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MEETING ABSTRACTS

Abstracts of papers presented at the 2015 UK Platelet Meeting held at the University of Leicester, 15–16 September 2015

DEVELOPMENT AND REFINEMENT OF A REMOTE PLATELET P-SELECTIN TEST FOR MEASUREMENT OF PLATELET FUNCTION IN DOGS AND CATS

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Background: It is increasingly apparent that dogs and cats suffer adversely due to the consequences of abnormal platelet function in a number of disease states. The use of anti-thrombotic therapy is increasingly common as a result. Unfortunately, measurement of platelet function and the efficacy of anti-thrombotic therapy is difficult in both species, due to limited availability of equipment and inability to delay platelet function analysis.
Aims: The aim of this study was to adapt, validate and refine test procedures and protocols previously developed for humans for use in dogs and cats.
Methods: Residual samples of citrate anticoagulated blood were obtained from 34 dogs and 4 cats presented to a specialist veterinary referral centre for various reasons unrelated to abnormal blood clotting. Initially the blood was stimulated using specific combinations of either arachidonic acid/epinephrine (AA/EPI) or ADP/U46619, designed to assess the effects of the anti-thrombotic agents aspirin and clopidogrel respectively. After 5 minutes of stimulation, the blood samples were fixed using a patented platelet fixative solution developed for human platelets, which allows the delayed analysis of P-selectin an established marker of platelet activation. Specific antibodies were selected for the recognition of canine and feline platelets. CD61 was used as a platelet identifier antibody while appropriate CD62P (P-selectin) antibodies for each species were also selected. Fixed samples were repeatedly analysed by flow cytometry at time points between 0 to 28 days following fixation to establish the stability of the fixed samples.
Results: High P-selectin expression was detected following stimulation with AA/EPI and ADP/U46619 in both dogs and cats following fixation. This was significantly different to unstimulated blood ($p<0.0001$). There was no significant difference in detectable P-selectin expression following storage of the fixed samples at any time-point up to 28 days. This confirmed the fixative was suitable as a preservative of canine and feline platelets. A limited number of dogs (n = 6) were evaluated whilst receiving anti-thrombotic medication. There was a significant difference in the activation of platelets in the dogs treated with either aspirin plus clopidogrel ($p<0.005$) or clopidogrel alone ($p<0.002$) compared with untreated dogs following stimulation with AA/EPI (dogs receiving aspirin) or ADP/U46619 (dogs receiving clopidogrel).
Conclusions: Our results demonstrate that fixation and delayed analysis of platelet function in dogs and cats is possible for up to 28 days. The results also demonstrate that anti-platelet medications inhibit platelet function in dogs and cats when assessed using this method. This demonstrates an exciting opportunity to analyse platelet function remotely and to determine the efficacy of thromboprophylaxis in animals presenting to clinics that do not have on-site platelet analysers.

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Declaration of interest: No conflicts of interest have been declared.

FLOW CYTOMETRIC MEASUREMENT OF PLATELET AGGREGATION AND PLATELET-LEUCOCYTE CONJUGATE FORMATION USING SMALL VOLUME OF FIXED WHOLE BLOOD

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Background: We have developed a method that allows simultaneous assessment of platelet aggregation and platelet-leukocyte conjugate (PLC) formation in a small volume of blood.
Aims: To evaluate the performance of this dual assay conducted in a 96 well plate format and its ability to assess both the effects of agents that inhibit platelet aggregation and platelet-leucocyte interactions.
Methods: Platelet function was assessed in whole blood obtained from healthy volunteers, using 96 well plates coated with the following agonists: arachidonic acid (AA, 0.03–1 mM), ADP (0.3–30 μM), collagen (0.1–10 μg/ml) and TRAP (0.1–10 μM), or vehicle. 46 μl of whole blood was added to each well and the plate was shaken for 5 min at 1000 rpm at 37 °C. A fixative solution AGGFix (Platelet Solutions Ltd UK) was applied which stabilizes samples for up to 9 days thus allowing flow cytometry analysis to be performed in a central laboratory. The effects of the platelet inhibitors, aspirin and cangrelor, the GPIIbIIIa blocker, MK-852, and of the inhibitor of PLC formation, a PSGL-1 blocker KPL-1 were investigated using this technique.
Results: Aggregation assessed in duplicate was robust and reproducible (CV<10%). As expected, cangrelor induced a profound inhibition of ADP-induced aggregation and aspirin dose-dependently inhibited platelet responses to AA. Collagen- and TRAP-induced aggregation was also impaired to a different

extent by in vitro treatment with either antiplatelet agent. PLC formation was readily measured in the same fixed whole blood samples and was reduced by platelet inhibition with aspirin and cangrelor and by KPL-1; however, platelet stimulation in the presence on MK-852 dramatically increased the PLC formation.
Conclusions: This new technique, which enables platelet aggregation and PLC formation to be assessed by flow cytometry in the same fixed whole blood samples, requires very small volumes of blood and would be especially useful in individuals where blood sample volume is limited.

PLATELET-DERIVED EXTRACELLULAR VESICLES ACT AS AN INTERCELLULAR COMMUNICATION MECHANISM WITH MONOCYTES

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Background: Platelets contain abundant microRNAs which can be packaged into extracellular Vesicles (EV) and released into the circulation. Platelets release two types of EVs; procoagulant microparticles (MP) and exosomes. EV's containing proteins and nucleic acids have been shown to transfer their contents to target cells, thereby acting as an important intercellular mediator.
Aims: We characterised the phenotype and miRNA content of EVs released from platelets stimulated by different agonists and demonstrated the transfer of platelet-derived EV to monocytes.
Methods: Washed platelets from healthy subjects were maximally stimulated with agonists specific for GPVI (CRP-XL), PAR1 (SFFLRN), PAR4 (AYGPKF), P2Y1/P2Y12 (ADP) or PAR1/PAR4 (thrombin), with/wo inhibiting COX-1 (aspirin), aggregation (GPIIb-IIIa mAb) or ADP (apyrase). Released EVs isolated by differential centrifugation were characterised by size (Nanosight) and for procoagulant activity (Annexin-V binding and Stago CAT assay), or the exosome-specific markers CD63 & HSP70. RNA was isolated from EV populations, reverse transcribed and amplified, and the miRNA profiled on TaqMan microRNA microarray cards. Platelet-derived EV produced using stimulation with PAR1-AP were stained using PKH67 (Sigma-Aldrich) and then incubated with the THP-1 monocyte cell line. EV uptake was measured using fluorescence microscopy and flow cytometry.
Results: Stimulation through GPVI produced a mixed population of MPs and exosomes, while all other agonists released predominantly exosomes. Of the inhibitors, only apyrase reduced EV release for all agonists, demonstrating the importance of ADP in regulating EV release. The EVs contained between 57–79 different miRNA with a core of 36 miRNA observed with all agonists. There is high correlation of agonist profiles ($r^2 > 0.98$ for all), and also with the total platelet miRNA content ($r^2 > 0.98$).
The 36 miRNA seen in all samples are predicted to have significant effects on the translation of proteins involved in endocytosis, cell cycle control and differentiation. miR-223 was most highly expressed in all samples and has previously been shown to affect myeloid lineage development and have anti-inflammatory and cardioprotective effects.
Platelet-derived EV uptake was observed in THP-1 cells using both flow cytometry and fluorescence microscopy. This suggests that platelets can play a key role in the control of monocyte protein expression through the microRNA transferred in EV
Conclusions: These data suggest that while the EV profile released from platelets is agonist-dependent, all agonists release exosomes with similar miRNA content. ADP plays an important role in the release of exosomes. The data also suggest exosomes as the most likely vehicle for miRNA release from platelets with these exosomes acting as intercellular messengers.

LIVE PLATELET ASSAY FOR THE DETECTION OF AMYLOID BETA PEPTIDE-DEPENDENT ROS GENERATION

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Background: Amyloid beta (Aβ) peptides are protein fragments present as insoluble polymeric depositions in the brain parenchyma and cerebral blood vessel walls of Alzheimer's disease (AD) patients. Studies on AD patients have demonstrated pre-activated platelets and established Aβ peptides' contribution to disease pathology. However, the exact intracellular mechanisms underlying platelet activation triggered by Aβ peptides are not well established. Emerging evidences suggested a strong correlation between Aβ peptide effects on platelets and reactive oxygen species (ROS) generation. Unfortunately, experimental conditions and protocols varied significantly amongst studies and a complete picture of the link between Aβ peptides and platelet redox homeostasis is still missing.
Aims: Establish/optimize a protocol for the analysis of ROS generation in live platelets and investigate the effect of synthetic Aβ peptides on ROS generation in human platelets.
Methods: Platelets were isolated from human whole blood then incubated with chloromethyl-dihydro-2′,7′-dichlorohydrofluorescein diacetate (CM-H2-DCFDA) or dihydroethidium (DHE) to detect intracellular ROS. 10 μg/ml Collagen and ROS scavenger, N-acetylcysteine (NAC, 1mM), were used as positive and negative controls, respectively. Commercially available synthetic Aβ25-35 peptide and in house-synthesized Aβ25-35 peptide were used as stimulus. Fluorescence levels generated were assessed using three different approaches including microplate reader, flow cytometry and confocal microscopy.
Results: Preliminary studies using microplate reader generated varied and inconsistent results. Live-platelet confocal microscopy with single cell kinetics analysis was successful in the detection of ROS generation rate increase induced by collagen. Nonetheless, this method was not pursued further due to its laboriousness and extremely low throughput. Flow cytometry using CM-H2-DCFDA resulted to be a more reliable and convenient approach for ROS detection. Robust increase in ROS generation was stimulated by collagen (10 μg/ml), which was significantly inhibited by 1mM NAC. Different concentrations of both commercial and in-house synthesized Aβ25-35 peptides (1 μM, 3 μM, 10 μM, 30 μM and 100 μM) were assessed. No significant differences were identified between commercial and in house-synthesized Aβ25-35 peptide. 30 μM and 100 μM of Aβ25-35 induced a significant increase in ROS generation, but not the lower concentrations.
Conclusion: We developed a reliable and convenient flow cytometry method for the analysis of ROS generation in human platelets. This assay will be used for the analysis of redox-dependent responses of human platelets in response to Aβ peptides. This will provide novel information on the cerebrovascular symptoms associated with AD.

OXIDATION OF C-REACTIVE PROTEIN BY HYPOCHLOROUS ACID LEADS TO THE FORMATION OF POTENT PLATELET ACTIVATOR

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Background: Currently available evidence indicates that C-reactive protein, a nonspecific marker of inflammation, has a potential causal role in the pathogenesis of atherosclerosis. However, it is still controversial whether pentameric CRP (native CRP; nCRP) or rather its monomeric form (mCRP; modified CRP) mediates proinflammatory action *in vivo*. Several mechanisms involved in generation of modified CRP have been identified so far. They include direct synthesis of mCRP by extrahepatic cells, dissociation of nCRP to monomers on activated blood platelets or exposure of native CRP to acidic or alkaline environments, such as those found at inflammation sites. Importantly however, the biological role for CRP modified by hypochlorous acid (HOCl), abundantly generated in vivo via myeloperoxidase/H_2O_2/Cl^- system, has not yet been elucidated.
Aims: In the present study we examined structural consequences of oxidative modification of C-reactive protein by hypochlorous acid and investigated the *in vitro* effects of the interactions of HOCl-modified C-reactive protein with washed blood platelets and plasma proteins.
Methods: Sodium dodecylsulfate-polyacrylamide gel electrophoresis and fluorescent spectroscopy were used to identify changes in CRP structure after HOCl-mediated oxidation. Functional analyses included flow cytometry, light transmittance aggregometry, ELISA and surface plasmon resonance, which we employed to reason on platelet function and interactions of HOCl-oxidized CRP with selected human plasma proteins (complement component C1q and immunoglobulin G).
Results: Exposure of CRP to HOCl led to protein unfolding, the increased surface hydrophobicity and formation of aggregates. The oxidatively modified CRP (HOCl-CRP, 50 μg/ml) significantly stimulated platelet activation (over 10-fold increased fraction of P-selectin-positive platelets compared to non-treated platelets, $P<0.008$, $n = 7$), enhanced the deposition of platelets onto immobilized fibrinogen (2-fold rise compared to the control, $P<0.0001$, $n = 4$), and induced spontaneous platelet aggregation (up to 79.5%). Contrary to nCRP, HOCl-CRP strongly bound to immobilized C1q and IgG. This points to possible HOCl-mediated structural changes in CRP molecule, resulting in the appearance of neo-epitopes and the formation of new ligand binding sites, both characteristic for proinflammatory monomeric form of CRP.
Conclusions: Oxidation of native CRP by HOCl seems to represent an alternative mechanism of CRP modification, by which CRP unmasks its proinflmmatory and prothrombotic properties. Hence, it may become the trigger in the pathogenesis of atherosclerosis.

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IBRUTINIB INHIBITS PLATELET INTEGRIN $\alpha_{IIb}\beta_3$ OUTSIDE-IN SIGNALING AND THROMBUS STABILITY BUT NOT ADHESION TO COLLAGEN

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Background: Ibrutinib is an irreversible Btk inhibitor approved for treatment of Waldenstrom's macroglobulinemia, chronic lymphocytic leukemia and mantle cell lymphoma that increases the risk of bleeding among patients. Platelets from ibrutinib-treated patients exhibit deficiencies in collagen-evoked signaling in suspension, however the significance of this observation and how it relates to bleeding risk is unclear, as platelets encounter immobile collagen *in vivo.*
Aims: We sought to clarify the effects of ibrutinib on platelet function, especially during adhesion, to better understand the mechanism underlying bleeding risk.
Methods: We treated platelets from healthy donors with ibrutinib to investigate its effects on platelet aggregation, Ca^{2+} elevation in suspension, phospho-blotting and clot retraction. We used these results to compare with the effects of ibrutinib during platelet adhesion to immobilised fibrinogen or collagen using various methods including single-cell Ca^{2+} imaging and thrombus formation on collagen under flow and phospho-blotting of signalling molecules.
Results: Ibrutinib inhibits aggregation and Ca^{2+} elevation evoked by the GPVI-specific agonist, CRP-XL (IC50 = 186 nM and 51 nM, respectively). Aggregation and $[Ca^{2+}]i$ elevation evoked by collagen were also inhibited by 1 μM ibrutinib whereas responses evoked by ADP, U46619 and thrombin were unaffected. Platelet adhesion to immobilised fibrinogen and CRP-XL was inhibited by 48% and 70% respectively by 1 μM ibrutinib, while adhesion to immobilised collagen was not significantly inhibited. Spreading on collagen was impaired (control = 48% vs. 1 μM ibrutinib = 35% platelets expressing lamelipodia) and Ca^{2+} elevation on collagen was partially impaired. Processes that are dependent on integrin $\alpha_{IIb}\beta_3$ outside-in signalling were strongly inhibited by ibrutinib: Ca^{2+} elevation evoked by adhesion to fibrinogen was abolished by 1μM ibrutinib and clot retraction was inhibited 2.1-fold. In vitro thrombus formation on collagen under arterial shear was inhibited by 61% after 10 minutes in the presence of 1μM ibrutinib due to the formation of unstable thrombi, although initial adhesion was unaffected. When 1 μM ibrutinib was combined with the P2Y12 antagonist cangrelor (1 μM) thrombus formation was inhibited additively (76% inhibition).
Conclusions: By comparing signaling in suspension and during adhesion to immobilised ligands we found that the collagen signaling deficiency caused by ibrutinib is milder during adhesion to immobilised collagen than it is when platelets are stimulated in suspension. We also found that platelets in whole blood treated with ibrutinib adhered to collagen under arterial shear but formed unstable thrombi, suggesting that the collagen signaling deficiency caused by ibrutinib may not be the predominant cause of bleeding *in vivo.* However, clot retraction and signaling evoked by platelet adhesion to immobilised fibrinogen were also inhibited by ibrutinib, indicating that integrin $\alpha_{IIb}\beta_3$ outside-in signaling is also effected in addition to GPVI signaling. When ibrutinib was combined with the $P2Y_{12}$ inhibitor, cangrelor, thrombus formation under arterial shear was inhibited additively. These findings suggest that (1) ibrutinib causes GPVI and integrin $\alpha_{IIb}\beta_3$ platelet signaling deficiencies that result in formation of unstable thrombi and may contribute toward bleeding observed *in vivo* and (2)

combining ibrutinib with $P2Y_{12}$ antagonists, which also inhibit thrombus stability, may have a detrimental effect on hemostasis.

THIOREDOXIN INHIBITORS ATTENUATE GPVI-MEDIATED PLATELET ACTIVATION AND THROMBUS FORMATION

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Background: Oxidoreductases contribute to a number of physiological and pathological processes by limiting damage caused by oxidative stress, as well as regulating protein structure and function through reduction/oxidation of disulphide bonds. Thioredoxin (Trx) is an oxidoreductase associated with a number of pathologies including cancer and inflammation, but its effect on thrombosis and haemostasis is largely unknown.
Aims: We set out to determine if, and to what extent, Trx contributes to platelet function.
Methods: Platelet function was assessed using standard platelet functional assays (aggregation, Ca^{2+} release, reactive oxygen species (ROS) generation). Thrombus formation in whole blood under flow was assessed using a Fluxion Bioflux200.
Results: Inhibitors of Trx function selectively attenuated CRP-XL-induced aggregation in PRP, Ca^{2+} release in washed, fura2am-loaded platelets, and ROS generation in a concentration-dependent manner; there was no effect on thrombin, ADP or U46619 responses. Kinetics was a factor as extended incubations enhanced the inhibitory effect of lower drug concentrations; thrombin-, ADP- and U46619-mediated effects were unaffected suggesting that loss of CRP-XL-induced effects was not due to toxicity.
Conclusions: Using anti-cancer drugs that inhibit Trx activity, we assessed the contribution of Trx to platelet reactivity and function. Loss of Trx activity affects GPVI-mediated platelet activation in a selective and concentration dependent manner. This translates to effects on thrombus formation in whole blood on collagen under physiological shear rates. Early indications suggest that these anti-cancer Trx inhibitors have potential as antiplatelet medications.

BIOGENESIS, SUBCELLULAR LOCALIZATION AND MOBILIZATION OF TWO ABUNDANT PLATELET THIOL ISOMERASES

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Background: Thiol isomerases facilitate the rearrangement of protein disulphide bonds required for the correct folding of secreted proteins. While typically localized in the endoplasmic reticulum (ER), in platelets they are believed to act at the cell surface to regulate platelet activation, although their origins and distribution in these cells has caused contention.
Aim: we explored the biogenesis of PDI and ERp57, and followed their fate from immature megakaryocytes (MK) to mature platelets, and their release to the platelet surface.
Methods and Results: Using confocal microscopy we observed that throughout MK development, PDI and ERp57 were organized into punctuate structures in the cytoplasm which become progressively smaller with cell maturation and were trafficked to proplatelets. PDI and ERp57 were transported separately from α-granule cargo proteins and from calnexin, an ER chaperone. In mature human or mouse platelets they were not found in α-granules, as also confirmed by sucrose gradient sub-cellular fractionation, but were co-distributed in distinct granular compartments that were present in Nbeal2-/- platelets that lack α-granules. PDI, which was also absent from lysosomes and δ-granules, has been reported to localize with TLR9 in electron-dense regions termed T-granules. We did not observe a significant degree of co-distribution between PDI and TLR9, suggesting that this thiol isomerase largely reside in compartments distinct from T-granules. When platelets were activated, thiol isomerases were secreted to the platelet surface and this, similar to α-granule secretion, was regulated by the polymerization of actin.
Conclusions: PDI and ERp57 are synthesized early in megakaryocyte development and packaged into secretory compartments that are distinct from known granules and from where they are released to the platelet surface via an actin-dependent mechanism.

PLATELET ACTIVATION AND FUNCTION IS IMPEDED BY THE SECRETOME OF HODGKIN'S LYMPHOMA CELLS

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Background: Lymphoma is commonly associated with platelet dysfunction, usually evidenced by the occurrence of thrombosis in lymphoma patients. However, active bleeding is also a frequent occurrence. Bleeding is typically attributed to thrombocytopenia which occurs due to impaired megakaryocyte production in the bone marrow. There is a growing body of evidence demonstrating that platelets can actively sequester growth factors, inflammatory proteins and even miRNAs from their microenvironment and this can affect platelet function. The secretome of lymphoma cells, which contains lymphoma-derived growth factors and cytokines could therefore contribute to bleeding symptoms in lymphoma patients via thrombocytopathy in the presence of adequate megakaryocyte production.
Aims: The study aimed to determine the effect of the secretome from L-1236 Hodgkin's lymphoma cells on the activation and function of platelets from healthy subjects.
Methods: L-1236 Hodgkin's lymphoma cells were cultured at specific cell concentrations (3×10^5–5×10^6) for specific times (24, 48, 72 and 96 hours) in RPMI growth media under normal growth conditions (37 °C, 5% CO_2 in air). Conditioned media was collected at the specified times. Platelet rich plasma (PRP) was purified from citrated whole blood samples from healthy volunteer donors. PRP was incubated with L-1236 conditioned media for 1 hour at 37 °C. Platelet aggregometry was performed following stimulation with 5 μM ADP. Platelet activation was assessed by analysis of CD62P and PAC-1 by flow cytometry.

Results: L-1236 conditioned media significantly inhibited the expression of CD62P and PAC-1 on the surface of platelets at all cell concentrations and all culture times tested. The inhibition appeared to be dose-dependent, with conditioned media collected from 5×10^6 cells after 96 hours causing maximal inhibition. This dose response effect was also true for the aggregometry results, with inhibition of ADP-stimulated aggregation.
Conclusions: Platelet activation and function is significantly affected by the secretome of L-1236 Hodgkin lymphoma cells. The results provide further evidence of a link between lymphoma and platelet function. Further work aims to characterise the specific proteins responsible for this moderation of platelet function.

DETECTION OF PLATELET SECRETION DEFECTS IN SUBJECTS WITH MUCOCUTANEOUS BLEEDING USING A RELIABLE AND SIMPLE DIAGNOSTIC APPROACH

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Background: Mild platelet function defects (PFDs) are complex, difficult to diagnose and often require extensive platelet function testing on freshly collected blood samples. The prevalence of PFDs might be underestimated due to complexity of the required testing and limited accessibility of diagnostic facilities across the world. Secretion defects are among the most common PFDs.
Aims: To assess the diagnostic value of a simple assay for platelet markers of dense (CD63) and α-granule (P-selectin) secretion that can be performed on blood taken locally and then sent to a central laboratory for analysis.
Methods: Subjects with impaired dense granule secretion ($n = 9$) were identified within the GAPP (ISRCTN 77951167) study population ($n = 61$) using the lumi-aggregometry (LA) approach. Platelet CD63 and P-selectin were assessed in unstimulated and stimulated (ADP/U46619, TRAP-6 and AA/epinephrine) whole blood samples that were stabilized with PAMFix (Platelet Solutions Ltd., UK) and analyzed using flow cytometry (FC) in a central laboratory within 9 days. Normal ranges were established using ROC curve analysis. For P-selectin measurements ROC curves were generated using the subjects with and without any defect, as determined by LA, and for measurements of CD63 subjects with and without a secretion defect. Healthy controls were studied in parallel ($n = 41$).
Results: All 9 patients with reduced secretion on LA presented with abnormal results on the FC assay. Two had a very high level of CD63 even in the unstimulated samples with reduced P-selectin, which is characteristic of Hermansky-Pudlak syndrome type 2. Four subjects with the lowest levels of secretion on LA had reduced CD63 with all three stimulants and reduced P-selectin with at least one stimulant; this pattern was not observed in subjects with other types of platelet defects ($n = 24$). In another 3 subjects with secretion defect both CD63 and P-selectin were reduced with at least one agonist. This pattern of results was seen in 6 of 24 subjects who were labeled as mildly abnormal by LA. Only 1 subject with normal platelet function on LA ($n = 28$) had CD63 expression slightly below and P-selectin expression within normal range. Using the set cut-off levels showed that 5 healthy controls presented with a test result with one agonist below the normal range.
Conclusion: Abnormal granule secretion can be identified using a new simple assay that could be performed remotely as first line diagnostic testing for PFDs.

DOES BLOOD GROUP AFFECT PLATELET INTERACTIONS WITH VON WILLEBRAND FACTOR?

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Background: Blood group is a risk marker for myocardial infarction; it is also a risk marker for bleeding, it is not clear why. ABO antigens expressed on platelets and von Willebrand Factor (VWF) may cause functional changes in either platelets or VWF.
Aims: To investigate the functional behaviour of platelets from donors with different blood groups we perfused blood from healthy normal donors over a surface of VWF purified from pooled human plasma at rates of arterial shear (1500s-1). A total of 80 normal donors were characterised.
Methods: Microlitre quantities of whole blood were fluorescently labelled and prefused through a parallel perfusion flow chamber. 1000 images were recorded in real time at a frame rate of 30 frames per second. Platelet motion was tracked and measured. Outputs recorded included the number of platelets translocating or rolling across the VWF coated surface, the number of platelets stably adhered to the surface, the distance travelled and speed of movement for each platelet. Plasma VWF levels for each donor were measured by ELISA.
Results: Blood from 80 volunteers was analyzed. There were 30 blood group O donors and 50 non-O donors. Mean plasma VWF was 684 IU, with a range of 138 to 2729 IU. Platelets from volunteers with blood group O travelled a distance of $9.2 \pm 3.4 \propto$M (mean ± SD) across the VWF surface compared to $7.4 \pm 3.2 \propto$M for non-O individuals ($p = 0.0004$). The speed of platelet translocation was also increased in Type O donors – $4.6 \pm 2.5 \propto$M/sec compared to $3.4 \pm 2.4 \propto$M/sec in non-O platelets ($p = 0.003$). Type O donors had more translocating than static platelets (ratio 1.4 ± 0.5 vs. 1.2 ± 0.4, $p = 0.05$).
Conclusion: The results of this investigation demonstrate that platelets from type O donors travel further and move faster when perfused over plasma derived human VWF. These results are independent of donor VWF plasma concentrations. Collectively, these data suggest a novel mechanism through which specific ABO blood groups may modulate risks for both thrombosis and bleeding respectively.

THE GASOTRANSMITTER HYDROGEN SULFIDE IS A NEGATIVE REGULATOR OF PLATELET AGGREGATION *IN VITRO* AND *IN VIVO*

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Background: Hydrogen sulfide (H_2S) is generated by a range of human tissues and mediates a variety of biological processes such as vasodilatation, inflammation and mitochondrial function. Studies to date with platelets suggest inhibitory roles following exposure to only non-physiological mM

concentrations of salts which release a short burst of H_2S. The functional impact of continuous exposure to H_2S that mimics physiological generation is unknown. In addition, the physiological relevance of endogenous H_2S to platelets *in vivo* remains undefined.

Aims: To determine whether and by which enzymes platelets generate H_2S. Secondly, to determine the impact of slow, consistent exposure to H_2S at physiological concentraions upon platelet function and to investigate the role of endogenous H_2S in regulating platelet function *in vivo*.

Methods: Expression, enzymatic activity and endogenous protein S-sulfhydration of the two principal H_2S generating enzymes cystathionine-γ-lyase (CSE) and cystathionine-β-synthase (CBS) were assessed by western blotting, thialysine, Bindschelder's assays and a tag-switch assay in human platelet lysates respectively. *In vitro* and *in vivo* platelet function was assessed by light transmission aggregometry and in a real-time mouse model of platelet thromboembolism with novel slow releasing H_2S donors and inert spent controls.

Results: Western blotting showed consistent and robust expression of CBS but not CSE in human platelets. Platelets also contained CBS but not CSE catalytic activity. H_2S generation by resting platelets could be detected by an H_2S specific probe and was significantly reduced upon pharmacological inhibition of CBS. Platelets contained S-sulfhydrated proteins. Exposure of platelets to the slow release donor GYY4137 led to inhibition of aggregation in the nM range of H_2S and the H_2S donor AP67 which releases H_2S at a greater rate that GYY4137 had higher potency. Treatment of mice with the CBS inhibitor aminooxyacetate significantly increased collagen-induced platelet aggregation *in vivo* compared to spent donor control treated mice.

Conclusions: Platelets generate H_2S catalytically from CBS which is associated with S-sulfhydration of as yet unidentified proteins. Slow consistent exposure to H_2S to mimic phsyiological release inhibits platelet aggregation in the nM range through mechanisms that remain to be identified. Finally, endogenous H_2S in a negative systemic regulator of platelet function and may exert anti-thrombotic activity *in vivo*.

CONSECUTIVE *SLFN14* MUTATIONS IN 3 UNRELATED FAMILIES WITH AN INHERITED BELLEDING DISORDER, THROMBOCYTOPENIA AND SECRETION DEFECTS

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Background: Inherited thrombocytopenias are a rare heterogeneous group of disorders characterised by a low platelet count and sometimes associated with excessive bleeding, ranging from mild to severe. A cohort of 36 patients and 17 family members all displaying a reduced platelet count were recruited to the UK-GAPP (Genotyping and Phenotyping of Platelets) study. All patients had a history of excessive bleeding. Critically, all major known causes of inherited thrombocytopenia (e.g. MYH9-related disorder, Bernard Soulier syndrome and Wiskott-Aldrich syndrome), had been ruled out prior to enrolment.

Aim: The aim of this project was to identify novel disease causing mutations in patients with inherited thrombocytopenia and/or platelet function defects. Once novel mutations had been identified, the mechanism through which the genetic mutation mediates thrombocytopenia/platelet dysfunction was to be investigated.

Methods: Platelet function analysis was performed using standard techniques including lumi-aggregometry examining both aggregation and ATP secretion in response to a panel of platelet agonists. P-selectin response was measured using flow cytometry. Alongside platelet phenotyping, whole exome sequencing was performed on all patients. Further characterisation of patient and healthy volunteer platelets was achieved using electron microscopy, Western blotting and protein localisation studies.

Results: We identified mutations in the novel gene *SLFN14* in 12 patients from three unrelated families. All patients displayed an analogous phenotype of moderate thrombocytopenia, enlarged platelets, decreased ATP secretion upon stimulation with platelet agonists and a dominant inheritance pattern. *SLFN14* codes for a protein of unknown function, Schlafen family member 14 (SLFN14). Three heterozygous missense mutations predicting p.K218E, p.K219N, p.V220D substitutions within an ATPase-AAA-4, GTP/ATP binding region were identified in affected but not unaffected family members. Platelets from all three families had a marked reduction in expression of endogenous SLFN14. This corresponded with expression studies in HEK293T cells which demonstrated a significant reduction in all three mutants relative to the wild type protein, suggesting instability. Electron microscopy studies demonstrated a significant reduction in the number of dense granules in platelets from affected patients relative to those from healthy volunteers, correlating with a decrease in measured ATP secretion using lumi-aggregometry.

Conclusions: Together these results identify mutations in *SLFN14* as causative gene for an inherited thrombocytopenia and significant bleeding diathesis, outlining a fundamental role of SLFN14 in platelet formation and megakaryopoiesis.

RETINOID X RECEPTOR LIGANDS EXHIBIT NON-GENOMIC EFFECTS TO INHIBIT COLLAGEN AND THROMBIN-MEDIATED PLATELET ACTIVATION

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Background: Retinoid X receptors (RXRs) are ligand bound transcription factors that are involved in regulation of vital biological processes such as cellular differentiation, haematopoiesis and embryogenesis. RXRs function by forming homodimers or heterodimers by binding to almost a quarter of the known human nuclear receptors. Platelets, despite being enucleated, express numerous nuclear receptors (such as liver X receptors, peroxisome proliferator activated receptors, and glucocorticoid receptors). Both the isoform of RXRs (α and β) have been reported to be present in platelets, which upon stimulation inhibit platelet activity mediated by Gq coupled ADP and TXA_2 receptors.

Aims: The present study aims to identify the effects of RXR ligands and the receptor on platelet function following stimulation by the major platelet agonists-collagen and thrombin and also to investigate its role in the regulation of outside-in signalling via the integrin $\alpha_{IIb}\beta_3$.
Methods: Washed human platelets were pre-treated with RXR ligands, 9-cis-retinoic acid (9cRA) or methoprene acid (1, 5, 10 and 20 μM) for five minutes and collagen (1 μg/ml) and thrombin (0.05 U/ml) induced aggregation was measured using light transmission aggregometry. Levels of calcium mobilisation were assessed by spectrofluorimetry using FURA-2AM loaded platelets after treatment with RXR ligands and stimulation with collagen related peptide (CRP-XL) or thrombin at concentrations of 0.25 μg/ml and 0.05 U/ml, respectively. Affinity up-regulation of integrin $\alpha_{IIb}\beta_3$ and α-granule secretion was measured using flow cytometry through the detection of levels of fibrinogen binding and P-selectin exposure on platelet surface, respectively. The extent of platelet adhesion and spreading on fibrinogen (100 μg/mL) after pre-treatment with 9cRA was studied using phalloidin Alexa-488 stained platelets and visualised by confocal microscopy.
Results: Both the RXR ligands significantly inhibited collagen and thrombin induced platelet aggregations in a dose-dependent manner with stronger inhibition of collagen or CRP-XL responses in comparison to thrombin. Both 9cRA and methoprene acid exhibited concentration-dependent inhibition of P-selectin exposure and fibrinogen binding indicating the suppression of α-granule secretion and up-regulation of integrin $\alpha_{IIb}\beta_3$ affinity, respectively, with significant reduction observed at concentration of 10 and 20 μM. Calcium mobilisation was also inhibited upon treatment with either of the ligands. Lastly, the effect of 9cRA on $\alpha_{IIb}\beta_3$ outside-in signalling in platelets was studied by examining platelet adhesion and spreading on fibrinogen. The number of platelets adhering to fibrinogen was significantly decreased upon incubation with 10 and 20 μM of 9cRA in contrast to untreated platelets. An inhibition of platelet spreading (formation of filopodia and lamellipodia) was also observed upon treatment with 9cRA compared to untreated controls.
Conclusions: RXR ligands negatively regulate both collagen and thrombin-mediated platelet activation and inhibit $\alpha_{IIb}\beta_3$ outside-in signalling. Future work will determine the mechanisms by which RXR and its ligands act.

BREAST CANCER CELLS STIMULATE THE RELEASE OF PRO-METASTATIC MICROPARTICLES FROM HUMAN PLATELETS: A NOVEL POSITIVE FEEDBACK MECHANISM FOR TUMOR SPREAD

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Background: The survival of breast cancer patients with distant metastasis is very low and, once detected, metastatic breast cancer is incurable. Circulating blood platelets support cancer metastasis through multiple mechanisms, including the protection of cancer cells from the immune system, the release of bioactive molecules that control metastasis and angiogenesis, and the promotion of cancer cell extravasation. Also platelet-derived microparticles (PDMPs) may represent important regulators of the metastatic potential of cancer cells in the bloodstream and an increased level of circulating PDMPs is associated to several malignancies, including breast cancer. However, the ability of cancer cells themselves to directly induce the release of PDMPs has never been documented.
Aims: In this study we have investigated and characterized the release of PDMPs induced in vitro by platelet exposure to a breast cancer cell line (MDA-MB-231) and we have determined their possible pro-metastatic effect.
Methods: Tumor cell-induced platelet aggregation (TCIPA) stimulated by breast carcinoma cells MDA-MB-231 was assessed by light transmission aggregometry. PDMPs released upon exposure of platelets to cancer cells were recovered by ultracentrifugation and analyzed by fluorescence microscopy, flow cytometry and protein quantification. The pro-metastatic effect of PDMPs on MDA-MB-231 cells was investigated by the analysis of cell migration in a transwell assay and in a wound healing test.
Results: MDA-MB-231 failed to induce the release of PDMPs and TCIPA in washed platelets, but the addition of a small amount of plasma was sufficient to support both events. TCIPA and tumor cell-induced PDMPs release were suppressed by the thrombin inhibitor PPACK. TCIPA was also prevented upon cAMP increase by PGE_1, ADP scavenging by apyrase, as well as by the simultaneous inhibition of fibrinogen binding to platelets and fibrin polymerization. PDMPs released by platelets upon exposure to MDA-MB-231 were able to stimulate the migration of the same cancer cells in a dose-dependent fashion.
Conclusions: MDA-MB-231 cells stimulate platelets to release PDMPs in association to a full platelet aggregation. The sole interaction between cancer cells and platelets is not sufficient to trigger these responses, which require the contribution of plasma components. Released PDMPs display a strong ability to potentiate the migration of MDA-MB-231 cells. In conclusion we described a possible novel pro-metastatic loop where breast cancer cells induce platelets to release microparticles that, in turn, stimulate their metastatic potential.

COMPARISON OF THE MULTIPLATE® WITH LUMI-AGGREGOMETRY FOR THE DIAGNOSIS OF PATIENTS WITH MILD BLEEDING DISORDERS

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Background: The Multiplate® platelet analyser (MPA) is an impedance aggregometer used for performing platelet function testing in whole blood. This approach offers the advantage of assessing platelet function in more physiological conditions and avoids the variables associated with the preparation of PRP. A number of studies have showed the utility of this method in assessing platelet inhibition in individuals taking antiplatelet agents e.g. aspirin and clopidogrel. Until now few studies have been performed to fully assess the potential of MPA for the diagnosis of patients with platelet bleeding disorders.
Aims: To evaluate the potential role of MPA for the routine diagnosis of bleeding disorders by comparison with Light transmission lumi-aggregometry (LTA)
Methods: 74 patients were recruited to the GAPP project with classical histories of mild bleeding disorders. A standardized bleeding assessment tool (ISTH-BAT) score was also performed and all clotting and VWF parameters were normal. Whole blood samples were anticoagulated with trisodium citrate from each centre and sent to Birmingham by courier for analysis. MPA was performed with a limited panel of agonists (ADP [10 and 2.5 μM], PAR-1 peptide [100 μM] and collagen [1 and 3 μg/mL]). LTA was

performed in platelet rich plasma (PRP) by lumi-aggregometry (Chrono-Log 460 VS aggregometer, LabMedics, UK) using a full panel of agonists (ADP [3, 10, and 30 μM], adrenaline [3, 10, and 30 μM], arachidonic acid [0.5, 1, and 1.5 mM], collagen [1 and 3 μg/mL], PAR-1 peptide [10, 30 and 100 μM], and ristocetin [1.5 and 2 mg/mL]). Additional agonists U46619 [1 and 3 μM] and CRP [1, 3, and 10 μg/mL] are also utilised when there were abnormal responses to arachidonic acid and collagen respectively.
Results: Out of a total of 74 patients, 5/74(6.8%) and 33/74 (44.6%) had abnormally low responses to one or more agonists by MPA and LTA respectively. 40 patients gave normal responses to all agonists by MPA and LTA. 4/5 patients with abnormal MPA were also abnormal by LTA. Of these 1 patient gave an abnormal response to collagen by MPA only. Normal results on MPA and LTA were found in 6/8 and 4/8 patients with mild to moderate thrombocytopenia (48–147 x 10^9/L) respectively. 4 patients with abnormal responses to ADP were identified by MPA compared to 17 patients by LTA. MPA identified only 3 defects in collagen responses compared to 10 patients by LTA. LTA also detected low levels of secreted ATP in 8/74 (10.8%) patients but with normal aggregation by both LTA and MPA. Other abnormal responses detected by LTA including patients with defects in responses to arachidonic acid (N = 9), low dose ristocetin (N = 1) and low dose PAR-1 peptide (N = 5). Only 1/9 patients with an abnormal response to arachidonic acid were abnormal with U46619.
Conclusion: MPA with a limited screening panel of agonists demonstrates a lack of sensitivity in identifying patients with abnormal platelet function and a history of clinical bleeding. Moreover, MPA is insensitive for detecting ATP secretion disorders detected by LTA. Further studies are required to fully determine the potential role for MPA in the diagnosis of bleeding disorders.

A COMPARISON OF TYROSINE PHOSPHORYLATION OF KEY SIGNALLING MOLECULES DOWNSTREAM OF CLEC-2 AND GPVI IN HUMAN AND MURINE PLATELETS

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Background: The platelet collagen receptor GPVI and podoplanin receptor CLEC-2 are two members of the (hem)ITAM family of receptors with distinct functional roles. ITAM motifs are amino acid sequences of YxxL6-12YxxL (e.g. GPVI), whereas hemITAM motifs contain only a single YxxL consensus sequence, downstream of a triacidic amino acid sequence (e.g. CLEC-2). The tyrosine residue(s) become phosphorylated upon receptor engagement, allowing the recruitment and phosphorylation of SH2 domain-containing proteins, notably Syk. Upon activation, Syk phosphorylates a number of downstream targets including LAT, leading to formation of a LAT signalosome and subsequent phosphorylation and activation of PLCγ2. There are a number of tyrosines within each signalling molecule, many of which have been shown to play distinct roles in supporting signalling. However, a detailed investigation of Syk, LAT & PLCγ2 tyrosine phosphorylation at the individual tyrosine level downstream of human and murine GPVI and CLEC-2 has not been undertaken.
Aims: We propose that Syk, LAT and PLCγ2 tyrosine residues are differentially phosphorylated downstream of platelet surface receptors GPVI and CLEC-2 in both murine and human platelets, and that this phosphorylation pattern differs both with time and between species.
Methods: Venous blood was collected in anticoagulant (citrate for human & acid citrate dextrose for murine platelets) and washed platelets prepared in modified Tyrodes buffer as previously described. For all studies, platelets were set at a final concentration of $4x10^8$/ml and aggregations were performed using 30μg/ml collagen or 300nM Rhodocytin. Samples were lysed using 5X reducing sample buffer after time points of 180, 90, 45, 15 & 0 seconds, denatured and run on 4–12% acrylamide gels. PVDF Western blots were probed using selected phosphospecific Syk (Y525/526, Y352, Y323) LAT (Y200, Y171, Y132) & PLCγ2 (Y1217, Y759) antibodies and imaged using the Li-Cor Odyssey FC system.
Results: Differences in patterns of tyrosine phosphorylation over time were observed between agonists within each species. For example, murine Syk Y519/520 shows a gradual increase in phosphorylation from 45 seconds with collagen stimulation, whereas rhodocytin gives an initial peak in phosphorylation at 45 seconds which decreases after 90 seconds. There were also observable differences in the patterns and levels of phosphorylation between species when stimulated with the same agonists. For example, Y323 within Syk (Y317 in murine Syk) is phosphorylated to a greater extent in human platelets, with an initial spike at 15 seconds and gradual reduction over time; murine platelets display a gradual, small increase over time.
Conclusions: Rhodocytin and Collagen stimulations induce different patterns of phosphorylation downstream of their respective receptors. There also appeared to be species differences in the patterns and levels of phosphorylation at certain tyrosine residues, within the Rhodocytin and Collagen stimulations. This work suggests that phosphorylation downstream of GPVI and collagen is a more gradual, sustained process whereas phosphorylation downstream of CLEC-2 and rhodocytin is a faster, phasic response. The data hints at underlying, intra-molecular differences in signalling downstream of the two platelet glycoprotein receptors.

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CIRCULATING PRIMERS INDUCE RESISTANCE TO ANTIPLATELET THERAPY WITH $P2Y_{12}$ AND TXA_2 ANTAGONISTS

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Background: Aspirin and $P2Y_{12}$ antagonists are clinically used antiplatelet compounds in patients with thrombosis. However, a sufficient inhibition of platelet function by aspirin and $P2Y_{12}$ blockade is not always achieved. This results in a population of patients who are 'resistant' to antiplatelet therapy and at risk of acute coronary syndromes (ACS). Often these patients express a hyperactive platelet phenotype and present with altered levels of circulating primers. Platelet primers cannot stimulate platelet activation, but in combination with physiological stimuli significantly enhance platelet function and contribute to platelet hyperactivity.
Aims: In this study, we explored the contribution of platelet primers to antiplatelet resistance and evaluated whether PI3K plays an important role in this process.
Methods: We used platelet aggregation, TXA_2 production and ex vivo thrombus formation as functional readouts of platelet activity. Platelets were treated with the potent $P2Y_{12}$ inhibitor, AR-C66096 (1 μM), aspirin (30 μM) or a combination of both, in

the presence/absence of platelet primers that are known to be elevated in ACS, insulin-like growth factor-1 (IGF-1; 100 nM), thrombopoietin (TPO; 50 μg/mL) or adrenaline (5 μM or 100 nM). The role of PI3K was evaluated using the PI3K inhibitors, wortmannin and LY294002.

Results: The data demonstrate that platelet primers can overcome the inhibitory effects of aspirin and ARC66096 on platelet functional responses. Furthermore, PI3K appears to drive IGF-1 and TPO-mediated resistance, whereas PI3K-dependent and independent mechanisms are responsible for adrenaline-mediated resistance to antiplatelet therapy.

Conclusion: Platelet primers can contribute to anti-platelet resistance through both PI3K dependent and independent pathways.

MECHANISMS OF PLATELET ACTIVATION BY THE BH3-MIMETIC DRUGS ABT-737 AND ABT-199

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Background: BH3-mimetic drugs are promising anti-cancer agents which act by inhibiting BCL proteins, e.g., BcL-2, thereby promoting cell apoptosis. However, these agents cause a rapid and dose-limiting thrombocytopenia. ABT-737 also inhibits BcL-XL which is found in platelets. This results in a substantially reduced lifespan and their removal from the circulation. By contrast, ABT-199 is reported to be selective for BcL-2 and may therefore spare platelets *in vivo* by inhibiting BcL-XL less. We have previously observed that at sufficiently high concentrations, ABT-737 directly activates platelets resulting in aggregation.

Aims: In this study, we compared ABT-737- and ABT-199-induced platelet activation in order to relate it to their inhibitory potency at BcL-XL. We also investigated the dependence of activation on the production of thromboxane A_2 (TxA_2).

Methods: Citrated fresh blood was collected from healthy human volunteers and washed platelets prepared. Platelets were activated with ABT-737 and ABT-199. Rate and extent of agonist-induced aggregation were measured using luminometric aggregometry. Production of thromboxane B_2 (TxB_2) was quantified using a commercial ELISA kit. Agonist-concentration response data were analysed using a four parameter logistic model. Data are reported as mean ± sem (n = 9 donors).

Results: ABT-737-induced rapid and full (81 ± 1%) irreversible aggregation. The pA_{50} of ABT-737 for the extent of aggregation after six minutes was 4.90 ± 0.04 (EC_{50} = 13 μM). ABT-199 also induced aggregation with a slightly lower potency: pA_{50} = 4.52 ± 0.07 (EC_{50} = 30 μM). However, the rate of ABT-199-induced aggregation was lower (maximum rate = 128 ± 28%/min) than that for ABT-737 (205 ± 8%/min). Aggregation induced by both compounds was abolished by the αIIbβ3 inhibitor, GR144053, with potency consistent with its action at the integrin (10–100 nM). *In vitro* treatment with aspirin (100 μM) attenuated but did not abolish the aggregation response to ABT-737. Interestingly, in the presence of aspirin, the quantitative difference between ABT-737 and ABT-199 was almost absent and they both acted similarly to ABT-199 in control platelets. TxB_2 production (which was abolished in the presence of aspirin) matched the rate of aggregation, with ABT-199 producing approximately one half of the amount of TxB_2 as ABT-737 at each concentration.

Conclusions: Both ABT-737 and ABT-199 activate platelets, albeit it at relatively high concentrations. The mechanism of activation remains unclear although our data indicate that ABT-737 is more effective at inducing thromboxane synthesis than ABT-199 suggesting that there is a specific difference in their action which is closely related to this activation pathway. How this mechanism relates to BcL-XL is also unclear. The concentrations at which these compounds activated platelets are higher than those reported in pharmacokinetic studies. However, inevitable fluctuations in drug concentration that occur between individuals and within different body compartments make it likely that platelets are exposed to drug concentrations higher than the mean values typically reported. Whether the rate or extent of thrombocytopenia induced by BH-3 mimetics could be reduced by the simultaneous use of an anti-platelet drug remains an interesting and unanswered question.

CHARACTERISATION OF THE INTERACTIONS OF PLATELETS AND ANTIPLATELET AGENTS ON FIBRIN CLOT MICROSTRUCTURE

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Introduction: Development of the platelet-rich fibrin clot at sites of atherosclerotic plaque rupture is central to atherothrombotic diseases. Configuration of the fibrin network affects the biological function of the clot and is a vital determinant of clot stability and susceptibility to fibrinolysis. Dense clots composed of thinner fibres and increased branch points are more prone to thrombosis. Platelets are also critical and have a central role in the cell-based regulation of the coagulation system. Aspirin and $P2Y_{12}$ inhibitors are currently prescribed to patients with cardiovascular disease. However, thrombotic events still occur and high on-treatment platelet reactivity despite these therapies is associated with adverse clinical outcome. The management of these at risk patients is hindered by a lack of technique that reliably assesses thrombotic risk and guides antiplatelet therapy. Given the interwoven nature of the coagulation system and platelet activation, a global marker of haemostasis could provide a more clinically useful tool than tests of platelet function alone. We therefore, sought to characterise interactions between platelets and fibrin-platelet clot characteristics and how these are affected by antiplatelet therapies.

Methods: Four groups of 8 healthy male volunteers received standard antiplatelet agents for 7 days. Regimes consisted of aspirin (75 mg) or prasugrel (10 mg) monotherapy, or DAPT (aspirin + prasugrel or aspirin + ticagrelor (90 mg)). Platelet responses were characterised by LTA, lumi-aggregometry and flow cytometry and were correlated with fibrin clot structure represented by the viscoelastic properties of incipient clots, relative mass and fractal dimension (Df) as determined by rheometry of whole blood.

Results: Treatment with aspirin did not affect ATP release, P-selectin or PAC-1 binding and there was no significant change in D_f (1.71 ± 0.01 to 1.69 ± 0.01, $p = 0.41$) or mean relative mass (0.70 ± 0.08 to 0.59 ± 0.1, $p = 0.47$). Prasugrel therapy, however, significantly reduced ($p<0.05$) collagen-induced ATP release, PAC-1 and p-selectin expression as well as D_f (1.72 ± 0.02 to 1.67 ± 0.01, $p = 0.03$) and mean relative mass (0.95 ± 0.23 to 0.45 ± 0.06, $p = 0.03$).

DAPT, aspirin + prasugel or aspirin + ticagerlor caused potent and significant inhibition ($p < 0.05$) of platelet responses to AA (1 mM), ADP (20 μM), collagen (4 μg/ml), U46610 (10 μM) and TRAP-6 (25 μM) in LTA and significantly affected p-selectin expression ($p<0.05$). This was reflected by decreases in D_f 1.73 ± 0.02 to 1.68 ± 0.02 ($p = 0.03$) and 1.72 ± 0.03 to 1.62 ± 0.02 ($p = 0.04$), respectively. This was reflected by significant reductions in mean relative mass values (1.19 ± 0.39 to 0.62 ± 0.16, $p = 0.04$) and (1.75 ± 1.14 to 0.31 ± 0.07, $p = 0.04$).

Conclusion: Our observations support that clot microstructure and relative mass is determined by multiple components including platelet reactivity and modifiable by antiplatelet therapy. There is diagnostic potential in characterising clot structure and modulation of clot architecture as a possible treatment for thrombosis. Further characterisation of the relationship between platelet parameters and fibrin clot properties could identify novel prothrombotic profiles, help to overcome the current limitation of platelet function tests and further our understanding of cardiovascular risk and therapies.

NUCLEAR RECEPTOR DEPENDENT FORMATION OF COATED PLATELETS, INHIBITS PLATELET RESPONSES TO STIMULI

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Background: A number of intracellular nuclear receptors have been identified in human platelets, including the Liver X receptors (LXRs) and Farnesoid X receptor (FXR) which are usually involved in the genomic regulation of cholesterol, lipid and bile acid metabolism. Ligands for either of these receptors have been shown to have non-genomic inhibitory effects on platelet activation, including inhibition of aggregation, granule secretion, adhesion, spreading and thrombus formation. Interestingly however, several pathological conditions associated with increased circulating levels of molecules that act as LXR and FXR agonists, such as hyperlipidemia, type two diabetes, metabolic syndrome and high cholesterol have all been related to platelet hyper-reactivity and increased platelet responses.

Aims: The work described here aims to gain an insight into any potential effects of the nuclear receptor agonists on the basal activity of resting platelets.

Methods: Resting human washed platelets were treated with increasing concentrations of LXR and FXR synthetic ligands (GW3965 and GW4064 respectively) and markers of platelet activation such as fibrinogen binding, P-selectin exposure, changes to intracellular calcium levels, procoagulant phosphatidylserine exposure, mitochondrial membrane depolarisation and serine protease activity were determined in comparison to vehicle treated platelets.

Results: Treatment of platelets with either GW3965 or GW4064 elicited a conversion of platelets to the procoagulant state, with both ligands capable of causing a concentration dependent increase in phosphatidylserine exposure, platelet swelling, depolarization of the mitochondrial membrane and microparticle release. Ligands for LXR and FXR were also capable of forming a subpopulation of procoagulant platelets known as COATED platelets, as GW3965 and GW4064 treated platelets showed a concentration-dependent increase in P-selectin exposure, fibrinogen binding, and fibrin generation, supported by increased serine protease activity at the platelet surface compared to vehicle-treated controls. This conversion following treatment with either GW3965 or GW4064 was found to be dependent on both the formation of reactive oxygen species and intracellular calcium mobilisation as studies with either a reactive oxygen species scavenger, DPPD, or an intracellular calcium chelator BAPTA-AM prevented COATED platelet formation. LXR and FXR dependent formation of COATED platelets further correlates with an increase in the initial kinetics of thrombus formation *in vivo* as thrombi formed significantly faster following nuclear receptor ligand treatment compared to vehicle treated controls, although thrombus formation overall was reduced.

Conclusions: The work presented here suggests that activation of LXR and FXR by their ligands converts a subset of platelets to the reactive procoagulant state, forming COATED platelets and causing platelet desensitisation through receptor cleavage. This makes these platelets unable to respond to platelet stimuli resulting in inhibition of responses to classical platelet agonists and thrombus formation *in vivo*.

This has clinical implications since nuclear receptor ligands are under development for the treatment of several pathological conditions. Understanding these non-genomic effects of the nuclear receptors and their ligands is essential to balance their genomic regulatory roles against their possible additional effects on platelet reactivity and function.

SUNITINIB UPTAKE INHIBITS PLATELET FUNCTION IN CANCER PATIENTS

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Background: Sunitinib is an oral receptor tyrosine kinase inhibitor used for treatment of several solid tumors. Patients treated with sunitinib are at higher risk of bleeding. As protein tyrosine kinases are essential for platelet function, we hypothesized that platelet functional properties are altered in patients treated with sunitinib.

Aims: Investigate the effect of sunitinib on platelet functions in vitro and in cancer patients on treatment.

Methods: The study was approved by the medical ethics committee from the Maastricht University Medical Centre. For in vitro measurements, blood platelets were isolated from healthy individuals, and preincubated with sunitinib (3.3–30 μM) or vehicle for 10 minutes at 37°C. To investigate the clinical significance of sunitinib treatment on platelet function, patients diagnosed with metastatic renal cell cancer (RCC) and eligible for sunitinib treatment, were included in this study. Also, healthy individuals were included of comparable age and gender. Blood samples from healthy volunteers ($n = 8$) and RCC patients ($n = 8$) before and 2 weeks on-treatment with sunitinib (monotherapy) were collected after informed consent. Immunofluorescence imaging, western blotting, light transmission aggregometry, whole blood perfusion over collagen, flow cytometry and ELISA were performed.

Results: Confocal microscopy indicated that platelets sequester sunitinib in vitro. Preincubation of healthy washed platelets with

sunitinib strongly reduced the tyrosine phosphorylation of multiple proteins induced by collagen. Furthermore, collagen and ADP-induced platelet aggregation were inhibited by sunitinib in a concentration-dependent manner. Thrombus formation on collagen under flow was also reduced in the presence of sunitinib. In addition, sunitinib reduced the secretion of PDGF and β-thromboglobulin upon collagen-induced platelet activation. In whole blood from advanced RCC patients before treatment with sunitinib, enhanced thrombus formation and procoagulant activity was observed under flow compared to healthy controls. Platelets from patients on-treatment for 14 days also demonstrated sunitinib uptake. Treatment with sunitinib was accompanied by a significant decrease in platelet count ($p<0.05$ compared to pre-treatment). Bleeding complications were observed in four patients. In patients treated with sunitinib, glycoprotein VI-induced platelet aggregation as well as activation of integrin αIIbβ3 and P-selectin expression were reduced compared to controls. Finally, sunitinib treatment reduced collagen-dependent thrombus formation and phosphatidylserine exposure under flow conditions.

Conclusion: Sunitinib uptake by platelets inhibits collagen receptor-induced aggregation and thrombus formation via reduced protein tyrosine phosphorylation and α-granule secretion. Given the general effect on tyrosine phosphorylation of multiple proteins, this suggests an inhibition by sunitinib early in the signaling cascade underneath the collagen receptor glycoprotein (GP) VI. Treatment with sunitinib reduces platelet count as well as platelet function, which together may contribute to the higher bleeding risk observed in patients treated with sunitinib.

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LIPID RAFTS AND TRANSGLUTAMINASE ACTIVITY MEDIATE TRANSLOCATION OF FACTOR XIII-A FROM THE STIMULATED PLATELET MEMBRANE ONTO ADJACENT FIBRIN FIBRES

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Background: We have previously shown that cellular factor XIII (FXIII-A) is exposed on the stimulated platelet surface and mediates extracellular cross-linking reactions, including cross-linking of α_2-antiplasmin (α_2AP) to fibrin[1] thereby protecting clots against fibrinolytic degradation. The externalisation mechanism for FXIII-A is currently unknown. It is also yet to be determined whether FXIII-A performs extracellular cross-linking from the platelet surface or via transfer into the fibrin network.

Aims: To determine the mechanisms involved in exposure of FXIII-A on stimulated platelets and study its translocation into the fibrin network.

Methods: Washed platelets were prepared from whole blood by centrifugation, in some cases platelets were pre-incubated with methyl-β-cyclodextrin (MβCD), cytochalasin D, eptifibatide, tirofiban, theophylline and prostaglandin E1 (PGE_1) or ARG-GLY-ASP-SER (RGDS). FXIII-depleted plasma ± isolated platelets formed static clots or model thrombi under flow ± a transglutaminase (TG) inhibitor. Incorporation of FITC-labelled fibrinogen allowed model thrombus lysis to be quantified as release of fluorescence. Lysed thrombi were dissolved and analysed by Western blots to detect cross-linked α_2AP. A FITC-labelled anti-FXIII-A antibody was used in confocal microscopy and flow cytometry.

Results: Addition of platelets to FXIII-depleted clots revealed movement of FXIII-A from the platelet membrane onto extending fibrin fibres. In contrast, in the absence of platelets no FXIII staining was evident and addition of purified FXIII generated a homogenous distribution.

Pre-incubation of platelets with PGE1 and theophylline, to prevent aggregation and degranulation, abolished FXIII-A exposure. Similarly, treated platelets were unable to stabilize FXIII-depleted thrombi and FXIII-A was undetectable on fibrin fibres in clots.

Pre-incubation of platelets with MβCD, an agent that disrupts lipid raft formation, prevented visualisation of FXIII-A on fibrin fibres. Likewise, the ability of washed platelets to stabilise FXIII-depleted thrombi against fibrinolysis was lost following MβCD pre-incubation. Addition of platelets to FXIII-depleted thrombi allowed detection of cross-linked α_2AP by Western blotting. Pre-incubation with MβCD abolished cross-linked α_2AP in thrombi, as did inclusion of a TG inhibitor. These data suggest lipid rafts function in translocation of FXIII-A from the activated platelet surface onto the fibrin network.

We previously reported that inhibiting TG activity abrogated the stabilising effect of platelets on FXIII-depleted thrombi. Similarly, including a TG inhibitor in FXIII-depleted clots with platelets revealed FXIII-A activity was required for movement from the platelet membrane onto adjacent fibrin fibres.

Blocking the fibrin-binding integrin, $\alpha_{II}b\beta_3$ with tirofiban, eptifibatide or RGDS reduced the presence of FXIII-A on adjacent fibrin fibres. This suggests movement of FXIII-A from the membrane requires platelet-bound fibrin and is mediated via $\alpha_{II}b\beta_3$. Pre-incubation of platelets with tirofiban or eptifibatide reduced their stabilising effect on FXIII-depleted thrombi and the presence of cross-linked α_2AP. Pre-treatment with cytochalasin D to inhibit actin polymerisation decreased detectable FXIII-A on fibrin and attenuated the ability of platelets to stabilise FXIII-depleted thrombi. These data indicate that cytoskeletal reorganisation upon platelet activation is implicated in transfer of FXIII-A from the cytoplasm onto the platelet-bound fibrin.

Conclusions: Activated platelets expose functional FXIII-A on their membrane which translocates onto bound fibrin via a lipid raft and $\alpha_{II}b\beta_3$-dependent mechanism that requires TG activity.

IDENTIFICATION OF NON-ENZYMATIC GLYCOSYLATION OF PLATELET MEMBRANE PROTEINS

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Background: Diabetes is characterized by chronic states with high glucose concentrations. Hyperglycemic conditions may lead to the non-enzymatic glycosylation (glycation) of proteins and formation of Amadori-products, which may significantly impair protein functions and enhance the development of diabetic complications. Protein glycation starts with a nucleophilic attack of the protein primary amine group to carbonyl group of a reducing sugar and the formation of the reversible Schiff base intermediate, which subsequently rearrange into a stable Amadori product (ketoamine).

Aims: Our aim was to identify platelet proteins that undergo non-enzymatic glycosylation to the highest extent with a main focus

on those of membrane integrin receptors, which play important roles in platelet adhesion.

Methods: *In vitro* glycation was achieved by the incubation of isolated platelets membranes with 300 mM glucose over 7 days at 37°C. In addition, to ease the protein identification, membranes were incubated with radiolabeled glucose, proteins were resolved on SDS-PAGE gel and further analyzed with the use of autoradiography. *In vivo* glycation part of the study involved isolation of membrane proteins from platelets originating from diabetic patients and control individuals. Subsequently proteins were resolved on SDS-PAGE gel, bands excised and after trypsinization analyzed with the use of LC-MS/MS.

Results: Based on autoradiography data ([^{14}C]-glucose incorporation into membrane proteins) we reasoned that one of the most abundant targets among platelet membrane proteins – GPIIb/IIIa ($\alpha_{IIb}\beta_3$), demonstrated the highest isotope labelling. The LC-MS/MS analysis of GPIIb/IIIa demonstrated a few glycation sites, which were detected in both diabetic and control samples. As expected, the number of the identified glycation sites remained slightly higher in diabetic samples compared to control samples. Interestingly, β_3 subunit of GPIIb/IIIa exhibited higher number of the glycation sites.

Conclusions: Under conditions of chronic and severe hyperglycemia, mimicking poorly controlled diabetes mellitus, the incorporation of glucose occurs to the highest extent in the GPIIb/IIIa complex, and particularly in the β_3 subunit of GPIIb/IIIa, as deduced from autoradiography data and the analysis with the use of LC-MS/MS.

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REGULATION OF NHERF1 LOCALIZATION BY SNX27 REDUCES PLATELET P2Y$_{12}$R FUNCTION

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Introduction: As part of the Genotyping and Phenotyping of Platelets (GAPP) consortium we have identified a number of mutations in GPCR genes that could contribute to bleeding tendency in patients, including mutations in the P2Y$_{12}$ receptor (P2Y$_{12}$R (1)). Through this approach we have shown that disruption of the PDZ ligand found at the extreme C-terminus of the P2Y$_{12}$R, results in loss of surface P2Y$_{12}$R and dysregulation of receptor recycling and resensitization to ADP in human platelets (2).

Aims: To identify proteins interacting with the PDZ binding motif of the P2Y$_{12}$R and characterize their ability to regulate P2Y$_{12}$R function platelets

Methods: P2Y$_{12}$R activity and surface expression was undertaken in in 1321N1 cells stably expressing HA-tagged P2Y$_{12}$R and washed mouse platelets as previously described (2,3).

Results: Mass spectrometry analysis revealed binding of two PDZ domain proteins sorting nexin 27 (SNX27) and NHERF1 which bound to the C-tail of the P2Y$_{12}$R. Studies in SNX27 (−/+) and NHERF1 (−/−) mice revealed that ADP-stimulated platelet aggregation was reduced in both mice lines. Intriguingly although acute P2Y$_{12}$R signalling was not attenuated in either mouse line there was a significant deficit in receptor resensitization following receptor desensitization accompanied by a reduction in receptor internalization. Previous studies from our laboratory have shown in cell lines that NHERF1 regulates receptor internalization(3). Knockdown of SNX27 in cells similarly attenuated P2Y$_{12}$R internalization. Further study in these cells revealed that loss of SNX27 expression ablated both basal and ADP-induced NHERF1 interaction with P2Y$_{12}$R. Surface biotinylation and subcellular fractionation of SNX27 depleted cells revealed a loss of NHERF1 expression at the plasma membrane. Importantly we also found reduced NHERF1 surface expression in SNX27 (−/+) mouse platelets.

Conclusions: In conclusion, we reveal a novel regulatory role of SNX27 in the membrane localization of NHERF1. Reduced NHERF1 surface expression in turn attenuates P2Y$_{12}$R function in both cell lines and mouse platelets.

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MODE OF ACTION OF THE P2Y$_{12}$R ANTAGONIST TICAGRELOR ON HUMAN PLATELETS: BLOCKADE OF THE PLATELET ENT-1 TRANSPORTER AND INVERSE AGONISM

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Background: Several P2Y$_{12}$R antagonists, including ticagrelor are used in the treatment of acute coronary syndromes. The mechanism of action of the drug has not yet been fully characterised. Recent studies have shown that in addition to antagonism of the P2Y$_{12}$R, ticagrelor also prevents platelet aggregation by inhibiting the endonucleoside transporter 1 (ENT1) on red blood cells. This results in an extracellular increase in adenosine levels which stimulates platelet G_s-coupled adenosine receptors, inhibiting platelet activity (1).

Aims: The aim of our study was to determine whether adenosine uptake inhibition can still occur in the absence of red blood cells and also fully characterize the mode of action of this drug.

Methods: We conducted a series of experiments on washed platelets, isolated from whole blood donated by healthy volunteers as previously described (2). The ability of ticagrelor to affect platelet activity was determined using Western blotting analysis of changes in vasodilator-stimulated phosphoprotein (VASP) phosphorylation. Changes in phosphoVASP (pVASP) levels are a sensitive measure of changes in platelet cyclic adenosine monophosphate (cAMP), whereby increased cAMP production or VASP phosphorylation correlates to inhibition of platelet activation (3). Data are expressed as mean ± SEM and analysis was performed using one-way ANOVA followed by Bonferroni's post-hoc test where applicable.

Results: Ticagrelor treatment of human platelets induced a time (0-60 min) and concentration-dependent (1 nM–10 μM) increase in pVASP levels. Ticagrelor treatment (10 μM; 60 mins) increased pVASP levels compared to vehicle controls (43.17 ± 11.5% vs 4.32 ± 1.5%; n = 5; $p<0.001$). Further investigations revealed that treatment with the selective A_{2A} adenosine receptor antagonist SCH442416 (1 μM; 60 mins) attenuated ticagrelor (10 μM; 60 mins)-stimulated increases in pVASP (69.37 ± 16.3% vs 7.36 ± 2.8%; n = 4; $p < 0.001$) whilst treatment with the A_{2B}-selective antagonist PSB 603 (1 μM) had no effect. Interestingly further studies revealed that following adenosine receptor blockade, there was still a significant increase in VASP phosphorylation induced by ticagrelor (10 μM; 60 mins) treatment compared to the vehicle controls (3.48 ± 0.6% vs 0.95 ± 0.2% increase in pVASP; n = 4; $p<0.001$), hinting at the possibility that the

drug may also be able to act as an inverse agonist at the Gi-coupled $P2Y_{12}R$ in human platelets. Further study in cell lines further confirmed that ticagrelor was indeed an inverse agonist at the $P2Y_{12}$ receptor decreasing agonist-independent receptor activity.

Conclusions: In summary, we provide evidence that ticagrelor can increase extra-platelet adenosine in the absence of red blood cells, likely by inhibiting a platelet-expressed ENT to activate platelet A_{2A}-adenosine receptors, thereby inhibiting platelet activity. In addition, ticagrelor appears to be able to modulate the basal activity of the $P2Y_{12}R$, behaving as an inverse agonist at this receptor.

This work was supported by the British Heart Foundation. SJM is a Senior BHF Research Fellow.

TETRASPANIN TSPAN18 REGULATES HAEMOSTASIS AND GPVI-INDUCED PLATELET ACTIVATION BY INTERACTING WITH THE ORAI FAMILY OF STORE-OPERATED Ca^{2+} ENTRY CHANNELS

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Introduction: The superfamily of tetraspanins consists of 33 transmembrane proteins in humans and are emerging as important regulators of the trafficking and/or clustering of certain membrane proteins with which they associate. There are at least 10 tetraspanins that are expressed on platelets, but little is known about the role of these proteins in platelets. We previously identified the platelet collagen receptor GPVI as tetraspanin-associated. However, it is not known which tetraspanins regulate GPVI-induced platelet activation.

Aims: To characterize Tspan18-deficient mice and investigate the role of Tspan18 in platelet activation.

Methods: Experiments were conducted in cell line models, human umbilical cord endothelial cells and in platelets from Tspan18-deficient mice.

Results: The previously uncharacterized platelet tetraspanin Tspan18 was found to be unique amongst tetraspanins in its activation of a Ca^{2+}-responsive signalling pathway in cell lines. We hypothesized that Tspan18 might regulate Ca^{2+} and/or GPVI signalling in platelets. To address this we performed the first analyses of Tspan18-deficient mice. Tspan18-deficient platelets were defective in aggregation and secretion via the GPVI-specific agonist collagen-related peptide, but aggregation via thrombin or the CLEC-2 receptor was normal. This GPVI-specific phenotype is similar to that previously reported for mice with platelets deficient for the store-operated Ca^{2+} entry channel Orai1. Consistent with a role for Tspan18 in regulating Orai1, store-operated Ca^{2+} entry was impaired in the absence of Tspan18, and Tspan18 specifically interacted with Orai1, as well as its family members Orai2 and 3. Unexpectedly, Tspan18-deficient mice exhibited defective haemostasis (five-fold more blood was lost than wild-type mice) in a tail bleeding assay. Using fetal liver chimeras of wildtype and Tspan18 deficient mice, non-haematopoietic cells were identified as the cause of the defective haemostasis. Store-operated Ca^{2+} entry is crucial for vWF/P-selectin release from Weibel-Palade Bodies (WPB) in endothelial cells when exposed to vaso-active compounds and we hypothesized Tspan18 is critical for this response. To evaluate the role of Tspan18 we analysed endothelial cells with silenced Tspan18 expression and found they had reduced Weibel-Palade Body release.

Conclusions: We have characterized a new platelet tetraspanin, Tspan18, that positively regulates GPVI signalling via interaction with the store-operated Ca^{2+} entry channel Orai1. Furthermore analysis of the Tspan18-deficient mice indicates that Tspan18 may regulate haemostasis through WPB release from the endothelium and not through platelet Tspan18.

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THROMBOSIS AND PLATELET FUNCTION IN FETAL GROWTH RESTRICTION

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Background: Fetal growth restriction (FGR) is a serious pregnancy complication, which affects 3–8% of pregnancies and is associated with increased perinatal morbidity and mortality, in addition to increased risk of cardiovascular disease later in life. In severe cases of FGR, placental vascular resistance is increased and blood flow reduced compromising oxygen and nutrient exchange between mother and fetus. Hypoxia and endothelial dysfunction, have both been demonstrated in FGR, and both have the ability to alter thrombosis and haemostasis.

Aim: The aim of this study was to determine whether there is increased platelet activation and thrombosis in FGR which may contribute to its pathogenesis.

Methods: Dual ex-vivo perfusions were performed on placentas from healthy preganancies ($n = 10$) and those affected by FGR ($n = 11$). Placentas were perfused with Earles bicarbonate buffer for ~5 hours prior to fixation with 4% NBF. Placental cross-sections were processed and stained with H&E and Martius Scarlet Blue for histological analysis or immunofluorescence was performed to analyse levels of fibrin and tissue factor. Full blood counts and platelet closure time using the PFA200 were assessed using fetal blood collected from the umbilical vein immediately following delivery.

Results: Placental sections from pregnancies affected by FGR exhibited significantly more thrombi (P0.004) within the fetal vasculature than healthy placental sections. Thrombi could be observed in both large conduit and small resistance vessels, with many small vessels completely occluded. In FGR, total fibrin deposition in placental tissue was significantly increased ($P = 0.0027$), as was fibrin expression specifically located within fetal vessels ($P = 0.0354$). In contrast, tissue factor levels remained unaltered. Blood samples from fetal growth restricted babies demonstrated increased closure time in platelet function assays (122 ± 12.6s) compared to healthy controls (90.10 ± 5.46s), and platelet counts were significantly reduced (P0.045). The red blood cell count, white blood cell count and mean platelet volume were not significantly different.

Conclusions: These data indicate that in FGR there is increased platelet activation and thrombosis in fetoplacental vessels, which may contribute to increased vascular resistance

through the occlusion of small resistance arteries. Antithrombotic therapies, which cross the placenta may therefore be beneficial in FGR to prevent placental thrombosis and improve pregnancy outcome.

LIPOPOLYSACHARIDE STIMULATES PLATELET α-GRANULE SECRETION AND INCREASES PLATELET AGGREGATION *VIA* AKT ACTIVATION

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Background: Bacterial lipopolysaccharide (LPS) promotes rapid thrombocytopenia, hypotension and sepsis. Although growing evidence indicates that platelets may contribute to these abnormalities, the mechanism of LPS-mediated platelet activation is unclear. LPS is not a typical platelet agonist and isolated platelets do not aggregate in its presence. However, in CRP and U46619-stimulated platelets, LPS induces aggregation and Akt phosphorylation.
Aims: In this study we sought to explore the signaling pathway(s) through which LPS enhance platelets.
Method: Blood was obtained from healthy human volunteers with written informed consent as approved by the University of Reading Research Ethics Committee. Washed platelets were prepared to a density of 4 x 10^8 cells/mL. The effect of LPS – *E.coli* (0.5–10 μg/ml) on platelet function in vitro was investigated including platelet aggregation, fibrinogen binding and P-selectin exposure following stimulation with CRP (0.25 μg/ml) or U46619 (0.25 μM). Concentration of agonist was chosen that produced approximately 50% of the maximal aggregation response. Akt activation was analysed by immunoblot analysis.
Results: LPS alone did not modify platelets function. However, in CRP and U46619- activated platelets, platelet aggregation with increased following pretreatment with the highest concentration of LPS (increase around 25% both CRP and U46169 stimulation) this was accompanied by marked rise in fibrinogen and P-selectin levels. Immunobloting analysis indicated the Akt phosphorylation of Ser473 was intensified by 2.5min-LPS incubation both in unstimulated platelets and in CRP and U461619-stimulated platelets.
Conclusions: Taken together, our data indicate that LPS raises platelet α-granule secretion, fibrinogen binding and aggregation. Elevated response was mediated through a mechanism that resulted in increased Akt signaling.

PHENOPYPING AND GENOTYPING INHERITED PLATELET DISORDERS: THE IBERIAN PENINSULA MULTICENTER PROJECT EXPERIENCE

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Background: Inherited platelet disorders (IPDs) comprise uncommon diseases characterized by abnormalities of platelet function/production giving rise to lifelong bleeding diathesis. Precise diagnosis of affected patients benefit their qualiy of life and clinical management, and prevent unappropriate treatments. However, characterization of IPD is challenging due to heterogeneous clinical and laboratory presentation; low specificity and complexity of platelet function assays; and limited access of clinical laboratories to genotyping. Multicentre projects on IPDs and reference centers networks, could help to overcome this challenge.
Aims: To provide a diagnostic tool to centers not having access to specific platelets studies in the Iberian Peninsula, we established the project ''Functional and Molecular Characterization of Patients with Inherited Platelet Disorders'' under the sponsorship of the Spanish Society of Thrombosis and Haemostasis
Methods: During seven years, a hundred unrelated patients with suspicion of IPDs were referred from hospitals of twenty cities of Spain and Portugal. Clinical features were assessed and bleeding manifestations graded by a unique simple scale. Blood samples from each patient and a parallel healthy control were delivered by express courier (18–24h) to our lab in Murcia. As an additional control, fresh blood was taken from a healthy volunteer from our facility the day functional studies were performed. Platelet phenotyping included: full blood count and film examination; light transmission aggregation (LTA); flow cytometry (FC) evaluation of major platelet receptors; and, in some patients, activation status (PAC-1, CD62, CD63), ^{14}C-serotonin uptake/release, clot retraction assay, and/or electron microscopy. In around 70 recruited patients (first 5 years), genotyping of candidate genes was guided by clinical and laboratory phenotyping, and it was performed by conventional PCR amplification and Sanger sequencing. More recently, we established a next generation sequencing (NGS) approach for simultaneous analysis of 110 genes known to be involved in IPDs or, for a few of them, in other bleeding disorders, using Illumina platform.
Results: In this cohort, clinical information review and phenoyping confirmed an IPD in 70% of cases. Definitive molecular diagnosis was achieved in 48 cases: 15 patients were diagnosed with Glanzmann Thrombasthenia, 11 with Bernard-Soulier syndrome, 2 with Chediak-Higashi, 3 with Hermansky-Pudlack, 3 with Gray platelet syndrome, 1 with Wiskot-Aldrich syndrome, 1 with P2Y12 receptor defect, 1 with TxA2 receptor variant, 1 with GATA-1 variation, 1 with RUNX1 alteration, 1 with congenital amegakaryocytic thrombocytopenia, 1 *ANKRD26-r*thrombocytopenia, 1 with Tubulin β1-related thombocytopenia, 1 with filaminopathy, and 5 with MYH9-RD. NGS gene panel in 29 patients identifed the causative molecular variation in 21 cases. Overall, 27 novel mutations were identified among these patients. This series of patients represent by far the largest series of IPDs ever described in the Iberian Peninsula.
Conclusions: This work illustrates the feasibility and usefulness of a multicenter project in the Iberian Peninsula to assist biological and molecular characterization of IPDs. Clinical and laboratory suspicion of severe IPD was confirmed by genetic studies in about half of the cases. Noteworthy, the NGS strategy identified the causative molecular defect in about 70% of the screeened cases and enabled a more rapid molecular diagnosis of IPDs.

MILD THROMBOCYTOPENIA ASSOCIATED WITH GPVI DEFICIENCY IN TWO UNRELATED PATIENTS

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Background: Acquired glycoprotein (GP) VI deficiency is a rare clinical condition that has been identified in just a few patients presenting with variable extent of thrombocytopenia and a bleeding diathesis, and usually in association with immune/tumoral diseases.

Aims: To investigate the potential role of platelet GPVI in the mechanism of chronic moderate thrombocytopenia in two unrelated patients.

Methods: Two unrelated patients with chronic moderate thrombocytopenia of unknown origin, mild bleeding diathesis and platelet dysfunction according to whole blood testing (PFA-100 and/or Multiplate) were referred to our clinic. Previous clinical records were reviewed and further biochemical, immunological and haematological test were done to discard potential immune/tumoral diseases. Blood samples were obtained for platelet studies which included: full blood count and film examination; light transmission aggregation (LTA); flow cytometry (FC) evaluation of platelet receptors; activation status (PAC-1, CD62, CD63, thromboxane A2 formation), ^{14}C-serotonin release, antiplatelet antibodies, and/or electron microscopy. Some of these assays are used to assess the effect of plasma and/serum of these patients on platelets from healthy volunteers or platelets from other patients (Glanzmann Thrombastenia > or afribrinogemia [AF]).

Results: Case 1 is a 37y female with lifelong moderate thrombocytopenia and mild bleeding diathesis. Case 2, also a 37y female, had 1y history of thrombocytopenia, menorrhagia and spontaneous ecchymoses, commencing few months after unremarkable childbirth. None of them displayed biochemical, coagulation or any other analytical or organ abnormalities, nor have family history of thrombocytopenia or bleeding diathesis. In Case 1 repeated sampling across 5y consistently showed spontaneous, rapid and temperature-dependent platelet clumping in tubes containing citrate or heparin but not EDTA or anti-αIIbβ3 antibody. Her platelets showed elevated levels of bound IgM, P-selectin, CD63 and -αIIbβ3 expression, TxA2 production, and reduced levels of GPIbα and GPVI. Soluble GPVI was 3-fold higher than in healthy donors. Her plasma/serum activated allogeneic platelets from healthy subjects, GT or AF, but not platelets from Case 2. Activation was blocked selectively by Src/Syk inhibitors, soluble GPVI-Fc fusion protein, or by immunodepletion of IgM from patient serum, consistent with an anti-GPVI IgM.

Case 2 displayed abnormal PFA-100 closure times (mainly with Col-Epi and P2Y cartridges) and defective aggregation responses to epinephrine, collagen, collagen-related peptide and convulxin. Aggregations with other agonists were normal. Her platelets showed normal expression of αIIbβ3, GPIb/IX and α2β1, but deficiency (~20%) of GPVI. No antiplatelet autoantibodies were detected in patient serum. Patient treatment with prednisone (1 mg/kg/day,1-month) normalized platelet count, GPVI expression and aggregation response, but thrombocytopenia and GPVI loss re-emerged on cessation of therapy.

Conclusions: These two cases illustrate that acquired GPVI deficiency may not be such a rare cause of moderate thrombocytopenia, even in patients without apparent autoimmune disease. Monitoring GPVI levels, GPVI functional status and presence of anti-GPVI autoantibodies may aid in the clinical evaluation of patients with unexplained acquired thrombocytopenias.

BLOOD PLATELETS' ADHESION TO ENDOTHELIUM IS INCREASED *IN VIVO* IN MICE WITH STREPTOZOTOCIN-INDUCED DIABETES

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Background: It has been shown in genetically modified mice spontaneously developing atherosclerosis that experimental decreasing of platelet count reduces the occurrence of atherosclerotic plaques in arteries. It is suggested that platelets' interaction with inflamed endothelium facilitates adhesion of monocytes to vascular wall, which is recognized as a first step of the developing of atherosclerotic lesions. This process could be potentially enhanced in the case of hyperreactive blood platelets. It is well recognized that reactivity of blood platelets is increased in individuals with diabetes.

Aims: Our aim was to test a hypothesis stating that adhesion of blood platelets to endothelium *in vivo* in animals with a severe chronic hyperglycemia is higher than that observed in the normoglycemic animals.

Methods: Experimental diabetes was induced in C57BL 8-week old mice by intraperitoneal injection of streptozotocin in a dose of 200 mg/kg b.w. Control mice were injected with a vehicle. STZ animals, in which blood glucose values exceeded 300 mg% seven days after STZ injection, were recruited to the study. Thirty days after injection mice were anesthetized, injected with platelet-specific fluorescent anti-GPIbβ antibodies and placed on a stage of upright microscope equipped with saline immersion objectives. Mesentery was exteriorized and fixed in a chamber allowing constant superfusion with saline. Imaging was carried out for 40 s with exposition time of 200 ms in at least 3 sites of mesenteric vascular bed in each mouse. Analysis of platelet adhesion was performed with the use of ImageJ software. The platelets, which did not change their position for at least two frames (400 ms), were included in the calculus and timespans of their adhesion were evaluated. Distributions of the adhesion timespans in both groups of animals were analyzed to determine whether platelets' in these groups differed in terms of their readiness to adhere. Basal activation state and reactivity of platelets in mice were also assayed by means of flow cytometry.

Results: Blood platelets in mice with experimental diabetes formed transient adhesions to vascular wall 4 times more often than in their normoglycemic littermates. Not only the number of adhesion events was higher, but also the averaged adhesion timespans were increased. When the distributions of these timespans were approximated to a Poisson distributions, the highest probability for control platelets to remain adhered occurred at approx. 13.6 s while that for 'diabetic' platelet was at over 21 s. Basal activation and reactivity of platelets in STZ mice were also increased, as deduced from flow cytometry analysis.

Conclusions: Blood platelets in mice with experimental diabetes are more prone to adhere to endothelium *in vivo*. This confirms

the notion that platelets in a diabetic state can play an important role in the development of complications related to endothelial dysfunction.

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ASSOCIATIONS BETWEEN ADHESION OF HUMAN BLOOD PLATELETS TO FIBRINOGEN AND VON WILLEBRAND FACTOR UNDER FLOW CONDITIONS AND FLOW CYTOMETRIC HALLMARKS OF PLATELET ACTIVATION AND REACTIVITY. STUDY IN DIABETIC PATIENTS AND NON-DIABETIC VOLUNTEERS

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Background: It is well recognized that reactivity of blood platelets is increased in individuals with diabetes. At the same time a burden of evidence suggests that blood platelets are also main players in early stages of development of atherosclerosis. It is suggested that their interaction with inflamed endothelium facilitates adhesion of monocytes to vascular wall. This process could be potentially enhanced in the case of hyperreactive blood platelets. Such an increased adhesion of blood platelets in diabetic patients could explain, to some extent, their vulnerability for facilitating of the development of atherosclerosis.
Aims: Our aim was to compare adhesiveness in flow conditions of blood platelets from non-diabetic and diabetic individuals. There are two proteins which are known to play main roles in a firm adhesion of platelets to inflamed endothelium, namely fibrinogen and von Willebrand factor (vWf). As far as atherosclerosis is known to affect mostly arteries, the adhesion to these proteins was assessed under the flow conditions resembling arterial shear forces. We also aimed at verifying whether some hallmarks of blood platelet activation and reactivity, as monitored with flow cytometry, may associate with their adhesion.
Methods: Citrated blood was diluted with donor's platelet poor plasma to obtain normalized platelet count of 100 000/μl and supplemented with the thrombin inhibitor PPACK and with PGE_1. Thus prepared blood samples were passed for 1 min through microchannels coated with either fibrinogen or vWf at the shear force equal to 20 dynes/cm^2. Firmly adherent blood platelets were stained with PE-conjugated anti-CD61 antibodies, visualized with the use of inverted fluorescence microscope and counted in four different fields of the channel. Platelets of each donor were also analyzed by flow cytometry to assess basal expression of the active form of GPIIbIIIa, bound fibrinogen and ability for binding of exogenous fibrinogen. Multivariate covariance analysis was applied to reveal to which extent diabetes affects the platelet ability to adhere and whether other covariates may influence the outcomes.
Results: Number of blood platelets adherent to fibrinogen was not significantly different between non-diabetic and diabetic individuals (206 ± 127 and 188 ± 101 respectively; $n = 8$-12) (mean ± SD). Diabetic platelets bound to higher extent to vWf (56 ± 35; $n = 12$) than those from non-diabetic subjects (37 ± 21; $n = 8$) (mean ± SD), but the difference was not statistically significant (one-tailed $p<0.08$). Adhesion to fibrinogen was significantly associated with two other covariates: the binding of exogenous fibrinogen as shown by correlation analysis ($p<0.001$) The analysis also revealed that adhesion to fibrinogen associated with blood hematocrit ($p<0.002$).
Conclusions: Our preliminary results suggest that diabetic platelets demonstrate the tendency of the increased binding to vWf. However, due to a high variability of the monitored variables, this difference did not reach statistical significance. The variability can be partially explained by the fact that adhesion is strongly dependent on the factors other than platelet readiness to adhere.

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ZINC IS A TRANSMEMBRANE AGONIST THAT INDUCES PLATELET ACTIVATION

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Background: Dietary Zinc (Zn^{2+}) deficiency results in a platelet-related bleeding disorder in rodents and humans. Following vascular injury, zinc (Zn^{2+}) is released from damaged cells, activated platelets and inflammatory cells, contributing to localised $[Zn^{2+}]_o$ increases in the vicinity of a growing thrombus. Zn^{2+} is also a platelet agonist, but the mechanisms by which Zn^{2+} activates platelets are not understood.
Aims: To investigate the role of Zn^{2+} as a platelet agonist and to further elucidate the mechanism of Zn^{2+}-induced aggregation, with a focus on intracellular signalling events.
Methods: The mechanism of Zn^{2+}-induced activation of washed platelets was assessed using aggregometry, fluorometry, confocal microscopy and Western blotting. In addition, platelet activation in flowing whole blood was quantified using a perfusion-based model of thrombus formation. Cation-induced platelet aggregation was assessed using a panel of cation salts.
Results: As previously demonstrated, exogenously applied Zn^{2+} ($ZnSO_4$ or $ZnCl_2$) induced aggregation of washed platelets in a dose-dependent manner. Aggregation was not apparent following treatment with Mn^{2+}, Mg^{2+}, Ca^{2+}, Li^{2+} or Co^{2+}, although Cu^{2+} initiated a partial response. Zinc-induced platelet aggregation was inhibited following intracellular ion chelation with BAPTA-AM or the Zn^{2+} chelator TPEN, and was abolished following inhibition of integrin $\alpha_{IIb}\beta_3$ or protein kinase C (PKC). In the presence of aspirin (cyclooxygenase inhibitor), MeSAMP (P2Y antagonist) and NF449 (P2X1 antagonist) aggregation was only partially inhibited. Sub-activatory levels of Zn^{2+} potentiated aggregation to collagen-related peptide (CRP-XL), thrombin and adrenaline. Fluorometry and confocal microscopy using fluozin-3-stained platelets revealed increases in cytosolic fluorescence following Zn^{2+} treatment. Zinc-induced aggregation was associated with a distinct pattern of tyrosine phosphorylation of platelet proteins, which differed to that induced by CRP-XL or thrombin. Pre-incubation of platelets with TPEN reduced aggregation in response to stimulation by CRP, thrombin or Zn^{2+}. Finally, using a physiologically relevant perfusion based model of thrombus formation in whole blood, TPEN-treated platelets displayed substantially reduced activation and subsequent thrombus formation was attenuated.
Conclusions: Our data are consistent with a signalling role for Zn^{2+} during platelet activation. Zn^{2+} is a transmembrane agonist, initiating platelet aggregation in a manner dependent on PKC activity, tyrosine phosphorylation and granule release. Interestingly, the pattern of tyrosine phosphorylation following Zn^{2+} activation is indicative of a novel signalling pathway.

Experiments using TPEN-treated cells demonstrates a requirement for intracellular Zn^{2+} in platelet activation during thrombus formation under physiologically relevant blood flow conditions. We propose a model where localised Zn^{2+} increases, following vascular injury, potentiates platelet responses to conventional agonists, and/or activates platelets directly. This work further supports a role for zinc during pathophysiological thrombus formation.

REAL-TIME MONITORING TECHNIQUES TO REDUCE AND REFINE ANIMAL USE IN RESEARCH

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Introduction: We have developed an animal model to study platelet aggregation *in vivo* which overcomes the use of mortality techniques known to inflict suffering and pain since they are conducted in conscious animals and require high numbers.
Methods: In our thromboembolic model blood is collected from anaesthetised donor mice by cardiac puncture and platelets are isolated and then radiolabelled with ^{111}Indium chloride. Radiolabelled platelets are infused *via* the femoral vein into anaesthetised recipient mice and platelet aggregation is measured as increases in platelet-associated counts in the pulmonary vasculature following intravenous injection of platelet agonists. Data are collected via a ''Single Point Extended Area Radiation'' detector positioned over the pulmonary vascular bed and recorded on a UCS-20 spectrometer using custom made software. Furthermore, we developed a simplistic alternative to our model which entails the measurement of circulating platelet counts at different time points following agonist stimulation. This model has been validated by treating platelets in the presence or absence of anti-platelet drugs.
Results: Data show that increases in platelet counts in the pulmonary vasculature following platelet stimulation is associated with a measurable drop in circulating platelet counts. Additionally, we identified three published studies which have investigated the cardioprotective effects of different drugs (i.e. Desmolaris, Aegyptin and sulforaphane) on platelet aggregation *in vivo* by using models of thromboembolism which use mortality as an end-point. We have established collaborations with these groups to promote the use of our refined model, which holds the potential of delivering more informative data by recording the complete time-course of the platelet response and reduces mouse use by 23% (Aegyptin) to 40% (sulforaphane) and avoids the use of painful procedures by conducting experiments under general anaesthesia. Our data have identified differences between the effects of test drugs in models of mortality compared to platelet monitoring experiments.
Conclusions: These findings suggest that caution should be used when using thromboembolic mortality models to predict platelet function *in vivo*. In addition, the development of more simplistic assays of thromboembolism will drive uptake of our refined methods by making them easier to implement in other research groups.

PLATELET INDUCED HEPATOCELLULAR CARCINOMA HEPG2 CELL PROLIFERATION AND ANGIOGENIC POTENTIAL IS INTEGRIN $\alpha_{IIb}\beta_3$ DEPENDENT

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Background: Hepatocellular carcinoma (HCC) is the fifth most common malignancy in the world, leading to an estimated one million deaths annually. Although several treatment options are available, the prognosis for HCC patients remains poor, largely due to rapid metastasis. Liver cancers are often highly vascularized, and both experimental and clinical data indicate that the progression of HCC is associated with increased angiogenesis, aiding in their metastatic potential. The vascular nature of HCCs gives them ample opportunity to recruit and interact with platelets. Platelets bind to cancer cells through a range of receptors including the integrin $\alpha_{IIb}\beta_3$, which is upregulated by platelet derived ADP and thromboxane A2 (TxA2). When activated, platelets release a large array of cytokines and growth factors that may induce angiogenesis and aid in the migration, invasion and proliferation of a range of tumour cells, but it is unclear if they support HCC progression. Identifying the roles platelets play in enhancing HCC proliferation and metastatic potential could provide novel treatment strategies to target HCC.
Aims: To determine the importance of platelet integrin receptor $\alpha_{IIb}\beta_3$ and released mediators ADP and TxA2 in platelet adhesion to the HCC cell line HepG2. To evaluate if $\alpha_{IIb}\beta_3$ dependent platelet adhesion enhances HCC proliferation and HCC induced angiogenesis.
Methods: Platelet adhesion to HepG2 cells was measured fluorescently. Activation of $\alpha_{IIb}\beta_3$ on adherent platelets was measured by fluorescent microscopy with PAC-1 antibody. HepG2 proliferation was determined using CellTitre-Glo and the upregulation of cyclins required for cellular growth were investigated by western blotting. The release of angiogenesis mediators from HepG2 cells treated with platelets was analysed by a R&D systems proteome profiler. The ability of conditioned media from HepG2 cells treated with and without platelets to induce endothelial cell proliferation and angiogenesis was determined with CellTitre-Glo and matrigel assays respectively.
Results: Platelets robustly adhered to HepG2 cells in a time dependent fashion. Pre-treatment with RGDS to inhibit $\alpha_{IIb}\beta_3$ reduced adhesion to $40 \pm 7\%$ of control. Combined treatment with apyrase and indomethacin, to block the effects of ADP and TxA2 respectively, reduced adhesion to $60 \pm 3\%$. Combined inhibition of $\alpha_{IIb}\beta_3$, ADP and TxA2 did not significantly further reduce adhesion. Platelet binding to HepG2 cells induced the activation of platelet $\alpha_{IIb}\beta_3$ as shown by upregulated PAC-1 binding, which was blunted by treatment with apyrase and indomethacin. Adhesion of platelets increased HCC proliferation by $180 \pm 30\%$ and increased the expression of cyclin B1 and D1, both key regulators of cellular proliferation. RGDS reduced proliferation to $103 \pm 8\%$. Conditioned media from HepG2 cells treated with platelets was enhanced with several angiogenesis regulatory cytokines including angiogenin, amphiregulin, Il-8 and VEGF. The conditioned media from HepG2 cells treated with platelets, but not with platelets pre-incubated with RGDS, robustly induced endothelial cell tube formation and branching.
Conclusions: Platelet adhesion to HepG2 cells is largely $\alpha_{IIb}\beta_3$ dependent, with released ADP and TxA2 potentiating this response. $\alpha_{IIb}\beta_3$ mediated platelet adhesion induces HepG2 cell proliferation, associated with upregulated cyclin expression. Additionally HepG2 cells treated with platelets release a raft of

angiogenesis mediators and induce endothelial cell angiogenic responses.

MECHANISMS OF PLATELET INHIBITION BY THE SELECTIVE SEROTONIN REUPTAKE INHIBITOR CITALOPRAM

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Background: Selective serotonin reuptake inhibitors (SSRIs) prevent serotonin uptake into platelets by blocking the serotonin reuptake transporter (SERT). SSRI treatment has been suggested to affect haemostasis and thrombosis. However, potential clinical benefits and risks are poorly characterised and useful *in vitro* data is limited.

Citalopram is a commonly prescribed SSRI. It is a racemic mix of two isomers, which differ in their potency for blocking SERT by approximately 30-fold. Citalopram has previously been reported to inhibit collagen-induced platelet activation *in vitro*, although whether this effect is mediated directly through blocking SERT or by some alternative mechanism is unclear.

Aims: The objective of the study is to quantify the inhibitory effects of citalopram and its isomers on platelets and determine the mechanism underlying these effects.

Methods Citrated fresh blood was collected from healthy human volunteers and washed platelets prepared, before incubation with either citalopram or its individual isomers. The rate and extent of platelet aggregation induced by collagen, U46619 and thrombin was quantified using turbidimetric aggregometry. Platelet adhesion to various ligands was measured using a static 96 well plate assay for acid phosphatase. SERT activity was measured by adding exogenous serotonin to washed platelets and quantifying the reduction in the concentration of extracellular serotonin over time. Serotonin was quantified using high pressure liquid chromatography. Agonist-concentration response data were analysed using a four parameter logistic model.

Results: At concentrations of 100–200 μM, racemic citalopram reduces aggregation induced by a maximal concentration of collagen by approximately 50%. In contrast, the rate of serotonin uptake into platelets is halved by approximately 5 nM citalopram, a value that is consistent with an action at SERT as reported in the literature. These observations suggest that citalopram-induced inhibition of platelet aggregation is not caused primarily by SERT blockade. This conclusion is further reinforced by data showing that the isomers of citalopram, with a reported 30-fold difference in potency for blocking SERT, inhibit platelet aggregation and adhesion to various ligands with similar potencies.

Conclusions: We conclude that platelet inhibition by citalopram is not mediated via SERT blockade and alternative mechanisms of action are more likely. This conclusion presents two additional questions: (1) by what mechanism does citalopram inhibit platelets, and (2) what effect does SERT inhibition have on platelet function, if any?

ROLE OF CONNEXIN 62 IN HUMAN PLATELETS

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Background: Connexins (Cxs) constitute a large family of proteins that are expressed by a wide variety of mammalian cells or tissues. They function by non-covalently oligomerising Cx into six units, resulting in a hexameric structure termed as a hemichannel or connexon. An intercellular channel is formed by the extracellular domains of two connexons belonging to adjacent cells. These intercellular channels arrange to form arrays of channels in plaques termed gap junctions that function by regulating the membrane permeability and trafficking. In this way, they provide a channel for regulated intercellular exchange of molecules up to around 1000 Daltons. Recent studies have reported the presence of connexin37 (Cx37) and connexin40 (Cx40), in human platelets and formation of hemichannels and gap junctions. The inhibition of both Cx37 and Cx40 (by selective inhibitors ^{43,37}Gap27 and ^{40}Gap27, respectively) significantly inhibited a wide range of platelet functions, such as aggregation, calcium mobilisation, α-granule secretion, and fibrinogen binding. In addition, a considerable reduction in clot retraction was observed, which indicates reduced outside-in signalling through integrin αIIbβ3. Notable levels of Cx62 transcripts (mRNA) in megakaryocytes were also reported in the same study. But, the role of this protein in platelets has not been explored.

Aim: The objective of this study was to determine the role of Cxs 62 in human platelets and its effects on platelet function.

Methods: Western blotting and immunohistochemistry were performed using anti-gap junction alpha-10 (Gja10) polyclonal antibodies to confirm the presence and localisation of Cx62 in human/mouse platelets and megakaryocytes. A selective inhibitor (^{62}Gap27) targeting the second external loop of the Cxs 62 was designed and synthesised along with a scrambled control peptide. Washed human platelets were treated with selective inhibitor ^{62}Gap27 for five minutes and aggregation responses were measured following stimulation by collagen-related peptide (CRP-XL) (0.25 μg/ml) or thrombin (0.05 U/ml) using light transmission aggregometry. The levels of calcium mobilisation were assessed by spectrofluorimetry using Fura-2-acetoxymethyl ester (FURA-2AM) loaded platelets in the presence or absence of ^{62}Gap27 upon stimulation with CRP-XL or thrombin. The affinity upregulation of integrin $\alpha_{IIb}\beta_3$ and α-granule secretion was measured using flow cytometry through the detection of levels of fibrinogen binding and P-selectin exposure on the platelet surface, respectively.

Results: Expression of Cx62 was confirmed in human and mouse platelets and megakaryocytic cell line (Meg01). The inhibitor designed to target Cx62 was found to significantly inhibit platelet aggregation in a dose-dependent manner with the highest reduction of 65% observed at 100 μg/ml of ^{62}Gap27. Concentration-dependent inhibition of the surface exposure of P-selectin exposure and fibrinogen binding was also observed, which indicates diminished α-granule secretion and $\alpha_{IIb}\beta_3$ integrin activation. The extent of calcium mobilisation was reduced compared to control following incubation with ^{62}Gap27.

Conclusion: Cx62 is expressed in human and mouse platelets and megakaryocytic cell line (Meg01) and its inhibition significantly affects platelet function. Future work would involve identifying

the nature of the signalling molecules that are transported by the Cx62 gap junction or hemichannels in platelets. Furthermore, the molecular pathways that regulate the conductance of these channels will be studied.

DIFFERENTIAL PHARMACOLOGICAL IMPACT OF ABACAVIR SULPHATE AND TENOFOVIR ON PLATELET AGGREGATION INDEPENDENT OF HIV INFECTION

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Background: Highly active antiretroviral therapy (HAART) is now so effective in the treatment of HIV that successfully treated individuals rarely progress to AIDS and live a near normal life expectancy. HIV infection is associated with increased cardiovascular morbidity and mortality and observational studies found the nucleoside reverse transcriptase inhibitor (NRTI) abacavir sulphate (ABC) to be associated with a reversible increased risk of myocardial infarction (MI), an effect not seen with other NTRI's such as tenofovir (TFV). It is not clear whether increased cardiovascular risk is driven pharmacologically by ABC or pathophysiologically by HIV and co-morbidities. Additionally, ABC has been reported to interrupt signalling by the endothelial mediator and inhibitor of platelet activation, nitric oxide (NO).

Aims: Since MI is platelet driven and platelets are inhibited by NO, our objective was to assess the impact of the ABC and TFV on platelet aggregation independent of HIV infection and investigate the mechanistic link between platelets, NO signalling and NRTIs.

Methods: Isolated human platelets from healthy male and female volunteers aged 20 to 50 were incubated for 10 minutes with either ABC (3 μg/ml), TFV (3 μg/ml) and the active metabolite of ABC, carbovir triphosphate (50 μM), then stimulated with collagen (0.1–2.5 μg/ml) or thrombin (0.01–0.1 U/ml). Additionally, carbovir triphosphate was incubated with PRP in the absence or presence of the NO-donor S-Nitroso-N-Acetyl-D, L-Penicillamine (SNAP) (0.5 or 5 μM) and stimulated with ADP (3 or 5 μM). Platelet aggregation was measured using light transmission aggregometry.

Radiolabelled platelet aggregation in response to a submaximal dose of collagen (50 μg/kg i.v.) was measured in real-time via external scintillation probes in anaesthetised mice 30 minutes or 4 hours after ABC or TFV (estimated plasma concentration of 30 μg/ml).

Results: TFV significantly inhibited platelet aggregation induced by an EC_{50} concentration of thrombin (0.03 U/ml) and collagen (0.6 μg/ml) compared to the vehicle control in vitro, however, no effect was detected following incubation with ABC. Carbovir triphosphate reversed NO-mediated inhibition of ADP induced platelet aggregation but no effect was observed for TFV.

ABC significantly enhanced collagen-induced platelet aggregation compared to the vehicle control in vivo. In contrast, no effect was observed following treatment with TFV in vivo. Neither ABC nor TFV had any effect of platelet aggregation in vivo 4 hours after administration.

Conclusions: Reported differences between the cardiovascular risk profile of ABC and TFV in patient studies may be due to retention of endothelial-derived platelet inhibition in the presence of TFV but reversible pharmacological blockade of this cardioprotective mechanism by ABC. Additionally, a direct and previously unreported inhibitory pharmacological effect of TFV on platelet activation may lower the risk of MI in patients receiving TFV.

A COMPARISON OF QUERCETIN AND ITS IN VIVO METABOLITES IN THE INHIBITION OF PLATELET FUNCTION

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Background: There is a well-established link between diet and CVD risk. Diets high in flavonoids, a class of plant secondary metabolites, have demonstrated cardio-protective effects, thought in part to be due to anti-platelet mechanisms. Quercetin is one of the main dietary flavonoids, and upon consumption, is extensively metabolised in the small intestine, liver and colon. Whilst some research has established the ability of quercetin to inhibit platelet function, interpretation of current data is limited due to a paucity of information regarding the actions of quercetin's physiological metabolites, the ''final effectors'' of dietary quercetin.

Aims: We investigated the ability of quercetin and two key methylated metabolites – isorhamnetin (3′ methylated) and tamarixetin (4′ methylated) – to inhibit platelet aggregation, fibrinogen:integrin αIIbβ3 interactions, α- granule release, platelet adhesion and spreading, intracellular calcium mobilization and clot retraction, and compared the potencies of these metabolites and the aglycone at physiologically achievable concentrations. The effect of a glucuronidated metabolite, quercetin-3-glucuronide, to inhibit platelet function was also investigated.

Methods: Platelet aggregation was performed by light transmission aggregometry, and fibrinogen binding and P-Selectin exposure measured by flow cytometry. Platelet adhesion and spreading on fibrinogen and CRP-XL (Collagen Related Peptide, cross-linked) were measured using confocal microscopy. Calcium mobilization was measured by fluorescence intensity in a plate reader using FURA 2-AM loaded platelets. Clot retraction was measured through analysis of clot weights after retraction post-stimulation with thrombin.

Results: Quercetin, tamarixetin and isorhamnetin inhibited platelet aggregation stimulated with collagen (5 μg/ml); significant inhibition was observed at flavonoid concentrations as low as 1 μM, with higher concentrations ($\geq$5 μM) resulting in $>$90% inhibition. Fibrinogen binding and P-Selectin exposure stimulated by CRP-XL (1 μg/ml) were also inhibited significantly at flavonoid concentrations low as 0.5μM; up to 95% inhibition was observed with higher ($>$5 μM) quercetin concentrations. Concentrations of an order of magnitude higher ($\geq$50 μM) were required to achieve comparable inhibition with quercetin-3-glucuronide, implicating the glucuronide moiety in reduced inhibitory potency. Quercetin and its methylated metabolites inhibited significantly the adhesion and spreading of platelets on both fibrinogen and CRP-XL at concentrations of 1 μM and above, with isorhamnetin displaying increased potency compared to tamarixetin and quercetin aglycone. Intracellular calcium mobilization stimulated by CRP-XL (1 μg/ml) was also inhibited significantly at 1 μM and above, and displayed the same order of potency; isorhamnetin $>$ quercetin $>$ tamarixetin. Quercetin, tamarixetin and isorhamnetin inhibited the process of clot

retraction stimulated by thrombin (1 U/ml) ≥2.5 μM, indicating an ability to inhibit platelet function significantly in the presence of plasma proteins at physiologically achievable concentrations.

Conclusions: Quercetin, as well as two methylated metabolites, tamarixetin and isorhamnetin, inhibit significantly platelet function at concentrations that are physiologically achievable through either dietary modulation or supplementation. Methylation on the 3′ position (isorhamnetin) results in improved platelet inhibitory activity, with glucuronidation at the 3-position resulting in significantly reduced potency. These data, that may help explain the reduction in CVD risk associated with elevated quercetin intake, will be incorporated into a wider systems biology project to establish the impact of quercetin metabolites in the regulation of cardiovascular function.

QUANTIFICATION OF BLOOD PLATELET REACTIVITY IN MODEL OF ADP-INDUCED NON-LETHAL PULMONARY THROMBOEMBOLISM IN MICE WITH THE USE OF LASER DOPPLER FLOWMETRY

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Background: The most often used techniques to study platelets reactivity *in vivo* are based on experimental disruption of endothelial layer, which inevitably leads to thrombus formation. On the other hand, there are experimental approaches aimed at studying platelets' activation under conditions of intact endothelial layer where venous injection of agonist serves as trigger for platelets activation. In the latter case there are two approaches to measure the effect of agonist. One of them is based on monitoring of the death rate of animals, and the other method is based on measurement of accumulation of radiolabelled platelets in pulmonary circulation.

Aims: In the presented studies we tested an alternative method for monitoring of *in vivo* agonist-induced platelet aggregation, which requires neither platelet extravasation nor radiolabelling. Since platelet aggregates can occlude small vessels and thus impair blood flow in a vascular bed, we hypothesized that platelet aggregation induced *in vivo* can be monitored by instant measuring of blood flow in this vascular bed with the use of Laser Doppler Flowmetry (LDF).

Methods: Mice of C57Bl strain were used in the study. ADP (2.5 mg/ml) was injected to *vena cava* utilizing the infusion pump and blood flow was monitored with the use of laser Doppler flowmeter in the mesentery. Measurements in platelet-depleted mice (5 μg of anti CD41 antibodies), mice pretreated with cangrelor (0.2 and 1 mg/kg b.w.) – the antagonist of ADP receptors and eptifibatide (0.5 and 4 mg/kg b.w.) – blocker of fibrinogen binding to GPIIbIIIa were conducted as the proof-of-concept in the performed experiments. Intravital microscopy and ex vivo imaging of organs was performed to identify the sites of aggregates formation in animals as an effect of ADP injection. The platelet-specific fluorescent anti-GPIbβ antibodies was used to visualize the blood platelets.

Results: Injection of ADP resulted in a dose-dependent reduction of blood flow in the mesentery. Area under the curve measured from the injection of the agonist to 99% plateau was chosen as the most discriminative variable between the tested doses of ADP (0.5; 1; 2; 3.5; 5 and 10 mg/kg b.w). These responses were fully attributable to blood platelets aggregation, as shown by lack of the effect in platelet-depleted mice (platelet count was lowered to 10% of control value) and significantly reduced responses in mice pretreated with cangrelor (by 53.1 ± 10.9% and 44.3 ± 14.5%, respectively in the presence of either 0.2 or 1 mg/kg cangrelor) and eptifibatide (to 23.0 ± 16.4% and 55.2 ± 8.3%, respectively in the presence of either 0.5 or 4 mg/kg eptifibatide). Intravital microscopy did not reveal platelet aggregates formation in mesenteric vessels while ex vivo imaging showed accumulation of fluorescent labelled platelets in the lung.

Conclusions: Measurement of blood flow cessation in the mesentery allows indirect measurement of ADP-induced pulmonary thromboembolism. We suggest that this approach can be useful for *in vivo* screening for antiplatelet drug candidates.

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EMERGING ROLES FOR TRP CHANNELS AND THE Na^+/Ca^{2+} EXCHANGER IN PLATELET Zn^{2+} INFLUX

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Background: Zinc (Zn^{2+}) is a biologically important cation that acts as a platelet agonist. Studies have shown rodents fed low Zn^{2+} diets develop a bleeding phenotype, which correlates with a reduction of the plasma zinc concentration. In addition, cancer patients, diagnosed with long term Zn^{2+} deficiency, have increased bleeding times, impaired aggregation responses and develop ecchymoses. Within our laboratory we have shown that platelet activation coincides with a rise of intracellular Zn^{2+} ($[Zn^{2+}]_i$), however, the pathways governing Zn^{2+}-influx remain unclear.

Aims: To investigate the potential routes for platelet Zn^{2+} influx using a range of pharmacological tools, aggregometry and fluorescence-based assays.

Methods: Contributions by candiate ion channels to Zn^{2+}-induced platelet activation were assessed using a range of channel blockers on washed platelet suspensions. Aggregometry was used to assess inhibition of Zn^{2+}-induced (1mM) aggregation, whilst flow cytometry was used to assess real time $[Zn^{2+}]_i$ responses of fluozin-3-stained platelets.

Results: We identified several Zn^{2+}-permeable channels, transporters and exchangers known to be expressed by platelets, based on literature searches and available proteomic data. Platelets possess ZIP3, ZIP7, ZnT5 and ZnT6 of the Zn^{2+} transporter family, however, a lack of specific blockers prevented further investigation. Therefore we focussed on the transient receptor potential (TRP) channel family of non-selective cation channels and the Na^+/Ca^{2+} exchanger (NCX). Zn^{2+}-induced platelet aggregation was substantially reduced by the TRP channel inhibitors SKF96365, flufenamic acid, $GdCl_3$ and $LaCl_3$, in a concentration-dependent manner. A similar effect was observed when platelets were pre-incubated with the reverse-mode NCX inhibitor KB-R7943. In isolation, application of 50μM SKF96365 or KB-R7943 reduced aggregation to 43.72% ± 3.77% and 59.77% ± 8.77% of control responses, respectively. Interestingly, co-application of these drugs completely abolished Zn^{2+}-induced aggregation (2.94% ± 2.75% of control), suggesting that multiple channels regulate Zn^{2+}-induced aggregation.

Conclusions: These data indicate that Zn^{2+}-induced platelet activation involves Zn^{2+}-permeable ion channels and exchangers. Given the lack of pharmacological tools to target Zn^{2+} transporter family proteins, we were unale to investigate their contributions to

platelet activation. Our data provide the first evidence for contributions by NCX and TRP channels to Zn^{2+}-mediated platelet activation. In addition, Zn^{2+}-induced aggregation was abrogated by co-application of KB-R7943 and SKF96365, suggesting that these proteins may interact to co-ordinate the platelet response to Zn^{2+}. Futher work is required to fully identify the cohort of Zn^{2+} entry pathways in the platelet and the role that these may play in cellular activation, aggregation and adhesion.

MEGAKARYOCYTE Ca^{2+}-ACTIVATED ION CHANNELS SENSITIVE TO THE TMEM16F BLOCKER $CaCC_{inh}$-A01 SHOW A MAJOR INTERSPECIES DIFFERENCE IN THEIR IONIC SELECTIVITY

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Background: Assymmetrical distribution of membrane phospholipids is maintained by ATP-dependent flippases and floppases. Platelet stimulation activates a Ca^{2+}-dependent phospholipid scramblase, which mediates phosphatidylserine exposure on the cell surface and thus procoagulant activity. Recently, TMEM16F was cloned and shown to possess Ca^{2+}-activated Cl^- channel and phospholipid scramblase activity. Missense mutations of the human TMEM16F gene cause the rare bleeding diathesis Scott syndrome. Interestingly, excised inside-out patch clamp recordings from $TMEM16F^{-/-}$ mouse megakaryocytes (MKs) reported the loss of a Ca^{2+}-activated cation channel (Yang *et al.,* 2012, Cell; 151, 111) rather than a Cl^- conductance. To date, no study has used whole cell recordings of MKs to monitor the membrane currents induced by the sustained $[Ca^{2+}]_i$ increases required to activate phospholipid scramblases.
Aims: To characterise the whole cell membrane currents of primary rodent MKs stimulated by sustained $[Ca^{2+}]_i$ increases.
Methods: Primary MKs were isolated from male adult wistar rats or C57/bl6 mice as described previously (Mahaut-Smith, 2004, Methods Mol. Bio.; 273, 277). Conventional whole-cell patch clamp recordings were carried out in K^+-free salines with an $[Ca^{2+}]_i$ of either $\approx$5nM or 100μM. A combination of voltage ramps and voltage steps were applied to MKs treated with the TMEM16F inhibitor $CaCC_{inh}$-A01 (A01) or DMSO as the vehicle control.
Results: Ca^{2+}-dependent activation of TMEM16F channels requires a sustained elevation of $[Ca^{2+}]_i$ (>5 minutes, $EC_{50} = 100\,\mu M$). Thus, Ca^{2+}-activated ion channel activity, was assessed over a 10 minute timecourse using voltage ramps from a holding potential of -80mV (−120 to +120 mV, 0.36 V/s), before applying a series of voltage steps (1s duration across the range −120 to +120 mV). In symmetrical NaCl recording solutions, 100 μM Ca^{2+}-activated currents developed within 6–7 minutes in both mouse and rat MKs. At $t = 10+$ minutes Ca^{2+}-activated outwardly rectifying currents were observed that reversed close to 0mV and were blocked by 20 μM A01. Ion substitution experiments were performed to determine the permeability of these conductances. Interestingly, lowering $[Cl^-]_o$ to 5 mM shifted the reversal potential for 100 μM $[Ca^{2+}]_i$ -activated currents of rat MKs to $\approx$+40 mV, indicative of a predominantly anionic conductance whilst mouse MK currents continued to reverse at $\approx$0 mV. Substitution of $[Na^+]_o$ with the large cation $NMDG^+$ shifted the reversal potential of these currents in mouse MKs to -8 ± 1.6 mV, further indicating a cation-permeable rather than anion-permeable conductance.
Conclusions: These data represent the first recordings of whole cell currents from primary mouse and rat MKs under conditions of sustained high $[Ca^{2+}]_I$ (100 μM) that are required to activate TMEM16F and thus phospholipid scramblases. The Ca^{2+}-dependent currents were activated after >5 minutes of dialysis with 100 μM $[Ca^{2+}]_i$, were outwardly rectifying and A01-sensitive, as expected from previous studies of TMEM16F channels. However, ion substitution experiments suggest that this conductance is principally permeable to Cl^- in rat MKs, whilst mouse MKs are cation-selective. Coupled with immunohistochemistry data, these data confirm expression of TMEM16F-like channels at the MK plasma membrane. However, given the interspecies difference of ion-permeability, the relationship between lipid scrambling and ion channel activity of TMEM16F channels require further investigation.

$P2Y_{12}$ INHIBITORS ATTENUATE SYSTEMIC INFLAMMATION AND ITS PROTHROMBOTIC EFFECTS IN A MODEL OF HUMAN SEPSIS

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Background: In the PLATelet inhibition and patient Outcomes (PLATO) study of patients with acute coronary syndromes, the novel platelet $P2Y_{12}$ inhibitor ticagrelor was associated with fewer deaths following sepsis and pulmonary infections than clopidogrel.
Aims: Determine the effects of ticagrelor and clopidogrel on systemic inflammation and its associated prothrombotic effects in a human sepsis model.
Methods: Thirty healthy volunteers were randomized to a normal dose of ticagrelor (n = 10), clopidogrel (n = 10) or no antiplatelet medication (controls; n = 10) for one week. We then used the well-established method of intravenous E. coli endotoxin administration. Platelet-leukocyte interactions, platelet function, cytokine release and fibrin clot structure were investigated before randomized treatment and up to 24 hours after endotoxin administration.
Results: Both ticagrelor and clopidogrel exhibited potent suppression of multiple inflammatory mechanisms, resulting in peak levels of TNFα, IL-6 and CCL2 that were 18–66% lower than control (all $p<0.05$). Ticagrelor, but not clopidogrel, also significantly reduced peak levels of IL-8 (29% reduction; $p = 0.001$) and G-CSF (51% reduction; $p<0.001$) and increased peak levels of IL-10 (54% increase; $p = 0.02$) compared to control. In the control group, fibrin clot maximum absorbance (an indicator of fibrin clot density), increased by $109 \pm 37\%$ at 6 hours after LPS administration compared to baseline ($p<0.001$). The percentage increase in maximum absorbance was significantly lower in the ticagrelor group ($33 \pm 19\%$; $p = 0.02$) compared to control, but was not significantly lower in the clopidogrel group ($67 \pm 20\%$; $p = 0.20$). In the control group, plasma levels of the fibrin degradation product D-dimer increased from 192 ± 36 μg/l at baseline to a peak of $2{,}217 \pm 447$ μg/l at 4 hours after LPS ($p<0.001$). Peak levels of D-dimer were 48% lower in the

ticagrelor group ($p<0.001$) and 19% lower in the clopidogrel group ($p=0.01$) compared to control.
Conclusions: $P2Y_{12}$ inhibitors markedly reduced systemic inflammation and its associated prothrombotic effects in a human sepsis model. The greater overall effect of ticagrelor on these pathways compared to clopidogrel provides critical mechanistic insight into the lower mortality following sepsis observed in the ticagrelor group compared to the clopidogrel group in the PLATO study.

TETRASPANIN TSPAN9 REGULATES PLATELET COLLAGEN RECEPTOR GPVI LATERAL MOBILITY AND ACTIVATION

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Background: The tetraspanins are a superfamily of 33 four-transmembrane proteins in humans that interact with specific 'partner proteins' and regulate their intracellular trafficking, lateral mobility in the plasma membrane and clustering. We previously identified Tspan9 as a tetraspanin that is expressed on platelets but not several other cell types tested. The function of Tspan9 remains unknown.
Aims: To generate a Tspan9-deficient mouse to study Tspan9 function.
Methods: Tspan9-deficient mice were generated using gene trap embryonic stem cells. Platelet function was investigated using a variety of *in vitro* and *in vivo* assays. Single particle tracking studies were performed on platelets using total internal reflection fluorescence (TIRF) microscopy.
Results: The mice were viable and had normal platelet count, size and levels of major platelet antigens. However, a specific role for Tspan9 in platelet collagen receptor GPVI-induced platelet aggregation, secretion and protein tyrosine phosphorylation was identified. This phenotype was mild because it could be overcome at high concentrations of the GPVI-specific agonist collagen-related peptide or by collagen. Additionally, no defect was detected for thrombus formation on collagen under *in vitro* flow conditions, or for haemostasis or thrombus formation *in vivo*. To address the mechanism by which Tspan9 regulates GPVI, evidence that the two proteins interact on human platelets was obtained by co-immunoprecipitation and confocal fluorescence microscopy. Furthermore, single particle tracking studies on mouse platelets revealed that GPVI lateral mobility was reduced by more than 60% in the absence of Tspan9. As a control, no such effect was observed for lateral mobility of the tetraspanin CD9. Finally, pre-treatment of Tspan9-deficient platelets with the GPVI monoclonal antibody GPVI, with the aim of pre-cross-linking GPVI, rescued the subsequent GPVI-induced aggregation defect in Tspan9-deficient platelets.
Conclusions: This study demonstrates that the platelet collagen receptor GPVI, a receptor with therapeutic potential for the treatment of thrombosis, is regulated by tetraspanins. We propose that tetraspanin Tspan9 promotes GPVI lateral mobility on the platelet surface to ensure its rapid clustering and activation upon engagement with collagen.

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IDENTIFICATION OF LOW MOLECULAR WEIGHT FACTORS RELEASED FROM PLATELETS THAT INDUCE *PROCR* IN MONOCYTES

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Background: Endothelial protein C receptor (EPCR) regulates haemostasis by supporting activation of Protein C by the thrombomodulin – thrombin complex. EPCR is primarily expressed on endothelial cells but previous studies have shown EPCR mRNA (*procr*) can also be induced in monocytes by GPVI-activated platelets.
Aim: To investigate the mechanism by which platelets regulate monocyte *procr* expression.
Methods: Platelets activated by the collagen peptide mimetic CRP-XL were allowed to interact with monocytes, then isolated using immune-magnetic beads, and relative expression of *procr* was measured in monocytes by RT-qPCR. Platelet releasate was prepared from washed platelets and separated on the basis of MW. Proteins were subjected to tryptic digestion and peptides analysed by electrospray ionization mass spectrometry (ESI-MS) using a Q-Exactive Orbitrap mass spectrometer. Raw data was processed using Proteome Discoverer 1.4 to identify and quantify the releasate proteins. Gene ontology analysis was performed using the program Panther.
Results: GPVI-activated platelets induced monocyte *procr* expression in a time-dependent manner, reaching maximum at 8h ($p=0.0028$; $n=4$). Preventing direct platelet-monocyte interaction with a CD62P-blocking antibody 9E1 did not change *procr* expression indicating the active component was released from the platelets. This was confirmed by incubating isolated monocytes with platelet releasate. Analysis of fractionated platelet releasate identified proteins ~10kDa as responsible for *procr* induction ($p=0.0192$; $n=9$). Gene ontology biological process analysis of platelet-released proteins identified by MS identified several candidates ~10kDa. Three of these groups, biological regulation, cellular process and response to stimuli, were considered as likely to contain regulatory proteins. A total of 6 proteins (Acyl CoA binding protein, CCL5, peptodyl prolyl isomerase, PF4, PF4v and platelet basic protein) were identified but only 3 of these (PF4, PF4v and PBP) were common to all 3 groups. Platelet Factor 4 (PF4; MW 11kD) was selected as the best candidate due to its abundance and its known affects on monocytes. Incubation of platelet releasate with heparin-agarose beads to remove PF4, attenuated *procr* expression in autologous monocytes compared to untreated releasate fraction ($p=0.0340$; $n=3$), although this could not be rescued with hPF4. Western blotting showed PF4 was not removed from platelet releasates using this method but the Ponceau stain did show sremoval of a low molecular weight protein. MS analysis of heparin agarose treated releasates identified 2 proteins of ~10kDa MW significantly decreased in treated compared to untreated releasates. Of these, regulated on activation, normal T-cell expressed and secreted (RANTES/CCL5) was completely removed ($p=0.0074$).

Conclusions: These results suggest that RANTES released from activated platelets induces expression of *procr* in monocytes. This may be a modifiable mechanism by which monocytes regulate coagulation within a clot.

NITRIC OXIDE INHIBITS RHOA AND RHOA KINASE SIGNALLING IN BLOOD PLATELETS TO REGULATE PLATELET SHAPE CHANGE

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Background: Platelet shape change results from a rapid reorganization of the cytoskeleton including formation of new actin filaments, a process that requires Rho family GTPase-dependent inhibition of myosin light chain (MLC) phosphatase and MLC phosphorylation. Here we investigated the regulation of MLC phosphorylation by cGMP signalling pathways.
Methods: Washed Platelets (WP) treated with S-nitroso-gluthatione (GSNO), a NO donor drug, prior to stimulation with thrombin. Signalling mechanisms were studied using Western blotting, while a Rho A-pulldown assay was used to detail RhoA activity.
Results: Platelet stimulation with thrombin led to shape change and MLC phosphorylation (ser^{19}) through pathways that are Ca^{2+} and Rho Kinase (ROCK) dependent. Activation of cGMP signalling by GSNO abolished shape change and MLC phosphorylation. Use of Y-27632 and BAPTA-AM to isolate the two pathways revealed that GSNO inhibited MLC phosphorylation in the presence of BAPTA-AM indicating that cGMP signalling inhibits ROCK-dependent MLC phosphorylation. These observations were replicated by 8-CPT-6-Phe-cGMP (PDEs-resistant cGMP analog) and prevented by the guanylyl cyclase inhibitor ODQ. To explore the potential mechanism of cGMP action we next examined RhoA. Rhotekin pulldown analysis revealed that thrombin stimulation rapidly increased the levels of GTP bound-RhoA, which was prevented by GSNO. To examine how this influenced ROCK signaling we examined the inhibitory phosphorylation of MYPT-1 on threonine (thr^{853}). Thrombin induced phosphoMYPT1-thr^{853} was blocked by pretreatment with GSNO. Consistent with these observations we found that GSNO stimulated the inhibitory phosphorylation of RhoA on ser^{188} in a time and concentration dependent manner. Phosphorylation of RhoA on ser^{188} is associated with inhibition of RhoA.
Conclusion: These results suggest that cGMP/PKG signaling prevents activation of RhoA which blocks agonist-induced inhibition of MLCP. The disinhibition of MLCP prevents the phosphorylation of MLC required for shape change.

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DOES NON-ENZYMATIC GLYCOSYLATION INVOLVE PLATELET GLYCOPROTEIN IIBIIIA AND AFFECT RECEPTOR-LIGAND INTERACTION? – A PRELIMINARY STUDY

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Background: Non-enzymatic glycosylation (glycation) is one of the most prominent posttranslational protein modifications involved in many cellular functions, including cell-cell and receptor-ligand interactions, immune response, apoptosis, and pathogenesis of many diseases. It has been suggested that glycation of platelet membrane proteins alters the structure and conformation of platelet membrane proteins and evokes changes in membrane lipid dynamics, which subsequently may lead to the increased platelet responsiveness to physiological stimuli and reduced sensitivity to platelet inhibitors. However, a direct relationship between the structure of platelet membrane proteins and their function has not yet been elucidated.
Aims: The aim of study was to examine whether a major platelet receptor, glycoprotein IIbIIIa (GPIIbIIIa), and its primary ligand (fibrinogen) may undergo glycation under *in vitro* conditions mimicking severe chronic hyperglycaemia. We also evaluated the effect of *in vitro* glycation on the receptor-ligand interaction.
Methods: Purified human GPIIbIIIa and fibrinogen were glycated by incubating a given protein (1 mg/ml) at 37 °C with 30 mM glucose in phosphate buffered saline, containing protease inhibitors and antibiotics. The glycations of receptor and ligand were carried out for up to 7 and 5 days respectively, according to the average platelet lifespan with respect to the receptor and half-life of fibrinogen. Control samples were processed the same way in the absence of glucose. At the end of the incubation period, all samples were washed, concentrated, and protein content determined. The identification and quantitation of glycated amino acid residues in the examined proteins was performed following their enzymatic digestion with trypsin with the use of mass spectrometry (LC-MS/MS). Interactions between receptor and ligand in glycated or non-glycated form were examined by enzyme-linked immunosorbent assay (ELISA), using the receptor in solid phase.
Results: Both, glycated and non-glycated proteins demonstrated the modifications with glucose, however, the extent of glycation was considerably higher in the proteins incubated *in vitro* with glucose compared to the control samples. Apart from 4 modifications detected in control fibrinogen, we showed additional 11 modifications in the *in vitro* glycated fibrinogen (9 lysine residues and 2 arginine residues). Likewise, in the glycated GPIIbIIIa 2 more modifications occurred in the course of *in vitro* glycation, in addition to 9 modifications found in the non-glycated receptor. Within the range of 0.078–5 μg protein/ml native fibrinogen bound to immobilized non-glycated and glycated GPIIbIIIa, and the differences were not statistically significant. Interestingly, native ligand bound slightly better to a native receptor as compared to glycated GPIIbIIIa. When glycated fibrinogen was incubated with the native receptor, no interaction was detected, unless the native ligand was used. It may indicate the influence of experimental procedure on ligand functionality.
Conclusions: During platelet life span platelet receptors, such as GIIbIIIa, may undergo glycation, similarly as its ligand–plasma fibrinogen. With respect to the number of potential sites of glucose incorporation into GPIbIIIa/fibrinogen, the structural changes were rather subtle what it is reason for further investigations needed to define the role of protein glycation in the binding of GPIIbIIIa with ligands, using more sophisticated techniques, such as surface plasmon resonance.

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CONTRIBUTION OF THE VOLTAGE-GATED POTASSIUM CHANNEL Kv1.3 TO FLOW-DEPENDENT PLATELET ADHESION

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Background: Kv1.3, a voltage-gated K^+-selective channel with major roles in lymphocytes and neuronal function, was recently shown to be responsible for the largest ionic conductance observed in whole-cell recordings of platelets and megakaryocytes (McCloskey *et al*. 2010 J.Physiol. 588, 1399). This conductance sets the membrane potential of the resting platelet, however its relevance for platelet function has not been further characterised. In other cell types, K^+ channels have roles in integrin regulation, thus the contribution of Kv1.3 to platelet responses may extend beyond an indirect influence on the driving force for Ca^{2+} entry.

Aims: To investigate a possible role for Kv1.3 in flow-dependent platelet adhesion, using blood from WT and Kv1.3$^{-/-}$ mice.

Methods: Blood was collected under Home Office UK regulations from the inferior vena cava of isoflurane-anesthetised mice into ACD for flow cytometry, and into 40μM PPACK for platelet adhesion studies. Surface glycoproteins were measured by flow cytometry of washed platelet suspensions incubated with antibody or isotype control, then analysed for mean fluorescence intensity (MFI) for each surface antigen. To examine platelet adhesion and thrombus formation, platelets in whole blood were loaded with 1μM $DiOC_6$ and perfused over coverslips coated with collagen (100μg/mL), collagen peptides (100 μg/mL) or fibrinogen (200μg/mL) at a shear rate of 1,800s^{-1} for collagen and collagen peptides, and 800s^{-1} for fibrinogen. Z-series of adherent platelets and thrombi were acquired on an Olympus FV1000 confocal microscope and exported to Image-J v1.49 to ascertain percent surface coverage, mean thrombus height and mean thrombus volume.

Results: Deletion of Kv1.3 had no significant effect on expression of GPIbα, GPIbβ, GPV, nor on expression of integrin subunits α_2, β_1 and β_3; however, the MFI for integrin α_{IIb} was significantly elevated on Kv1.3-deficient platelets (6.98 ± 0.44 vs. 4.82 ± 0.25 for Kv1.3$^{-/-}$ and WT respectively; $n = 10$, $P<0.001$). Despite this increase, platelet adhesion to a surface coated with the $\alpha_{IIb}\beta_3$ ligand fibrinogen was not significantly different between the two genotypes (mean surface coverage $11.70\% \pm 1.03$ vs.$14.35\% \pm 2.02$, $n = 5$, $P > 0.05$ for Kv1.3$^{-/-}$ and WT). In contrast, flow-dependent platelet adhesion to fibrillar collagen was significantly reduced in Kv1.3-deficient platelets ($5.90\% \pm 1.57$ vs. $14.90\% \pm 3.21$, $n = 4$, $P<0.05$). Kv1.3-deficient platelets that successfully adhered to collagen formed significantly smaller thrombi (mean thrombus height $0.10\,\mu m \pm 0.04$ vs. $0.38\mu m \pm 0.11$, $n = 4$, $P < 0.05$). Analysis of platelet adhesion using collagen peptides that are specific for integrin $\alpha_2\beta_1$ or GPVI receptors, found that platelets from Kv1.3$^{-/-}$ mice had significantly lower surface coverage on coverslips coated with VWF-III + GFOGER (VWF A3 domain- and $\alpha_2\beta_1$–specific peptides) ($13.92\% \pm 2.31$ vs. $21.26\% \pm 1.29$, $n = 5$, $P<0.05$). In contrast, adhesion on coverslips coated with VWF-III + CRP-XL (GPVI-specific peptide) was not significantly different ($18.90\% \pm 0.65$ vs. $16.96\% \pm 1.31$, $n = 5$, $P > 0.05$ for Kv1.3$^{-/-}$ and WT). Furthermore, Kv1.3-deficient platelets had fewer, and shorter filopodia when adhered to VWF-III + GFOGER (mean number of filopodia per platelet 3.684 ± 0.34 vs. 4.47 ± 0.20, $P < 0.05$; and mean filopodia length $2.58\mu m \pm 0.14$ vs. $3.37\,\mu m \pm 0.14$, $P<0.005$).

Conclusions: These results demonstrate an important role for Kv1.3 in the regulation of platelet adhesion and thrombus formation via integrin $\alpha_2\beta_1$.