platelet GPVI-specific stimulation resulted in rapid upregulation in surface expression of CD16 and CXCR4 ($p<0.05$ for both) on CD14+ monocytes and down regulation of CCR2 ($p<0.05$); consistent with adoption of pro-inflammatory phenotype. Isolated PMCs, compared to isolated monocytes, appear to adhere more readily under flow to ICAM-1 ($p=0.0517$) and demonstrate altered migration to monocyte chemoattractant protein 1. Furthermore, increased abundance of PMCs was associated with enhanced monocyte extracellular trap (MET) formation in response lipopolysaccharide (LPS; 50ng/ml).
Conclusion:
Our preliminary data suggests that binding of activated platelets, and the formation of platelet-monocyte complexes shifts monocytes towards a more pro-inflammatory phenotype. Additional work is required to explore these changes and to further characterise platelet-monocyte complexes.

Abstract Number - P16

Linking platelet acetyl-CoA carboxylase and inflammatory response in septic patients

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Background:
Sepsis is characterised by major endothelial dysfunction, microvascular alterations, and coagulopathy. Platelets promote immunothrombosis by generating cytokines and lipid mediators of inflammation. Previously, we observed that platelet acetyl-CoA carboxylase 1 (ACC1), responsible for de novo lipid synthesis, is phosphorylated and thus inhibited by AMP-activated protein kinase (AMPK) upon thrombin stimulation, resulting in platelet lipidome changes.
Aim:
To investigate the role of AMPK-ACC signalling in the regulation of the platelet lipidome during sepsis and its impact on inflammation.
Method:
Platelets and plasma were isolated from 48 septic and 48 control patients. In parallel, WT and DKI mice injected intraperitoneally with lipopolysaccharide were used as an endotoxemia model. DKI mice express a mutated form of ACC that is no longer phosphorylatable by AMPK. ACC1 phosphorylation/expression levels in platelets were measured by western blot. Lipidomic analysis was performed by untargeted liquid chromatography-mass spectrometry or on the commercial Lipidyzer platform. Plasma factors were measured by Enzyme-linked immunosorbent assays (ELISA).
Results:
ACC1 expression and phosphorylation in platelets was increased in both septic patients and mice. Lipidomic analysis revealed significant changes in lipids in human and murine platelets during sepsis, with a substantial reduction in phosphatidylcholines and phosphatidylethanolamines containing long polyunsaturated fatty acid chains (ω3 and ω6). These phospholipids are crucial for the generation of pro- and anti-inflammatory lipid mediators. In addition, our data suggest that elevated ACC phosphorylation is associated with a higher content of these phospholipids as well as a plasma reduction in myeloid inflammatory parameters. Consistent with this, ACC DKI mice have higher levels of LPS-induced inflammatory cytokines.
Conclusion:
Our data reveal that critical changes in the platelet lipidome occur during sepsis and might contribute to the pathophysiology of the disease. Our results also suggest that ACC status can influence the lipid composition of platelets as well as the inflammatory response of subjects.

Abstract Number - P17

Triggering ACKR3 and CXCR4 heterodimerization in platelets by targeting ACKR3

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Background:
Platelets are critically involved in regulating inflammation in various disease states and therefore have a variety of surface receptors regulated by coagulation factors and mediators like chemokines. One prominent receptor is the C-X-C chemokine receptor 4 (CXCR4) promoting cell migration and autocrine platelet activation. Atypical chemokine receptor 3 (ACKR3, formerly CXCR7) functions as counterpart to CXCR4 and promotes platelet survival, decreases platelet activation, and attenuates thrombus formation. Since ACKR3 lacks a functional G-protein binding domain, it has been shown to mediate signaling via β-arrestin recruitment but also via direct phosphorylation by G protein-coupled receptor kinase 2 (GRK2). CXCR4 and ACKR3 have the potential to heterodimerize.
Aim:
Here we aimed to analyze the effect of CXCR4/ACKR3 heterodimerization in platelets.
Methods:
We established the immunofluorescence proximity ligation assay Duolink® (Sigma Aldrich) for analyzing platelet ACKR3 and CXCR4 heterodimerization. Effects on platelet activation, degranulation and survival were measured via flow cytometry. Furthermore, we investigated platelet aggregation using a light-transmission aggregometer and in vitro thrombus formation was observed via flow chamber experiments.
Results:
We used the proximity ligation assay to screen for CXCR4-ACKR3 heterodimerization modulating compounds. We found that activation of ACKR3 using a potent ACKR3 agonist significantly increases the formation of heterodimers. Interestingly, inhibition of direct ACKR3 phosphorylation by a GRK2 inhibitor also resulted in a significant increase in CXCR4-ACKR3 heterodimers. Co-incubation with both compounds enhanced heterodimer formation even further. Both, the ACKR3 agonist and the GRK2 inhibitor, revealed significant effects on platelet function resulting in decreased platelet activation and degranulation, reduced platelet aggregation and attenuated thrombus formation in vitro. In addition, platelet survival was increased after pre-incubation with the agonist as well as the inhibitor.
Conclusions:
We were able to show that targeting ACKR3 using an ACKR3 agonist or inhibiting ACKR3 -phophorylation triggers heterodimerization and increases platelet inhibition and therefore platelet survival. These findings indicate, that the formation of CXCR4-ACKR3-heterodimers is strongly dependent on ACKR3 and ACKR3 is able to reduce the platelet activating function of CXCR4 by heterodimerizing.

Abstract Number - P18

Characterising the enhancing effect of oestrogen on procoagulant platelet formation .

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Background:
Oestrogen containing medication is commonly taken as contraceptives and hormone replacement therapy (HRT). Therapeutic oestrogens are associated with increased risk of thromboembolism. Activated platelets form proaggregatory and procoagulant (PS-exposing) subpopulations. Studies on the effect of oestrogen on proaggregatory platelets has been variable with no clear outcome. Little is known about the effect of oestrogen on procoagulant platelet formation. Mitochondrial transition pore opening is key in procoagulant platelet formation, with inhibitors of procoagulant platelets such as cyclosporin A indicating a role for CypD. Oestrogen has been shown to promote CypD interaction with its regulator, OSCP, in rat brain mitochondria. Therefore, oestrogen may influence procoagulant platelet formation.
Aim:
Investigate whether oestrogen enhances procoagulant platelet formation and if so by what mechanism.
Methods:
Washed human platelets were stimulated using various platelet activators. Annexin V was used to detect PS exposure as a marker of procoagulant platelet formation and analysed by flow cytometry.
Results:
Oestrogens enhanced procoagulant platelet formation. In a screen of different physiological /pharmacological forms, we found that E2 (main form of oestrogen in HRT) and EE (pharmacological oestrogen in the combined contraceptive pill) showed the greatest enhancement. The enhancing effect was at concentration > 1 μM. Progesterone reversed the enhancing effect. In addition, treatment with cyclosporin A prevented E2-enhanced procoagulant platelet formation, indicating that this process is still regulated by CypD. Cellular Thermal Shift Assays (CETSA) revealed that E2 does not bind directly to CypD. Co-immunoprecipitation of OSCP-CypD is planned to determine whether oestrogen promotes this interaction in platelets.
Conclusions:
Oestrogens at high concentrations enhance procoagulant platelet formation. This enhancing effect required CypD and further implicates CypD as the key regulator of procoagulant platelets. Future research into drugs that influence CypD or its potential partners may provide future therapeutics against procoagulant platelets and therefore arterial thrombosis. The concentrations of oestrogen in this study are supraphysiological and suprapharmacological but over short periods. In future work we will investigate whether prolonged exposure to oestrogen at lower concentrations also enhances procoagulant platelet formation.

Abstract Number - P19

Risk factors for cardiovascular disease (CVD) alter the composition of the vascular extracellular matrix (ECM), promoting thrombogenicity

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Background:
The rupture or erosion of an atherosclerotic plaque exposes ECM proteins, leading to platelet adhesion and atherothrombosis. Studies to investigate thrombus formation and identify novel antithrombotic targets routinely use Type I HORM collagen for in vitro studies or vessels from young healthy mice in in vivo studies. These standard approaches fail to consider vascular dysfunction and its impact on ECM composition.
Aim:
The aim of this study was to identify changes in the ECM produced by human coronary artery endothelial cells (HCAECs) following treatment with common CVD risk factors and determine the impact on platelet function.
Methods:
HCAECs were cultured for 7-days then treated for a further 3-days with TNF-α or cigarette smoke extract (CSE). Decellularisation was performed, leaving an intact matrix for platelet adhesion, spreading and thrombus formation assays. The ECM generated by HCAECs was isolated and characterised using mass spectrometry. Proteomic analysis was performed using Proteome Discoverer, DAVID bioinformatics tool and Graphpad Prism.
Results:
HCAECs produced an abundant and consistent coverage of Type I and Type III collagen. Despite this, thrombi generated on the native matrix were significantly smaller compared with Type I HORM collagen ($P<0.01$) but more abundant ($P<0.01$), consistent with results from platelet adhesion assays. Platelet adhesion was enhanced ($P<0.05$) and significantly larger thrombi formed ($P<0.05$) on the ECMs associated with TNF-α treatment compared to control. ECM composition varied significantly following treatment with TNF-α and CSE. A reduction in structural collagens ($P<0.001$) and an upregulation of Galectin-9 ($P<0.01$) was observed, in addition to significant increases in coagulation proteins associated with the ECM, including tissue factor, plasminogen and Factor XIII ($P<0.0001$).
Conclusions:
Endothelial dysfunction significantly alters ECM composition, influencing platelet-matrix interactions and thrombogenicity. Understanding thrombus formation on ECMs representative of CVD may help to identify novel antithrombotic targets which disrupt thrombosis but not haemostasis.

Abstract Number - P20

Characterising the role of platelets in the host immune response to invasive candidiasis

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Background:
Invasive candidiasis is a nosocomial fungal infection with an associated mortality rate of up to 70%. C.albicans is the pathogen most commonly associated with infection and can invade tissues via the bloodstream. Platelets are increasingly being recognised for their role in immunity during infection, through their antimicrobial activity and interaction with leukocytes. Despite this, their role in C.albicans infections is largely unknown.
Aim:
The aim of this study was to elucidate whether platelets interact with the yeast and hyphal forms of C.albicans, activate, and release mediators that can fuel the immune response.
Methods:
Platelet-rich plasma was prepared from whole blood by centrifugation, before being added to yeast or hyphal forms of C.albicans. Platelet aggregation experiments were performed using a 96-well plate aggregometry assay, while platelet interaction with C.albicans was visualised using fluorescent microscopy. Markers of platelet activation were measured by flow cytometry and cytokines and other immune mediators released by platelets were measured using a multiplex assay.
Results:
Platelet aggregation was significantly enhanced by C.albicans at lower concentrations of thrombin receptor activating peptide 6 (TRAP-6) and Type I collagen ($P<0.05$). Microscopy demonstrated that platelets were aggregating around hyphae or yeast. When platelets where activated with low concentrations of TRAP-6, C.albicans enhanced alpha and dense granule secretion and the release of inflammatory and immune mediators DK-K1, sCD40L and IFN-α ($P<0.05$). The activation of platelets was partially abrogated in presence of TLR4 inhibitor TAK-242, thus suggesting that platelet immune response to C.albicans is at least in part mediated by the TLR4 receptor.
Conclusion:
Platelets, alongside leukocytes, may be first responders in invasive fungal infections and play a key role in innate immunity against fungal pathogens. Further work is needed to be able to dissect the platelet receptors and mediators involved, and to enable the discovery of new drug targets and improve the survivability of patients following infection.

Abstract Number - P21

A computational framework reveals the importance of the spatial distribution of PtdIns in the regulation of phosphoinositide recycling in platelets.

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Background:
Phosphoinositides are a group of interconvertible membrane-bound and soluble lipids that form a complex network of reactions that regulate many aspects of a platelets's response to its environment. Mathematical models have long been utilised to provide insight into such complex pathways where they can illuminate experimentally opaque steps. But, given the difficulty in measuring the dynamic changes to phosphoinositides mathematical models of the network have yet to be validated against consistent, time-dependent data.
Aims:
To develop a computational framework that can utilise time-dependent experimental data describing the phosphoinositides to shed light on the underlying dominant mechanisms of regulation.
Methods:
We utilise a new method to generate a large dataset that characterises the changes in five membrane bound phospoinositides and a single soluble inositol phosphate downstream of a platelet's GPVI receptor. Eleven mathematical models were developed, each capturing competing interpretations of the dominant mechanisms that regulate the phosphoinositide pathway. All models (in the form of ordinary differential equations) were inferred and tested against the experimental data using a computational Bayesian framework.
Results:
We found that while four out of eleven mechanisms were able to generate the data only one, that incorporates an additional pool of cytosolic PtdIns, is consistent with the data and able to successfully predict the effects of an inhibitor (GSK-A1). Using this model we predicted that changes in the shape and magnitude of the events that stimulate alterations in the phosphoinositides have differential effects on membrane species with PtdIns(4,5)P2 maintaining abundance in the face of changing levels of stimulation.
Conclusions:
We identified that the spatial distribution of PtdIns is an important regulator of phosphoinositide recycling in platelets and predict that measures of the ratio of PtdIns(4,5)P2 to PtdIns(4,5,3)P3 are indicative of differential levels of stimulation.

Abstract Number - P22

Structural and functional consequences of calpain-dependent cleavage of platelet connexin 62

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Background:
Platelets express multiple connexin isoforms that assemble as hemichannels at the plasma membrane or dock to form gap junctions with adjacent cells, facilitating release or transfer of bioactive molecules. Selective inhibition of connexin 37 (Cx37), Cx40 or Cx62 reduces thrombus formation in vivo and limits platelet activation in vitro. However, mechanisms that underpin platelet connexin activation remain unclear.
Aim:
To investigate the role of calpain-mediated proteolysis of Cx62.
Methods:
Immunoblotting was performed to assess levels of intact Cx62, calpain activation and the activation of key signalling molecules. Cx62 hemichannel activation was assessed by calcein efflux assay and dye transfer experiments were performed to evaluate gap junction activity during thrombus formation. Finally, we modelled the structural consequences of Cx62 cleavage using in silico prediction techniques.
Results:
Stimulation of platelets led to a reduced immunoblot detection of Cx62 that coincided with the appearance of a lower molecular weight band. We hypothesised that this was mediated by proteolytic cleavage and was blocked by chelation of either cytosolic or extracellular calcium. Cx62 cleavage was only detected in samples with active calpain-1. Sequence analysis predicted an N-terminal calpain cleavage site on the first extracellular loop. To probe the functional consequence of calpain cleavage, we designed a mimetic peptide using the predicted cleavage site (62Pept-NT) as a decoy substrate. Incubation with 62Pept-NT inhibited Cx62 cleavage and delayed calcein efflux from activated platelets without impacting platelet aggregation. Calpain-dependent cleavage of Cx62 was prevented following activation of PKA (protein kinase A) or PP2A (protein phosphatase 2A). Our decoy peptide reduced the extent of dye transfer between platelets in thrombi, suggesting that calpain may also regulate gap junction function. Finally, we modelled the impact of calpain cleavage on the structure of Cx62. Using in silico prediction tools, we modelled the cleaved and intact hemichannel and observed an increase of minimum pore diameter from 12.5 Å to 29.6 Å.
Conclusion:
Our data indicate a novel calpain-dependent mechanism for the gating of platelet Cx62. Activation of Cx62 promotes platelet activation and intercellular communication. These channels may therefore represent a potential antithrombotic target.

Abstract Number - P23

Increasing Receptor Expression and Cholesterol Levels Enhances Agonist-Independent Activity at the Platelet P2Y12 Receptor

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Introduction:
The platelet P2Y12 receptor (P2Y12R) plays a critical role in the feed-forward amplification of platelet responses in both haemostasis and thrombosis. This receptor is an established anti-platelet therapeutic target in the treatment of acute coronary syndromes (ACS). The P2Y12R displays a high degree of constitutive activity1. Patients with ACS exhibit altered expression of P2Y12R, and it is postulated that P2Y12R upregulation increases platelet hyperreactivity in patients2. Cholesterol-enriched domains, or lipid rafts, at the plasma membrane, are also critical for P2Y12R signalling3. We hypothesise that altering receptor expression and the cholesterol environment may affect both ligand-dependent and independent P2Y12R activity, investigated using a BRET based approach.

Methods:
Agonist-dependent and -independent P2Y12R activity in HEK293T cells was assessed using a BRET2-based G protein reporter system using varying amounts of receptor. Surface receptor expression of P2Y12R was assessed using ELISA. Plasma membrane cholesterol was enriched via incubation with cholesterol.

Results:
As expected ELISA assays showed an increase in surface receptor expression by increasing the amount of transfected P2Y12R. Constitutive P2Y12R activity as assessed by measuring netBRET which was significantly enhanced by increasing the level of receptor expression ($F(3.385), p<0.05$). This increased receptor expression was accompanied by an increased response to the inverse agonist ticagrelor (Emax was 0.114 ± 0.218 and 0.0713 ± 0.115 A.U. in 2µg and 0.5µg of P2Y12R respectively, one-way ANOVA,N=3) and a decreased response to the agonist ADP. Enrichment of membrane cholesterol also enhanced P2Y12R constitutive activity which was accompanied by increased ticagrelor responsiveness.

Conclusion:
Increasing the surface expression of the P2Y12R enhances ligand-independent activity of P2Y12R. This further supports the hypothesis that upregulation of P2Y12R expression as seen in ACS patients may be linked to platelet hyperreactivity. Enriching membrane cholesterol also increases agonist-independent P2Y12R activity. Ongoing studies in human platelets aim to further our molecular understanding of ligand-dependent and independent P2Y12R activity and its implication in patient pathology.

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Abstract Number - P24

Priming of maternal platelets during pregnancy – the role of pregnancy specific beta-1-glycoprotein 11 in platelet activation

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Background:
Pro- and anticoagulant mechanisms play an important role during gestation to guarantee a successful implantation of the blastocyst, but also when it comes to term to avoid haemorrhage. Pregnancy is a state of hypercoagulation and platelets underlie certain dynamic changes during pregnancy, like a decrease of the platelet count towards the end of pregnancy.
Previous studies revealed that adherence of maternal platelets to the placental villous surface is a common process even in very early stages of gestation, and therefore a tightly regulated cross talk between platelets and the placenta seems to be of high importance.
Pregnancy-specific glycoproteins (PSGs) are the most abundant trophoblast-derived proteins in the maternal blood during human pregnancy and several studies indicate that PSGs play a critical role in the regulation of the immune response and platelet activation.
Aim:
Here, we test the hypothesis whether the pregnancy-specific glycoprotein 11 (PSG11) is selectively taken up by platelets and whether platelet priming is a crucial process during pregnancy.
Methods:
Platelets were isolated from whole blood samples of either healthy pregnant women in the first and third trimester, pregnant women suffering from preeclampsia or healthy non-pregnant women. Afterwards, isolated platelets were subjected to proteomics analysis as well as to RNA Sequencing in order to elucidate dynamics changes of the platelet proteome and transcriptome over the course of gestation and in case of preeclampsia. Furthermore, isolated platelets from non-pregnant women were incubated with plasma from healthy pregnant women or with supernatant from placental tissue cultures and subsequently analysed on protein level as well as via electron microscopy.
Results:
Our proteomics data showed an abundance of PSG11 in platelets from pregnant women and an accumulation over the course of pregnancy. These results could be verified via Western blot and immunohistochemistry. Furthermore, we could detect PSG11 in platelets from healthy non-pregnant women after incubation with plasma from pregnant women.
Conclusion:
Our data suggests that platelets sequester increased concentrations of PSG11 over gestation, which tempts us to speculate that platelet priming by placenta-derived factors is a common process during gestation and might play an important role in the tightly regulated platelet activation.

Abstract Number - P25

Thyroid hormones and analogues promote the acute release of platelets from megakaryocytes: from blood donor biology to the production of platelets in vitro

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Background:
Many improvements have been made over the past decade in the safety and quality of platelet units intended for transfusion but significant problems still remain. Often there is no alternative for severely ill patients but to be given allogenic platelets which have very limited efficacy and uncertain immunomodulatory responses and so there is a critical need for alternative treatments. Unfortunately, our current knowledge of thrombopoiesis is limited and the mediators that are directly involved remain elusive.
Aims:
To elucidate soluble factors in the blood that promote platelet formation and can be used as therapeutic treatments or to upscale in vitro platelet production for transfusion purposes.
Methods:
19 plateletpheresis donors who regularly donate we recruited to analysis the dynamics of platelet recovery post-acute loss of platelets. Full blood counts were performed on various timepoints before and after donation to identify the most relevant timepoints to analyse further. Metabolomic, proteomic and cytokine/chemokine/growth factor analyses were performed on the plasma/serum of these donors to identify differentially expressed analytes in these relevant timepoints compared to baseline levels. These analytes were screened in platelet production assays to identify novel targets.
Results:
Using metabolomics and proteomics screening methods, thyroid hormones have been identified as potent mediators of platelet production. Triiodothyronine (T3) as well as thyroid hormone analogues, GC-1 (Sobetirome), KB2115 (Eprotirome) and MGL-3196 showed a significant effect on platelet production, as well as proplatelet formation in vitro in both primary derived- and iPSC derived-megakaryocytes. These platelets that are produced under the influence of thyroid hormones are functionally active, showing degranulation (P-selectin exposure) and incorporation into thrombi.
Conclusions:
We have identified for the first time that thyroid hormones directly promote platelet production which offers very interesting therapeutic opportunities.

Abstract Number - P26

Attenuation of hyperglycaemic-induced increase in platelet-derived reactive oxygen species (ROS) and procoagulant activity by polyphenols

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Background:
Polyphenols have reported benefits at reducing cardiovascular risk, including modulating glycaemic response. However, the effects of these compounds on platelet-derived reactive oxygen species (ROS) and procoagulant activity in hyperglycaemia are unknown.
Aim:
To investigate the effect of polyphenols on platelet ROS, mitochondrial density, procoagulant phenotype and fibrin clot structure under normo- and hyperglycaemic conditions.
Methods:
Selected polyphenols (resveratrol, hesperetin, epigallocatechin gallate (EGCG) and quercetin), shown to modulate glycaemic response in vitro, were analysed. Platelets were isolated in buffer containing 5 or 25mM glucose then treated with polyphenols (20μM; 1hr). Platelet ROS was measured using DCFDA cellular ROS kit in isolated platelets and mitochondria density determined using the citrate synthase assay in platelet lysates. Confocal microscopy was used to determine procoagulant platelet number (PP; annexin-V) and fibrin fiber density (AlexaFluor488 fibrinogen) in platelet-rich plasma (PRP) clots in normo- (5mM glucose; 20mins) and hyperglycaemia (25mM glucose; 20mins). Clot pore size was determined by permeation in PRP under the same conditions. Tyrosine kinase inhibitors (PRT-060318, ibrutinib and dasatinib) shown to alter clot structure and PP number were used in bioenergetic analysis as a comparator.
Results:
Hyperglycaemia increased platelet ROS and PP number. Platelet ROS was decreased by resveratrol, quercetin and EGCG in normo- (43±15%, 60±9% and 49±15%, respectively) and hyperglycaemia (72±7%, 71±15% and 57±12%, respectively). Quercetin and EGCG reduced mitochondrial density by 36% by (±11 and 3%, respectively). In normoglycaemia, PRT-060318 and dasatinib decreased platelet ROS (38±8% and 34±19%, respectively). Tyrosine kinase inhibitors showed no significant changes in ROS in hyperglycaemia or in mitochondrial density under either condition. Resveratrol reduced PP number in normo- (22±3 and 39±7 for resveratrol and control, respectively) and hyperglycaemia (32±5 and 48±5 for resveratrol and control, respectively). Polyphenols showed no significant effects on fibrin fiber clot density or clot pore size.
Conclusion:
Our data show that polyphenols attenuate platelet bioenergetics and procoagulant activity in normo- and hyperglycaemia. These findings may have important implications for strategies aimed at preventing and decreasing thrombosis risk in patients with diabetes.

Abstract Number - P27

PLATELET ACTIVATION INDUCED BY CpG-RICH OLIGODEOXYNUCLEOTIDES: THE INVOLVEMENT OF THE C-TYPE LECTIN RECEPTOR CD93

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Background:
CpG-oligodeoxynucleotides (CpG-ODNs) are short single-stranded synthetic DNA molecules containing unmethylated CpG motifs, which mimic bacterial DNA and act as pathogen associated molecular patterns (PAMPs). CpG-ODNs exhibit potent immunostimulatory properties in vertebrates and are extensively exploited in clinical trials as adjuvants for new anticancer therapies and vaccines. As other PAMPs, CpG-ODNs induce platelet activation, but very little is known on the molecular mechanisms responsible for this process. Platelets are emerging players in innate immunity and express several pathogen recognition receptors (PRRs) involved in pathogen sensing and immune response. The C-type lectin (CTL) receptor CD93 is a type I transmembrane glycoprotein which plays a prominent role in inflammation, angiogenesis, and cancer. In nucleated cells, the CTL domain of CD93 has recently been shown to bind CpG-ODNs, suggesting a possible function for CD93 as a novel unidentified PRR.
Aim:
In this study we have investigated the role of CD93 in platelet activation induced by CpG-ODNs.
Methods:
Type C CpG-ODNs (ODN2395) were selected for this study and their effect on control wild type (WT) and CD93-knockout (KO) murine platelets was investigated. Integrin αIIbβ3 activation and granule secretion were analysed in whole blood by flow cytometry. Platelet aggregation and protein phosphorylation were analysed on purified platelets by light transmission aggregometry and immunoblotting, respectively.
Results:
CpG-ODNs induced aggregation of WT platelets, accompanied by integrin αIIbβ3 activation and α-granule release. Platelets lacking CD93 exhibit a significant impairment in α-granule secretion, whereas integrin activation and platelet aggregation were only marginally affected. Immunoblotting analysis revealed that the CpG-ODNs stimulated the tyrosine phosphorylation of different signalling proteins in WT platelets, including PLCγ2 and Pyk2. These responses were significantly defective in the absence of CD93. Moreover, activation of the AKT/GSK3 axis and of the MAP kinases cascade by CpG-ODNs were markedly dependent on CD93 expression.
Conclusion:
CD93 is involved in platelet stimulation induced by CpG-ODNs by regulating the activation of protein kinase-dependent pathways. Further studies are required to clarify the relevance of CD93 in functional platelet responses to CpG-ODNs in thrombosis and immunity.

Abstract Number - P28

CD36 mediated uptake and utilisation of fatty acids in platelets.

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Background:
As platelets circulate in the blood, they require the mitochondrial oxidation of glucose and fatty acids (FA) to meet the energy demands of survival and vascular surveillance. Little is currently known about the uptake and metabolic processing of FA.
Aims:
We sought to understand FA uptake, trafficking, and utilisation in blood platelets, and to study downstream effects on platelet metabolism and biology.
Methods:
We used a combination of fluorescent flow cytometry (FFC), confocal laser scanning microscopy (CLSM), bioenergetic measurements (Seahorse XF metabolic analyser) and light-transmission aggregometry (LTA) to profile the platelets in response to FA.
Results:
The incubation of washed platelets with FA analogue BODIPY™ 558/568 C12 (1-10 μM) led to a concentration dependent uptake of FA, incubation of platelets with thrombin or GPVI agonists did not significantly increase uptake. Using CLSM it was observed that FA accumulated within the cells and co-localised with mitochondrial markers. To examine the effects of internalised FA on platelet function, platelets were treated with the C16 FA palmitate (0 -100 μM). Bioenergetic studies indicated that palmitate increased both basal and maximal respiration, with negligible effects on glucose uptake and glycolysis. The increase in mitochondria activity was associated with an increase in mitochondrial membrane potential ($\Delta m\Psi$), measured by FFC, an effect that was blocked by the CPT inhibitor etomoxir. In contrast, mitochondrial superoxide and total ROS were not affected by palmitate supply. Pharmacological blockade of CD36 with established inhibitors led to a reduction in the palmitate-mediated increase in respiration suggesting that CD36 may be the responsible fatty-acid transporter on platelets. Palmitate, in contrast to the CD36 ligand oxLDL, did not induce CD36 signalling suggesting that the effects on metabolism were likely due to FA uptake. The reprogramming of platelet metabolism by FA through CD36 led to a change in sensitivity to GPVI, but not PAR, mediated platelet activation measured by LTA.
Conclusions:
The incubation of platelets with FA led to CD36 mediated uptake and resulted in increased mitochondrial respiration and elevated $\Delta m\Psi$ but did not alter ROS production or glucose uptake. This redressed state of metabolic balance led to a shift in platelet phenotype with reduced sensitivity to GPVI mediated platelet activation.

This project was funded by the British Heart Foundation.

Abstract Number - P29

TP and P2Y12 receptors display reciprocal regulation of surface expression.

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Background:
Dual antiplatelet therapy (DAPT) targeting both TP- and P2Y12-mediated pathways is standard following percutaneous coronary intervention (PCI) in coronary artery disease patients. Trial data remain inconclusive but suggest redundancy of aspirin in the presence of strong P2Y12 inhibitors such as ticagrelor or prasugrel [1].

Aims:
To explore potential interplay between the Gαq-coupled TP and Gαi-coupled P2Y12 receptor pathways.

Methods:
Ligand-induced calcium flux and platelet receptor surface expression was assessed in acute coronary syndrome (ACS) patients at the end of DAPT with ticagrelor, and again 4 weeks after cessation of P2Y12 inhibitor treatment(n=20). P2Y12 and TP receptor surface expression were assessed by flow cytometry. Receptor-receptor interactions were assessed via co-immunoprecipitation and densitometric Western blot. Receptor activity in HEK293T cells was assessed using a BRET2-based G protein reporter system.

Results:
Longitudinal comparisons of platelet responses from patients at the end of DAPT revealed significant reduction in U46619-induced, TP-mediated calcium flux with ticagrelor. Ticagrelor treatment was also associated with a significant increase in both P2Y12($p=0.0179$) and TP($p=0.0178$) platelet surface expression as assessed by flow cytometry. This was recapitulated ex vivo using healthy donor platelets, and in HEK293T cells where enhanced surface expression of each receptor was observed upon co-expression($p=0.0364$). This appeared specific, since no such enhancement was seen upon substitution of P2Y12 with the similarly Galphai-coupled muOR. Tagged, overexpressed P2Y12 and TP receptors were demonstrated to interact in reciprocal co-immunoprecipitation experiments, while TP receptor activity was significantly and specifically reduced following ticagrelor treatment of cells co-expressing P2Y12($p<0.0001$).

Conclusions:
Together, these data suggest co-trafficking and direct receptor-receptor interaction of P2Y12 and TP receptors which can be modulated through inhibition of P2Y12 by ticagrelor. These data further highlight the complex relationship between P2Y12 and TP receptor biology and help inform the debate on the merits of DAPT versus ticagrelor monotherapy in patients with acute coronary syndromes.

References:
1. Johnson T, Baos S, Collett L, Hutchinson JL, Aungraheeta R, Reilly-Stitt C, Mundell SJ, Baumbach A, and M

Abstract Number - P30

Assessment in vitro of potential effects of therapeutic oligonucleotides on platelet function

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Background:
Multiple antisense oligonucleotides (ASOs) have been developed and some approved for various indications. However, in toxicological assessment and clinical studies a dose- and sequence-dependent thrombocytopenia (TP) of varied severity has been reported. This has been mainly attributed to pro-inflammatory and platelet activating effects of the ASOs, leading to enhanced platelet sequestration.
Aim:
Accordingly, we aimed to establish reliable and high throughput platelet function assays that would aid safety assessment of ASOs earlier in drug development.
Methods:
Important reference agents are ODN2395, known to stimulate platelets in vitro, and inotersen, an ASO that produced TP in toxicology and clinical studies. We have used these compounds to develop and validate test systems suitable for screening for effects of ASOs on platelet function. Effects of the two compounds were assessed directly and in combination with collagen related peptide (CRP). Both platelet aggregation, using platelets in plasma and platelets isolated in protein-free buffer, and platelet activation in whole blood were used.
Results:
All three assays showed an increase in potency of CRP in the presence of ODN2395 and interest. Effects of the compounds quantified by EC50 shift suggest that aggregation in washed platelets provides the most sensitive assay to investigate the effects of ASOs on platelets, followed by aggregation in platelet rich plasma. The platelet activation assay appeared to be the least sensitive. However, since it is performed in whole blood on fixed samples, it can be easily applied to testing ex vivo in samples from pre-clinical and clinical studies of ASOs.
Conclusion:
Platelet function testing can be used to de-risk selection and progression, or as a tool to identify individuals at risk of ASO-induced TP. Availability of a range of sensitive, reproducible, plate-based test systems allows platelet function testing to be considered as part of a screening strategy.

Abstract Number - P31

Detection of hyperfunctioning platelets and novel phenotypic platelet subtypes

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Background:
Platelets are known to be heterogeneous in phenotype markers such as size, density and receptor content as well as their function with both highly reactive platelets as well as hard to activate platelet subtypes. However, while many clinical methods exist to detect hypofunctioning platelets, methodology for the detection of hyperfunctioning platelets is limited.
Aim:
We aim to design a highly polychromatic flow cytometry panel to detect platelet subtypes in hyperfunctioning platelets.
Methods:
We designed a novel multi-colour flow cytometry panel that utilises modern innovations in flow cytometers and new dyes to map distinct platelet phenotypes in hyperfunctioning platelets. Our ultimate goal is to identify clinically relevant subtypes of platelets that predict thrombosis risk more accurately in individuals with a higher risk due to smoking, obesity and diabetes so that anti-platelet treatment can be tailored to those patients with a clinically relevant high risk. Additional difficulty in designing a panel is the fact that some antibodies activate the platelets.
Results:
We designed an 19 colour panel with antibodies against CD9, CD29, CD31, CD32, CD36, CD41, CD42a, CD42b, CD47, CD49b, CD61, CD62P, CD63, CD107a, CD154, b2M, CLEC2, GPVI and PAC-1. This combination of markers has the potential to detect known and novel subtypes of platelets in healthy volunteers and can be used in patient samples in the future.
Conclusion:
Detecting multiple subtypes of platelets brings clinically relevant detection of hyperfunction of platelets a step closer and can lead us to find specific subtypes to target with anti-platelet treatment in the future.

Abstract Number - P32

Pim kinase inhibition attenuates platelet-leukocyte aggregate formation

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Background:
Thrombosis, the development of an occlusive clot, is the result of an interaction between various cell types within blood vessels. Platelets, besides their physiological roles in haemostasis and wound healing, actively modulate immune responses via leukocyte interaction, playing pathological roles in inflammation and atherothrombosis. Platelet-leukocyte aggregates are heterotypic combinations of at least one platelet with one leukocyte, primarily formed through platelet surface expression of p-selectin binding to p-selectin glycoprotein ligand 1 on activated leukocytes and are essential for endothelial leukocyte recruitment. Platelet-leukocytes represent markers of thrombo-inflammatory diseases, particularly cardiovascular disease.
Aim:
Pim kinases have been shown to modulate platelet function, however their role in platelet-mediated leukocyte recruitment and interaction is unknown. We therefore aimed to determine the regulatory role for Pim kinase in platelet-leukocyte aggregation.
Methods:
Whole blood was stimulated with platelet agonists U46619, and TRAP-6 and treated with ADZ1208, a pan Pim kinase inhibitor. Platelet and leukocyte activation and platelet-leukocyte aggregation were quantified using flow cytometry and fluorescently labelled antibodies against platelet and leukocyte surface antigens, namely CD45 (leukocytes), CD66b/CD11b (neutrophils), CD14 (monocytes), and CD42b/CD62P (platelets).
Results:
After blood stimulation with U46619 (10uM) and TRAP-6 (10uM), ~40% of neutrophils and monocytes were found within platelet-neutrophil (CD66b+CD42b+) and platelet-monocyte (CD14+CD42b+) aggregates. Interestingly AZD1208 treatment was not observed to reduce percentage of neutrophil or monocyte-platelet aggregates formed but did reduce the median fluorescence intensity of CD42b in both U46619 and TRAP-6 stimulated platelet-neutrophil and platelet-monocyte aggregates, indicating reduced platelet-leukocyte binding. This inhibition in binding was also associated with and could be explained by AZD1208-mediated reductions in platelet P-selectin exposure, and reduction in surface expression levels of neutrophil/monocyte activation markers.
Conclusions:
These findings identify a potential role for Pim kinase in mediating platelet-leukocyte interaction and indicates that Pim kinase inhibitors could be repurposed for use alongside anti-thrombotic agents for the prevention of inflammation-driven thrombosis and cardiovascular-related events.

Abstract Number - P33

Quercetin, isoquercetin and zafirlukast enhance the actions of the P2Y12 antagonist Cangrelor

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Background:
Inappropriate activation of platelets leads to arterial thrombosis leading to myocardial infarction and ischaemic stroke, and treatments reducing thrombosis can cause unnecessary bleeding. Previous studies have shown that the flavonoids quercetin and isoquercetin, and the leukotriene receptor antagonist, zafirlukast, inhibit platelet function through related mechanisms. Quercetin has been shown to inhibit the activity of a range of platelet kinases while isoquercetin and zafirlukast inhibits thiol isomerases such as protein disulphide isomerase (PDI).
Aim:
We sought to determine whether these agents, used in combination with the P2Y12 antagonist cangrelor, were able to enhance the effects of cangrelor allowing its use at lower concentrations.
Methods:
Platelet activation was measured in washed platelets, which were incubated with varying concentrations of either quercetin, isoquercetin, or zafirlukast and then stimulated with collagen. Fibrinogen binding and P-selectin exposure were measured by flow cytometry. Whole blood was used for thrombus formation in vitro where thrombus volume, height, and fluorescence intensity were measured. Western blot was used to determine the effects of the compounds on tyrosine kinase- and thiol isomerase-dependent signalling.
Results:
Platelet aggregation, fibrinogen binding and P-selectin exposure were reduced by each compound in a concentration dependent manner. Thrombus formation in vitro illustrated that compounds reduced thrombus volume, height, and fluorescence intensity up to 50%, with zafirlukast showing a greater efficacy. Western blot revealed differences in the level of inhibition of kinases and thiol isomerases, indicating which signalling pathways were modulated. Similar signalling profiles were observed with isoquercetin and zafirlukast, while distinct and potent effects on kinase signalling were observed with quercetin. In thrombus formation in vitro, pre-treatment with each agent synergised with cangrelor resulting it a greater efficacy at lower concentration.
Conclusion:
Our study indicates that selective inhibition of platelet thiol isomerases enhances the actions the P2Y12 cangrelor.

Abstract Number - P34

Optimisation of the Alt-R™ CRISPR-Cas9 System in imMKCLs to generate an in-vitro model for the evaluation of platelet disorder genes

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Background:
Various in vitro cell models for megakaryocytes (MK) are available, but challenges exist around genetic tractability, limited longevity of primary cell models, and the ability to extrapolate findings from immortal cell lines. The iPSC-derived immortalised megakaryocyte cell line (imMKCLs) from the Eto laboratory (Kyoto University, Japan)3 has emerged as a CRISPR amenable tool. However, as this is a recently established cell line there is limited experience of different CRISPR-Cas9 systems available to create tractable modified imMKCL models.
Aim:
To determine if the Alt-R™ CRISPR-Cas9 System was effective in the imMKCLs and to optimise the protocol to maximise editing efficiency using GP1BA and ITGA2B as model targets.
Methods:
Gene targeting was performed using the Alt-R™ CRISPR-Cas9 System in which recombinant Cas9 enzyme and chemically synthesised sgRNAs are delivered directly into the cell as a ribonucleoprotein (RNP) by nucleofection using the CRIMSON protocol from Montenont et al. Three sgRNAs for both GP1BA and ITGA2B were evaluated alongside a scrambled sgRNA control. Optimisation steps included altering the total amount of Cas9 nuclease, the Cas9 to sgRNA ratio, total quantity of RNP used and the electrical settings for nucleofection. Gene targeting was evaluated by Sanger sequencing and analysis with TIDE and Synthego software, and by flow cytometry to measure target protein expression with positive gates defined using unstained controls.
Results:
The three test sgRNAs all enabled editing of the target genes with efficiencies ranging from 16-87% in polyclonal nucleofected imMKCLs. Selecting the most efficient guide and optimisation of the protocol demonstrated a reduction of GP1BA expression; from 92% of control nucleofected imMKCLs expressing GP1BA reduced to 2.5% in the targeted cells, indicating efficient knockdown of GP1BA. Similarly, for ITGA2B 94% of control cells expressed ITGA2B compared to only 22% of targeted cells, representing a 72% reduction in ITGA2B expression, as determined by flow cytometry.
Conclusion:
We have validated the Alt-R™ CRISPR-Cas9 System as a viable and efficient method to edit imMKCLs and established a robust and optimised protocol for future gene editing. We have identified the importance of trailing a range of sgRNAs, as large variation exists between them. This methodology provides a robust means of generating clones, and future experimental study will focus on culturing single colonies.

Abstract Number - P35

A high-throughput, antibody-free method for measuring platelet activation and dense granule release by flow-cytometry

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Background:
Flow-cytometry is a useful tool for measuring platelet activation and the effects of inhibitors and drugs on platelet function. We have previously developed a method for high-throughput platelet function analysis using micro-titre plates with freeze-dried agonists and fluorescently labelled antibodies (Dunster et al, 2021). This method is ideal for measuring platelet responses in large cohorts or for screening panels of drugs, however the cost of the antibodies that are required is considerable and can even limit the feasibility of such studies.
Aim:
The aim of our study was to explore the application of this plate-based assay without the use of antibodies.
Methods:
We compared changes in Forward and Side Scatter with changes in activation markers such as fibrinogen binding, P-selectin exposure and CD63 exposure (measured using fluorescently labelled antibodies) and also mepacrine release.
Results:
We were able to use Forward or Side scatter measurements to plot dose-response curves for end-point assays and time-dependent activation in real-time flow-cytometry assays instead Median Fluorescence Intensity. This method can be used with undiluted Platelet-Rich Plasma (PRP), which reflects more closely platelet activation in vivo.
Conclusion:
We have adapted our plate-based platelet function assay for high-throughput use without the need for expensive antibodies. We are currently investigating the use of this method for drug screening purposes and for testing platelets from various clinical groups. If successful, this method would be a useful tool for identifying compounds that interfere with vesicle release and may have the potential to be developed as novel anti-platelet therapies.

Reference:
Multiparameter phenotyping of platelet reactivity for stratification of human cohorts. Dunster et al, 2021. Blood Adv 5: 4017-4030.

Abstract Number - P36

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 and over 500,000 cases/year globally. The highest case burden occurs in Sub-Saharan Africa, which accounts for over 75% of these cases, an increasing number of which 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.

Abstract Number - P37

Generation of universal platelets from HLA-null human pluripotent stem cell (hPSC) lines

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Background:
Alloimmunisation, the process of developing an immune response to "non-self" (foreign) antigens after exposure to genetically different cells or tissues, increases in occurrence in patients undergoing transplant or repeated transfusions; and in multiparous women. Human Leukocyte Antigens (HLA) expressed on cells, such as platelets, distinguish between "self" and "non-self" antigens. Patients that develop HLA-alloimmunisation and require transfusions to prevent bleeding, undergo HLA-typing to receive HLA-compatible platelets from a restricted pool of recallable donors. However, locating a perfect match is not always possible and partial matches result in shorter survivability of circulating platelets.
Aim:
Platelets are formed and released from Megakaryocytes (MKs). Our aim is to develop HLA-null hPSC lines to produce functional MKs, which would then generate 'HLA-universal donor' platelets. These platelets would circumvent alloimmunity concerns and help alleviate the cost of transfusion management for HLA-alloimmunised patients.
Methods:
CRISPR-Cas9n technology was employed with guides targeted to the β2-microglobulin (B2M) locus to generate HLA-knock out (KO, null) hPSC lines. Whole genome sequencing was performed to analyse the effect of CRISPR-mediated HLA-KO in hPSCs. MKs and platelets differentiated from HLA-KO hPSC lines by either doxycycline-induction or the forward programmed method (PMID 27052461) were characterised with flow cytometry and immunofluorescence microscopy.
Conclusion:
We have successfully generated HLA-null hPSC lines. These lines maintain pluripotency (TRA+/SSEA+) in chemically defined cultures and do not express HLA in the presence of interferon gamma (IFNγ), a known inducer of HLA proteins. Genome sequencing analysis reveals no obvious off-target effects or significant changes in mutation signatures. HLA-KO hPSCs can be differentiated into highly purified MKs that are polyploidy and generate proplatelets and platelets. Our work here progresses towards delivering an alternative and universal platelet source, with the potential of using these cells as site-specific delivery vehicles of components with added clinical benefits.

Abstract Number - P38

Creation of a murine myocardial infarction model for in vivo study of therapeutic effects of bio-engineered platelets

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Background:
Myocardial infarction (MI) is a major cause of mortality and platelets have been shown to play multiple roles during MI. Platelets participate in the thrombosis and haemostasis contributing to the coronary arterial occlusion and ischemia. In acute MI, activated platelets accumulate at sites of ischemic myocardium and release numerous soluble mediators which contribute to vasoconstriction and inflammatory processes in the ischemic myocardium. Beyond the known pathological role of platelets in MI, platelets also are increasingly recognised as potential key players have beneficial effects on the process of MI repair and remodelling.
Aim:
To test the potential therapeutic effect on MI of our bio-engineered platelets which are produced in vitro from iPSCs we created a murine MI model through surgical ligation of the coronary left anterior descending (LAD) branch and preliminary results showed this murine MI model is a valuable tool in study of many platelet-derived factors and their roles during MI inflammatory and remodelling process.
Methods:
Adult mice (8-10 week-old) were anaesthetised with isoflurane and subject to intra-tracheal intubation and ventilation. Left thoracotomy was carried out to expose the heart and the LAD was ligated with 6-0 suture to created ischemia to the left ventricle. The wound was closed and animals were allowed to recover with analgesia provision. Cardiac functions were monitor on 1 and 2 weeks post MI with echocardiography and ischemic injury of the left ventricle myocardium was assessed with histological staining of cardiac sections.
Results:
More than 90% of mice survived by the end of 2 week ischemia and heart function measured as ejection fraction (EF) and fractional shortening (FE) were reduced to 28% (EF) and 13% (FE), compared to normal values of 58% (EF) and 31% (FS), respectively. Immuno-histochemistry (IHC) staining showed massive platelet infiltration in ischemic myocardium 24h post MI, and extensive fibrosis and collagen accumulation in the damaged left ventricle 1 week post MI. In vivo labelled platelets were shown to concentrate to sites of infarcted tissue in the hearts of mice with induced MI. Also, donor platelets (derived from donor mice or engineered) could be labelled prior to intravenous infusion into mice at the time of induced MI, and could be detected in the ischemic myocardium by IHC 24h post MI.
Conclusion:
We have successfully created a murine model of MI

Abstract Number - P39

AICAR induced AMPK activation increases antioxidant capacity but activates platelets and reduces agonist reactivity during storage

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Background:
Human platelet concentrates (PCs) are used in transfusion medicine. In Belgium, these concentrates can be transfused up to 5 days after donation. During storage, platelets undergo platelet storage lesions (PSL) with the production of reactive oxygen species among other things. AMP-activated protein kinase (AMPK) is known for its antioxidant properties.
Aim:
The present study was designed to evaluate the impact of 5'-aminoimidasole-4-carboxamide-1-β-d-ribofuranoside (AICAR), an activator of AMPK, on oxidative stress and activation of platelets during storage time.
Methods:
Platelet bags were obtained from healthy blood donors of the Belgian Red Cross Blood Service. The platelet bags were prepared from 5 buffy coats from 5 different donors to obtain one platelet bag. Two platelet bags were mixed and then separated into two equal parts. 0.3 mM AICAR was added to the test groups. Normal saline was added to the control groups. AMPK and acetyl-coA carboxylase (ACC) phosphorylation/expression, activation and aggregation of platelets were assessed by standard methods at 3, 5 and 7 days of platelet storage. Antioxidant capacity was quantified electrochemically using disposable devices and expressed as ascorbic acid equivalent concentration.
Results:
Treatment of platelets with AICAR increased the phosphorylation of AMPK and its bona fide substrate, ACC, with maximal phosphorylation observed on day 3 and maintained until day 7. In terms of antioxidant power assessment, the mean results for pools of buffy coat-derived platelet concentrates were 36.50 ± 12.02 and 79.00 ± 0.00 μmol/L eq. ascorbic acid before and after AICAR treatment, respectively. However, treatment potentiated CD62p exposure on the platelet surface between days 3 and 7. In addition, thrombin- and collagen-induced aggregation was significantly reduced on day 5.
Conclusion:
Antioxidant capacity is significantly increased in AICAR-treated platelet concentrates. However, this is associated with increased platelet activation and impaired agonist reactivity after 3 days and 5 days of storage respectively, which calls into question the use of AMPK activators to reduce PSL during storage.

Abstract Number - P40

The effect of maternal platelets on the differentiation of extravillous trophoblasts

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Background:
During human placentation extravillous trophoblasts (EVTs) migrate into the maternal endometrium and form plugs within the lumen of uterine spiral arteries preventing maternal blood cells from entering the intervillous space. However, by the middle of first trimester, cells within trophoblast plugs become loosely cohesive leading to the formation of narrow capillary-sized channels. Due to their small size, maternal platelets may be the first amongst maternal blood cells to pass such narrow intercellular gaps of trophoblast plugs.
Aim:
After previously showing platelets in between EVTs in anchoring villi of a human first trimester placenta via electron microscopy, we aimed to investigate the hypothesis if platelets and release of platelet-derived factors into the intercellular space of EVTs play a significant role in their differentiation and behaviour.
Methods:
The human trophoblast cell line ACH-3P was used to establish a proper 3D cell culture model in creating spheroids which were either co-cultured with platelets directly or treated with released factors from activated platelets. The difference in the morphology of spheroids was visualized by immunofluorescence staining, confocal microscopy and transmission electron microscopy. Their diversity on molecular level was measured using standard RNA sequencing (RNA-seq) analysis, qPCR and western blotting.
Results:
Upon spheroid formation in the presence of maternal platelets, the latter were enclosed into a cavity of the trophoblast spheroids. However, some platelets were also found in intercellular spaces of the trophoblasts, mirroring in vivo circumstances. RNA-seq revealed that platelet-derived factors cause a significant deregulation of certain genes of trophoblast spheroids. Out of the significantly deregulated genes we identified those in relation to trophoblast development and used them as promising candidates for further investigation, in particular LAIR2 and HAND1.
Conclusion:
We established a 3D cell culture model using the human trophoblast cell line ACH-3P, which upon co-culture with human platelets will change their morphology. When treated with platelet-derived factors significant changes on the molecular level could be detected. LAIR2 and HAND1, two genes that supposedly play an important role in early placental development, were significantly upregulated and will be further investigated.

Abstract Number - P41

Multivalent Nanobodies : Novel Agonists for Platelet Activation

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Background:
The platelet surface receptors GPVI, CLEC-2 and FcγRIIa stimulate platelet activation through interactions with their endogenous ligands collagen, podoplanin and immune complexes respectively. These receptors signal through an ITAM which induces sequential activation of tyrosine kinases and platelet aggregation. PEAR1, an orphan receptor activated by sulphated polysaccharides, signals though a related tandem YxxM sequence. Clustering of all of these platelet receptors is a pre-requisite for platelet activation.
Current ligands used to activate the receptors have an undefined valency with significant batch variation e.g. collagens, snake venom toxins, fucoidans. Thus, there is a need for a standardised multivalent ligand for receptor mediated platelet activation. We have previously raised nanobodies against the ligand binding domains of GPVI, CLEC-2, FcγRIIa and PEAR1.
Aims:
To modify nanobodies against GPVI, CLEC-2, FcγRIIa and PEAR1 to create divalent, trivalent and tetravalent variants with the aim of generating ligands that can activate their respective receptors.
Methods:
Multivalent nanobodies were expressed in bacterial or mammalian systems then purified using column chromatography. Platelet aggregometry and ATP secretion assays were performed to investigate their effect. Receptor binding affinity measurements were determined by SPR.
Results:
Trivalent nanobody ligands to GPVI, CLEC-2 and PEAR1 induce potent platelet aggregation (EC50 0.98, 1.79, 0.65 nM respectively) and secretion indicating that clustering of 3 receptors is sufficient for activation. In contrast, tetravalent nanobodies were required to activate FcγRIIa (EC50 11.78 nM) suggesting a required cluster of 4 receptors. SPR confirmed the multivalent nanobodies have sub-nanomolar affinity (KD 0.05 - 0.53 nM) for their respective receptors.
Conclusions:
Our novel multivalent nanobodies provide well-defined, standardised agonists for these 4 major platelet receptors with greater potency and reproducibility compared to currently used agonists. Therefore, these nanobodies have applications as research tools and in clinically validated assays.

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Abstract Number - P42

Staying on track: collagen fibres orientation strongly affects platelet adhesion at high shear

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Background:
Subendothelial matrix in mammals shows specific spatial and biochemical organisation. Collagen fibres right under the endothelium are oriented mostly parallel to the blood flow and are composed predominantly of type III and type I collagen in both arteries and arterioles. However, it is not clear if such organisation is important for triggering rapid hemostatic response upon vascular injury.
Methods:
To get deeper insight into the possible role of collagen fibre orientation during primary hemostasis we took advantage of the microfluidic model of thrombus formation.
Hirudinated human whole blood was perfused through a microfluidic system with the immobilised type I collagen fibres oriented either parallel or perpendicular to the blood flow.
Results:
At a high shear rate of 2000 s-1 thrombi height and surface coverage were higher on the parallel fibres. High-speed epifluorescence microscopy revealed that platelets achieved stable adhesion only after interacting with the surface for several seconds and translocating for several micrometres for both fibres orientations. However, analysis of all single platelet adhesion attempts showed that generally platelets interacted longer and translocated farther upon interaction with parallel fibres at high shear.
Immunofluorescence analysis showed that only a small portion of collagen fibres adsorbed plasma vWF abundantly. Importantly, these fibres were also stained by type III collagen antibodies and supported platelet translocation at high shear.
Conclusions:
Taken together, our results imply that an admixture of type III collagen in fibres oriented parallel to the flow facilitates stable platelet adhesion at high shear rates as platelets can “stay on track” during their flow-directed translocation along the fibre. Thus, axial orientation of subendothelial collagen fibres might be of a physiological relevance for triggering rapid hemostatic response at high shear. Our findings also imply that collagen immobilisation procedure significantly influences the outcome of microfluidic-based experiments and thus should be considered in their design and analysis.

Abstract Number - P43

Comparison of platelet activation between established and novel FcγRIIA ligands

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Background:
Platelet activation via FcγRIIA underlies thrombosis and thrombocytopenia in immune-complex driven pathologies, including heparin-induced thrombocytopenia (HIT) and vaccine-induced immune thrombotic thrombocytopenia (VITT). Immune complexes consisting of platelet-factor-4 (PF4) and anti-PF4 antibodies in VITT and heparin, PF4 and anti-PF4 antibodies, in HIT, cluster FcγRIIA inducing activation. Despite similar exposure to heparin or vaccine, only a low proportion of patients develop HIT/VITT, suggesting donor heterogeneity. The monoclonal antibodies (mAbs) to PF4; 5B9 (HIT-like antibody) and 1E12 (VITT-like antibody) are tools generated to aid diagnosis and study HIT/VITT mechanisms. Recently, we have generated nanobodies (Nbs) raised against tandem Ig domains of FcγRIIA and crosslinked these to form agonists of defined valency.
Aims:
To explore if platelet activation in individuals varies in response to different FcγRIIA stimuli, including isolated VITT patient IgG and a novel FcγRIIA Nb.
Methods:
We have characterised 20 FcγRIIA Nbs and produced a potent tetravalent Nb (Nb17-2-Fc) that induces platelet activation and compared this with the anti-PF4 mAbs 5B9, 1E12 and a purified VITT patient IgG (6Ab), in order to test their ability to stimulate platelet activation in washed platelets and test for donor variability.
Results:
Nb17-2-Fc binds to recombinant FcγRIIA with a KD=0.054 nM measured by surface plasmon resonance and mediates platelet activation from 3 nM. Mono-, di- and trivalent forms of Nb17 do not induce activation. At high concentrations (>100 nM), Nb17-2-Fc induced robust aggregation in almost all donors (90%). However, variation was observed from 10 nM, with aggregation mediated in 40% donors (n=10). 1E12 (VITT-like Ab) stimulated full aggregation and increased CD62P expression in all donors tested at high concentrations (10 μg/mL, n=15), with more variable responses at lower doses. 5B9 (HIT-like Ab) stimulated aggregation and CD62P expression in 70% of donors in the presence of 0.5 U/mL heparin. 6Ab in presence of PF4, stimulated aggregation in 73% of donors and CD62P expression in 83% of donors (n=8-15). Some low responders were consistent across the different FcγRIIA stimuli, but not all.
Conclusions:
A high but not complete degree of similarity in platelet activation between FcγRIIA stimuli in individuals indicates ligand- and platelet-specific variables in governing responsiveness.

Abstract Number - P44

Description of three new variants in glycoprotein Ib platelet subunit beta (GP1BB) associated with a monoallelic form of Bernard-Soulier Syndrome

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Background:
The GPIb-IX-V glycoprotein complex is the platelet receptor for von Willebrand factor (VWF). Homozygous or double heterozygous variants in GP1BA, GP1BB or GP9, the genes encoding for the complex subunits, are at the origin of Bernard-Soulier syndrome (BSS), a bleeding disorder characterised by macrothrombocytopenia and platelet dysfunction, due to a diminished or dysfunction GPIb-IX-V. Recent reports show a moderate phenotype, transmitted as an autosomal dominant state. GP1BB variants account for 28% of BSS cases and to date, only 16 variants were reported as associated to this monoallelic form.
Aim:
We report 3 novel heterozygous variants in GP1BB in three unrelated families with autosomal dominant macrothrombocytopenia phenotypes.
Methods:
Platelet (PLT) counts, mean platelet volume (MPV) and immature platelet fractions (IPF) were assessed. Aggregations studies, including Ristocetin-induced platelet agglutination (RIPA), were performed. By flow cytometry, the GPIb/IX expression was measured, as well as the PLT size (Forward scatter index). Genetic studies were accomplished by conventional sequencing of GP1BB. The variant's pathogenicity was assessed by ACMG recommendations, segregation studies and in-silico predictions.
Results:
Platelet counts showed moderate thrombocytopenia (median 97.5×103/µL), increased MPV (median 14.5fL) and IPF (median 19%) in all probands and available family members, in a total of 8 cases. The expression of CD42b and CD42a glycoproteins (GPs) was shown to be diminished in all patients, with median values of 55.3% and 54.5%, respectively. Platelet function tests showed normal to slightly increased occlusion times, and normal (in one family) or slightly decreased RIPA. Two nonsense substitutions (Y113*, E136*), classified as likely pathogenic, and 1 missense variant of unknown significance (E134K), were found in leucine-rich repeat cytoplasmic (LRR-CT) domain of GPIbβ.
Conclusion:
Recently, monoallelic variants in GP1BB have been increasingly associated with milder forms of BSS. We report 3 new heterozygous variants in GP1BB implicated in an autosomal-dominant form of BSS, with a moderate macrothrombocytopenia phenotype. These patients show mild to absent bleeding symptoms (BAT score of 0-4), variable but altered PLT counts and indexes, and reduced RIPA and GPs quantifications. Further studies may lead to new insights into how GP1BB variants contributes to impaired VWF ligation and platelet function.

Abstract Number - P45

Clinical, phenotypic and genetic overview of inherited platelet disorders diagnosed in a single Portuguese reference centre for Congenital Coagulopathies.

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Background:
Inherited platelet disorders (IPDs) comprise an heterogenous group of about sixty rare diseases caused by molecular variants in more than 75 genes implicated in platelet formation and function. Platelet dysfunction, thrombocytopenia, bleeding diathesis, but also multisystemic syndromic features and/or increased predisposition for myelodysplasia, comprise the wide clinical spectrum of IPDs. Given their complex pathogenesis, an accurate diagnosis requires a detailed clinical and laboratory assessments, including genetic screening.
Aim:
Phenotypic and molecular characterization of IPDs diagnosed in a single centre.
Methods:
200 unrelated families with quantitative and/or qualitative platelet (PLT) defects, excluding acquired causes, were included and characterized. Laboratory investigations include PLT counts, size, and morphology; assessments of PLT function (lumiaggregometry); and flow cytometry (glycoproteins expression, PLT size). Patients' DNA samples were analysed by direct Sanger sequencing of suspected causative genes, or by a high-throughput sequencing haemostasis-related genes panel. In selected cases, Multiplex Ligation-dependent Probe Amplification (MLPA) was applied.
Results:
A definitive diagnosis was achieved in ≈51% of the cohort. PLT dysfunction disorders were diagnosed in 16% of the families: 8 Glanzmann Thrombasthenia; 7 Hermansky-Pudlak syndrome and 1 CalDAG-GEFI deficiency. More than 84% showed thrombocytopenia and/or PLT dysfunction: 15 ITGB3/ITGA2B-related thrombocytopenia (RT); 10 ANKRD26-related disease (RD); 10 TUBB1-RT; 8 ACTN1-RT; 6 MYH9-RD; 7 monoallelic and 5 bialellic Bernard-Soulier syndrome; 4 RUNX1-RD; 4 GFI1B-RD; 3 WAS/X-linked thrombocytopenia; 2 RUSAT2; 2 Noonan syndrome; 2 STXBP2-RD; 1 TARS; 1 Gray Platelet syndrome; 1 TTP; 1 DiGeorge syndrome; 1 PT-VWD; 1 DIAPH1-RD and 1 Sitosterolemia family.
Conclusions:
The pathogenesis of IPD has proved to be complex and heterogeneous. Complete clinical, morphologic, functional and molecular screening are crucial for accurate diagnosis, for prognosis and clinical managements of patients with syndromic entities. Following our diagnostic strategy, 51% of our cohort (101 families) had their IPD identified, contributing towards the understanding of these disorders, their clinical presentations, and underlying defects in our population. The undiagnosed cases remain under investigation and high-throughput sequencing approaches (genes panel and/or WES) are intended to be applied.

Abstract Number - P46

Identification of Polo-like Kinase 3 in Platelets and its Role in the Regulation of Hemostasis and Thrombosis

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Background:
Polo-like kinase 3 (Plk3) is a serine/threonine kinase involved in cell cycle regulation. Its expression and function in platelet are not known.
Aim:
To elucidate the role of Plk3 in platelet activation and function.
Methods:
Plk3 expression in platelets was evaluated by western blot and immunofluorescence imaging. Platelet aggregation was performed using lumi-aggregometer. Dense-granule secretion was measured using 14C serotonin. Alpha-granule release and alphaIIbeta3 activation was assessed using flow cytometry. Age-matched congenic Plk3 knockout and C57BL/6 mice of both genders were used for in-vivo studies. To evaluate hemostasis, tail-bleeding and liver bleeding assays were performed. To determine thrombosis FeCl3-induced carotid artery injury and collagen/epinephrine-induced pulmonary thromboembolism assays were performed. Ex-vivo thrombus formation was performed under arterial flow on collagen surface.
Results:
We found that Plk3 is expressed in platelet and localizes to the filopodia. Deletion of Plk3 in mice (Plk3-/-) showed a significantly (P<0.05) delayed tail bleeding time (325 s), compared to WT mice (130 s) suggesting a role for Plk3 in hemostasis. In-vivo thrombosis was also significantly affected in Plk3-/- mice (vessel occlusion time ~14 min), compared to WT (7-9 min; P<0.001). Furthermore, Plk3-/- mice were protected from thromboembolism compared to WT mice (P<0.001). Moreover, we found significantly reduced in-vitro thrombus formation under arterial flow (800s-1) in the absence of Plk3. Thrombin-induced platelet aggregation (P<0.0001), TxA2 generation (P<0.05), secretion of both alpha- and dense- granules was significantly reduced (P<0.01) in Plk3-/- mouse platelets compared to WT, consistent with the observed anti-thrombotic phenotype in-vivo. Furthermore, Plk3-/- null platelets failed to retract fibrin clot. Interestingly, thrombin induced intracellular Ca2+ rise was unaffected, suggesting that Plk3 operates downstream of Ca2+ mobilization.
Conclusion:
These results suggest that Plk3, plays a significant role downstream of agonist induced Ca2+ rise to regulate platelet activation thus affecting the process of hemostasis and thrombosis.

Abstract Number - P47

PIM Kinase: A novel regulator of thromboxane A2 receptor signalling and platlelet Function

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Background:
Pim kinases have recently been identified to play a role in the regulation of platelet function and thrombosis. Deletion or inhibition of Pim-1 results in reduced thrombus formation without altered hemostasis suggesting it is a desirable antiplatelet target. We have previously described that Pim-1 regulates platelet function via regulation of TPαR signalling. The mechanism by how Pim-1 regulates TPαR signalling in platelets has yet to be elucidated.
Methods:
HEK293T cells transfected with WT or mutant Flag tagged TPαR or GFP-Tagged TPαRs were treated with Pim kinase inhibitor (AZD1208). Site directed mutagenesis was utilised to mutate predicted Pim phosphorylation sites (S→A) within the intracellular loops and tail. Receptor levels (surface and total) were then assessed using both flow cytometry and microscopy. TxB2 generation was measured by ELISA and calcium mobilisation downstream of TPαR was measured to determine effects on downstream signalling.
Results:
No alteration in platelet TxB2 generation following treatment with pharmacological pan-pim kinase inhibitors indicates Pim kinase regulates platelet TxA2 receptor signalling independently of COX1 regulation. Pim kinase inhibition causes reduced surface expression of the TPαR, which is mediated by increased receptor internalisation when assessed using flow cytometry and fluorescence microscopy. Site prediction modelling, using the Pim kinase consensus sequence, identified 5 putative phosphorylation sites within the intracellular loops and C-terminal tail. Site directed mutagenesis of these sites identifies a novel potential Pim phosphorylation site within the first intracellular loop of the TPαR which shows reduced surface expression and is resistant to further internalisation in the presence of Pim kinase inhibition.
Conclusion:
Inhibition of Pim kinase attenuates TPαR signalling via receptor internalisation. We have identified a serine in the first intracellular loop of TPαR as a novel site of Pim kinase phosphorylation and a key player in mediating the internalisation of the receptor. Due to unresponsiveness of aspirin in a subset of high risk patients, Pim kinase inhibition may be a desirable anti platelet target as an alternative mechanism of TPαR inhibition.

Abstract Number - P48

Hypochlorous acid inhibits platelets through reversible oxidation of cysteine thiols in cytoskeletal and signalling proteins

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Background:
Hypochlorous acid (HOCl) is an antimicrobial oxidant produced by neutrophil and monocyte-derived myeloperoxidase (MPO) during oxidative stress. Modification of amino acid side-chains leading to protein aggregation is typically induced by HOCl within phagosomes. However, MPO is also present in the extracellular environment during inflammation and following strenuous exercise. The roles of extracellular MPO and HOCl are controversial as both detrimental and, more recently, protective effects have been described. MPO is considered a biomarker in atherosclerosis, however, HOCl is also reported to inhibit platelet aggregation in platelet-rich plasma. Whether HOCl directly targets platelets is not yet known, nor are the pathways involved.
Aim:
To characterise the effects of HOCl on platelet aggregation and spreading in washed platelets, and to identify and verify protein targets mediating these effects.
Methods:
Aggregation of washed human platelets was measured using light transmission aggregometry following pre-incubation with a combination of HOCl, the reactive oxygen species scavenger methionine, and antioxidants glutathione and ascorbic acid. Platelets were activated with TRAP6 and U46619. Proteomics as well as immunoprecipitation and Western blotting were used to identify redox activated proteins. Platelet spreading on fibrinogen coated surfaces were analysed using confocal microscopy.
Results:
0.5-2mM HOCl dose-dependently reduced platelet aggregation in response to TRAP6 and U46619. The inhibitory effects of HOCl were reversible with glutathione but not with ascorbic acid suggesting that HOCl effects on platelets are mediated by oxidation of cysteine thiols. Proteomic analysis of HOCl treated platelets revealed 68 proteins with reduced thiol levels compared to controls. 18 of these proteins are associated with actin binding and cytoskeletal organisation. In agreement, defective platelet spreading was demonstrated in the presence of 0.025-0.1mM HOCl. Furthermore, activation of Rap1b and RhoA, critical for platelet aggregation and shape change, were reduced and the inhibitory cAMP signalling pathway was stimulated following treatment with HOCl.
Conclusion:
HOCl attenuates platelet aggregation and spreading by oxidising cysteine residues in intracellular platelet proteins involved in cytoskeleton regulation and GTPase activity. Thus, extracellular HOCl can directly target platelets and prevent their activation during oxidative stress.

Abstract Number - P49

Platelet aggregation test, between doubts and certainties: Frequent Ask Questions

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Background:
The light transmission aggregation (LTA) test is considered as a gold standard test and is currently the most widely used test for the evaluation of platelet functions. This technique measures the aggregation of citrated platelet-rich plasma (cPRP), obtained after low-speed centrifugation of the patient's blood sample collected on sodium citrate. Aggregation is measured by a photometric technique, after addition of different platelet activators (aggregating agents).
Results:
The relatively restrictive pre-analytical and analytical conditions (blood collection conditions, transport and storage of the sample, time frame for the examination, protocol for obtaining PRP, centrifugation conditions, quality control used, analysis of aggregation profiles, etc.) make this technique difficult to implement by laboratories that are not experienced in this field.
Questions from potential future users are numerous and recurrent, often related to a lack of knowledge of platelet aggregation test. Answers can be provided to circumvent some of these pitfalls (type of instrument, variation and conversion algorithm of the aggregation signal, limit of use of PRP from thrombopenic or thrombocythemic patients, which type of agonist at what concentration, Heparin-Induced Thrombocytopenia (HIT) diagnostic pitfalls, hemolytic, icteric or lipemic plasmas, choice of quality controls, etc.) so that a greater number of users can appropriate this reference technique while keeping in mind its limitations.
Conclusions:
Despite these inherent limitations described here, LTA remains the cornerstone of platelet function investigation as a valuable choice in first-line to diagnose defects predisposing patients to bleeding symptoms, high platelet reactivity in cardiovascular thrombotic risk and monitoring of anti-platelet drugs or in transfusion medicine.

Abstract Number - P50

Scott syndrome with novel ANO6 deficiency and associated impaired PS exposure and platelet dysfunction

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Background:
Scott syndrome is a mild platelet-type bleeding disorder, first described in 1979 and with only 4 unrelated patients identified. The syndrome is characterized by impaired surface exposure of procoagulant phosphatidylserine (PS) on platelets after stimulation. To date, full platelet function and thrombin generation in this condition has not been extensively characterised.
Aims:
To elucidate the cause of a family with a bleeding history and functionally characterise the defect in platelets and whole blood from the patient and family members.
Methods:
Genetic and functional studies was undertaken in a consanguineous family with a history of excessive bleeding of unknown cause. A targeted gene panel of previously known bleeding and platelet genes was used to identified plausible genetic variants. Deep platelet phenotyping, flow adhesion, image stream flow cytometry, whole blood and PRP thrombin generation and specialized extracellular vesicle measurements were performed.
Results:
We detected a novel homozygous frameshift variant (c.1943del; p.Arg648Hisfs*23) in ANO6 encoding Anoctamin 6, in a patient with a bleeding history, but interestingly normal protein expression. Deep platelet phenotyping studies revealed function defects in the patient including, reduced number of δ granules (****p<0.0001), reduced platelet aggregation and secretion in response to ristocetin and reduced P-selectin expression after stimulation with low dose agonists. Platelet surface receptor levels remained normal. PS exposure was absent on spread platelets from the Scott patient and thrombi formed over collagen at 1500/s (****p<0.0001). Reduced thrombin generation in the Scott patient was also observed in platelet-rich-plasma and in whole blood using a new thrombin generation assay.
Conclusions:
In this study, we present a comprehensive report of a Scott syndrome patient with a novel frameshift mutation in AN06, which is associated with no PS exposure on platelets and thrombi, and markedly reduced thrombin generation in whole blood, explaining the significant bleeding phenotype observed.

Abstract Number - P51

Characterization of platelet subpopulations in mouse models of regenerative thrombocytopenia

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Background:
Circulating platelets differ in age, size and functionality. Upon release into the bloodstream, they are rich in RNA and receptors that enhance their hemostatic reactivity. This hemostatic capacity diminishes with age, as protein content, metabolic activity and organelles decrease. Dysregulation of platelet turnover and subpopulation alterations are linked to a variety of diseases.
Aims:
We aimed to investigate phenotypic differences of platelet subpopulations in two mouse models of regenerative thrombocytopenia.
Methods:
Platelets were either depleted via diphteria toxin (DT) induced megakaryocyte apoptosis of megakaryocyte-specific DT receptor transgenic mice or via intravenous injection of an anti-GPIbα antibody. After platelet nadir was reached, blood was drawn on five consecutive days and platelet subpopulations flow cytometrically characterized by RNA staining, desialylation, surface receptor as well as integrin expression prior to and after activation.
Results:
In the regeneration phase, the megakaryocyte apoptosis model displays a higher proportion of reticulated platelets compared to the antibody-induced platelet depletion model, caused by excessive platelet production. Principal component analysis revealed a striking difference in expression patterns between young and old platelets as well as between the two models. We found distinct differences of GPIIb/IIIa levels in reticulated platelets in the megakaryocyte apoptosis mouse model, while P-selectin expression upon platelet activation remained unaltered in reticulated platelets.
Conclusion:
While both platelet depletion models led to a rise in reticulated platelets, integrin expression and activation levels differ between diphtheria toxin and antibody-induced regenerative thrombocytopenia models, possibly due to changes in protein production and packaging during megakaryopoiesis.

Abstract Number - P52

Pathophysiological activation of platelet pannexin-1 channels

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Background:
Pannexin-1 (Panx1) channels amplify platelet activation via feed-forward stimulation of P2X1 receptors. Inhibition of Panx1 improves outcomes in models of myocardial infarction and ischemic stroke, and knockout mice have a thromboprotective phenotype. Platelet activation is enhanced by shear stress and inflammation, conditions that reportedly activate Panx1 channels in other cell types. Whilst aspirin and cangrelor are widely used antiplatelet agents, they are not effective for all patients. Panx1 is sensitive to clinically used drugs (e.g. spironolactone (Spiro) and carbenoxolone (cbx)) that could be repurposed to manage thromboinflammatory conditions.
Aim:
To determine contribution(s) by Panx1 to platelet activation, thrombus formation and structure under (patho)physiological conditions.
Methods:
Platelet activation was assessed by flow cytometry and nucleotide release measured by luminescence assay. Thrombus formation was performed at indicated shear rates and porosity assessed using fluorescently labelled dextrans. Thromboinflammatory responses were assessed using TNF-α stimulated HUVEC (human umbilical vein endothelial cells) in a vessel injury on a chip model.
Results:
Inhibition of Panx1 by Spiro reduced integrin activation and release of α granules, ADP and ATP. We next evaluated the effect of Panx1 blockers on platelets treated with aspirin plus cangrelor. At intermediate concentrations of platelet agonists, Panx1 blockers further reduced fibrinogen binding and CD62P expression. We explored whether platelet Panx1 is activated under increasing shear rates using a calcein efflux assay. Increasing levels of Panx1 activation were observed in response to venous (400s-1), arteriolar (1000s-1) and pathological (2,000s-1) shear rates. Inhibition of Panx1 increased the porosity of thrombi formed under arteriolar shear. Finally, we explored the role of Panx1 channels under thromboinflammatory conditions in vitro. In the presence of Spiro, the number of HUVEC-associated thrombi reduced by one third.
Conclusion:
Spiro and Cbx are clinically used drugs that also target Panx1 channels that reduce platelet activation under (patho)physiological conditions. Panx1 may be a useful target for thromboinflammatory conditions.

Abstract Number - P53

Role of FPR2/ALX in the modulation of thromboinflammatory responses

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Background:
The identification of formyl peptide receptors (FPRs) in platelets and their function in regulating platelet reactivity has shed light on the role of platelets in coordinating thrombosis and inflammation. For example, LL37, an antimicrobial peptide, activates platelets directly through FPR2/ALX receptor and induces thromboinflammatory responses. However, the contribution of platelets to the resolution of thromboinflammation has not been studied in detail. Here, we utilised a selective FPR2/ALX antagonist, WRW4 to determine the impact of this receptor in modulating platelet activity and subsequent platelet-mediated inflammatory responses.
Aim:
The aim of this study is to investigate the impact of an FPR2/ALX selective antagonist, WRW4 in the modulation of platelet-mediated thromboinflammatory responses.
Methods:
A range of platelet functions was assessed by flow cytometry, optical aggregometry and microscopy-based experiments in the presence and absence of various concentrations of WRW4 in human platelet-rich plasma and isolated platelets. To determine the impact of WRW4 in blood clotting, rotational thromboelastometry and a coagulation analyser were used to assess its effects in whole blood and plasma, respectively.
Results:
Our results demonstrate that pre-incubation of WRW4 with human platelet-rich plasma or isolated platelets inhibited platelet adhesion, activation, and aggregation upon stimulation with ADP (a P2Y12 receptor agonist) or LL37 (FPR2/ALX agonist) in a dose-dependent manner. In addition, WRW4 extended the clotting time in whole blood, and prothrombin and activated partial thromboplastin times in plasma.
Conclusions:
These results suggest that the blockade of FPR2/ALX with WRW4 affects a range of platelet functions as well as blood clotting highlighting the significance of this receptor in regulating platelet reactivity. Further experiments are underway to underpin the significance of FPR2/ALX and WRW4 in the modulation of platelet-mediated inflammatory responses. The outcomes of this study will facilitate the use of FPR2/ALX as a powerful therapeutic target and WRW4 and its structural analogues as potential agents to control thromboinflammatory conditions under diverse pathophysiological settings.

Abstract Number - P54

Differential effects of two distinctive snake venoms on the modulation of platelet reactivity and blood clotting

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Background:
Viper venoms are notorious for causing haemotoxic effects by interfering with platelet function and coagulation factors. Understanding the impact of these venoms on blood clotting is not only important for comprehending their pathological complications but also holds the potential for bioprospecting. Despite in-depth studies on viper venoms, the effects of elapid snake venoms on platelet function and blood clotting remain understudied. Here, we examined the distinct effects of venoms from an elapid snake (Pseudechis guttatus) and a viper snake (Agkistrodon piscivorus leucostoma) on blood clotting and platelet function.
Aim:
The aim of this study is to determine the impact of the venoms of Pseudechis guttatus and Agkistrodon piscivorus leucostoma in the modulation of human platelet reactivity and blood clotting cascades.
Methods:
We used a wide range of biochemical, platelet functional (flow cytometry and optical aggregometry), and clotting (rotational thromboelastometry and coagulation experiments) assays to determine the effects of various concentrations of Pseudechis guttatus and Agkistrodon piscivorus leucostoma venoms on human platelet-rich plasma, isolated platelets, and whole blood.
Results:
Both venoms displayed varying enzymatic activities (phospholipase A2, serine and metalloproteases). Interestingly, these two venoms showed opposing results on platelet activation and aggregation. Pseudechis guttatus venom showed an inhibitory effect on ADP (a P2Y12 receptor agonist)-induced platelet activation, whereas Agkistrodon piscivorus leucostoma venom exhibited a procoagulant effect by activating platelets on its own. However, both venoms have extended the clotting time in whole blood and activated partial thromboplastin time in plasma without causing significant changes to prothrombin time.
Conclusions:
These results suggest that Pseudechis guttatus venom possesses anti-coagulant effects as it inhibited platelet activation and blood clotting. However, Agkistrodon piscivorus leucostoma venom exhibits activatory effects on platelets but inhibitory effects on clotting experiments. Further investigations are underway to characterise individual toxins that affect platelet function and clotting and underpin the underlying molecular mechanisms. This study will pave the way to understand the haemotoxic effects of an elapid snake in comparison with a viper and explore their pathological complications as well as the potential for bioprospecting.

Abstract Number - P55

IPF as a surrogate marker for reduced response to aspirin in children post-cardiac surgery?

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Background:
Although studies have documented wide variation in aspirin response in adults, studies are lacking in children. This is particularly important in children with congenital heart disease in whom thrombosis may be life threatening. This phenomenon of inadequate response to aspirin may be defined as a failure of antiplatelet drugs to inhibit platelet function in an "in vitro" test.
A high platelet turnover rate might produce a population of platelets that could confer this inadequate response through several different mechanisms.
Aims:
(i)To investigate the relationship between residual platelet aggregation and platelet turnover in patients taking aspirin by evaluating the fraction of immature platelets (IPF) as a marker for turnover. (ii) To test the predictive ability of IPF to stratify immature platelet populations according to their response to aspirin.
Methods:
Sixty paediatric patients divided into two age categories (< =90 days, >90 days of age) were included in this prospective observational study. Patients were then stratified into tertiles based on their IPF level. Platelet studies included thromboelastography with platelet mapping (TEGPM).
Results:
The overall incidence of 'inadequate response to aspirin' (aggregation > = 50% or maximal amplitude > = 40mm in response arachidonic acid (AA) by TEGPM) was 38% in our patient cohort recently post cardiac surgery a consequence that warrants further study. The frequency of inadequate response to aspirin was higher in the upper tertile of IPF when compared to the lower tertile, (88%) versus (4%) respectively (P<0.001). The 'cut off' for IPF was determined to be 3.9% with a sensitivity of 95.7%, and a specificity of 92.9% (area under the curve of 0.955 [CI 0.896-1.014, P <0.001]).
Conclusion:
This study demonstrates that inadequate response to aspirin occurs in approximately 38% of patients undergoing specific high-risk congenital cardiac procedures using the dosing practice of a national centre. This study supports the hypothesis that an elevated platelet turnover as measured by IPF may render aspirin less effective in patients that are recently post cardiac surgery. The potential for the use of IPF as a screening test with MAAA as a confirmatory test has the added benefit of requiring low blood volume in a paediatric population and should be further investigated. Our findings suggest that greater surveillance of patients post cardiac surgery on aspirin monotherapy is warranted.

Abstract Number - P56

Dual antiplatelet therapy is associated with high alpha-tubulin acetylation in circulating platelets from coronary artery disease patients

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Background:
Platelet inhibition is the main treatment strategy to prevent atherothrombosis. Despite dual antiplatelet therapy (DAPT) combining aspirin and a P2Y12 receptor inhibitor, high on-treatment platelet reactivity (HPR) persists in some patients due to poor response to treatment and is associated with ischemic risk. It remains unknown if circulating platelets in high-risk patients have distinct morphological characteristics which could contribute in their pro-thrombotic potential.
Aim:
Knowing the key role of alpha-tubulin acetylation on lysine 40 (K40) in regulating platelet shape change, we investigated whether this post-translational modification could differ according to antiplatelet therapy and on-treatment platelet reactivity.
Methods:
Platelets were isolated from arterial blood samples of 187 patients admitted for coronary angiography and alpha-tubulin K40 acetylation levels were evaluated by immunoblotting. Platelet reactivity was assessed in whole blood using multiplate analysis. 141 (75%) patients were taking aspirin among which 30 (16%) were treated with an additional P2Y12 inhibitor. HPR was detected in 7 out of 30 DAPT-treated patients. Participants provided written informed consent and the study was approved by the institutional ethics committee.
Results:
Platelet alpha-tubulin K40 acetylation was significantly increased in DAPT-treated patients ($p<0.001$). The minority of non-treated patients (11%) exhibited high alpha-tubulin K40 acetylation level (third acetyl K40 alpha-tubulin tertile). In contrast, high level of alpha-tubulin K40 acetylation was observed in most patients on DAPT without high residual platelet reactivity (83%). Interestingly, the proportion of patients with high acetyl K40 alpha-tubulin level (43%) was drastically reduced among DAPT-treated patients with HPR. Multivariate logistic regression further supported that DAPT achieving platelet inhibition is independently associated with a 33-fold increase in the likelihood of being in the highest acetyl K40 alpha-tubulin tertile ($p<0.001$).
Conclusion:
We highlighted the role of high platelet alpha-tubulin K40 acetylation as a marker of platelet inhibition in response to DAPT, which could contribute to maintain resting morphology of circulating platelets.

Abstract Number - P57

Effects of curcumin on platelet activation promoted by amyloid peptides

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Background:
Deposition of amyloid peptides in brain parenchyma and cerebral vessels are correlated to Alzheimer's disease (AD). Fibrillar amyloid peptides, not only contribute to the progression of dementia, but also induce platelet activation, aggregation, and ROS generation increasing the risk of vascular complications. The possibility to modulate amyloid peptide-induced platelet activation is an important issue in the improvement of life quality in AD patients, and different natural compounds such as curcumin and its derivatives, are currently under investigation. Curcumin is a bioactive molecule derived from turmeric (Curcuma longa) and is a good candidate for its anti-inflammatory, anti-oxidant and anti-amyloidogenic effects.
Aim:
The aim of this study is to investigate the possible effects of curcumin in platelet activation induced by standard agonists and by amyloid peptides.
Methods:
Amyloid peptides 40 and 42 and corresponding scrambled peptides were diluted in phosphate buffered saline at 37°C for 24 hours to promote amyloid fibril formation. Platelet aggregation was analysed by light transmission aggregometry, and protein phosphorylation was evaluated by immunoblotting with specific phospho-antibodies.
Results:
Platelet aggregation and protein phosphorylation of selected substrates induced by thrombin and convulxin were impaired by curcumin in a dose-dependent manner, and 25 microM curcumin inhibited thrombin-induced aggregation of about 50%. Fibrillar amyloid peptides, but not scrambled peptides, promote platelet aggregation, which is strongly reduced by preincubation with curcumin. Curcumin also reduced MAP kinases and PI3K/Akt/GSK3 signalling pathways induced by stimulation with fibrillar amyloid peptides, without affecting the formation of TxA2 through cPLA2 activation.
Conclusion:
These results suggest a possible role of curcumin in the modulation of platelet activation induced by amyloid peptides and encourage to continue investigating the signalling pathway regulated by this natural compound in platelets.

Abstract Number - P58

Time- and distance-resolved robotic imaging of fluid flow in vertical microfluidic strips: a new technique for quantitative, multiparameter global measure of haemostasis

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Background:
The complex haemostasis process is central to cardiovascular health and disease, yet measurement of haemostasis remains challenging due to assays that require specialized equipment or personnel with specific technical training.
Aim:
Here, we propose a test for the global measure of haemostasis, analysing the combined effect of platelets and coagulation under physiologically relevant flow conditions by combining inexpensive mass-produced microfluidic devices with open-source robotic instrumentation.
Methods:
A time- and distance-resolved fluid flow analysis by Raspberry Pi imaging integrated with controlled illumination and sample testing, provides multiplexing (120 samples within 5 minutes) and automation at a fraction of the cost of standard haematology/coagulation analysers. Vertical capillary rise during stimulation of blood coagulation was measured in microfluidics having a mean internal diameter of ~160, 200 or 270 μm and pre-loaded with stimuli, differing from horizontal microfluidic blood analysis devices proposed to date as vertical capillary rise depends on more fluid properties (viscosity, density, and interfacial properties).
Results:
In our approach blood experienced theoretical wall shear rates of 100-600 s-1 during the initial stage of rise (0 to ~20mm), similar to conditions found in the human body. Critical fluid properties were derived from flow kinetics using a pressure balance model validated with glycerol:water mixtures. Time-resolved imaging revealed changes in fluid properties in a time scale of seconds that were unclear when extracted only based on endpoint equilibrium analysis. Instantaneous superficial fluid velocity during capillary rise was found to be independent of capillary diameter at initial time points. We demonstrated the application of time- and distance-resolved microfluidic imaging to the measurement of dynamics of haemostasis using thrombin-loaded microcapillaries. This slowed vertical fluid velocity in a manner consistent with a rapid increase in viscosity.
Conclusion:
We concluded that open-source instrumentation expands the capability of affordable microfluidic devices for haematological testing, a step towards the high-throughput multi-parameter microfluidic blood analysis.

Abstract Number - P59

Development of an in vitro model of ischemic stroke and reperfusion injury

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Background:
Ischaemic stroke, and subsequent reperfusion injury is a multicellular process, with limited treatment options. The successful translation of novel therapies is currently hampered by the lack of existing cellular models that reflect human disease and consider the multicellular nature of ischaemic stroke. In this project we developed a simple, cost effective and adaptable model of the human neurovascular unit, that is compatible with flow to enable the pathological basis of ischaemic stroke and neuroinflammation to be investigated. The model also offers a novel non-animal-based platform to identify and test novel therapeutic strategies for the treatment and prevention of ischaemic stroke and reperfusion injury.
Aims:
To characterise the role of activated platelets and inflammatory mediators on cells of the neurovascular unit to assess their involvement in ischemic reperfusion injury.
Methods:
Monocultures of human microglia (HMC-3), astrocyte-like glioma (U87) and microvascular endothelial cells (hCMECs/D3) were treated with activated platelet releasate, and cell activation, proliferation, migration, and survival were monitored. Human washed platelet releasate was prepared by centrifugation. Quantitative PCR (qPCR) in the presence of SYBR Green I was performed using CFX PCR system. High resolution 3D imaging. HMC-3 (Red), U87 (green) and HMEC/D3 (Blue) were incubated with cell tracker in serum free media for 30 minutes. HMC-3 and U87 were seeded within a 1.5 mg.ml collagen rat tail matrix in a microfluidic chamber. HMEC/D3 cell line was seeded on top of the collagen matrix within the fluid chamber. 3D Z stack images taken with Stellaris 5 Confocal microscope.
Results:
Treatment of the different neurovascular cell types with hypoxia, TNFα and platelet releasate were also found to alter expression of key mediators of neuroinflammation (IL-1B, IL-6) and thrombosis (vWF and CD39) alongside targets of interest for the research team (LXRα, LXRβ, Pim-1, Pim-2, Pim-3 and SIRT1, P2Y2), identifying potential roles for these mediators in the regulation of neuroinflammation following ischaemic stroke and reperfusion injury

Abstract Number - P60

Impact of fever and acidosis on platelet-mediated thromboinflammation

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Background:
Pathophysiological settings such as infections and inflammation are often associated with not only fever and tissue acidosis but also profound platelet function defects. Altered hemostatic and platelet-mediated immunomodulatory functions, such as increased platelet-leukocyte aggregates are associated with disease outcome and feed into a vicious cycle of amplifying both platelet and leukocyte activation. However, the precise impact of these factors on platelets as well as the mechanism of platelets as contributors to thromboinflammation are still unclear.
Aim:
We want to elucidate the impact of fever and acidosis on platelet reactivity as well as platelet-mediated leukocyte functions.
Methods:
We investigated the effect of fever (40°C), alone or in addition to acidosis (pH 6.9) on human platelet function, platelet-leukocyte interactions, myeloid tissue factor (TF) expression and neutrophil extracellular trap (NET) formation in vitro. Platelet reactivity (activated GPIIb/IIIa, CD62P, CD63, CD40L) as well as platelet-mediated immunomodulatory functions such as the formation of platelet-neutrophil/monocyte aggregates and platelet-induced leukocyte activation (activated CD11b, CD62L, TF) in response to TRAP-6 and ADP stimulation was investigated by flow cytometric analysis.
Results:
Platelet activation was only mildly affect at 40°C, while acidic conditions led to increased platelet granule exocytosis. This hyperreactivity of platelets was accompanied by increased platelet-leukocyte aggregate formation. Under acidic conditions, platelets strongly enhanced TF expression by monocytes, which was abolished by blocking direct platelet-monocyte interaction via P-selectin.
Conclusion:
Our results indicate that platelet activation as well as platelet-mediated immunomodulatory functions are only mildly affected by elevated body temperature, while acidosis drives platelet degranulation and amplifies thromboinflammation.

Abstract Number - P61

Differential role of the Rac1-binding proteins Fam49b and Cyfip1 in platelet spreading

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Background:
Platelet function at the sites of vascular injury involves a tight regulation of the actin cytoskeleton. When platelets bind to adhesive substrates, they form a broad circular branched actin network, the lamellipodium. Platelet lamellipodia formation was shown to be dispensable for hemostasis and thrombus formation, but crucial for vascular surveillance by platelet migration. The WAVE regulatory complex (WRC), which drives spreading-associated lamellipodia formation via Arp2/3 activation, is activated by interaction of GTP-bound Rac1 with WRC subunit Cyfip1. We previously demonstrated that Cyfip1 deficiency abolishes platelet lamellipodium formation. Recently, Fam49b (family of unknown function 49) was identified as a highly conserved regulator of actin-rich cellular protrusions. It was shown that Fam49b negatively regulates the WRC and binds activated Rac1 via a domain which is shared with Cyfip1.
Aim:
To better understand how Fam49b fits into the framework of the control of actin dynamics, and in particular of lamellipodial protrusions, by positive and negative feedback loops, we capitalized on platelet-specific Fam49b-/-, Cyfip1-/-, and Cyfip1/Fam49b-/- mice.
Methods:
We assessed platelet biogenesis and activation via whole blood analysis, flow cytometry and transmission electron microscopy. Using live-cell and fluorescence microscopy we analyzed the spreading ability and morphology of mutant platelets on biofunctionalized surfaces and micropatterns of platelet-activating ligands.
Results:
Platelet biogenesis was largely unaltered in all mutant mice. Cyfip1-/- and Cyfip1/Fam49b-/-, but not Fam49b-/-, platelets displayed a moderately reduced activation. On fully fibrinogen-coated surfaces Fam49b-/- platelets spread faster than control platelets, yet Cyfip1/Fam49b-/- platelets were unable to form lamellipodia and displayed a phenotype similar to Cyfip1-/- platelets. In contrast, on isotropically distributed µm-spaced ligands Fam49b-/- platelets displayed reduced spreading compared to Cyfip1-/-, Cyfip1/Fam49b-/- and control platelets, likely due to impaired filopodia outgrowth.
Conclusions:
Though we show that Fam49b plays a central role in the regulation of platelet spreading, the phenotypical similarity of Cyfip1-/- and Cyfip1/Fam49b-/- platelets suggests that Cyfip1 is the decisive factor for lamellipodia formation. We also demonstrate that the surface distribution of immobilized ligands strongly influences platelet spreading and morphology.

Abstract Number - P62

Glycocalicin is released and trapped in a retracted thrombus

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Background:
GPIbα is a unique and abundant receptor in platelets essential for adhesion to extracellular matrix proteins at high shear rates. Following strong and sustained activation, metalloproteinase ADAM17 catalyzes GPIbα proteolysis thereby releasing the soluble ectodomain glycocalicin. We recently demonstrated that this is a slow process that seems irrelevant to primary hemostasis. In addition, the GPIbα substrate subpopulation is uniquely located inside the platelets because surface GPIbα is insensitive to shedding.
Aim:
Examine GPIbα proteolysis and spatial distribution of released glycocalicin during in vitro thrombus formation.
Methods:
Thrombus formation was induced by addition of 15mM $CaCl_2$ to platelet-rich plasma (PRP). Following platelet coagulation and retraction (±100 minutes), the thrombus was washed and homogenized. Paired samples with metalloproteinase inhibitors were included as a control. Samples were interrogated for glycocalicin content using SDS-PAGE western blotting and ELISA.
Results:
The plasma volume that contained the retracted thrombus contained 15 nM (2 µg/mL) of glycocalicin at endpoint. This was not significantly different from the initial concentration in PRP ($p = 0.971$), suggesting glycocalicin was not released from platelets within or on the thrombus, nor from free platelets. The latter were in fact very rare at endpoint, because undetectable by standard blood counting (detection limit: 104 platelets per µL) and extremely low by flow cytometry (<200 CD61+ events/µL). Glycocalicin content inside uninhibited thrombi was 3.4-fold higher than in thrombi containing ADAM17 inhibitors ($p=0.011$, $n=10$). We calculated that an average thrombus contained ~62 nM of glycocalicin which is almost 10-fold higher than the median glycocalicin concentration in plasma of healthy blood donors that we determined as 6.5 nM [5.1-7.7 nM] (median [IQR]; n=413).
Conclusion:
GPIbα is shed from platelets during coagulation initiated by Ca^{2+} only. Platelet agonists were not required to cause shedding in this model, in vitro. All platelets got trapped in the retracted thrombus and glycocalicin is not released in surrounding plasma. Instead, glycocalicin remains trapped inside the thrombus at supraphysiologic concentrations.

Abstract Number - P63

Platelet dysfunction in Noonan Syndrome

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Background:
Noonan Syndrome (NS) is an autosomal dominant genetic disorder with multiple anomalies, including bleeding diathesis. Although the bleeding phenotype is mild, surgical management is often required including procedures with high bleeding risk. CBC, PT, aPTT and F XI are recommended at diagnosis, however platelet function abnormalities were rarely reported in these patients.
Aim:
The aim is the characterization of hemostatic and platelet function abnormalities in NS patients.
Methods:
PT, aPTT were determined with IL Werfen automated coagulation analyzer. ISTH-BAT was administered to patients. Platelet function was investigated using PFA 100 system within collagen/Epinephrine and collagen/ADP test cartridges, light transmission aggregometry (LTA) and PRP stimulated by ADP, collagen, epinephrine, PAR1 activating peptide (AP). Maximal aggregation (MA, %) for all agonist and lag phase (LP, seconds) for collagen were measured. Platelet secretion was investigated by flow cytometry using MoAbs directed against CD63 and CD62p on ADP and PAR1-AP stimulated platelets. Mean fluorescence intensity (MFI) was measured.
Results:
The study population included 24 patients and 14 controls. Platelet count, PT and fibrinogen were not significantly different in the 2 groups, 30% of NS had aPTT prolongation. Noonan patients had significant reduction of platelet aggregation with ADP 2 µM (91,6% with MA <60%), ADP 4 µM (79,1 % with MA<60%), collagen 2 µg/ml (41,6 % with MA<75%, 87% with LP>50 sec), epinephrine 5 µM (87% with MA< 60% at 180 sec, 62.5% with MA <70% at 300 sec), PAR1-AP (45.8% with MA<75%). Collagen-EPI and collagen-ADP tests by PFA-100 show that 90% and 60% of NS patients, respectively, have a longer response time to agonists than normal ranges (collag-EPI: 140 seconds; collag-ADP 120 seconds). A significant reduced expression of activation-dependent markers CD63 and CD62p was found after PAR1-AP stimulation, and of CD62p after ADP stimulation. (Fig.1 and 2)
Conclusions:
In the present cohort, large majority (>90%) of Noonan pts showed platelet dysfunction, whereas their platelet count was normal and only 30% of NS had aPTT prolongation. Platelet secretion defect is suggested by activation-dependent CD63 and CD62p marker exposure. In addition, PFA analysis can be useful to identify NS patients at greater risk of bleeding. Platelet dysfunction is the most frequent hemostasis abnormality in NS.

Abstract Number - P64

Identification of distinct platelet populations upregulated in sepsis patients from UKHSA study

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Background:
Sepsis is a complicated life-threatening condition of organ dysfunction caused by a dysregulated host response to infection. In a recent UKRI funded project, a metanalysis of previously published severe inflammatory response syndrome (SIRS) and sepsis datasets identified gene expression patterns highlighting platelet involvement in sepsis but not SIRS[3]. Platelets are crucial in innate immunity and possess complex roles within infection, with roles including leukocyte recruitment, protection against pathogen-induced host tissue damage and direct antimicrobial roles[1-2]. Here, we highlight the presence of 3 distinct platelet populations within gene expression data taken from human sepsis patients.
Methods:
A list of platelet specific markers was compiled using a literature search and the genelist imported into Agilent Genespring Version 14.9, to analyse the UKHSA microarray SIRS/Sepsis dataset. This study involved analysis of gene expression data from sepsis (pulmonary sepsis n=74, abdominal sepsis n=50), SIRS patients (n=38) and healthy controls (n=30) with samples taken at days 1, 2, 5 and upon discharge from ICU (n=125). The UKHSA dataset was analysed using a One-Way ANOVA on using the Benjamini/Hochberg false discovery rate (BHFDR) multiple testing correction and a SNK post-hoc test on the filtered expression platelet entity list. Statistical analysis showed that 92/92 platelet entities satisfied the corrected p-value (BHFDR) at acut-off of $p \leq 0.05$.
Results:
The filtered, statistically significant platelet biomarker list from the analysis of the UKHSA SIRS/Sepsis dataset revealed two main platelet clusters; cluster 1 showed significant platelet biomarker upregulation in SIRS, whilst cluster 2 showed significant upregulation in sepsis. Importantly, unlike in SIRS the sepsis platelet cluster differentiates into a further three subclusters. Interestingly, these 3 subclusters are characterised by different gene expression profiles with each sub-cluster showing selective expression of two distinct, platelet-specific biomarkers. In all cases these markers were significantly upregulated in sepsis patients compared to both healthy control and SIRS patients.
Conclusion:
Here for the first time, we identify at least 3 distinct populations of platelets based on gene expression profiles. Considering the multifaceted role of platelets in sepsis-associated infections, identification of sub-cluster specific biomarkers provides potential insight into the function of different platelet populations and their association with disease progression and severity.

[1]Am J Respir Cell Mol Biol 2018,58(2):331-340; [2]Cardiovascular Research 2015,107(3):386-395; [3]Front Immunol 2020,11:380

Abstract Number - P65

Complementary roles of platelets and red blood cells in high-throughput whole-blood thrombin generation

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Background:
Platelets play a crucial role in coagulation, but this role is incompletely understood in a whole-blood (WB) context. We developed a WB thrombin generation (TG) method, to approach physiology and provide new insights into the coagulation process.
Aims:
To evaluate the contribution of platelets to the process of coagulation in the presence of red blood cells (RBC) through thrombin generation curves.
Methods:
Using a newly developed WB-TG method, traces of TG were measured in parallel in WB and PRP. Triggering was dose-dependently with tissue factor or factor Xa activator, while the intrinsic or extrinsic coagulation pathway was blocked as required. Effects were studied of platelet glycoprotein VI (GPVI) stimulation with CRP-XL; of thrombin receptor (PAR) inhibition; of integrin αIIbβ3 inhibition with tirofiban; or phosphatidylserine blockage with annexin A5.
Results:
whole blood than in PRP upon triggering with low tissue factor, Russell's viper venom, factor Xa or thrombin. Inhibition of the intrinsic pathway but not of factor VII particularly delayed WB-TG, when using the appropriate triggers. Platelet activation with CRP-XL had a limited effect on WB-TG in comparison to PRP-TG. Similarly, platelet inhibition with tirofiban, PAR1, PAR4 or Syk inhibitors more strongly suppressed TG in PRP and in WB. Freshly isolated RBC showed significant phosphatidylserine expression, which could be blocked by annexin A5. Markedly, treatment of WB with annexin A5 substantially increased the effects of CRP-XL and platelet inhibitors to levels seen in PRP. Enhanced sensitivity to platelet modulators was also seen when PRP was reconstituted with annexin A5-treated RBC.
Conclusion:
In WB, the role of activated platelets in TG is preceded and complemented by RBC, in a manner downregulated by exogenous annexin A5. The findings point to a complementary role of phosphatidylserine-exposing RBC, next to platelets, which is expected to be relevant for the RBC-rich clotting in venous thromboembolism.

Abstract Number - P66

The alarmin S100A12 induces the activation of a subpopulation of platelets and the generation of highly activated microvesicles

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Background:
Neutrophil extracellular trap (NET) formation is associated with the release of multiple damage-associated molecular patterns including the alarmins S100A8/A9 and S100A12. We have recently shown that S100A8/A9 induces the formation of procoagulant platelets and potentiates thrombosis. S100A12 acts as a chemokine promoting inflammation and cell apoptosis. Moreover, plasma levels of S100A12 are increased in both sepsis and major thrombotic complications, however, the direct contribution of S100A12 to platelet activation and thrombosis is currently unknown.
Aim:
The aim of this study is to assess the effect of recombinant S100A12 on platelet function and thrombosis in vitro.
Methods:
S100A12 binding to washed human platelets was assessed by flow cytometry. Platelet activation and the formation of procoagulant platelets were assessed using an anti-P-selectin antibody as a marker of α-granule secretion, glycoprotein (GP) IIb/IIIa activation, fibrinogen binding and phosphatidylserine (PS) exposure. Platelet aggregation was assessed by light transmission aggregometry.
Results:
Recombinant S100A12 binds to human platelets in a dose-dependent manner. Despite a strong binding to platelets, S100A12 induces a slow increase in P-selectin expression and rapid activation of GPIIb/IIIa activation and fibrinogen binding in a subpopulation without inducing platelet aggregation. S100A12 did not induce PS exposure as assessed by Annexin-V binding. Strikingly, S100A12 triggered the release of platelet microvesicles with highly activated GPIIb/IIIa and high fibrinogen binding capacity.
Conclusion:
Recombinant S100A12 binds to platelets and induces slow α-granule degranulation and rapid GPIIb/IIIa activation without platelet aggregation. Importantly, S100A12 induces the release of platelet microvesicles with activated GPIIb/IIIa and high fibrinogen binding capacity. Non-classical platelet activation and the formation of a subpopulation of activated platelets and microvesicles might contribute to NET-mediated thrombosis.

Abstract Number - P67

Chemical degradation of Focal Adhesion Kinase utilising PROTACs with different linker lengths; effect on human platelet function.

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Background:
Focal Adhesion Kinase (FAK) is a scaffolding protein and kinase, however its roles in human platelets remains largely unelucidated, with many studies relying on genetic mouse models and non-selective inhibitors. We recently demonstrated that PROteolysis Targeting Chimeric molecules (PROTACs) can successfully generate a targeted protein 'knock-out' in human platelets. PROTACs are chimeric molecules that bind both the protein of interest and an E3 ligase, leading to protein ubiquitination and degradation.
Aim:
To 1) investigate the efficacy and specificity of FAK-targeting PROTACs with different linker lengths, and 2) the effect of FAK degradation upon platelet function.
Method:
Washed platelets and small molecule inhibitors were incubated with PROTACs for 4 hours. The platelet samples were then utilised in western blot analysis with the relevant antibodies, flow cytometry analysis and platelet spreading assays. Confocal imaging was used to assess the stages of platelet spreading. The statistical tests were completed using a two-way ANOVA with Dunnett's multiple comparisons test. This work was funded by the NC3R's and BHF.
Results:
Immunoblotting demonstrated that PROTACs degrade FAK in platelets, lowering levels of FAK by > 75% at nanomolar concentrations. The results indicate shorter chemical linkers give greater specificity/efficacy to the PROTACs for FAK over PYK2. PROTAC 8 shows the greatest selectivity as it induces less than 15% degradation of PYK2 at higher concentrations. Our results furthermore confirm that PROTACs rely on the proteasome for targeted protein degradation. Flow cytometry indicated that the incubation of the platelets with the FAK PROTAC has no effect on integrin activation and P-selectin expression when stimulated by GPVI and PAR-1. FAK degradation attenuated human platelet lamellipodial generation and spreading on a fibrinogen-coated surface.
Conclusion:
This study demonstrates that optimised PROTACs induce degradation of FAK, with limited effect on PYK2, thereby attenuating platelet lamellipodia formation and spreading on fibrinogen.

Abstract Number - P68

Ubc13-UBE2N E2 Ubiquitin ligase, and Lys63 Ubiquitin linkages positively regulate platelet function

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Background:
Ubc13, an E2 ubiquitin-conjugating enzyme (UBE2N) expressed in human platelets, catalyses the attachment of Lys63 polyubiquitin chains to target proteins. Unlike Lys48-linked ubiquitin which targets proteins to the proteasome for degradation, Lys63-linked polyubiquitination plays roles in protein activation, protein interactions, and subcellular targeting of proteins. Whilst genetic deletion of Ubc13-UBE2N has been shown to lead to reduced platelet counts, the role of Ubc13-UBE2N in the regulation of platelet function is unknown.
Aim:
To investigate the role of Ubc13-UBE2N in platelet function and signalling.
Methods:
Pharmacological inhibitors of Ubc13-UBE2N; NSC 697923 and BAY 11-7082 were used to investigate the effects of loss of Ubc13-UBE2N function. Human platelets were pretreated with increasing concentrations of NSC 697923 and BAY 11-7082 (0, 0.01, 0.1, 1, 10 μM) and a range of platelet function and signalling assays performed.
Results:
Treatment with Ubc13-UBE2N inhibitors was associated with decreased global platelet functional responses, with no alterations in platelet integrity. NSC 697923 and BAY 11-7082 were observed to cause a dose dependent inhibition of platelet aggregation and granule secretion to intermediate and threshold concentrations of ADP, Collagen, TRAP-6 and U46619. In addition, platelet adhesion and spreading on both fibrinogen and collagen were also found to be reduced in the presence of NSC 697923 and BAY 11-7082 compared to vehicle treated control. With an increased proportion of platelets unable to form lamellipodia. Inhibition of Ubc13-UBE2N was also found to attenuate clot retraction.
Conclusions:
These findings identify novel anti-platelet activity of Ubc13-UBE2N E2 ubiquitin ligase inhibitors and identifies novel positive regulatory roles for the E2 ubiquitin ligase Ubc13 in the regulation of platelet function. These findings highlight the need for further investigation and insight into the non-canonical roles of ubiquitination and how the posttranslational addition of Lys63 Ubiquitin linkages regulate platelet signalling and function.

Abstract Number - P69

Immunophenotyping of platelets using spectral flow cytometry

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Background:
Platelet function is mediated through expression of specialised surface markers and dedicated signalling pathways, with changes in them shown to underlie haemostatic responses and thrombus formation.
Aim:
To develop an assay to immunophenotype platelets using spectral flow cytometry.
Methods:
Blood was taken from healthy volunteers (n=21), centrifuged to produce platelet-rich plasma, and incubated (20min., 37°C) with ADP, U46619 (thromboxane A2 mimetic), TRAP-6 amide (SFLLRN), PAR-4 amide (AYGKPF), cross-linked collagen-related peptide (CRP-XL), or vehicle. Spectral flow cytometry (Cytek Aurora 5-laser) was used to simultaneously determine the expression levels of 14 markers in platelets comprising cell surface receptors and activation-dependent proteins. Multi-dimensional data were analysed on a global and single platelet level using a combination of NovoExpress and GraphPad Prism software, and bioinformatics and machine learning approaches.
Results:
Following platelet activation, changes in surface marker expression varied according to agonist stimulation. In general terms, expression of 9 markers (CD9, CD36, CD63, CD107a, CD62P, CD29, CD41, CD61, PAC-1) increased following activation, whilst the expression of 4 markers (CD31, CD42a, CD42b, GPVI) decreased, and that of CLEC-2 remained unchanged. The largest increase in expression of CD62P was in response to TRAP-6 (9818±2140 to 53186±2165, $p<0.0001$), and that of PAC-1 to PAR-4 (633±90 to 4866±494, $p<0.0001$). The largest decrease in CD42b was in response to PAR-4 (14014±1171 to 6447±530, $p<0.0001$). Interestingly, at the individual platelet level, those with the highest expression of degranulation markers were largely different from those with the greatest decrease in surface receptors.
Conclusion:
Using spectral flow cytometry, we have developed an assay that can concurrently characterise expression of 14 platelet surface markers in a low-volume sample. Our assay reliably detects changes in response to activation that are consistent with existing literature. Such an assay will be a powerful tool not only in investigating basal and activation-dependent platelet heterogeneity, but also in detecting abnormalities in surface marker expression associated with conditions such as inherited bleeding disorders or major trauma.

Abstract Number - P70

(Epi)genetic comparison of hiPSCs and their ability to differentiate into megakaryocytes and platelets

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Background:
Currently, donations of platelets are required for addressing clinical need such as thrombocytopaenia caused by medical treatments or extensive trauma. However, this model is limited by the short shelf-life and storage requirements of platelet units. Multiple groups are attempting to generate in vitro platelets that would avoid the complications associated with donor recruitment and recipient compatibility, often using human induced pluripotent stem cells (hiPSCs) as a starting point. However, it is commonly known that the input hiPSC line has a significant effect on the quality and quantity of megakaryocytes (MKs) produced by the techniques currently in use worldwide.
Aim:
Therefore, to identify the differences between hiPSC lines, we have been comparing the genetic and epigenetic signatures of “good” (>10 MKs/input hiPSC) and “bad” (<5 MKs/input hiPSC) hiPSCs.
Methods:
To achieve this aim, we have utilised whole genome sequencing for genetic DNA, RNA sequencing for analysis of the transcriptome, ATAC-sequencing for assessing regions of open chromatin, and CUT&Tag sequencing to investigate regions of chromatin enriched with H3K4me3, H3K27ac, or CTCF.
Results:
Consistent with previous reports, QOLG1, FFDK1, PODX1, and Ff_I01S04 have been identified as "good" sources of MKs whereas BIMA1, YZWJ513, YZWJ5217, and TKDN-SeV2 have been identified as "bad" sources of MKs generated by viral forward programming techniques.
Conclusions:
The ongoing work will result in genetic and epigenetic information from 10 different hiPSC lines generated by different groups from different sources.

Abstract Number - P71

SIRT3-mediated deacetylation of cyclophilin D lowers procoagulant platelet formation

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Background:
The formation of procoagulant platelets is regulated through cyclophilin D (CypD), which opens the mitochondrial permeability transition pore (mPTP) when the mitochondrial Ca2+ concentration exceeds a certain threshold. Sirtuin 3 (SIRT3) is the major deacetylase in mitochondria. In nucleated cells, CypD has been demonstrated to be deacetylated by SIRT3, thereby increasing the threshold for mPTP opening. We hypothesize that SIRT3-mediated CypD deacetylation inhibits platelet mPTP opening and hence lowers procoagulant platelet formation.
Aim:
To test whether SIRT3 inhibition with 4'-bromo-resveratrol leads to reduced deacetylation of CypD and an increased procoagulant formation.
Methods:
To inhibit SIRT3, washed human platelets were incubated with 30 µM 4’-bromo-resveratrol prior to activation with CRP-XL and/or α-thrombin. Acetylated CypD was detected using IP and western blot. Platelet activation was measured by P-selectin expression and integrin αIIbβ3 activation using flow cytometry. Procoagulant platelet formation was measured using flow cytometry upon dual agonist activation, by analyzing AnnexinV+/P-selectin+ platelets, mPTP opening and the mitochondrial membrane potential. The procoagulant platelet dependent clotting time was determined using a modified aPTT assay.
Results:
SIRT3 inhibition resulted in 1.3-fold higher levels of acetylated CypD compared to control. SIRT3 inhibition had no effect on normal platelet activation, but significantly increased the formation of procoagulant platelets compared to vehicle control (respectively 38.8% ± 6.5 and 22.1% ± 3.1 AnnexinV+/P-selectin+ platelets). This corresponded with an accelerated clotting time upon SIRT3 inhibition in comparison to vehicle control (103.7sec ± 6.2 compared to 148.9sec ± 9.3 for vehicle control).
Conclusion:
Inhibition of SIRT3 resulted in increased levels of acetylated CypD and increased procoagulant platelet formation. These findings suggests that SIRT3-mediated CypD deacetylation increases the threshold for mPTP formation in platelet mitochondria, consequently decreasing platelet procoagulant activity. Experiments using platelet-specific SIRT3 deficient mice are ongoing to further support these data.

Abstract Number - P72

Effects of the venom of a spitting elapid snake, Hemachatus haemachatus on the modulation of platelet reactivity and blood clotting

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Background:
For a long time, venoms have been a therapeutic resource to develop new treatments for various diseases, including cardiovascular diseases such as thrombotic conditions. Tirofiban is an example of a commercially available venom-derived anti-platelet drug which was developed based on a similar peptide present in the venom of Echis carinatus (the saw-scaled viper). Hemachatus haemachatus (Rinkhals) is a venomous elapid snake that is a distant relative of the cobra and delivers venom by spitting. Its venom was previously reported to possess three different types of three-finger toxins that act as potent inhibitors of platelet aggregation. However, their underlying molecular mechanisms and effect on a range of platelet functions as well as whole blood clotting have not been established.
Aim:
We aim to fully characterise the effects of Rinkhals venom on the modulation of human platelets and whole blood clotting and establish the underlying molecular mechanisms.
Methods:
We used a range of techniques such as flow cytometry, optical aggregometry, rotational thromboelastometry and coagulation analyser to determine the effects of various concentrations of Rinkhals venom on human platelet-rich plasma, isolated platelets, and whole blood.
Results:
The results demonstrate that the Rinkhals venom inhibits platelet activation and aggregation induced by different agonists such as U46619 (a Thromboxane A2 analogue), TRAP 6 (a PAR1 agonist), ADP (a P2Y12 receptor agonist) and collagen (an agonist for Glycoprotein VI and integrin $\alpha 2\beta 1$) in a dose-dependent manner. In line with these results, the Rinkhals venom has extended the clotting time in whole blood and prothrombin time and activated partial thromboplastin time in plasma.
Conclusions:
Our study suggests that the Rinkhals venom has the potential to inhibit platelet activation and control human blood clotting. Therefore, this venom could be useful in bioprospecting to identify and develop potential anti-platelet and anti-coagulant agents to treat/prevent thrombotic diseases.

Abstract Number - P73

Generation of Novel PROTAC Tools For Targeting Protein Kinase Degradation in Human Platelets

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Background:
Platelets are small cells in the blood that play a critical role in stopping bleeding but when inappropriately activated can also contribute to thrombosis and cardiovascular disease. Even though platelets are implicated in a range of diseases, identifying anti-platelets targets is a challenge due to their lack of nucleus as genetic engineering can not be used. The Hers group has recently demonstrated that platelet proteins can be targeted and degraded by PROteolysis TArgetting Chimera (PROTACs). PROTACs are heterobifunctional molecules consisting of three key components: an E3 Ligase Ligand, a flexible linker, and a protein of interest ligand (warhead).
Aim:
To design, synthesise and test novel PROTAC compounds that degrade specific kinases in human platelets, thereby allowing further research into new therapeutic treatments and understanding of biological pathways.
Methods:
PROTAC compounds were tested to degrade the tyrosine protein, focal adhesion kinase (FAK). Results were found via western blotting analysis and observed via densitometry.
Results:
Densitometry data analysis showed that degradation was significant between control and
Conclusion:
A range of different PROTAC compounds work in platelets. This means that PROTAC compounds can be used as an investigative tool to look at specific signalling mechanisms in platelets that are currently unknown. It also allows for a new exciting therapeutic potential as PROTAC’s may be able to cure certain diseases caused by inappropriately activated platelets.

Abstract Number - P74

Histone subfamilies elicit distinct platelet responses

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Background:
Circulating histones are implicated in the pathophysiology of various inflammatory diseases. Histones regulate gene expression within the nucleus but when released into the extracellular space by damaged cells, they act as damage-associated molecular patterns. Previous work has shown that extracellular histones can induce platelet aggregation, formation of procoagulant platelet balloons and release of extracellular vesicles. However, it remains unclear how individual histone subfamilies affect platelets.
Aim:
To determine how different histone subfamilies affect platelet function
Methods:
Washed platelets were prepared from healthy donors and incubated with recombinant human histones (50 µg/mL), Thrombin Receptor Activator Peptide 6 (TRAP-6) (10 µM) or vehicle for 30 minutes at 37C under gentle agitation. Samples were analysed using standard or spectral flow cytometry, or confocal microscopy. Data from $n \geq 3$ experiments and presented as mean ± s.d.
Results:
Histones H2B, H3 and H4 induced robust platelet activation, with a significant increase in P-selectin expression on the surface of platelets (H2B: 48.6±10.4%; H3: 89.1±12.2%; H4 78.1±18.7%, $p<0.05$ vs vehicle). Histones H3 and H4 also resulted in a marked increase in Annexin V binding, which was not seen following stimulation with histone H2B (H3: 68.1±28.1%, H4: 46.8±23.2%, $p<0.05$ vs vehicle; H2B: 5.2±3.6%, $p=0.17$ vs vehicle). Conversely, H1 and H2A exposure did not increase surface P-selectin or Annexin V binding. Detailed immunophenotyping using spectral flow cytometry indicated differences in lysosomal release but not α-degranulation in H3 and H4 treated platelets compared to TRAP6, evidenced by a lack of increase in CD107a expression ($p<0.05$ vs TRAP-6) but similar CD62P expression. Confocal imaging revealed disruption to the supporting microtubule ring following exposure to either H3 or H4, whereas H2B stimulated platelets had a morphology similar to those activated by TRAP6 with no microtubule disruption.
Conclusion:
Histones H3 and H4 induce a procoagulant platelet phenotype, while H2B causes degranulation only. Neither H1 or H2A induce changes in platelet behaviour. These results suggest that interactions between histones and platelets, and downstream activation are dependent on subfamily-specific molecular characteristics. Greater understanding of these interactions is required but support the development of targeted therapeutic agents to modulate histone subfamilies in disease.

Abstract Number - P75

Hierarchy and interactions of Syk, Btk, PKC and MAPKs in GPVI-dependent human platelet activation

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Background:
Glycoprotein VI (GPVI) stimulation activates platelet spleen tyrosine kinase (Syk) and Bruton´s tyrosine kinase (Btk), resulting in phospholipase Cγ (PLCγ) and protein kinase C (PKC) activation. However, their functional hierarchy and crosstalk in platelets are not well understood.
Aims:
To investigate the hierarchy of Syk, Btk and PKC with respect to their downstream effectors in GPVI-stimulated human platelets.
Methods:
Aggregation and signaling of washed human platelets was time-dependently (10-300s) monitored by light transmission aggregometry and immunoblotting in response to the GPVI agonist convulxin. Syk, Btk and PKC were inhibited by PRT-060318, acalabrutinib and GF109203X, respectively. Site-specific antibodies were used to quantify phospho-sites of Syk (S297, Y352, Y525/526), Btk (S180, Y223, Y551), PLCγ2 (Y759, Y1217), LAT (Y220), Akt (T308, S473) and MAPKs (MEK1/2 S217/221, Erk T202/Y204, p38 T180/Y182).
Results:
Strong platelet aggregation induced by convulxin was abolished by 1 µM PRT or 5 µM acalabrutinib. Convulxin induced a rapid, transient upregulation of multisite Y/S-phosphorylation with a clear kinetic hierarchy. Tyrosine phosphorylation (pY) of Syk, Btk, PLCγ2, LAT preceded serine phosphorylation (pS) of Syk, Btk, MEK1/2, Erk, p38 and Akt. PRT did not affect convulxin-induced Syk pY352, but inhibited all other Y-phospho-sites studied. Acalabrutinib inhibited convulxin-induced Btk pY223 (autophosphorylation), PLCγ2 pY759/pY1217, MEK1/2 S217/221, Erk pT202/Y204, p38 pT180/Y182, Akt pT308/S473, Syk pS297 and Btk pS180, but not Syk pY352, pY525/526, LAT pY220 and Btk pY551. GFX did not reduce convulxin-induced tyrosine phosphorylation of Syk, Btk, LAT and PLCγ2. Interestingly, GFX abolished convulxin-induced serine phosphorylation of Syk and Btk and inhibited MEK1/2 and Erk but not p38.
Conclusion:
GPVI activation results in a Btk-dependent activation of PLCγ2, PKC, MEK1/2 and Erk, whereas p38 and Akt activation are Btk- but not PKC-dependent. Specific phospho-sites can be well used as markers for certain kinase activities: Syk pY352 as SFK marker; Syk pY525/526, LAT pY220 and Btk pY551 as Syk marker; Btk pY223 and PLCγ2 pY759/p1217 as Btk marker; Syk pS297 and Btk pS180 as PKC marker. Implications of differential inhibition of the Syk-Btk-system and their downstream effectors on platelet granule secretion are investigated.

Abstract Number - P76

Lipid Mediators in Immunothrombotic Platelet Response and its Noncanonical Regulation by CXCR7

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Background:
FcγRIIA mediated platelet activation in heparin induced thrombocytopenia-(HIT) and other acute immune response like COVID-19 drives immunothrombosis and is associated with hypercoagulatory complications. Although anticoagulants are frequently administered, effective antiplatelet strategies are still under validation.
Aim:
Current investigation explores the involvement of lipid mediators and the therapeutic potential of CXCR7 in modulating immunothrombotic platelet response.
Methods:
We performed lipidomics analysis by UHPLC-QTOF-MS/MS, platelet degranulation, αIIbβIII-integrin activation, procoagulant phosphatidylserine exposure, platelet-neutrophil aggregate formation by flow cytometry, thrombin generation by calibrated automated thrombinoscopy, phosphorylation of SykTyr525/526, SykTyr323 by immunoblot analysis.
Results:
IgGs from HIT, severe and non-severe COVID-19 patients substantially induced platelet degranulation, αIIbβIII-integrin activation, like collagen related peptide (CRP), which were significantly countered by a pharmacological CXCR7-agonist. Moreover, active-CD11b surface expression on neutrophils and formation of platelet-neutrophil aggregates induced by IgGs and CRP were also decreased by CXCR7-agonist. We detected enhanced platelet procoagulant activity in terms of thrombogenic phospholipid phosphatidylserine exposure and consequently thrombin generation upon treatment with IgGs and CRP, that were significantly reduced by CXCR7-agonist. Lipidomics analysis revealed that alteration to the platelet lipidome (e.g. oxylipins) effected by IgGs was distinctively different from that induced by CRP. Moreover, generation of thrombo-inflammatory lipids through platelet COX-1 (TxA2), 12-LOX (12-HETE) and leukocyte 5-LOX (5-HETE), 15-LOX (15-HETE) was induced upon treatment with HIT+ sera and downregulated by CXCR7-agonist. Presence of CXCR7-agonist also intercepted transcellular lipid metabolism between platelet and leukocytes (5,12-diHETE, 12,20-diHETE, 13-HODE, 9-HODE). Like 12-LOX, Syk is a major player and therefore considered a potential therapeutic target downstream of FcγRIIA mediated platelet activation. CXCR7-agonist countered SykTyr525/526, SykTyr323 phosphorylation triggered by IgGs from HIT and COVID-19 patients.
Conclusion:
Considering its dual impact on 12-LOX and Syk, therapeutic targeting of CXCR7 may modulate immunothrombotic complications arising from FcγRIIA induced platelet activation.