Virtual Platelet Society Meeting 2021

Poster Abstracts

Keele University

Poster Communications

Platelet and Megakaryocyte Cellular Signalling

PC1. Regulation of $P2Y_{12}$ receptor expression and activity by cAMP mediated PKA signalling in human platelets; a novel mode of action of anti-platelet drugs?

J. S. Khalil[1], K. Naseem[2], S. Mundell[1]

[1]*School of Physiology, Pharmacology and Neurosciences, University of Bristol, UK*
[2]*Leeds Institute of Cardiovascular and Metabolic Medicine, University of Leeds, UK*

E-mail: jawad.khalil@bristol.ac.uk

Introduction: Pharmacological blockade of platelet G protein-coupled receptors (GPCRs), including the $P2Y_{12}$ receptor ($P2Y_{12}R$) forms a powerful therapeutic tool in the treatment and prevention of arterial thrombosis. Clarification of the intracellular regulation of these clinically important receptors may provide a sound basis for the development of novel and specific drugs aimed at selected elements of this receptor system. The main aim of this study is to fully characterize the interplay between two critical platelet signalling systems: the cyclic nucleotide signalling system, a negative regulator of platelet reactivity [1] and the platelet expressed $P2Y_{12}R$ that provides a critical feed-forward signal to amplify platelet reactivity [2]. Further study aims to characterize whether specific clinically used antiplatelet drugs potentially activate the cyclic nucleotide system to dampen $P2Y_{12}R$ activity.

Methods: Cell based studies including BRET and receptor immunoprecipitation were undertaken in HEK293T cells transiently expressing FLAG-tagged $P2Y_{12}R$ to assess changes in receptor function. Further studies including pull-down assays, flow cytometry and GTPase-activity assays were used to examine $P2Y_{12}R$ function.

Results: Initial studies in cell lines and platelets revealed that the $P2Y_{12}R$ is phosphorylated in response to cAMP-elevating agents suggesting that the receptor is a target for cAMP signalling. Platelet $P2Y_{12}R$ co-immunoprecipitated with PKA isoforms suggesting that $P2Y_{12}R$ is a novel PKA type I binding protein in platelets. cAMP pull-down assays in platelets also confirmed an interaction of the $P2Y_{12}R$ with PKA type I. Receptor surface expression (flow cytometry) and activity assays (BRET and GTPase-Glo) revealed that activation of PKA directly attenuated ADP-mediated $P2Y_{12}R$ expression and responsiveness in both cell lines and platelets. Intriguingly ticagrelor a potent inhibitor of the $P2Y_{12}R$ with efficient antithrombotic activity [3] also decreases receptor activity in a PKA-dependent manner.

Conclusion: Overall these data reveal that cAMP mediated PKA signalling negatively regulates $P2Y_{12}R$ activity. Further we have demonstrated that this negative feedback loop may also play a potential role in the mode of action of specific clinically used $P2Y_{12}R$ antagonists.

References: 1. Raslan Z et al., (2015) The Spatiotemporal Regulation of cAMP Signaling in Blood Platelets-Old Friends and New Players. Front Pharmacol. 6:266-72. 2. Cunningham MR et al., (2017) Pathophysiological consequences of receptor mistraffic: Tales from the platelet P2Y12 receptor. Mol Cell Endocrinol. 449:74-81 3. Aungraheeta R et al., (2016) Inverse agonism at the P2Y12 receptor and ENT1 transporter blockade contribute to platelet inhibition by ticagrelor. Blood. 128:2717-2728.

This study is funded by British Heart Foundation

PC2. Role of formyl peptide receptor 2 (FPR2/ALX) and its anti-inflammatory ligand, lipoxin A4 analogue (BML-111) in the modulation of platelet function

Shatha AlOmar[1], Darius Widera[1] and Sakthivel Vaiyapuri[1]

[1]*School of Pharmacy, University of Reading, UK.*

E-mail: s.a.a.alomar@pgr.reading.ac.uk

Background and Objective: Formyl peptide receptor 2/ALX (FPR2/ALX), a member of seven transmembrane G protein-coupled receptors superfamily, exhibits dual effects of pro- and anti-inflammatory responses based on the nature of stimulating ligand type. Recently, FPRs have been shown to be present in platelets and regulate their function. However, the role of FPR2/ALX and the effect of its anti-inflammatory ligands on the regulation of platelet function have not yet been addressed. Platelets play significant roles in the regulation of inflammation and host defence in addition to haemostasis and thrombosis. They act as sentinels through regulating inflammatory responses due to their large number in circulation and their ability to rapidly release different kinds of immunomodulatory cytokines, and other inflammatory mediators. Given the anti-inflammatory effects of FPR2/ALX ligands, we sought to study the role of its anti-inflammatory ligand, Lipoxin A4 analogue, BML-111 in the modulation of platelet activation and thrombus formation.

Methods and Results: The expression of FPR2/ALX in human and murine platelets as well as megakaryocytes was confirmed using immunoblot analysis. The subcellular distribution of FPR2/ALX in platelets was demonstrated by immunocytochemistry. BML-111 inhibited a range of platelet function such as aggregation, fibrinogen binding to integrin $\alpha_{II}b\beta_3$, α -granule secretion, dense granule secretion, platelet spreading, and clot retraction. These data suggests that BML-111 may act as a potential modulator of platelet function using FPR2/ALX as a target.

Conclusion: This study demonstrates the significance of FPR2/ALX and its anti-inflammatory ligand, BML-111 in the modulation of platelet function. This suggests that FPR2/ALX may act as novel target to control thromboinflammatory conditions.

PC3. Collagen binds and activates monomeric GPVI

Joanne C. Clark[1,2], Raluca A. I. Neagoe[1,3], Malou Zuidscherwoude[1,2], Deirdre M. Kavanagh[1,2], Alexandre Slater[1], Eleyna M. Martin[1], Mark Soave[2,4], David Stegner[3], Bernhard Nieswandt[3], Natalie S. Poulter[1,2], Johan Hummert[1,2,5], Dirk-Peter Herten[1,2,5], Michael G. Tomlinson[2,6], Stephen J. Hill[2,4], Steve P. Watson[1,2]

[1]Institute of Cardiovascular Sciences, Level 1 IBR, College of Medical and Dental Sciences, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK

[2]Centre of Membrane Proteins and Receptors (COMPARE), The Universities of Birmingham and Nottingham, The Midlands, UK

[3] Institute of Experimental Biomedicine I, University Hospital and Rudolf Virchow Center, University of Wurzburg, Wurzburg, Germany

[4]Division of Physiology, Pharmacology and Neuroscience, School of Life Sciences, University of Nottingham, Queen's Medical Centre, Nottingham, NG7 2UH, UK

[5]Department for Physical Chemistry, Heidelberg University, Heidelberg, Germany

[6]School of Biosciences, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK.

E-mail: jcc709@student.bham.ac.uk

Background: The platelet collagen receptor, GPVI has emerged as a promising therapeutic target in thrombosis. It has been proposed that collagen binds to a unique epitope in dimeric GPVI and that GPVI dimerisation increases upon platelet activation. However, the binding site for the synthetic collagen, CRP, on the D1 Ig domain is remote from the site of dimerisation and recombinant domains D1 and D2 do not show dimerisation in solution.

Aims: The aim was to investigate whether GPVI is expressed as a monomer or dimer, and the requirement of dimerisation in supporting activation by collagen.

Methods: We have used mutagenesis to generate D2-deficient and -substituted (with an Ig domain from monomeric CD2) GPVI constructs to investigate the requirement of dimerisation for binding and activation by collagen using a cell adhesion assay and NFAT-luciferase reporter assay in DT40 cells. We have used NanoBRET with Nanoluc and Halotag-GPVI and FCS and photobleaching with GPVI-eGFP to determine the organisation of GPVI in HEK293T cells.

Results: Both D2-mutated GPVI constructs supported adhesion to collagen. Collagen and CRP stimulated an increase in NFAT activity over basal with D2-deleted GPVI. The D2-substituted GPVI signalled constitutively. GPVI was shown to be expressed as a mixture of monomers and dimers under basal conditions in transfected HEK293T cells using nanoBRET, single molecule microscopy photobleaching and FCS.

Conclusions: We provide evidence using mutagenesis and advanced microscopy in cell lines that GPVI is expressed as a mixture of monomers and dimers and that dimerisation is not essential for adhesion or activation.

PC4. Using CRISPR-Cas9 to study the structure/function relationship of PLCγ2 in platelets

Safa Gayes[1], Selorm P.Segbefia[1], Sophie H. Nock[1], Alice Y. Pollitt[1] and Craig E. Hughes [1]

[1] *Institute for Cardiovascular and Metabolic Research (ICMR), School of Biological Sciences, University of Reading, Reading, UK*

Email: s.gayes@pgr.reading.ac.uk

Background: A number of platelet signalling pathways result in the activation of phospholipase C gamma 2 (PLCγ2). These include the collagen receptor GPVI which activates via an ITAM (immunoreceptor tyrosine-based activatory motif), and the podoplanin receptor CLEC-2, which activates through similar mechanism known as a hemITAM.

PLCγ2 also acts downstream of ITAM-linked receptors in other cells which have a similar signalling pathway. In patients who develop resistance to ibrutinib, mutations in PLCγ2 have been identified. Some of these have been shown to be gain-of-function mutations, but for the others the effect of the mutation is unknown. It is also unknown whether these mutations arise in the platelets, and what the functional effect on the platelets might be.

Aim: To use CRISPR-Cas9 to study the functional effect of PLCγ2 mutations downstream of (hem)ITAM receptors.

Methods: CRISPR-Cas9 was used to generate PLCγ2 knock-out and knock-in point mutations in DT40 cells. To characterise the functional effect of the mutations, calcium assays, NFAT assays, and tyrosine phosphorylation was examined. PLCγ2 and Btk inhibitors were used as a positive controls.

Results: PLCγ2 knock-out DT40s were successfully generated. Calcium responses and NFAT signalling in response to GPVI and CLEC-2 activation were abolished in these cells. Similar results were seen with a PLCγ2 or a Btk inhibitor in both DT40 cells and platelets. Knock-in point mutations have thus far been unsuccessful.

Conclusion: Thus far these results confirm the essential role for PLCγ2 downstream of (hem)ITAM receptor signalling. Work is ongoing to generate knock-in point mutations. Future work will move into human megakaryocytes and into zebrafish for in vivo analysis of PLCγ2 function.

PC5. A humanised CLEC-2 mouse model used to test anti-human CLEC-2 biologics *in vivo* shows that human CLEC-2 can be depleted

Helena Brown[1,2,3], Sarah Beck[1], Stefano Navarro[1,3], Steve Thomas[2,3,4], Steve Watson[2,3,4], Bernhard Nieswandt[1,3], David Stegner[1,3]

[1] *Institute of Experimental Biomedicine I, University Hospital and Rudolf Virchow Center of Experimental Biomedicine, University of Wuerzburg, Wuerzburg, Germany*

[2] *Institute of Cardiovascular Sciences, College of Medical and Dental Sciences, University of Birmingham, Birmingham, UK*

[3] *Targeting Platelet Adhesion Receptors in Thrombosis (TAPAS) Marie Sklodowska-Curie Innovative Training Network.*

[4] *Centre of Membrane Proteins and Receptors (COMPARE), Universities of Birmingham and Nottingham, The Midlands, UK*

E-mail: Brown_H@ukw.de

Background: Platelet C-type lectin-like receptor-2 (CLEC-2) and its endogenous ligand, podoplanin, play a role in blood and lymphatic vessel separation. Nevertheless, CLEC-2 has also been proposed as a potential anti-thrombotic target as antibody-mediated receptor depletion prevents occlusive thrombus formation in mice, suggesting an additional, intravascular, CLEC-2 ligand. However, it is unknown whether human CLEC-2 can also be depleted and there are currently no methods to test potential human therapeutics *in vivo*.

Aims: We aimed to generate a humanised CLEC-2 (hCLEC-2^{KI}) mouse model to test potential therapeutics *in vivo*.

Methods: The murine *Clec1b* gene was replaced with the human variant and receptor depletion was induced using monoclonal anti-hCLEC-2 antibodies (either AYP1 or the novel antibody HEL1).

Results: hCLEC-2^{KI} mice were phenotypically and functionally normal with comparable glycoprotein receptor expression, activation and aggregation to wildtype platelets and no evidence of blood-lymphatic defects. Injection of either antibody resulted in transient thrombocytopenia and prolonged CLEC-2 depletion on returning platelets for at least 10 days with CLEC-2 levels returning to normal after 20 days. Although haemostasis was unaltered, delayed vessel occlusion was observed in untreated hCLEC-2^{KI} mice following $FeCl_3$-induced vessel injury.

Conclusions: We have shown that a humanised CLEC-2 mouse model could be used to test potential therapeutics *in vivo*, and that human, like mouse CLEC-2, can be depleted from platelets, albeit over a longer timeframe.

PC6. Inhibition of Src, Syk and Btk reverse protein phosphorylation but does not block platelet aggregation mediated by GPVI and CLEC-2

Yam Fung Hilaire Cheung,[1,2,3] Luis Moran,[1,4] Albert Sickmann,[2] Johan W.M. Heemskerk,[3] Angel Garcia[1,4] and Steve P. Watson[1]

[1]Institute of Cardiovascular Sciences, College of Medical and Dental Sciences, University of Birmingham, UK

[2] Leibniz-Institut für Analytische Wissenschaften-ISAS-e.V., 44227 Dortmund, Germany

[3]Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, 6229 ER Maastricht, The Netherlands

[4]Center for Research in Molecular Medicine and Chronic Diseases (Cimus), Universidade de Santiago de Compostela, Spain.

E-mail: Hilaire.cheung@isas.de

Background: The collagen receptor GPVI initiates platelet activation in the injured vessel driving shape change, secretion of secondary agonists and integrin activation. The podoplanin receptor CLEC-2 is gaining interest as a therapeutic target for its role in arterial and venous thrombosis. Both GPVI and CLEC-2 signals through Src family, Syk and Btk tyrosine kinases leading to formation of a LAT signalosome and activation of PLCγ2.

Aims: To investigate the contribution of GPVI, CLEC-2, and Src, Syk and Btk tyrosine kinases to the maintenance of platelet aggregation measured by light transmission aggregometry.

Methods: Platelets were stimulted in a Born aggregometer and protein phosphorylation analysed using phosphospecific antibodies.

Results: Platelet aggregation and tyrosine phosphorylation of Syk, LAT and PLC · 2 induced by collagen and rhodocytin are sustained for up to 50 min, the longest time point investigated. The addition of the Src inhibitors, PP2 and dasatinib, after 150 sec, when aggregation had reach a plateau, causes rapid inhibition of phosphorylation of all three proteins but has no effect on aggregation. A similar result is seen with the Syk inhibitor, PRT-060318, although phosphorylation of Syk is maintained. Platelet aggregation is also sustained in the presence of the Src and Syk inhibitors combined with apyrase and indomethacin which block the effect of ADP and thromboxane A_2, respectively.

Conclusions: These findings indicate that signalling by GPVI, CLEC-2 and the secondary agonists are not required for sustained aggregation as measured by light transmission aggregometry.

PC7. OCRL phosphatase controls actin reorganization during human and mouse platelet spreading

Ana Bura[1], Leopoldo Staiano[2], Valentina Morra[2], Maria Antonietta De Matteis[2,3], Antonija Jurak Begonja[1]

[1] *Department of Biotechnology, University of Rijeka, Rijeka, Croatia*
[2] *Theleton Institute for Genetics and Medicine, Naples, Italy*
[3] *University of Naples Federico II, Italy*

E-mail: ana.bura@uniri.hr

Background: Phosphatidylinositol-4,5-bisphosphate [$PI(4,5)P_2$] is a phosphoinositide found at the plasma membrane which regulates actin reorganization. Lowe oculocerebrorenal syndrome protein (OCRL) dephosphorylates $PI(4,5)P_2$ and its mutations cause Lowe syndrome which is characterized by congenital cataracts, central hypotonia, and renal proximal tubular dysfunction. Aberrant primary hemostasis was shown in some Lowe syndrome patients. Platelets (PLT) recognize the site of injury and undergo extensive actin reorganization for their full activation.

Aims: We tested if the inhibition or deficiency of OCRL leads to impaired PLT function due to changes in the actin cytoskeleton.

Methods: Human PLTs with pharmacologically inhibited OCRL and mouse platelets deficient in OCRL were examined for their ability to spread on fibrinogen. In addition, the effect of the OCRL inhibition was tested in human PLT for the phosphorylation status of several signaling pathways after thrombin activation.

Results: Human OCRL-inhibited PLTs failed to fully spread on fibrinogen and contained more actin nodules in all tested time points of activation compared to the untreated PLTs. Actin nodules in OCRL inhibited PLTs colocalized with vinculin, ARP2/3, as well as with phosphotyrosine suggesting dysregulation of the cytoskeleton remodeling. When activated with thrombin, OCRL-inhibited PLTs showed reduced phosphorylation of MLC, but not of p38 or ERK. Impaired spreading on fibrinogen was further confirmed with OCRL deficient mouse PLTs that exhibited increased filopodia when compared to wild type PLTs.

Conclusions: Our data suggest that OCRL might control actin cytoskeleton rearrangements during platelet activation and thus their proper function which could be mediated by reduced MLC phosphorylation.

PC8. C3G: A new player in platelet signaling

Sara Gutiérrez-Herrero[1], Luis Hernández-Cano[1], Cristina Fernández-Infante[1], Óscar Herranz[1], Almudena Porras[2] and Carmen Guerrero[1,3]

[1]Instituto de Biología Molecular y Celular del Cáncer (IMBCC), University of Salamanca-CSIC, Salamanca, Spain; Instituto de Investigación Biomédica de Salamanca (IBSAL), Salamanca, Spain.
[2]Departamento de Bioquímica y Biología Molecular, Facultad de Farmacia, Complutense University of Madrid. Instituto de Investigación Sanitaria del Hospital Clínico San Carlos (IdISSC), Madrid, Spain.
[3]Departamento de Medicina, University of Salamanca, Salamanca, Spain.

E-mail: cguerrero@usal.es

Background: C3G (RAPGEF1) is a guanine nucleotide exchange factor for Rap1b, a protein essential for platelet activation and aggregation. C3G plays a role in angiogenesis, tumor growth and metastasis through the regulation of platelet secretome [1] and contributes to megakaryopoiesis and thrombopoiesis [2]. Additionally, C3G is the GEF that mediates the activation of Rap1b by PKC in response to thrombin and PMA [3].

Aims: Since C3G is a Rap1 GEF, we wanted to explore the contribution of C3G to signaling pathways that lead to Rap1b activation in platelets.

Methods: we have used transgenic and knockout mouse models for C3G expression to analyze platelet activation and aggregation, as well as C3G expression and phosphorylation. Platelets were treated with thrombin, PMA or ADP, in combination with specific inhibitors, such as bisindolylmaleimide, PP2, clopidogrel, wortmannin, SB203580, U0126, SHP099 and aspirin. C3G expression and phosphorylation was analyzed by immunofluorescence. Rap1 activation was also analyzed by pulldown under different conditions.

Results: Platelet C3G is phosphorylated in Tyr504 by a mechanism involving PKC-Src. This phosphorylation is positively regulated by ERKs through the inhibition of Sph2 tyrosine phosphatase. C3G participates in the ADP-P2Y12-PI3K-Rap1b pathway, and mediates the thrombin-TXA2 actions. Moreover, it inhibits the synthesis of TXA2 through cPLA2 regulation [4].

Conclusions: C3G contributes to major platelet pathways leading to Rap1b activation and is the main GEF in the thrombin-PKC-Rap1b pathway.

[1] Oncotarget 8, 110994-111011, 2017
[2] Cell Commun Signal 16, 101, 2018
[3] BBA Mol Cell Res 1823, 1366-1377, 2012
[4] Signal Transduct Target Ther 5, 29, 2020

PC9. Characterising platelet collagen receptor GPVI in its native environment using polymer-based nanodiscs

Xueqing Wang,[1,3] Sarah C. Lee,[2] Alexandre Slater,[1] Naomi L. Pollock,[2] Neale Harrison,[2] Tim R. Dafforn,[2] Ángel García,[3] Steve P. Watson[1] and Michael G. Tomlinson[2]

[1]*Institute of Cardiovascular Sciences, College of Medical and Dental Sciences, University of Birmingham, UK*

[2]*School of Biosciences, College of Life and Environmental Sciences, University of Birmingham, UK*

[3]*Center for Research in Molecular Medicine and Chronic Diseases (Cimus), Universidade de Santiago de Compostela, Spain.*

E-mail: x.wang.12@bham.ac.uk

Background: The platelet-activating collagen/fibrin(ogen) receptor GPVI activates platelets via associated FcRγ chains and is a promising anti-thrombotic target. However, the stoichiometry of the GPVI/FcRγ complex is unclear. Traditionally, membrane protein characterisation relied on detergent extraction, which strips away surrounding lipids/proteins that can be important for protein structure and function. Styrene meleic acid co-polymer lipid particles (SMALPs) are an emerging methodology designed to solve these problems. Membrane fractions are encapsulated into polymer-belted nanodiscs with ~10 nm diameter, which are readily amenable to structural and functional applications.

Aims: To develop SMALPs as a tool to investigate GPVI stoichiometry.

Methods: For HEK-293T cells transfected with His-tagged GPVI and FcRγ, SMALPs were generated, purified via nickel affinity columns and gel filtration, and characterised by nativePAGE "SMA-PAGE" and collagen-binding assay. For platelets, SMALPs were generated and isolated by co-immunoprecipitation.

Results: In HEK-293T cells, GPVI in SMALPs were functional in binding collagen. SMA-PAGE revealed GPVI/FcRγ SMALPs migrating at distinct molecular weights from approximately 85 kDa to 720 kDa, possibly because of the existence of monomers, dimers and oligomers of GPVI/FcRγ complex. In platelets, GPVI and FcRγ were co-immunoprecipitated in SMALPs, without evidence of co-precipitating proteins. NativePAGE will need to be performed to provide stoichiometry information.

Conclusions: Our study established a methodology in HEK-293T cells where full-length, functional GPVI/FcRγ were encapsulated into SMALPs. Different oligomerisation states of GPVI/FcRγ have been suggested to exist in this cell line; this can potentially be extended to investigate the stoichiometry of endogenous proteins, including GPVI/FcRγ and other major platelet membrane receptors.

PC10. Platelet CLEC-2 contributes to thrombi stability in arterial thrombosis

Joshua H. Bourne[1], Christopher W. Smith[1], Ying Di[1], Helena Brown[1], Julie Rayes[1, 2] and Steve P. Watson[1, 2]

[1]*Institute of Cardiovascular Sciences, College of Medical and Dental Sciences, University of Birmingham, Birmingham, B15 2TT, United Kingdom*
[2]*Centre of Membrane Proteins and Receptors (COMPARE), Universities of Birmingham and Nottingham, The Midlands, United Kingdom*

E-mail: JHB844@student.bham.ac.uk

Background: C-type lectin-like receptor 2 (CLEC-2) is a platelet receptor critical in lymphatic development and immunomodulation during thromboinflammation and infection. Distinctly activated through a hemi-immunoreceptor tyrosine-based activation motif (hemITAM), CLEC-2 to date has an ill-defined role in arterial thrombosis, with minor roles in haemostasis. Many anti-platelet drugs induce bleeding defects in patients. If the contribution of CLEC-2 to arterial thrombosis could be elucidated, it could be a promising therapeutic target, without increasing the risk of bleeding.

Aims: To determine how CLEC-2 contributes to thrombus growth in a novel, platelet-specific (GPIba-cre) knockout mouse model, and investigate the translational potential of CLEC-2-inhibtion in human blood.

Methods: Thrombus growth in CLEC-2-deficient mice was analysed by intravital microscopy post-ferric chloride injury of the carotid artery, and laser injury of cremaster arterioles. Aggregate formation under flow at arterial shear was also assessed in human blood treated with CLEC-2-blocking Fab' fragments of AYP1 antibody.

Results: In this study, we observe a reduction in thrombi stability in CLEC-2-KO mice compared to littermate controls following ferric chloride and laser injury, with no vessel occlusion. This result is independent of a reduction in platelet count, confirmed using a GPIb platelet-depletion antibody to match platelet counts between the Cre-positive and -negative mice. In preliminary studies in human blood, we observe a reduction in thrombi surface coverage in AYP1 $F(ab)_2$-treated blood, compared to the untreated control.

Conclusions: We conclude that CLEC-2 may contribute to thrombi stability in mouse and human blood, and could present a novel therapeutic target.

PC11. Exposure of platelet-derived FXIII-A on the activated membrane is dependent on $\alpha_{IIb}\beta_3$ and intracellular signalling via caspases and Src family kinases

Fahad Alshehri[1], Claire S Whyte[1], Nicola J Mutch[1]

[1]*Aberdeen Cardiovascular & Diabetes Centre, School of Medicine, Medical Sciences and Nutrition. Institute of Medical Sciences, Foresterhill, Aberdeen, AB25 2ZD*

E-mail: r01fsma@abdn.ac.uk

Background: Platelets contain an abundance of cellular FXIII-A in their cytoplasm which is released upon stimulation and participates in extracellular cross-linking reactions. As FXIII-A lacks a signal release peptide, the mechanisms involved in its externalisation remain unclear.

Aims: To identify the mechanism of exposure of FXIII-A on the platelet surface following stimulation.

Methods: Washed human platelets were activated with (thrombin 100 nM + convulxin 100 ng/ml) ± $\alpha_{IIb}\beta_3$ inhibitor (tirofiban 1 µg/ml), pan-caspase inhibitor (ZVAD 100 µM), caspase-3 inhibitor (Z-DEVD 100 µM), Src family kinases (SFKs) inhibitor (dasatinib 4 µM) or PTP1B inhibitor (MSI-1436 2 µM). FXIII-A and phosphatidylserine (PS) exposure were detected by flow cytometry and confocal microscopy. Model thrombi formed from FXIII deficient plasma + FITC-labelled fibrinogen ± platelets, transglutaminase inhibitor (1mM) or the inhibitors described above. Thrombi were bathed in tissue plasminogen activator (1 µM) and lysis rate determined as fluorescence release.

Results: The number of platelets expressing FXIII-A and PS was significantly increased following strong dual activation with thrombin + convulxin (PS 79.6 ± 6.6 vs. 3.4 ± 1.1 %; FXIII-A 74.3 ± 6.3 vs. 5.4 ± 1.6 %) compared to resting platelets. Flow cytometry and confocal microscopy revealed a significant reduction in FXIII-A and PS exposure after inhibition of $\alpha_{IIb}\beta_3$, caspases, caspase-3 and SFKs. Inhibition of PTP1B had no effect on FXIII-A or PS exposure. Platelet FXIII-A stabilized FXIII-depleted thrombi however, this stabilising effect was reduced upon inhibition of $\alpha_{IIb}\beta_3$, caspases, caspase-3 and SFKs.

Conclusions: Intracellular signalling of the integrin $\alpha_{IIb}\beta_3$, caspases and SFKs are required for externalisation and activity of FXIII-A.

PC12. Targeting human GPVI with novel monoclonal antibodies inhibits platelet function

Eva M. Soriano Jerez[1,2,4], S. Navarro[2,3,4], B. Nieswandt[2,4], D. Stegner[2,4], Jonathan M. Gibbins[1,4], C. E. Hughes[1,4]

[1] *Institute for Cardiovascular and Metabolic Research (ICMR), School of Biological Sciences, University of Reading, Reading, UK*
[2] *Institute of Experimental Biomedicine I, University Hospital and Rudolf Virchow Center, University of Würzburg, Würzburg, Germany*
[3] *Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, 6229 ER Maastricht, The Netherlands*
[4] *Targeting Platelet Adhesion Receptors in Thrombosis ITN*

E-mail: e.sorianojerez@pgr.reading.ac.uk

Background:
Glycoprotein receptor VI (GPVI) is the major collagen receptor. Ligand binding induces GPVI clustering, which initiates a tyrosine kinase-based signalling cascade via an immunoreceptor tyrosine-based activation motif. GPVI has been shown to play roles in both the initiation and growth of thrombi, although GPVI deletion is not associated with significant bleeding. Therefore, modulating GPVI pathway would be a prospect to overcome the bleeding risk associated with current therapies.

Aims:
Using novel monoclonal α-human GPVI antibodies (mAbs) and their Fab fragments, we aim to study their effect on platelet activation and determine their mode of action and to which epitopes or regions of the protein they bind this will be useful for rational drug discovery/development.

Methods:
The functional effect of mAbs were investigated using platelet aggregometry and flow cytometry assays. Structure-function relationships were studied using Bio-Layer Interferometry (BLI), in combination with recombinant chimeras.

Results:
Four mAbs were tested; one blocked GPVI-mediated aggregation and another reduced this by ~50%. Three of the Fabs inhibited fibrinogen binding and P-selectin exposure in response GPVI agonists while the response to PAR agonists was normal. All mAbs bound to monomeric rather than dimeric GPVI (KD ~1-5 nM). Recombinant chimeric GPVI has been cloned and cell-line studies are in progress.

Conclusions:
GPVI-mediated platelet activation was inhibited by multiple Fab fragments and one mAb suggesting these have potential as a novel α-GPVI therapy. BLI suggested that the mechanism of inhibition is not through prevention of clustering. How and to which epitopes they bind are questions we will approach with cell-line and crystallography studies.

PC13. Effects of platelet contractility outside of clot compaction

Martin Kenny[1], Sebastian Lickert[2], Viola Vogel[2], Ingmar Schoen[1]

[1]*Laboratory of MechanoVascular Biology, School of Pharmacy and Biomolecular Sciences and Irish Centre for Vascular Biology, RCSI, 123 St Stephen's Green, Dublin*

[2]*Laboratory of Applied Mechanobiology, Department of Health Sciences and Technology, ETH Zurich, Vladimir-Prelog-Weg 4, 8093 Zurich, Switzerland*

E-mail: martinkenny@rcsi.com

Background: Actin cytoskeleton reorganization upon platelet activation is a prerequisite for the development of platelet contractility, which drives thrombus compaction. Defective platelet contractility gives rise to bleeding diathesis and increases the likelihood of thrombus embolization. However, how platelet contractility affects platelet behaviour apart from clot compaction is poorly understood.

Aims: To determine the consequences of compromised contractility on platelet adhesion, cytoskeletal (re)organization, granule secretion and aggregation in solution.

Methods: Blood was obtained under RCSI Research Ethics Committee approval (REC1405). Myosin IIa activity was dose-dependently inhibited by 0-100 μM blebbistatin. Platelet responses were assessed by micropost array traction force microscopy, actin cytoskeletal morphology and super-resolution imaging of spread washed platelets on fibrinogen, flow cytometry of P-selectin expression on platelets activated with 5 μg/ml collagen-reactive peptide (CRP), and aggregation of washed platelets induced by 33 μM arachidonic acid, 10 μM thrombin receptor-activating peptide, or 5 μg/ml CRP.

Results: Platelet traction forces on fibrinogen were significantly reduced by doses as low as 1 μM blebbistatin. Actin filament bundling and vinculin recruitment to focal adhesions in spread platelets were decreased at low blebbistatin doses, whereas cytoskeletal rearrangements were inhibited only above 16 μM blebbistatin. LTA measurements of platelet aggregation were unaffected by blebbistatin. However, 40 μM blebbistatin prevented the reduction of platelet diameters induced by agonist stimulation within aggregates but also in suspension. Alpha granule secretion was unaffected by blebbistatin treatment.

Conclusions: Our results suggest that contractility affects multiple aspects of platelet biomechanics which go undetected by standard functional assays.

PC14. Vps34 localizes to the nucleolus and mediates early megakaryopoiesis

Ivana Bertović[1], Antonija Jurak Begonja[1]

[1]*University of Rijeka, Department of Biotechnology, Rijeka, Croatia*

E-mail: ibertovic@uniri.hr

Background: Phosphoinositides are membrane phospholipids implicated in membrane trafficking and cellular signaling. Phosphatidylinositol 3-monophosphate (PI3P), mainly produced by the Vps34 kinase, is largely confined to endosomal compartments where it regulates membrane trafficking, as well as autophagy and mTOR signaling. However, PI3P localization was also observed within the nucleolus (Gillooly et al., 2000 EMBO J), a nuclear subcompartment responsible for transcription and processing of ribosomal RNAs (rRNAs).

Aims: We aimed to investigate the localization of Vps34 and its potential role in the nucleolus in primary mouse megakaryocytes (MKs).

Methods and results: We demonstrated that Vps34 kinase localizes to the nucleus of immature MKs, as shown by immunofluorescence and cell fractionation experiments. Further analysis by confocal microscopy revealed that Vps34 is confined to the nucleolus, and co-localizes with upstream binding factor (UBF), a transcription factor required for rRNA expression. Nucleolar Vps34 localization was also confirmed in human mononuclear cells, as well as in the Balb3T3 cell line. Pull-down of GFP-UBF co-immunoprecipitated Vps34 from Balb3T3 cells. In MKs, inhibition of RNA polymerase I with actinomycin D, abolished Vps34 nucleolar localization, and Vps34 binding to UBF. Moreover, pharmacological inhibition of Vps34 from early stages reduced the size of MKs and expression of GPIb in a dose-dependent manner, indicating abnormal maturation.

Conclusions: Collectively, these data indicate that Vps34 might play an important, still undescribed, role in the nucleolar function, that could control the early stages of MK development *via* ribosome biogenesis. Additional studies are underway for a better understanding of these events.

PC15. Mechanisms of Zn^{2+}-induced platelet activation

Niaz Shahed Ahmed,[1] Kirk Taylor,[2] and Nicholas Pugh[1]

[1] School of Life Sciences, Anglia Ruskin University, Cambridge, UK
[2]Institute for Cardiovascular & Metabolic Research, School of Biological Sciences, University of Reading, Reading, UK
E-mail: niaz.ahmed@aru.ac.uk

Background: Exogenous zinc (Zn^{2+}) evokes platelet activation at high concentrations, whilst sub-activatory concentrations amplify platelet activation responses. We have shown that Zn^{2+} gains access to the platelet cytosol, but the channels responsible for Zn^{2+} influx are unknown. We performed experiments to determine the identity of channels responsible for Zn^{2+} into platelets, and to investigate the mechanisms by which elevated intracellular Zn^{2+} ($[Zn^{2+}]_i$) affects platelet activation.

Aims: To evaluate platelet Zn^{2+} entry pathways and interplay between $[Zn^{2+}]_i$ and $[Ca^{2+}]_i$ (intracellular Ca^{2+}).

Methods: Zn^{2+}-induced platelet activation was assessed using light transmission aggregometry, fluorometry, and flow cytometry. The molecular identity of Zn^{2+} entry pathways were evaluated using pharmacological tools.

Results: Pre-treatment of platelets with ion channel inhibitors revealed that Zn^{2+} influx was regulated by TRP channels and the sodium-calcium exchanger (NCX) operating in reverse mode. Depletion of the dense tubular system upon thapsigargin treatment also mediated Zn^{2+} entry in a manner that may be analogous to store-operated Ca^{2+} entry. A reduction in $[Ca^{2+}]_i$ elevation coincided with increased $[Zn^{2+}]_i$ elevation, demonstrating that $[Ca^{2+}]_i$ signalling is regulated by $[Zn^{2+}]_i$.

Conclusions: These data demonstrate a role for TRP channels and NCX during platelet Zn^{2+} entry. Finally, we report a regulatory role for Zn^{2+} in Ca^{2+} responses.

PC16. The fibrinogen αC-region is critical for the binding of platelet GPVI, which is mediated by avidity

Rui-Gang Xu[1§], Julia S. Gauer[1§], Stephen R. Baker[1,2], Alexandre Slater[3], Eleyna M. Martin[3], Helen R. McPherson[1], Cédric Duval[1], Iain W. Manfield[4], Arkadiusz M. Bonna[5], Steve Watson[3], Robert A. S. Ariëns[1]

[1] *Discovery and Translational Science Department, Institute of Cardiovascular and Metabolic Medicine, University of Leeds, Leeds, LS2 9LU, UK.*

[2] *Department of Physics, Wake Forest University, Winston Salem, NC 27103, USA.*

[3] *Institute of Cardiovascular Sciences, College of Medical and Dental Sciences, University of Birmingham, Birmingham, B15 2TT, UK.*

[4] *School of Molecular and Cellular Biology, Faculty of Biological Sciences, University of Leeds, Leeds, LS2 9LU, UK.*

[5] *Department of Biochemistry, University of Cambridge, CB2 1GA, UK.*

[§] *These authors contributed equally to this work.*

E-mail: r.xu1@leeds.ac.uk

Background: Recent findings suggest that GPVI contributes to platelet aggregation and thrombus growth through interactions with fibrin(ogen). However, the mechanisms of this interaction are poorly defined, including which fibrinogen region is responsible for GPVI binding. In addition, there are discrepancies in the literature whether monomeric or dimeric GPVI binds to fibrinogen at high affinity.

Aims: To gain further understanding of the molecular mechanisms of GPVI-fibrin(ogen) interaction and identify fibrinogen regions important for GPVI binding.

Methods: The interactions of wild-type and non-polymerising fibrinogen, X/D/E/αC-fragments and D-dimer to GPVI were characterised by Microscale Thermophoresis, Surface Plasmon Resonance and enzyme-linked immunosorbent assay. GPVI-fibrinogen interaction was visualised at the single molecule level by Atomic Force Microscopy.

Results: We observed that dimeric GPVI binds to fibrinogen with much higher affinity and shows a slower dissociation rate than its monomer form. Moreover, we found that the highest affinity interaction of GPVI dimer is with the αC-region of fibrinogen, while there is no binding to fibrinogen X-fragment (lacks αC). We further show that GPVI monomer and dimer interact with immobilised fibrinogen and fibrin variants at comparable levels, including a non-polymerising fibrin variant, suggesting that GPVI binding is independent of fibrin polymerisation.

Conclusions: The higher affinity and slower dissociation of dimeric over monomer GPVI for fibrinogen suggests that the binding is mediated by avidity. Moreover, the high affinity binding of GPVI dimer for the fibrinogen αC-fragment, and elimination of binding to fibrinogen X-fragment suggests that the fibrinogen αC-region is essential for GPVI binding.

PC17. Human platelets express the 75kDa pan-neurotrophic receptor that structurally differs from its brain counterpart

Samuel Fleury,[1,2] Imane Boukhatem,[1,2] Jessica Le Blanc,[1,2] Mélanie Welman,[1] Marie Lordkipanidzé[1,2]

[1] Research Center, Montreal Heart Institute, Montreal, QC, Canada
[2] Faculty of Pharmacy, Université de Montréal, Montreal, QC, Canada

E-mail: samuel.fleury@umontreal.ca

Background: The 75kDa pan-neurotrophic receptor ($p75^{NTR}$) is a receptor involved in neuronal survival and apoptosis that can bind all neurotrophins. The brain-derived neurotrophic factor (BDNF) is one such protein. BDNF is involved in learning and memory in the brain, but is found in greater quantities in blood, where it is predominantly stored within platelets. This led us to hypothesize that platelets would express the $p75^{NTR}$ receptor that would bear similarities to its brain counterpart.

Aims: Characterize the structure and distribution of the $p75^{NTR}$ in healthy human platelets and compare it to the $p75^{NTR}$ found in brain.

Methods: Washed platelets were obtained from whole blood of healthy donors. The presence of the $p75^{NTR}$ receptor was assessed by immunoblotting. Its location was investigated by flow cytometry and confocal microscopy. Glycosylation profile was assessed by enzymatic deglycosylation followed by band shift analysis on western blot.

Results: The $p75^{NTR}$ protein was identified in healthy human platelets by western blotting. The receptor is found at the platelet membrane, suggesting that it could be involved in platelet interaction with neurotrophins, as well as inside the platelet intracellular compartment. The glycosylation profile of this receptor in platelets differs from its brain counterpart. Whether these structural discrepancies reflect a functional difference remains to be investigated.

Conclusions: The $p75^{NTR}$ receptor is found at the membrane and within healthy human platelets, and the receptor bears an alternative glycosylation profile to the receptor found in brain. Functional assays are warranted to determine the role of this receptor in platelet biology.

PC18. Coagulation and anticoagulation factors affect agonist-induced platelet activation independently of thrombin and fibrin.

Ilaria De Simone [1,2], Jonathan M. Gibbins[2], Hugo ten Cate [1,3] , Johan W.M. Heemskerk[1] , Chris I. Jones[2] and Paola E.J. van der Meijden[1,3]

[1] *Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University,*

[2] *Institute for Cardiovascular and Metabolic Research, School of Biological Sciences, University of Reading, Reading, UK.*

[3] *Thrombosis Expertise Center, Heart and Vascular Center, Maastricht University Medical Center, Maastricht, The Netherlands*

E-mail: i.desimone@maastrichtuniversity.nl

Background. The contribution of coagulation processes beyond thrombin and fibrin to platelet functions has not been considered. Coagulation-related factors known to bind platelets include factor (F)XIIIa and activated protein C (APC), but their roles in platelet activation remain to be established.

Aims. To investigate thrombin-independent effects of coagulated plasma on platelet responses.

Methods. Modulating effects of hirudin-treated, coagulating plasma on platelets were evaluated via well plate-based aggregation and flow cytometry (PAC-1 binding). Fura-2 loaded platelets were activated with CRP-XL concentrations that induced submaximal platelet activation, in combination with FXa, FXIIIa or APC, and changes in cytosolic $[Ca^{2+}]_i$ were assessed. Spreading assays were performed on surfaces coated with fibrinogen, FXIIIa and/or APC and were assessed by fluorescence microscopy.

Results. Platelets exposed to hirudin-treated coagulating (fibrin-depleted) plasma showed increased aggregation and surface activation markers, compared to resting plasma. Purified FXa, FXIIIa and APC each enhanced CRP-XL-induced rises in platelet cytosolic $[Ca^{2+}]_i$. Markedly, the potentiating effect of FXa was completely abolished by addition of different thrombin inhibitors. While FXIIIa and APC alone did not induce platelet activation in solution, immobilisation of FXIIIa or APC caused spreading. Furthermore, platelet spreading was enhanced by co-coating FXIIIa or APC together with fibrinogen, while pretreatment with soluble FXIIIa or APC left spreading on fibrinogen unaltered. Interestingly, PAR-1 inhibition diminished spreading on APC, but not on FXIIIa.

Conclusions. Coagulating plasma promotes agonist-induced platelet activation. Measurements of calcium rises and spreading point to a role of FXIIIa and APC, but not of FXa herein, partly through thrombin-independent PAR-1 activation.

PC19. MAS9 – a novel small molecule inhibitor of the CLEC-2-Podoplanin interaction

Marcin A. Sowa[1,2], Jan van Groningen[3], Ying Di[4], Jonathan M. Gibbins[1], Ángel García[2] & Alice Y. Pollitt[1]

[1]*Institute for Cardiovascular and Metabolic Research (ICMR), School of Biological Sciences, University of Reading, Reading, UK*
[2]*Platelet Proteomics Group, Center for Research in Molecular Medicine and Chronic Diseases (CIMUS), Universidad de Santiago de Compostela, and Instituto de Investigación Sanitaria (IDIS), Santiago de Compostela, Spain*
[3]*Pivot Park Screening Centre, Oss, The Netherlands*
[4]*Institute of Cardiovascular Sciences, College of Medical and Dental Sciences, University of Birmingham, Edgbaston, Birmingham, UK*

E-mail: m.a.sowa@pgr.reading.ac.uk

Background: The C-type lectin-like receptor-2 (CLEC-2) is a platelet receptor for the endogenous ligand Podoplanin. This interaction contributes to a number of pathophysiological roles, such as lymphangiogenesis, preservation of blood and lymphatic vessel integrity, organ development and tumour metastasis. Activation of CLEC-2 leads to the phosphorylation of its cytoplasmic hemITAM domain and initiates a signalling cascade involving Syk and PLCγ2 .

Aims: The aim of this study was to identify a small-molecule inhibitor of the CLEC-2-Podoplanin interaction and to characterise their effect on human platelet activation.

Methods: AlphaScreen-based high-throughput screening identified a small-molecule inhibitor of the CLEC-2-Podopolanin interaction. Light transmission aggregometry, platelet spreading and phosphorylation assays were used to evaluate the effect of the small molecule on CLEC-2 mediated platelet activation.

Results: 18,476 small molecules were screened resulting in 14 candidates. Following the secondary screening, one small molecule (MAS9) was taken forward for further characterisation. 20 μM of MAS9 inhibited platelet aggregation in response to the CLEC-2 agonist Rhodocytin. MAS9 dose-dependently inhibited platelet spreading and adhesion on immobilized Podoplanin and Rhodocytin. 30 μM MAS9 inhibited the phosphorylation of Syk, PLCγ2 and Src in platelets activated by Rhodocytin. Partial inhibition of GPVI mediated aggregation and spreading was observed but MAS9 did not impact GPVI mediated phosphorylation.

Conclusions: MAS9 potently inhibits CLEC-2-mediated aggregation, platelet spreading and phosphorylation, showing selectivity on CLEC-2 inhibition over GPVI. Further pharmacological and functional experiments will be carried out to establish the potential of MAS9 as a lead compound to identify a novel anti-platelet drug with therapeutic effects in thrombosis and cancer.

PC20. Defining the molecular features of inverse agonism: insights from the $P2Y_{12}$ receptor and the antiplatelet drug ticagrelor.

Sukhvinder Kaur Bancroft[1], Jawad Khalil[1], Stuart Mundell[1]

[1]*School of Physiology, pharmacology and Neuroscience, Faculty of Life Sciences, University of Bristol.*

E-mail: sb14282@bristol.ac.uk

Background: Although many G-protein-coupled receptors (GPCRs) show varying degrees of constitutive activity, a detailed molecular understanding of this phenomena is lacking. Recent studies have revealed that the platelet expressed $P2Y_{12}$ receptor ($P2Y_{12}R$) displays a high degree of constitutive activity and that ticagrelor, a clinical antiplatelet drug, is an inverse agonist at this receptor (Aungraheeta et al., 2016).

Aims: Use of molecular dynamic simulations (MDs) alongside bioluminescence resonance energy transfer (BRET) assays to further our understanding of the molecular determinants underlying GPCR constitutive activity.

Methods: 1μs MDs of several P2Y12-ligand receptor complexes, employing the ff14SB forcefield. Residues believed to be important for regulating activity were mutated and transiently transfected into HEK293 cells where receptor/G protein coupling was assessed by BRET.

Results: MDs revealed that ticagrelor binds to a region of the receptor similar to that of AZD1283 and 2MeSADP, but not ADP. Ticagrelor interacts with transmembrane domains (TM) 3 and 5-7. ADP sits in an alternative region contacting TM1-3, TM5 and TM7. Principal component analysis reveals that ticagrelor induces movements in TM5 resulting in a shift at the intracellular end, towards TM3. Experimental mutation of C194 to an alanine produced a 64% decrease in ticagrelor inverse agonism.

Conclusions: The orthosteric cavity of the $P2Y_{12}R$ can be divided into two pockets with 2MeS-ADP, AZD1283 and ticagrelor binding in a distinct pocket to ADP. Ticagrelor induces a distinct conformation in TM5 bringing it into closer proximity with TM3. This likely occludes G-protein binding and in part defines the ability of ticagrelor to act as an inverse agonist.

Aungraheeta, R., Conibear, A., Butler, M., Kelly, E., Nylander, S., Mumford, A. and Mundell, S. (2016). Inverse agonism at the P2Y12 receptor and ENT1 transporter blockade contribute to platelet inhibition by ticagrelor. *Blood*, 128(23), pp.2717-2728.

PC21. Highly selective Btk inhibition has less potent effect on GPVI-mediated platelet function than ibrutinib

Christopher W Smith, Lourdes Garcia-Quintanilla, Maan H Harbi, Helena Brown, Kieran Rookes, Mark R Thomas, Steve P Watson and Phillip LR Nicolson

Institute of Cardiovascular Sciences, University of Birmingham, Birmingham, UK

E-mail: c.smith14@bham.ac.uk

Background: First-generation Btk inhibitor ibrutinib is associated with increased bleeding in patients, and correlates with platelet GPVI inhibition. Btk deficient patients however do not bleed excessively. Moreover, we have previously shown ibrutinib blocks GPVI-mediated platelet function due to off-target effects.

Aims: To compare the anti-platelet effects of ibrutinib with a novel, potent and highly selective Btk inhibitor and determine the effect of selective Btk inhibition on GPVI function.

Methods: GPVI and GPCR-mediated platelet function and signalling were analysed in healthy human donor platelets by lumi-aggregometry, flow cytometry, adhesion under flow and Western-blot following *ex vivo* inhibitor treatment. Platelet activation and $FeCl_3$-induced thrombosis were also assessed in *in vivo*-treated mice.

Results: Less potent inhibition of collagen-induced platelet aggregation and secretion to the novel inhibitor than ibrutinib was observed in washed platelets, with degree of inhibition correlating with phosphorylation blockade of Src but not Btk. No effect on PAR1 or TP receptor-mediated platelet function was found to either inhibitor. In whole blood, high concentrations of both inhibitors were required to inhibit GPVI, with ibrutinib showing more marked inhibition of platelet activation (P-selectin and activated integrin αIIbβ3 expression) and aggregate formation over collagen at arterial shear ($1000s^{-1}$). *In vivo* oral dosing of mice resulted in high Btk occupancy and marked reduction of GPVI-mediated platelet activation, but no effect on $FeCl_3$-induced thrombosis was observed with either inhibitor.

Conclusions: The novel and highly selective Btk inhibitor shows less marked inhibition of GPVI-mediated platelet function than ibrutinib despite their similar potency, likely due to fewer off-target effects.

PC22. Annexin V binding detects platelet procoagulant commitment but is not sensitive to reductions in the level of platelet PS exposure

Sarah L Millington-Burgess, Matthew T Harper

Department of Pharmacology, University of Cambridge

E-mail: slb95@cam.ac.uk

Background: Procoagulant platelets expose phosphatidylserine (PS) on their surface, where it supports thrombin generation, through activation of a scramblase protein (TMEM16F). Inhibiting platelet PS exposure could be a novel anti-thrombotic approach, though currently there are no known selective inhibitors of platelet PS exposure. Platelet PS exposure is commonly quantified by the % of platelets that bind annexin V (AV %+ve). This detection and anlaysis method, though convenient, may not be the most sensitive assay for screening novel inhibitors of platelet PS exposure.

Aims: Characterise the sensitivities of different PS exposure assays.

Methods: Washed human platelets were incubated with R5421 or DMSO. Scramblase and flippase activity were measured by flow cytometry using NBD-PS. PS expsoure following stimulation with 10μM A23187 was measured using multiple assays: a plate-based luminesence AV-binding assay, end-point and real-time flow cytometry assays using AV-FITC, lactadherin-FITC, or FRET pair AV-eGFP/AV-Alexa594. Liposomes containing different %PS were detected using each assay.

Results: Liposomes containing different %PS demonstrated that end-point AV binding by flow cytometry was the least sensitive measure of membrane PS composition. Decreased PS exposure following treatment with R5421 was not detectable using single colour AV %+ve analysis but could be detected using NBD-PS-, lactadherin-, AV FRET- and luminesence-based assays. Alternative analysis of single colour AV binding could convey the inhibition of PS exposure by R5421.

Conclusions: Anlaysis of platelet PS exposure by AV %+ve measures the commitment of platelets to becoming procoagulant but is not sensitive to differences in the extent of PS exposure.

PC23. Development of new ligands of the platelet CLEC-2 receptor

Luis A. Moran,[1,2,3] Eleyna Martin,[1] Ying Di,[1] Lourdes Garcia, [1] Marcin Sowa, [2,3] Lidia Hermidia-Nogueira, [2,3] Maria N Barrachina, [2,3] David Moreira,[2] Eduardo Dominguez,[2] Mabel Loza,[2] Angel Garcia, [2,3] Steve P. Watson,[1,4]

[1] Institute of Cardiovascular Sciences College of Medical and Dental Sciences University of Birmingham, Birmingham UK.
[2] Center for Research in Molecular Medicine and Chronic Diseases (CIMUS), Universidade Santiago de Compostela, Spain.
[3] Instituto de Investigación Sanitaria de Santiago (IDIS), Universidade Santiago de Compostela, Spain.
[4] Centre of Membrane Proteins and Receptors (COMPARE) Universities of Birmingham and Nottingham The Midlands UK

E-mail: LAM831@student.bham.ac.uk

Background: CLEC-2 is a novel target in thrombo-inflammation diseases such as deep vein thrombosis and infection. The minimal role of CLEC-2 in haemostasis indicates that blocking agents may have an advantage over current antiplatelets.

Aims: To develop new ligands for CLEC-2 based on small molecules and nanobodies against CLEC-2

Methods: We have screened a small molecule chemical library on an ALPHA screen platform assay based on the podoplanin and CLEC-2 interaction to identify small molecules ligands. We have screened high-affinity nanobodies raised against CLEC-2 with a potential role blocking CLEC-2 activation. The most potent nanobody has been optimised through multimerisation.

Results: We have identified a small molecule, KLM-1 which binds to CLEC-2 and activates platelets through a Src and Syk kinase pathway. We have identified a nanobody Nb 4, that blocks activation of platelets by podoplanin-expressing cells. SPR has shown that the Nb 4 binds to CLEC-2 with an affinity of ~100nM. Dimerisation increases the affinity to 1nM and generates a potent-blocking agent.

Conclusions: KLM-1 and dimer Nb-4 are new tools to probe CLEC-2 function.

PC24. C3G modulates platelet adhesion and spreading

Cristina Fernández-Infante[1], Luis Hernández-Cano[1], Óscar Herranz[1], Almudena Porras[2] and Carmen Guerrero[1,3]

[1]Instituto de Biología Molecular y Celular del Cáncer (IMBCC), University of Salamanca-CSIC, Salamanca, Spain; Instituto de Investigación Biomédica de Salamanca (IBSAL), Salamanca, Spain.
[2]Departamento de Bioquímica y Biología Molecular, Facultad de Farmacia, Complutense University of Madrid. Instituto de Investigación Sanitaria del Hospital Clínico San Carlos (IdISSC), Madrid, Spain.
[3]Departamento de Medicina, University of Salamanca, Salamanca, Spain.

E-mail: crisferin@usal.es

Background: C3G is one of the main activators of Rap1 GTPases, being the Rap1b isoform an essential player in most platelet functions. C3G mediates platelet activation and aggregation, [1] but also platelet-mediated pathological angiogenesis and metastasis [2]. Moreover, C3G has a role in megakaryopoiesis and thrombopoiesis [3].

Aims: Since C3G regulates cytoskeleton remodeling and platelet secretome [2], we wanted to explore its contribution to platelet adhesion and spreading.

Methods: We used transgenic and knockout mouse models for C3G expression to analyze platelet adhesion and spreading. We used different substrates such as fibrinogen, collagen, fibronectin, laminin, vitronectin and osteopontin. Platelets were treated with thrombin and PMA, in combination with inhibitors, such as cytochalasin D, latrunculin A, bisindolylmaleimide, 1A-166 and PP2. We have also analysed granule secretion by flow cytometry and the interaction of C3G with proteins of the secretory machinery by immunoprecipitation and Western blot and by immunofluorescence.

Results: C3G transgenic and knockout platelets showed alterations in adhesion and spreading on collagen type I, fibronectin and vitronectin, but not on fibrinogen and laminin. Besides, C3G modulates α-granule, but not δ-granule, secretion through its interaction with VAMP7, SNAP23 and syntaxin-11.

Conclusions: C3G modulates platelet adhesion and spreading through its interaction with proteins of the secretory machinery. Further studies will aim at characterizing the precise participation of C3G in the mechanisms that regulate the differential secretion of the content of platelet α-granules.

[1] Signal Transduct Target Ther 5, 29, 2020
[2] Oncotarget 8, 110994-111011, 2017
[3] Cell Commun Signal 16, 101, 2018

PC25. The Brain Derived Neurotrophic Factor induces platelet aggregation

Imane Boukhatem[1,2] ,Samuel Fleury[1,2],Mélanie Welman[1],Jessica Le Blanc[1,2],Bruce Allen[1,3],Marie Lordkipanidzé[1,2]

[1] Montreal Heart Institute, Research Centre, Montreal, Canada
[2] University of Montreal, Faculty of pharmacy Montreal, Canada
[3] University of Montreal, Faculty of medicine Montreal, Canada

E-mail: imane.boukhatem@umontreal.ca

Background: The Brain-Derived Neurotrophic Factor (BDNF) is found in platelets in concentrations reaching up to a 1000-fold those of neurons. We discovered that BDNF induces complete and irreversible platelet aggregation in washed platelets through an unknown mechanism.

Aims: We aimed to investigate BDNF signaling in human platelets

Methods: TrkB presence on platelets was assessed by immunoblotting, flow cytometry and confocal microscopy. BDNF signaling pathways were investigated through light transmission aggregometry and phospho-blotting.

Results: Immunoblotting showed a 95kDa-band, consistent with a truncated form of TrkB lacking its tyrosine kinase (human cortex cell lysate and TrkB-Fc used as positive controls). Approx. 20% of platelet TrkB is expressed on the surface while 80% appears intracellular. Consistent with the absence of the tyrosine kinase domain on the truncated TrkB receptor, 1µM GNF-5837 (tyrosine kinase inhibitor) and up to 50 µM Cyclotraxin B (tyrosine kinase domain allosteric modulator) had no effect on BDNF-induced platelet aggregation. However, BDNF-induced aggregation was abrogated with a higher concentration of GNF-5837 (30µM) which inhibits most cellular kinases. 10µM of RhoGTPase Rac-1 inhibitor (NSC23766) and 10µM of PKC inhibitor (BIM-1) showed consistent inhibition of BDNF-induced aggregation. So did 100nM wortmannin (PI3K inhibitor) suggesting that downstream signalling engages the PKC-PI3K/Akt pathway.

Conclusions: We demonstrate that BDNF activates platelets through a truncated TrkB receptor and engages the PKC/PI3K pathways, through a Rac-1-dependent mechanism.

PC26. Role of C3G in the regulation of platelet levels

Luis Hernández-Cano[1], Óscar Herranz[1], Cristina Fernández-Infante[1], Almudena Porras[2] and Carmen Guerrero[1]

[1]Instituto de Biología y Celular del Cáncer (IBMCC), University of Salamanca-CSIC, Salamanca, Spain; Instituto de Investigación Biomédica de Salamanca (IBSAL), Salamanca, Spain.
[2]Departamento de Bioquímica y Biología Molecular, Facultad de Farmacia, Complutense University of Madrid. Instituto de Investigación Sanitaria del Hospital Clínico San Carlos (IdISSC), Madrid, Spain.
[3]Departamento de Medicina, University of Salamanca, Salamanca, Spain.

E-mail: luishn220@gmail.com

Background: C3G is a guanine nucleotide exchange factor for Rap1 that regulates platelet functions, such as activation, aggregation and release of a-granules content [1,2]. Overexpression of C3G in megakaryocytes increases its maturation and differentiation in response to trombopoyetin (TPO), but is not reflected in an increase in platelet production [3].

Aims: To Characterize the role of C3G in megakaryocyte maturation and platelet production in physiologycal and pathological conditions.

Methods: We have used C3G knockout mice, in which C3G is specifically eliminated in megakaryocytes. Bone marrow was cultured in the presence of TPO + citokines (SCF, IL-3, IL-6 and IL-11) to determine the production of mature megakaryocytes by flow cytometry. In addition, we conducted megakaryopoiesis regeneration studies by treatment with 5-Fluorouracil (5-Fu). We have also analyzed the role of C3G in the activity of Cbl, an ubiquitin ligase involved in the regulation of platelet levels by the TPO/Mpl pathway.

Results: C3G ablation results in normal platelet counts in blood, and in normal levels of bone marrow megakaryocytes in a physiological context. However, C3G-KO mice showed a delayed platelet regeneration after 5-FU-induced myelosupression and an impaired downregulation after the recovery. In addition, phospho-Cbl levels are decreased in C3G-KO platelets.

Conclusions: C3G contributes to platelet rebound after 5-FU-induced myelosupression but prevents the subsequent platelet downregulation, probably through the modulation of Cbl activity. This suggests a role of C3G in the TPO/Mpl pathway.

[1] Signal Transduct Target Ther 5, 29, 2020
[2] Oncotarget 8, 110994-111011, 2017
[3] Cell Commun Signal 16, 101, 2018

PC27. Finding the "switch" in platelet activation. Prediction of key mediators involved in platelet hyperreactivity using a network biology approach.

Lemmens TP[1], Coenen DM[1], Niessen ICL[1], Swieringa F[1], Coort SLM[2], Koenen RR[1], Kutmon M[2,3], Cosemans JMEM[1].

[1]*Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, Maastricht, the Netherlands*

[2]*Department of Bioinformatics -BiGCaT, School of Nutrition and Translational Research in Metabolism (NUTRIM), Maastricht University, Maastricht, the Netherlands*

[3]*Maastricht Centre for Systems Biology (MaCSBio), Maastricht University, Maastricht, the Netherlands*

E-mail: Tp.lemmens@maastrichtuniversity.nl

Background: The healthy endothelium controls platelet activity through release of prostaglandin I2 (PGI2) and nitric oxide. The loss of this natural brake on platelet activity can cause platelets to become hyperreactive. PGI2 attenuates adenosine diphosphate (ADP) platelet activation through stimulating of cyclic adenosine monophosphate (cAMP) production and subsequent phosphorylation changes by protein kinase A (PKA). We hypothesize that downstream the cAMP-PKA pathway are proteins or processes that are involved in platelet hyperactivity and that some of these can serve as a "switch" in platelet activation and inhibition.

Aims & methods: We aimed to design a network biology approach based on existing phosphoproteomics datasets. To explore the entangled platelet signalling pathways downstream of PGI2 and ADP we incorporated pathway information, a quantitative platelet proteome dataset, relative RNA expression of hematopoietic cells, the likelihood of the proteins being phosphorylated by PKA, and the DrugBank database in our network.

Results and conclusions: We distilled 30 proteins from existing phosphoproteomics datasets (PXD000242, PXD001189) that can be "turned on" after ADP-mediated platelet activation and subsequently switched "off" after platelet inhibition with iloprost. A protein-protein interaction network with 20 additional interactors was created to also include indirectly related proteins. Gene ontology enrichment analysis revealed biological processes related to vesicle secretion and cytoskeletal reorganization to be overrepresented coinciding with topological clusters in the network. Next to expected proteins like phosphodiesterase 3A, our method highlighted several novel proteins related to vesicle transport, platelet shape change and small GTPases as potential switch proteins in platelet activation and inhibition.

PC28. Galectin-9 Activates Platelet Collagen Receptor GPVI

Zhaogong Zhi[1], Natalie J. Jooss[1], Yi Sun[1,2], Julie Rayes[1], Natalie S. Poulter[1], Alexandre Slater[1], Steve P. Watson[1,2] and Asif J. Iqbal[1]

[1] Institute of Cardiovascular Sciences (ICVS), College of Medical and Dental Sciences, University of Birmingham, Birmingham, B15 2TT, United Kingdom
[2] Centre of Membrane Proteins and Receptors (COMPARE), Universities of Birmingham and Nottingham, Midlands, United Kingdom

E-mail: ZXZ925@student.bham.ac.uk

Background: Platelets, are multifunctional cellular mediators in many pathological processes including thrombosis, angiogenesis, and inflammation. Several members of the galectins, a family of carbohydrate binding proteins with a broad range of immunomodulatory actions, have been reported to activate platelets.

Aims: The aim of this study was to characterise the role of Galectin-9 (Gal-9) in platelet activation and to identify novel receptors involved in this interaction.

Methods: AVidity-based EXtracellular Interaction Screen (AVEXIS) and competitive ELISA-based displacement assay were used to identify potential Gal-9 receptors on platelets. Wild-Type (n=5), GPVI-knockout ($GP6^{-/-}$)(n=5) and GPVI and CLEC-2-double knockout mice (n=5) were also included. Platelet spreading, aggregation and P-selectin secretion were all measured in washed platelet suspensions via static adhesion assay, light transmission aggregometry and flow cytometry, respectively.

Results: We have shown that Gal-9 is capable of inducing aggregation dose-dependently in both human and murine washed platelets. Murine WT platelets adhere and spread on immobilised recombinant Gal-9, and externalize P-selectin to the surface following the addition of soluble Gal-9 *in vitro*. AVEXIS identified GPVI as a binding partner for Gal-9. Displacement assay showed that Gal-9 competitively inhibited the binding of GPVI to collagen (IC_{50}=220 nM). Compared to Wild-Type, GPVI-deficient platelets exhibited significantly impaired aggregation in response to Gal-9 (P=0.002). This inhibition was further augmented in GPVI and CLEC-2-double deficient platelets (vs. WT, P<0.0001; vs. $GP6^{-/-}$, P=0.0099).

Conclusions: The data generated from this study demonstrates that Gal-9 is a novel platelet agonist which induces activation via interacting with receptors GPVI and CLEC-2.

PC29. Platelet inhibitors PAPA-NONOate and PGI_2 have differential effects on platelet sensitivity and response capacity to agonists

Neline Kriek,[1] Tanya Sage,[1] Joanne Dunster,[1] Jonathan Gibbins[1]

[1]*Institute for Cardiovascular and Metabolic Research, School of Biological Sciences, University of Reading, Reading, UK, RG6 6EX*

E-mail: n.kriek@reading.ac.uk

Background: Platelet function underpins haemostasis and thrombosis and differential responsiveness of individuals' platelets may impact drug efficacy. We have previously found that, in a healthy population, platelet responses varied widely in both sensitivity (EC_{50}) and capacity (maximal response) and that these measures were independent.

Aims: To study in detail the variation in the effect of platelet inhibitors PAPA-NONOate and PGI_2 on platelet function in a cohort of 36 healthy individuals.

Methods: We have used our high-throughput flow-cytometry assay to measure Fibrinogen binding and P-selectin exposure in response to ADP, CRP and TRAP-6 in the presence of 100µM PAPA-NONOate or 100pg/ml PGI_2 and determined the effect of these inhibitors on sensitivity to agonists and capacity to respond.

Results: We found that PAPA-NONOate and PGI_2 both reduced the sensitivity to ADP, CRP and TRAP-6 with a greater change in platelets that displayed greater sensitivity to the agonist and that this was similar for Fibrinogen binding and P-selectin exposure. The inhibition by PGI_2 of the capacity to respond was similar for all three agonists and for both fibrinogen and P-selectin exposure. However, the effect of PAPA-NONOate was more complex, as it reduced the capacity to ADP (38% of vehicle) more than to CRP and TRAP-6. For CRP and TRAP-6, the capacity for fibrinogen binding (70% and 47% of vehicle respectively) was reduced more than the capacity to expose P-selectin (79% and 75% respectively).

Conclusions: PAPA-NONOate and PGI_2 reduce platelet responses to ADP, CRP and TRAP-6 with differential effects on sensitivity and response capacity.

PC30. SARS-CoV2 spike protein can activate platelets through integrin $a_{IIb}b_3$.

Chris M. Williams[1], Jawad S Khalil[1], Tony G. Walsh[1], Kapil Gupta[2], Elizabeth W. Aitken[1], Stuart J Mundell[1], Imre Berger[2], Alastair W. Poole[1] and Ingeborg Hers[1]

[1]School of Physiology, Pharmacology and Neuroscience and
[2]School of Biochemistry: Biomedical Sciences Building, University Walk, BS8 1TD, Bristol, United Kingdom.

E-mail: chris.m.williams@bris.ac.uk

Introduction: Patients with severe coronavirus disease 19 (COVID-19) are at increased risk of thrombosis, which can be a challenge to manage and is associated with elevated mortality. The virus responsible for COVID19, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV2), is unique amongst coronaviruses that infect humans in that its envelope spike protein includes an arginine-glutamate-aspartate (RGD) peptide sequence in its receptor-binding domain (RBD). In platelets, RGD sequences are recognised by the main platelet activation and adhesion integrin, $\alpha_{IIb}\beta_3$, triggering integrin activation, outside-in signalling, and platelet activation.

Aim: To investigate whether the RGD sequence in the spike protein is able to activate platelets through integrin $a_{IIb}b_3$.

Methods: Fibrinogen, collagen, purified spike and RBD protein were coated on glass slides and platelets were allowed to adhere under static or flow conditions. Slides were washed, stained platelets with ActinGreen and imaged by confocal microscopy.

Results: Purified SARS-CoV2 spike protein and RBD protein triggered platelet spreading and this was blocked by incubating the platelets with the clinically used non-peptide RGD mimetic $\alpha_{IIb}\beta_3$-integrin blocker, tirofiban. In an *in vitro* thrombosis model using healthy donor blood, we find surprisingly that this ability to activate platelet integrins does not translate into an enhancement in thrombus formation on collagen, and platelets cannot form thrombi on the spike protein under arterial or venous conditions.

Conclusions: We conclude therefore that although SARS-CoV2 spike contains an RGD sequence that can activate platelet integrins, whether this contributes to enhanced thrombosis under pathological conditions in Covid-19 patients is not known. Structural analysis of the RGD site suggests a buried location in the spike, which may be revealed by other activatory mechanisms and receptors, and which will require further study.

PC31. Exploiting the role of GPVI and Syk inhibition in thrombus stability

Gina Perrella,[1,2] Helena Brown,[1,3] *David Stegner,[3] Mark Thomas,[1] Johan W.M. Heemskerk,[2] Steve P. Watson[1,4]*

[1]Institute of Cardiovascular Sciences, College of Medical and Dental Sciences, University of Birmingham (UK)
[2]Dept. of Biochemistry, CARIM, Maastricht University, Maastricht (NL)
[3]Institute of Experimental Biomedicine I, University Hospital, University of Würzburg, Würzburg (DE)
[4]COMPARE, The Universities of Birmingham and Nottingham, The Midlands (UK)

E-mail: GXP760@student.bham.ac.uk

Background: GP (glycoprotein) VI is a platelet immunoglobulin receptor with a key function in arterial thrombosis. In the exposed subendothelium, GPVI binds to collagen leading to Syk phosphorylation. Blockage of GPVI blocks thrombus occlusion leading to the finding of GPVI as receptor for fibrin(ogen). GPVI-deficiency, described only in a few individuals worldwilde, does not cause severe bleeding raising interest for targeting GPVI pathway in arterial thrombosis.

Aims: To investigate the role of GPVI and Syk inhibition in thrombus stability.

Methods: Anticoagulated human and mouse whole blood was perfused on collagen-coated surfaces using the Maastricht microfluidics device at 1000 s^{-1}. Thrombus stability was challenged by post-perfusing the aggregates with nb against GPVI and the Syk inhibitor PRT-060318. Protein phosphorylation was observed in collagen- and thrombin-stimulated human platelets, alongside with platelet aggregation.

Results: Under flow, blockage of GPVI and Syk promotes de-aggregation of preformed aggregates in human blood to the same extent, with no effect on platelet adhesion. A similar results in observed for Syk inhibition in mouse platelets. Syk phosphorylation is maintained for up to 50 min in thrombin-aggregated platelets in the absence of flow and lost on addition of a Syk inhibitor, although aggregation is maintained. Blocking of fibrinogen binding to integrin αIIbβ3 inhibits protein posphorylation of Syk in thrombin-stimulated platelets.

Conclusions: Syk regulate thrombus stability under flow. Upon platelet activation, Syk is continously phosphorylated and the process is mediated through GPVI in human. Fibrinogen interaction with integrin αIIbβ3 supports GPVI activation where collagen is no longer available. This findings suggest that greater understanding of GPVI pathway in the thrombus may reveal new ways to prevent thrombus growth and the risk of embolisation.

PC32. Endothelial COX-2 protects against thrombosis through endothelial function alteration and independently of local prostacyclin

Maria E Lopes-Pires[1]; Fisnik Shala[1]; Lulu Wang[1]; Hime H Gashaw[1]; Jane A Mitchell[1]; Nicholas S Kirkby[1]

[1] *National Heart Lung Institute, Imperial College of London*

E-mail: m.lopes-pires@imperial.ac.uk

Background: Non-steroidal anti-inflammatory drugs (NSAIDs) like celecoxib and ibuprofen work by blocking cyclo-oxygenase-2 (COX-2). Their use is associated with ~30% increase the risk of a thrombotic event. Although COX-2 is not expressed in most arteries, constitutive COX-2 is present at other discrete tissue locations.

Aims: In this study we used novel endothelium-specific COX-2 knockout (EC-COX-2 KO) mice to understand if EC-COX-2 protects against thrombosis and by what mechanisms this occurs.

Methods: EC-COX-2 KO mice were generated by a Cre-loxP approach. Thrombosis *in vivo* was measured using a $FeCl_3$ carotid artery injury model. Endothelial function was analysed by myograph. Prostacyclin release was measured by ELISA and COX-2 expression by RT-qPCR. All procedures were carried out under PPL1576048.

Results: Thrombotic occlusion time after $FeCl_3$ carotid artery injury was reduced by ~70% in EC-COX-2 KO animals. This was not associated with any change in endothelium-dependent relaxation or prostacyclin release from the carotid artery *ex vivo*. In wild-type mice, COX-2 mRNA was detectable in the colon, lung, thymus, kidney and brain but not aorta. Total COX-2 mRNA levels were reduced by EC-COX-2 deletion in the colon but not other studied tissues.

Conclusions: Taken together, our data confirm that EC-COX-2 provides anti-thrombotic protection independent of local prostacyclin release. Instead, these data suggest that EC-COX-2 expressed at a distant site, possibly including the colon, regulates systemic thrombotic tone. Considering that the gut is a well-established target tissue for NSAID-associated damage, future studies should explore the links between gut EC-COX-2 and NSAID cardiovascular side effects.

PC33. Modelling the importance of receptor number in clustering of single transmembrane receptors transmembrane receptors

Zahra Maqsood[1,2,3], Yam Fung Hilaire Cheung[1,4,5], Sean ET Watson[1], Jeremy A Pike[1,6], Bodo M. H. Lange[2], Dylan Owen[6,7], David Stegner[3], Lloyd Bridge[8], Christoph Wierling[2] and Steve P. Watson[1,5,6]

[1]Institute of Cardiovascular Sciences, IBR Building, College of Medical and Dental Sciences, University of Birmingham, Birmingham B15 2TT, UK.

[2]Alacris Theranostics GmbH, Max-Planck-Straße 3, 12489 Berlin, Germany.

[3]Rudolf Virchow Center for Experimental Biomedicine and Institute of Experimental Biomedicine, University of Würzburg and University Hospital of Würzburg, Würzburg, Germany.

[4]Leibniz-Institut fur Analytische Wissenschaften-ISASe. V., Dortmund, Germany.

[5]Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, Maastricht, The Netherlands.

[6]Centre of Membrane Proteins and Receptors (COMPARE), Universities of Birmingham and Nottingham, The Midlands, UK.

[7]Institute of Immunology and Immunotherapy, IBR Building, College of Medical and Dental Sciences, University of Birmingham, Birmingham B15 2TT, UK.

[8]Faculty of Environment & Technology – Department of Engineering, Design & Mathematics, University of the West England, Frenchay Campus, BS16 1QY, UK.

E-mail: zxm850@student.bham.ac.uk

Background: Single transmembrane platelet receptors such as GPVI, CLEC-2, FcγRIIA and PEAR1 are activated via clustering.

Aim: Here, we aimed to understand the mechanism of clustering single transmembrane receptors and key parameters governing this.

Methods: Two modelling approaches were developed to simulate the interaction of single transmembrane receptors. The binding of monovalent, bivalent and tetravalent ligands was modelled using ordinarily differential equations (ODEs). Agent-based modelling (ABM) was used to model receptor dimerisation and higher order clustering in the presence of a divalent ligand, along with a tandem SH2-domain containing protein.

Results: ODE models identify the relationship between ligand concentration and maximum number of bound epitopes as a bell-shaped curve for multivalent ligands, suggesting that receptors at higher ligand concentrations are packed sparsely. ABM was used to simulate spatial dynamics of signalling proteins based on a series of autonomous, decision-taking agents, for a system comprising of dynamically dimerising single transmembrane receptors in the presence of a divalent ligand and a dimeric SH2-domain-containing protein. ABM results showed that an increase in receptor number raised constitutive receptor dimerization levels, which when coupled with a dimeric crosslinker or a dimeric ligand generated micro-clusters. The concomitant presence of both a divalent ligand and a crosslinker was seen to expedite receptor clustering and resulted in the generation of macro-clusters of considerable size.

Conclusions: For single transmembrane receptors that signal via clustering, receptor number and receptor dimerisation levels are key parameters governing cluster size, which is amplified in the presence of crosslinking species.

Platelets in Health & Disease

PC34. Estimating the causal association between body mass index and platelet properties: evidence for a positive association with immature platelet count

Lucy J. Goudswaard[1, 2, 3], Parsa Akbari[4], Laura J. Corbin[2, 3], Adam S. Butterworth[4], Nicole Soranzo[5], Nicholas J. Timpson[2, 3], Ingeborg Hers[1]

[1] *Physiology, Pharmacology and Neuroscience, University of Bristol, UK*
[2] *Population Health Sciences, University of Bristol, UK*
[3] *Medical Research Council (MRC) Integrative Epidemiology Unit at the University of Bristol, UK*
[4] *Department of Public Health and Primary Care, British Heart Foundation Cardiovascular Epidemiology Unit, University of Cambridge, UK*

[5] *Wellcome Sanger Institute, Hinxton, UK*

E-mail: lg14289@bristol.ac.uk

Background: Higher body mass index (BMI) is a recognised risk factor for thrombotic disorders. Platelets are essential for haemostasis but also contribute to thrombosis when activated pathologically. Changes in platelet properties may reflect platelet function changes.

Aims: We hypothesise that an increase in BMI may lead to changes in platelet properties.

Methods: We examined the effect of BMI on platelet traits measured by Sysmex XN-1000 in 33,388 adults from the INTERVAL study. Linear regression models were used for observational analyses between BMI and platelet characteristics. Mendelian randomisation (MR) was implemented, where a genetic risk score for BMI (using 654 single nucleotide polymorphisms associated with BMI ($P<5x10^{-8}$) was used to estimate the causal association.

Results: Observationally, higher BMI was positively associated plateletcrit (PCT, 0.12 SD higher per SD BMI, 95% CI 0.11-0.13, $P = 9.2x10^{-88}$), platelet count (PLT), immature platelet count (IPC, 0.06 SD higher per SD BMI, 95% CI 0.05-0.08, $P=4.8x10^{-22}$) and side fluorescence (SFL, a measure of mRNA content). MR provided evidence for a causal association between BMI and both SFL (0.08 SD higher per SD BMI, 95% CI 0.03-0.14, P = 0.003) and IPC (0.06 SD higher per SD BMI, 95% CI 0.006-0.12, P = 0.03).

Conclusions: This study provides evidence for a positive causal association between BMI and immature platelet count, suggesting that a higher BMI may increase platelet production. Immature platelets have been suggested to be prothrombotic, which might explain, in part, the increased risk of thrombosis as a result of a higher BMI.

PC35. Persistent long-term platelet activation in post-menopausal women affected by Takotsubo Syndrome

Patrizia Amadio[1], Benedetta Porro[1], Viviana Cavalca[1], Silvia Stella Barbieri[1], Sonia Eligini[1], Susanna Fiorelli[1], Alessandro Di Minno[1,3], Alessandra Gorini[1,2], Mattia Giuliani[1], Josè Pablo Werba[1], Nicola Cosentino[1], Paolo Olivares[1], Simone Barbieri[1], Fabrizio Veglia[1], Elena Tremoli[1], Daniela Trabattoni[1].

[1] Centro Cardiologico Monzino I.R.C.C.S., Milan, Italy
[2] Department of Oncology and Hemato-Oncology; University of Milan, Milan, Italy
[3] Present address: Department of Pharmacy Federico II University Naples, Italy

E-mail of the Presenting Author: pamadio@ccfm.it

Background: Takotsubo syndrome (TTS), a reversible form of ventricular dysfunction, resembles clinical presentation of acute coronary syndrome (ACS) in the absence of significant stenosis. Based on its clinical presentation, treatment with acetylsalicylic acid (ASA) has been initially recommended and generally maintained also at long-term. However, ASA efficacy in TTS patients has been recently challenged.

Aims: To investigate if ASA treatment is useful in this clinical setting, we evaluated thromboxane formation and platelets response in TTS patients at long-term.

Methods: 28 women patients with a history of TTS and 23 age-matched women with a history of ACS were enrolled. For comparison, we enrolled also 26 control women with no TTS or clinically evident coronary artery disease, with no ASA treatment. Thromboxane measurement was assessed in serum, while platelets aggregation was performed on platelet rich plasma.

Results: Despite ASA treatment, thromboxane was significantly higher in TTS vs CAD patients, even though significantly lower vs controls. This result was coupled with platelet hyperactivity. Indeed, PRP aggregation ADP-induced, was significantly greater in TTS compared to CAD, but similar to controls. In the same way, the response to epinephrine or norepinephrine alone, or combined with ADP, was enhanced in TTS patients compared to CAD. Moreover, the slopes of primary aggregation, induced by the same stimuli, were increased in TTS vs CAD patients, indicating a greater aggregation velocity.

Conclusions: The residual thromboxane production and platelet aggregation observed in our cohort, still leaves open the question about the use of ASA in TTS patients at long-term.

PC36. Maternal and offspring high-fat diet leads to platelet hyperactivation in male mice offspring

Renato S. Gaspar[1], Amanda J. Unsworth[2], Alaa Al-Dibouni[1], Alexander P. Bye[1], Tanya Sage[1], Michelle Stewart[3], Sara Wells[3], Roger D. Cox[4], Jonathan M. Gibbins[1], Dyan Sellayah[1], Craig Hughes[1]

[1] Institute for Cardiovascular and Metabolic Research, School of Biological Sciences, University of Reading, Reading, UK.

[2] Department of Life Sciences, Faculty of Science and Engineering, John Dalton Building, Manchester Metropolitan University, Manchester, UK, M1 5GD

[3] MRC Harwell Institute, Mary Lyon Centre, Harwell Campus, Oxfordshire, OX11 0RD, UK.

[4] MRC Harwell Institute, Genetics of type 2 diabetes, Mammalian Genetics Unit, Harwell Campus, Oxfordshire, OX11 0RD, UK.

E-mail: renatosgaspar@gmail.com

Background: Maternal over-nutrition increases the risk of diabetes and cardiovascular events in offspring. While prominent effects on cardiovascular health were observed, the impact on platelet physiology has not been addressed.

Aims: Here, we examined if maternal high-fat diet (HF) ingestion affects the platelet function in lean and obese mice offspring.

Methods: C57BL6/N mice dams were given a HF or control (C) diet for 8 weeks before and during pregnancy. Male and female offspring received C or HF diets for 26 weeks. Experimental groups were: C/C, dam and offspring fed standard laboratory diet; C/HF dam fed standard laboratory diet and offspring fed HF diet; HF/C and HF/HF. Phenotypic and metabolic tests were performed and blood collected for platelet studies.

Results: Compared to C/C, offspring HF groups were obese, with fat accumulation, hyperglycaemia and insulin resistance. Female offspring did not present platelet hyperactivity, hence we focused on male offspring. Platelets from HF/HF mice were larger, hyperactive and presented oxidative stress when compared to C/C.

Conclusions: Maternal and offspring HF diet results in platelet hyperactivation in male mouse offspring, suggesting a novel 'double-hit' effect.

PC37. Vitamin D-Independent Inter-individual variation in platelet responsiveness in normal healthy donors

Alfatani, E.[1] and Moran, N.[1]

[1]*School of Pharmacy and Biomolecular Sciences, Royal College of Surgeons in Ireland,123 St Stephens Green, Dublin 2;*

Emails: nmoran@rcsi.com and ebtihalalfatani@rcsi.com

Background: Pathological platelet activation underlies thrombotic cardiovascular disease and is responsible for up to 33% of all deaths in the western world. Longitudinal studies have demonstrated that increased cardiovascular mortality and morbidity is associated with vitamin D deficiency (Wang et al, Circulation 2008 29;117(4):503-11).

Aims: To correlate *ex-vivo* platelet responsiveness with serum levels of Vitamin D in a normal healthy population, in order to ascertain if platelets play a role in the cardiovascular pathology of Vitamin-D.

Methods: Dose-dependent responses of platelets from 70 normal healthy volunteers were assessed following activation by: thrombin receptor activating peptide (TRAP; 2.9-33uM); thromboxaneA2-mimmetic, (U46619; 0.1-15 uM); and collagen-related peptide (CRP; 0.1-2 ug/ml). Specifically, we examined the potency and capacity of each individual's responsiveness in platelet aggregation (Light Transmission Aggregometry; BioData) and ATP secretion (Luminometry) assays. Serum Vitamin-D levels were assessed using standard clinical assays.

Results: The range of responsiveness across individual donors, as measured by ATP secretion assays, varied by >order-of-magnitude for both capacity and potency, following activation by all agonists as shown in the Table.

	Max ATP released (pmoles/10^6 platelets; mean±SD)	Range	EC_{50} (mean±SD)	Range
TRAP(uM)	0.91±0.88	0.11->4.67	15.57±4.63	8.05->32.48
U46619(uM)	0.54±0.55	0.08->2.71	4.5±2.71	1.26->15.41
CRP(ug/ml)	1.02±0.82	0.27->4.97	0.52±0.29	0.16->1.67

Serum Vitamin-D levels varied from 13-122 nmol/L in this healthy population. However, Pearson's correlation analysis demonstrates no correlation between platelet responsiveness and their serum Vitamin-D level (R^2= 0.008).

Conclusions: Substantial inter-individual differences in ATP secretion were observed for all agonists but are independent of Vitamin-D status in this cohort of normal healthy donors.

PC38. Acute coronary heart disease is characterised by circulating procoagulant platelets and hyposensitivity to prostacyclin

Beth A. Webb,[1] Matthew S. Hindle[1], Benjamin Spurgeon[1], Clare Wilson[1], Jianping Lu[1], Erica Watson[1], Jennifer Rossington[2] and Khalid Naseem[1]

[1]*LICAMM, University of Leeds, Leeds, UK*

[2]*Department of Cardiology, Leeds General Infirmary, Leeds, UK*

E-mail: umbaw@leeds.ac.uk

Background: Previously, we have shown that platelet activation and thrombosis in models of dyslipidaemia are associated with hyposensitivity to prostacyclin (PGI_2). Subjects with coronary artery disease (CAD) are characterised by a prothrombotic state in which platelet hyperactivity is proposed to play a central role. However, the mechanism of platelet hyperactivity is unclear.

Aims: To determine if platelet hyperactivity in subjects is characterised by dysregulation of PGI_2 sensitivity and whether this is related to disease severity.

Method: Platelet activation in whole blood from subjects with stable CAD and post myocardial infarction (MI) were examined by multiparameter flow cytometry.

Results: In basal or activated cells, we found no significant differences in fibrinogen binding or P-selectin expression between groups. In contrast, PGI_2-mediated inhibition of fibrinogen binding was compromised in CAD patients compared to control. Whereas, inhibition of P-selectin expression by PGI_2 was not statistically different. PGI_2 hyposensitivity was associated with diminished cAMP signalling as evidenced by reduced phosphoVASP. Next, platelet function post-MI was examined using a four-parameter activation panel. At basal, in acute patients, 57.14±8.3% of platelets were positive for Annexin V (AnnV) compared to control 15.09±3.4%, suggesting the presence of circulating activated platelets in post-MI patients. Previously, we have found that AnnV binding as a marker of phosphatidylserine (PSer) exposure is highly sensitive to the inhibitory actions of PGI_2. However, in post-MI platelets, AnnV binding was insensitive to the effects of PGI_2.

Conclusions: These data suggest that MI is associated with circulating procoagulant platelets that are insensitive to PGI_2.

The authors would like to thank Heart Research UK (RG.2659) and the British Heart Foundation (PG/18/81/43110 and RG/16/5/22350) for funding this study.

PC39. The Effect of Platelets On Thrombin Generation In The Premature Infant; The Event Study

Claire Murphy[1,2], Elaine Neary[3,4], Barry Kevane[5], John O'Loughlin[6], Daniel O'Reilly[2,] Afif EL-Khuffash[1,2], Patricia Maguire[7], Fionnuala NíAinle[5,7,8], Naomi McCallion[1,2]

[1]*Department of Paediatrics, Royal College of Surgeons in Ireland, Dublin, Ireland*
[2]*Department of Neonatology, Rotunda Hospital, Dublin, Ireland*
[3]*Department of Neonatology, Liverpool Women's Hospital, Liverpool, United Kingdom,*
[4]*Department of Health and Life Sciences, University of Liverpool, Liverpool, United Kingdom*
[5]*Department of Haematology, Mater Misericordiae University Hospital, Dublin, Ireland*
[6]*Department of Pathology, Rotunda Hospital, Dublin, Ireland*
[7]*Conway-SPHERE Research Group, Conway Institute, University College Dublin, Ireland*
[8]*Department of Haematology, Rotunda Hospital, Dublin, Ireland*
E-mail: claireamurphy@rcsi.com

Background: Premature infants are at high risk of haemorrhage and have reduced coagulation factors and hypo-reactive platelets. In spite of this, plasma thrombin generation in preterm infants, is similar or greater than term infants, when measured with Calibrated Automated Thrombography (CAT) [1, 2].
Aims: To describe the effect of platelets on thrombin generation in the premature infant.
Methods: This was a prospective observational study, performed in platelet rich plasma (PRP) from umbilical cord blood. Premature infants (24 - 31 weeks) and term controls were recruited. CAT was performed using PRP reagent (contains tissue factor only), and is thus dependent on the phospholipid content of PRP.
Results: These preliminary results (n=9 preterm, n=17 term) show no difference in any of the thrombin generation parameters in PRP between preterm and term infants.

Median values	Preterm N=9	Term N=17	p
Gestational age (weeks)	29.6	39.3	<0.01
Birth weight (g)	1375	3830	<0.01
Whole blood platelet count (x10^9/L)	247	254	0.35
Mean platelet volume (fL)	8.3	7.6	0.04
PRP platelet count (x 10^9/L)	105	107	0.28
Lag time (mins)	3.77	4.28	0.16
Endogenous thrombin potential (nM.min)	1003	970	0.4
Peak thrombin (nM)	81	77	0.49
Time to peak thrombin (mins)	9.44	10.45	0.13

Table 1: Results of CAT in PRP

In a subset of infants (n=5 term, n=2 preterm), thrombin generation was assessed in both platelet-poor and platelet-rich plasma using PRP reagent. There was no difference in any of the thrombin generation parameters, suggesting there is sufficient phospholipid present in PPP for thrombin generation to occur, likely from extracellular vesicles.

Conclusion: Our early findings suggest that thrombin generation in PRP is similar in preterm and term infants. In spite of reported "hypo-reactive" preterm platelets, it appears there is adequate phospholipid present to allow thrombin generation to occur. This supports the findings by Haidl *et al.*, that neonatal thrombin generation has very little platelet dependency [3]. We plan to further evaluate thrombin generation in PRP in larger numbers and in peripheral blood.

1. J Thromb Haemost, 2015. 13(11): p. 2021-30.
2. Thromb Res, 2020. 185: p. 96-101.
3. Sci Rep, 2019. 9(1): p. 8014.

PC40. The Brain-Derived Neurotrophic Factor mitigates the association between platelet dysfunction and cognitive impairment in coronary artery disease

Jean-Christophe Bélanger,[1,2] Bianca D'Antono,[1,3] Jessica Le Blanc,[1,2] Louisia Starnino,[1] Mélanie Welman,[1] Marie Lordkipanidzé,[1,2]

[1] Research center, Montreal Heart Institute, Montreal, Qc, Canada
[2] Faculty of Pharmacy, Université de Montréal, Montreal, Qc, Canada
[3] Département de psychologie, Faculté des arts et des sciences, Université de Montréal, Montreal, Qc, Canada

E-mail: jean-christophe.belanger@umontreal.ca

Background: Upon activation, platelets release Brain-Derived Neurotrophic Factor (BDNF), a neurotrophin protective against cognitive decline. Platelet hyperactivity however, is deleterious in coronary artery disease (CAD), requiring lifelong antiplatelet therapy, and is associated with worse cognitive outcomes.

Aims: Given these apparently opposing effects of platelet activation on cognitive health, we investigated whether BDNF levels intercede in the relationship between platelet activation and cognitive function; and whether this relationship is moderated by the presence of CAD.

Methods: In this cross-sectional study, 1280 participants with (n=673) and without CAD (n=607) completed the Montreal Cognitive Assessment (MoCA). Plasma BDNF and soluble P-selectin levels were assessed using multiplex flow cytometry.

Results: The relationship between sP-selectin and BDNF concentrations was stronger for individuals without CAD (b=0.71, p<0.0001) than for CAD participants (b=0.43, p<0.0001, pinteraction<0.0001). In a simple mediation model, platelet activity was negatively associated with MoCA scores (direct effect: b=-0.26, p=0.01). However, when BDNF levels were introduced as a mediating factor, platelet activity associated with higher plasma BDNF concentrations (b=-0.56, p<0.0001), and higher BDNF concentrations associated with higher MoCA scores (b=0.26, p<0.05), resulting in a positive indirect effect (b=0.14, p<0.05). The negative association of platelet activation with cognition was abrogated when BDNF levels were accounted (total effect: b=-0.13, p=0.13).

Conclusions: BDNF released from platelets could be a mitigating factor in a negative association between platelet activity and cognitive function.

PC41. Expanding the genetic spectrum of *TUBB1*- related thrombocytopenia

Verónica Palma-Barqueros[1,#], Loredana Bury[2,#], Shinji Kunishima[3], María Luisa Lozano[1], Agustín Rodríguez Alen[4], Nuria Revilla[5], Natalia Bohdan[5], José Padilla[5], María P. Fernández-Pérez[5], María Eugenia de la Morena-Barrio[5], Ana Marín-Quiles[6], Rocío Benito[6], María F. López-Fernández[7], Shally Marcellini[8], Ana Zamora-Cánovas[5], Vicente Vicente[1], Constantino Martínez[5], Paolo Gresele[2], *José M. Bastida[9,10], *José Rivera[1,10]

[1]Servicio de Hematología y Oncología Médica, Hospital Universitario Morales Meseguer, Centro Regional de Hemodonación, Universidad de Murcia, IMIB-Arrixaca, CIBERER-U765, Murcia 30003, Spain.
[2] Department of Medicine, University of Perugia, Perugia, Italy
[3] Department of Medical Technology, Gifu University of Medical Science, Seki, Japan
[4] Servicio de Hematología y Hemoterapia, Hospital Virgen de la Salud, Complejo Hospitalario de Toledo, Spain
[5] Servicio de Hematología y Oncología Médica, Hospital Universitario Morales Meseguer, Centro Regional de Hemodonación, Universidad de Murcia, IMIB-Arrixaca
[6] IBSAL, IBMCC, CIC, Universidad de Salamanca-CSIC
[7] Servicio Hematología y Hemoterapia, Complejo Hospitalario Universitario ACoruña, Spain
[8] Servicio Hematología, Hospital General, Segovia, Spain
[9] Departamento de Hematología, IBSAL-Hospital Universitario de Salamanca, Salamanca, Spain
[10] On behalf of the "Inherited Platelet Disorders Project", Grupo Español de Alteraciones Plaquetarias Congénitas (GEAPC), Spanish Society of Thrombosis and Haemostasis (SETH)

E-mail: veronica_93@hotmail.es

Background: β1Tubulin mediates pro-platelet formation and platelet shape. Limited families with autosomal dominant *TUBB1* variants have been reported.

Aims: Description of 9 unrelated families (38 patients) with macrothrombocytopenia (57-134 x 10^9 /L; MPV 13.3-15.0 fL) enrolled in the Spanish project "Functional/molecular characterization of patients with Inherited Platelet Disorder".

Methods: Platelet phenotyping included: platelet aggregation, glycoproteins and activation by flow cytometry; cytoskeletal proteins in resting/spreading platelets by immunofluorescence; ultrastructure by electron microscopy. Molecular analysis involved HTS-gene-panel. Proplatelet-formation was evaluated in megakaryocytes differentiated from CD34+ peripheral blood cells; the pathogenicity of candidate variants was assessed in a CHO cell transfection model.

Results: Molecular analysis identified six β1tubulin variants: p.Cys12Leufs12*, p.Thr107Pro, p.Gln423*, p.Arg359Trp, p.Gly269Asp and p.Gly109Glu, the last two novel. Segregation studies showed incomplete penetrance of these variants in platelet traits. Most carriers showed macrothrombocytopenia, some only increased platelet size and a minority no abnormalities. For the p.Gly109Glu variant, only homozygous carriers displayed macrothrombocytopenia, highlighting the weight of allele burden in the phenotypic expression of TUBB1-RT. Transfection of *TUBB1* missense variants in cells altered β1tubulin incorporation into the cytoskeleton. p.Arg359Trp, p.Gly269Asp and p.Gly109Glu affected β1tubulin localization into the marginal ring in resting platelets. These variants have negligible effect on platelet activation/secretion or spreading, suggesting that β1tubulin is dispensable for these processes. In contrast, these *TUBB1* variants markedly impaired proplatelet formation.

Conclusions: This study expand the genetic spectrum of TUBB1-RT and highlight a remarkable heterogeneity in its clinical presentation, indicating that allelic burden or combination with other genetic or environmental factors modulate the phenotypic impact of rare TUBB1 variants.

PC42. Expanding the clinical features of the tyrosine kinase SRC-related thrombocytopenia disorder

Nuria Revilla[1,2], Ana Galera[3], Verónica Palma-Barqueros[1] , Natalia Bohdan[1] , Jose Padilla[1] , Agustín Rodríguez Alén[4] , Ana Marín[5], Rocio Benito[5], Vicente Vicente[1] , Maria Luisa Lozano[1] , Jose María Bastida[5,6,7], Jose Rivera[1,7.]

[1] Servicio de Hematología y Oncología Médica, Hospital Universitario Morales Meseguer, Centro Regional de Hemodonación, Universidad de Murcia, IMIB-Arrixaca, CIBERER-U765, Murcia 30003, Spain.
[2]Hospital Universitario Ramón y Cajal. Madrid, Spain.
[3]Hospital Universitario Virgen de la Arrixaca, Murcia, Spain.
[4]Hospital Virgen de la Salud, Complejo Hospitalario de Toledo, Spain.
[5] IBSAL, IBMCC, CIC, Universidad de Salamanca-CSIC.
[6] Departamento de Hematología, IBSAL-Hospital Universitario de Salamanca, Salamanca, Spain
[7] On behalf of the "Inherited Platelet Disorders Project", Grupo Español de Alteraciones Plaquetarias Congénitas (GEAPC), Spanish Society of Thrombosis and Haemostasis (SETH)

E-mail: nrecal@gmail.com

Background: The heterozygous germline gain-of-function E527K variant in SRC has been associated to a rare form of inherited thrombocytopenia (IT), namely SRC-RT, in three unrelated families. Their clinical features ranging from isolated thrombocytopenia to a complex syndrome with thrombocytopenia, bleeding, myelofibrosis, splenomegaly and bone pathologies. Recently, DNA analysis in two sibling with enrolled in the Spanish project "Functional/molecular characterization of patients with Inherited Platelet Disorder", identified the E527K variant.

Aims: To characterize the fourth SRC-RT family.

Methods: The E527K variant was segregated in the three-generation pedigree by Sanger sequencing.The syndromic phenotype of familly members was reassessed. Platelet phenotyping included peripheral smear, platelet aggregation, glycoproteins, activation and granule secretion by flow cytometry and electron microscopy.

Results: Seven E527K carriers were identified, all showing thrombocytopenia, anisocytosis, alpha-granule deficiency and defective collagen-induced platelet aggregation. Flow cytometry showed normal glycoproteins but reduced TRAP-induced P-Selectin expression. The four adults E527K carriers underwent splenectomy after misdiagnosis of refractory ITP. E527K carriers had a syndromic phenotype, including recurrent bronchitis. One carrier developed post-splenectomy portal cavernomatosis with several variceal bleeding events. Another four had bone pathologies and three cases had teeth abnormalities. Four cases showed neurological manifestations, including language impairment, behavior abnormalities, or severe epilepsy.

Conclusions: We report a fourth family with SRC-RT, expanding the clinical features of the disorder. This rare syndrome can associated with neurological manifestations from autism to epilepsy. SRC may be critical for processes underlying physiological and pathological plasticity. Mutant SRC can account for this familial neurological syndrome.

PC43. Strategies for analysis of novel RUNX1 mutations as a cause of familial platelet disorder with predisposition to acute myeloid leukemia(FPD/AML)

Palma-Barqueros Verónica[1], Ruiz-Pividal Juan Francisco[1], Bastida Jose María[2], Bohdan Natalia[1], López-Andreo Maria José[3], Teruel-Montoya Raúl[1], Rodriguez-Alén Agustín[4], Padilla Jose[1], Revilla Nuria[1], Marín-Quilez Ana[2], Zamora-Cánovas Ana[1], Benito Rocío[2], González-Porras Jose Ramón[2], Vicente Vicente[1], Lozano Maria Luisa[1,5], Rivera José[1,5]

[1]Servicio de Hematología y Oncología Médica, Hospital Universitario Morales Meseguer, Centro Regional de Hemodonación, Universidad de Murcia, IMIB-Arrixaca, CB15/00055-CIBERER.
[2]Unidad de Trombosis y Hemostasia. H. Universitario de Salamanca-IBSAL, Salamanca.
[3]Sección de Biologia Molecular, Área Científica y Técnica de Investigación, Universidad de Murcia, Plataforma de Biología Molecular, IMIB-Arrixaca, Murcia.
[4]Servicio de Hematología y Hemoterapia, Hospital Virgen de la Salud, Complejo Hospitalario de Toledo, Spain.
[5]On behalf of the Project "Caracterización funcional y Molecular de trastornos plaquetarios congénitos", Grupo de Trabajo de Patología Hemorrágica de la SETH.
E-mail: veronica_93@hotmail.es

Background: Germline mutations in *RUNX1* cause FPD/AML. Overestimating the pathogenicity of new variants can lead to unnecessary worry about a potential risk to develop malignancies.

Aims: To evaluate the pathogenicity of new *RUNX1* variants identified in three women (P1, P2, P3) with congenital thrombocytopenia and moderate bleeding.

Methods: We performed platelet phenotyping (flow cytometry, aggregation studies), and molecular analysis by high-through-sequencing (HTS). Ultrapure-platelets were isolated by filtration+immunoselection (patients, and 5 controls). Ultrapure-platelet RNA was analyzed using Clariom-D Array (≈540 000 transcripts). In P1 we evaluate proplatelet formation in megakaryocytes differentiated from CD34+ peripheral blood cells.

Results: P1 and P2 showed altered aggregation/granule secretion and GPIa levels. HTS identified new heterozygous RUNX1 variants: c.802C<T (P1); c.586A>G (P2); c.476A>G (P3). The expression analysis of 15 genes recognized as RUNX1 targets was confirmed to be altered in P1 and P2 (30.8% and 38.5% similarity to controls), but not in P3 (92.7% resemblance). Similarly, the evaluation of 100 genes reported downregulated in a RUNX1 patient (*Sun, JTH 2007*), showed 74.7%, 67.7% and 7.1% of them had lower expression in P1, P2, and P3, respectively. P1 showed reduced pro-platelet formation. Transcriptome analysis (P1/P2 vs. controls) revealed that of the top 120-downregulated genes: 18%, 40% and 30% encode for cytoskeletal, signaling and related-cell cycle/oncogenic events proteins, respectively.

Conclusions: Our study supports the pathogenicity of the *RUNX1* variants identified in P1 and P2, but not in P3. Platelet transcriptome became a useful tool in the pathogenic characterization of new variants affecting transcription factors; and it allows the identification of novel target genes.

PC44. The acute impact E-cigarette extract on *in vitro* platelet function.

Jordan Vautrinot,[1] Chris Williams[1,] Alastair Poole[1]

[1]*University of Bristol*

E-mail: BH19541@bristol.ac.uk

Background: In recent years electronic cigarette use has become more prevalent, in both smoking and non-smoking populations with people perceiving it as a risk-free smoking alternative, however, little is known about their effects on human health. Recent data suggest that they have association with cardiovascular pathology, which may include an impact upon haemostasis, platelet function and thrombosis.

Aims: In this study we therefore aimed to determine the acute effects of e-cigarette extract on acute platelet function.

Methods & results: Using *in vitro* thrombosis, we showed no statistical difference in thrombus formation in whole blood. We also showed no statistical difference in PS exposure in washed platelets with thrombin and CRP agonist, platelet-neutrophil interaction in whole blood with or P selectin and integrin $a_{IIb}b_3$ levels in PRP when measured by FACS.

Conclusions: In conclusion we find that E-cigarette extract has no acute effects on platelet function in an in vitro setting. This is in contradiction with previously published data, suggesting that E-cigarette use may not pose as much of a health risk in respect to platelet function and associated thrombotic events as previously thought. It is however important to note that more physiologically relevant methods need to be studied further and the chronic effects of E-cigarette use could still pose a risk to public health.

PC45. Prospective study reveals increased platelet function associated with multiple myeloma and its treatment

*Mitchell, JL[1], *Khan, D[2], *Rana, R[1], Kriek, N[1], Unsworth[4], AJ[1], Sage, T[1], Bye, AP[1], Laffan, M[5], Shapiro, S[2], Thakurta,A[6,7], Grech, H[3], Ramasamy, K[2], and Gibbins, JM[1].

* authors contributed equally

[1]*Institute for Cardiovascular and Metabolic Research, School of Biological Sciences, University of Reading, Reading, UK.*
[2]*Oxford University Hospitals NHS Foundation Trust, Blood Theme Oxford Biomedical Research Centre, Oxford, UK.*
[3]*Royal Berkshire Hospitals NHS Foundation Trust, Reading, UK.*
[4]*Department of Life Sciences, Manchester Metropolitan University, Manchester, UK.*
[5]*Centre for Haematology, Department of Medicine, Imperial College London, London, United Kingdom.*
[6]*Bristol Myers Squibb, Princeton, NJ, USA.* [7]*University of Oxford, Oxford, UK.*

Email: j.l.mitchell@reading.ac.uk

Background: Multiple Myeloma(MM) is a bone marrow cancer preceded by premalignant, benign Monoclonal Gammopathy of Undetermined Significance(MGUS). MM patients have an increased risk of thrombosis. MM treatments include Immunomodulatory drugs(iMiDs), e.g. Lenalidomide, which targets the protein Cereblon and improves patient outcomes whilst also further increasing risk of thrombosis.

Aims: Determine whether MM and its treatment affect platelet function.

Methods:Platelet-rich plasma(PRP) was isolated from healthy controls(controls; n=31), MGUS(n=18) and MM(26) patients. The MM group was divided into; (1)-no treatment, (2)-proteasome inhibitor(PI) with Dexamethasone(Dex), and (3)-PI, Dex, iMiD and direct oral anticoagulant.

Platelet aggregation, fibrinogen and P-selectin binding were measured in PRP stimulated with various agonists. Platelet activation and receptor levels were measured by flow cytometry. Cereblon was detected by Western Blot. *In vitro* thrombus formation on collagen was performed in DiOC6-labelled whole blood from controls at shear rates from $200s^{-1}$ to $1500s^{-1}$ in the presence and absence of Lenalidomide(1μM).

Results: Platelet receptor expression was altered in MGUS and MM. Cereblon was present in platelets. ADP stimulation increased fibrinogen($P<0.01$) and P-selectin($P<0.05$) binding in MGUS and MM. All agonists enhanced platelet aggregation after iMiD/Dex treatment in MM compared to pre-treatment. Aggregation($P<0.001$) and P-selectin($P<0.001$) were increased in Lenalidomide treated control platelets in response to ADP, CRP and TRAP. Thrombus formation was increased *in vitro* in Lenalidomide treated control blood at arterial shear rates of $1000s^{-1}$($P<0.01$) and $1500s^{-1}$($P<0.05$).

Conclusions: Platelet reactivity is increased in MM and upon iMiD treatment. The presence of Cereblon in platelets and the ability of lenalidomide to modulate platelet function directly, reveals new avenues for investigation to determine the underlying mechanism of action.

PC46. CD36 is a biomarker of platelet GPVI hyperreactivity specific to women

Alexander P Bye[1], Joanne L Dunster[1], Neline Kriek[1], Carly Kempster[1,2], Tanya Sage[1], Joanne L Mitchell[1], Joana Batista[2], Harriet McKinney[2], Patrick Thomas[2], Chris I Jones[1], Amanda J Unsworth[3], Kate Downes[2], and Jonathan MGibbins[1]

[1]Institute for Cardiovascular and Metabolic Research, School of Biological Sciences, University of Reading, UK
[2]Department of Haemotology, University of Cambridge, UK
[3]School of Healthcare Science, Manchester Metropolitan University Manchester, UK.

E-mail: alex_p_bye@hotmail.com

Background: High platelet reactivity is associated with high on-treatment reactivity, which limits the efficacy of antiplatelet therapy for a subset of patients. GPVI inhibition is a promising new strategy for reducing atherothrombosis but must contend with the same variability in efficacy seen with aspirin and P2Y12 antagonist therapies.

Aim: To identify biomarkers of GPVI reactivity and develop a predictive model to facilitate personalised therapy with GPVI inhibitors.

Methods: Blood was taken from 550 healthy donors aged 18 to 75. Fibrinogen binding and p-selectin exposure evoked by ADP, CRP-XL and TRAP-6 was measured using high-throughput flow cytometry and plate based aggregometry, expression of surface proteins was measured by flow cytometry. A random forest machine learning approach was used to identify variables that were predictive of GPVI reactivity. Linear regression and hierarchical cluster analysis were used to explore relationships between GPVI reactivity and predictor variables. These variables were used to generate predictive models of GPVI reactivity.

Results: Variability in GPVI reactivity was greater than for P2Y or PAR1 receptors and was strongly correlated with GPVI surface expression and age in both males and females. However, surface expression of the scavenger receptor, CD36 was specifically identified as a biomarker in females over 60.

Conclusions: CD36 is an important biomarker of GPVI reactivity in women over 60, a group that has traditionally been underserved by cardiovascular research. These findings may have implications for personalised therapy with drugs that target GPVI and to the understanding of atherothrombosis in women more broadly.

PC47. Obstetric Neuraxial Anesthesia in the setting of Immune Thrombocytopenia and Low Platelet Counts: Call to Participation in an International Registry

A. Kinga Malinowski,[1,2] Maha Othman[3,4]
on behalf of the International Society on Thrombosis and Haemostasis' Women's Health Issues in Thrombosis and Haemostasis SSC, Platelet Physiology SSC, and Platelet Immunology SSC

[1]*Department of Obstetrics & Gynaecology, Division of Maternal-Fetal Medicine, Lunenfeld-Tanenbaum Research Institute, Sinai Health System, Canada*

[2]*Department of Obstetrics & Gynaecology, University of Toronto, Canada.*

[3]*Department of Biomedical & Molecular Sciences, School of Medicine, Queen's University, Kingston, Canada*

[4]*School of Baccalaureate Nursing, St. Lawrence College, Kingston, Canada*

E-mail: ann.malinowski@sinaihealth.ca

Background: Immune Thrombocytopenia (ITP) affects 1-2/1,000 pregnancies. While platelet count thresholds associated with morbidity have not been established, fears of neuraxial haematoma restrict Obstetric Neuraxial Anaesthesia (OBNA) eligibility for those with ITP and platelet counts <70-80x10(9)/L. This results in sub-standard analgesia, which is typically unacceptable in other clinical settings. To provide a definitive answer with respect to neuraxial haematoma risk in this setting, an international effort is required.

Aim: Establishment of an international registry to prospectively record outcomes of OBNA in women with ITP and platelet counts below 100x10(9)/L, in partnership with the Women's Health Issues in Thrombosis and Haemostasis, Platelet Physiology, and Platelet Immunology Scientific Standardization Committees of the International Society on Thrombosis and Haemostasis.

Methods: Eligible study population includes pregnant individuals with ITP and peripartum platelet counts below 100x10(9)/L who received OBNA in the form of spinal, epidural, or combined spinal-epidural. No lower range platelet count restriction has been placed for inclusion, as occasional reports of OBNA at even lower platelet counts are available, and all such reports will add valuable data. Individual entries are non-identifiable and ethics approval from Sinai Health System, Toronto, Canada has been obtained; though, local ethics practices apply.

Results/Conclusion: To assure registry success, its wide-dissemination to attract global engagement is paramount. This is a call to participate in the Registry, available electronically via REDCap at: https://redcap.isth.org/surveys/?s=7PPAY4CDCA. Each contribution is indispensable to the accrual of data for this highly clinically-relevant, yet relatively rare question, and we hope that this endeavour will eventually allow for the development of evidence-based recommendations for this population.

PC48. PKC-Delta-Dependent Pathways Contribute to the Exacerbation of the Platelet Activity in Crohn's Disease

Younes Zaid[1,2], Loubna Khalki[3], Youness Limami[2], Nabil Zaid[1], Abdallah Naya[4], Mounia Oudghiri[4]

[1] *Biology Department, Faculty of Sciences, Mohammed V University, Rabat, Morocco*

[2] *Research Center of Abulcasis University of Health Sciences, Rabat, Morocco*

[3] *Research Center, Mohammed VI University of Health Sciences (UM6SS), Casablanca, Morocco*

[4] *Immunology and Biodiversity Laboratory, Department of Biology, Faculty of Sciences Ain Chock, Hassan II University, Casablanca, Morocco*

E-mail: younes_zaid@yahoo.ca

Background: Platelets are widely recognized for their role in the prevention of bleeding and promotion of hemostasis; However, accumulating evidence points to a nonhemostatic role for platelets in chronic inflammatory conditions. This stems from the ability of activated platelets to secrete many immunomodulatory cytokines and chemokines. In addition, it is becoming increasingly clear that an elevated platelet count and reactivity are considered as a marker of inflammatory bowel disease (IBD) activity. Crohn's disease (CD) is one of the most severe gastrointestinal disorders classified as an IBD.

Aims: To elucidate platelet hyperactivity in Crohn's patients and to assess the role of PKC delta during inflammation.

Methods: PKC-delta (PKCδ) isoform is expressed in platelets and plays distinct roles in regulating platelet function. In this study, pharmacological and molecular genetic approaches were used to investigate the functional role of PKCδ isoform and downstream effectors in modulation of molecular inflammatory mechanisms during CD pathogenesis.

Results: In human platelets, pre-treatment with the specific PKCδ inhibitor δ(V1-1)TAT significantly decreased platelet activation in patients with Crohn's disease (CD). Analysis of PKCδ phosphorylation on Tyr^{311} indicates that it is positively regulated by the mitogen-activated protein kinase (MAPK) pathway. Importantly, PKCδ null mice were refractory to acute dextran sodium sulphate (DSS)-induced colitis, suggesting a target role of the PKCδ isoform during chronic intestinal inflammation pathogenesis.

Conclusions: These findings highlight a new inhibitory mechanism in the regulation of platelet-induced inflammation that could be the basis for translational research and drug development.

New techniques in platelet research

PC49. Development of a novel "arterial thrombosis-on-a-chip" microfluidic device.

Jess Berry[1], François J. Peaudecerf[2], Nicole A. Masters[3], Keith B. Neeves[3], Raymond E. Goldstein[4], Matthew Harper[1]

[1]*Department of Pharmacology, University of Cambridge, U.K.*
[2]*Department of Civil, Environmental, and Geomatic Engineering, ETH Zürich, 8093 Zürich, Switzerland*
[3]*Department of Bioengineering, Department of Pediatrics, Section of Hematology, Oncology, and Bone Marrow Transplant, Hemophilia and Thrombosis Center, University of Colorado Denver | Anschutz Medical Campus, Aurora, CO*
[4]*Department of Applied Mathematics and Theoretical Physics, University of Cambridge, U.K.*

E-mail: jb747@cam.ac.uk

Background: Myocardial infarction is triggered by occlusion of coronary arteries by platelet-rich thrombi. Development of new anti-platelet drugs to prevent myocardial infarction depends on accurate models of thrombosis. *In vivo* animal models produce variable results and only have limited relevance to human disease. Few *in vitro* models using human blood generate occlusive thrombi; those that do generate occlusive thrombi do not allow quantitive assessment of antithrombotic compounds.

Aims: Development of a novel "arterial thrombosis-on-a-chip" microfluidic system that allows quantitative measurement of occlusion time.

Methods: A microfluidic chip was iteratively designed, and fabricated using soft lithography. Within the chip, a collagen and tissue factor patch triggers thrombosis in whole human blood flowed at arterial shear. Thrombus growth is monitored using confocal microscopy. Occlusion time is measured in a simple, robust way using a balance.

Results: Initial experiments confirmed that addition of a bifurcation into a microfludic chip allows occlusion to occur. However, further analysis highlighted that this occlusion can be caused by off-site coagulation, obscuring the effect of anti-platelet drugs. We therefore designed a microfluidic device that generates biologically relevant occlusive thrombi by quenching downstream coagulation. We validated our device by using the approved anti-platelet drug eptifibatide, demonstrating that our device can be used to monitor the effect of antithrombotic drugs on occlusion time in an unbiased manner.

Conclusions: We have developed a novel arterial thrombosis-on-a-chip device that allows biologically relevant occlusive thrombi to form, and that can be used to assess the effect of anti-thrombotic compounds on occlusion time.

PC50. Vessel-on-a-chip model reveals rapid on and off mechanisms of collagen- and thrombin-induced platelet activation

Isabella Provenzale,[1,2] Sanne L.N. Brouns,[1] Claudia Schönichen,[1] Delia I. Fernández,[1] Jonathan M Gibbins,[2] Paola E.J. van der Meijden,[1,3] Chris Jones,[2] Johan W.M. Heemskerk,[1]

[1] Dept of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, Maastricht (NL)
[2]Institute for Cardiovascular and Metabolic Research (ICMR), School of Biological Sciences, University of Reading, Reading (UK)
[3] Thrombosis Expertise Center, Heart and Vascular Center, Maastricht University Medical Center+ , Maastricht (NL)

E-mail: i.provenzale@maastrichtuniversity.nl; i.provenzale@pgr.reading.ac.uk

Background: Microfluidic assays represent established *in-vitro* thrombosis models. To understand the mechanisms and kinetics of endothelial antiplatelet and anticoagulant processes, endothelialised microfluidic models are needed.

Aims: To establish an *in-vitro* model to assess the endothelial control of platelet activation and coagulation under flow.

Methods: Calcium fluxes were measured in Calcium6-loaded platelets pre-incubated with human umbilical vein endothelial cells (HUVECs) and activated with thrombin or collagen-related peptide (CRP-XL). HUVECs, cultured in microfluidic channels coated with collagen and tissue factor, were subjected to whole blood perfusion (shear rate 1000/s). Fluo4-loaded platelets and labelled fibrinogen were added to measure Ca^{2+} fluxes and fibrin formation.

Results: Platelet preincubation with HUVECs in stasis suppressed intracellular Ca^{2+} responses to thrombin or CRP-XL. Whole blood perfusion over sub-confluent HUVECs resulted in low platelet adhesion with a surface area coverage (SAC) of 4.6±3.4% (n=3) compared to experiments without HUVECs (40.4±6.9 %SAC,p-value<0.001). HUVECs treatment with Tumor Necrosis Factor-α (TNFα) resulted in enhanced platelet adhesion (20.9 ±22.4 %SAC). In both cases the thrombi were restricted to areas in between HUVECs. The presence of HUVECs delayed fibrin formation, this lessened when treated with TNFα. Platelet Ca2+ responses in thrombi were suppressed by the presence of untreated HUVECs, this effect was reverted by TNFα treatment.

Conclusions: HUVECs suppressed *in-vitro* thrombus formation rapidly and locally. TNFα treatment partially impaired inhibition, pointing to a distinct behaviour in an inflammatory context. The developed model shows potential to further study thrombus formation in contexts where the presence of a dysfunctional endothelium can distinctively modulate platelet reactivity.

PC51. Efficient labelling of human platelets using fusogenic liposomes

Carly Kempster[1], Josh Cull[1], Jonathan M Gibbins[1], and Alice Pollitt[1]

[1.] *Institute for Cardiovascular and Metabolic Research (ICMR), School of Biological Sciences, University of Reading, Reading, UK*

E-mail: c.r.kempster@pgr.reading.ac.uk

Introduction: Platelets play a critical role at sites of vascular injury to prevent bleeding, yet hyper-responsive platelets can be causative to heart attacks and stroke. Understanding platelet molecular mechanisms is therefore important to identify novel drug targets and improved therapeutics. Since platelets lack a cell nucleus, conventional methods used to identify processes in nucleated cells cannot directly be applied.

Aims: This project aims to use biocompatible liposomes with a unique fusogenic nature to deliver cargo directly into the cytoplasm of platelets. This will enable biological processes and molecular mechanisms to be studied directly in human platelets.

Methods: Granule secretion was measured using P-selectin as a marker of platelet activation. Platelet adhesion and spreading on Fibrinogen was quantified using ImageJ. Annexin V binding was used as a measure of apoptotic platelets.

Results: Fluorescently labelled biocompatible fusogenic liposomes efficiently fuse with the membrane of human platelets. Spontaneous fusion with platelets does not lead to platelet activation, with no significant increase in P-selectin surface expression, or the induction of phosphatidylserine translocation to the outer platelet membrane as measured by Annexin V binding. Normal platelet behaviour is unaffected by liposome fusion as measured by agonist induced P-selectin exposure and platelet spreading.

Conclusions: These results demonstrate that fusogenic liposomes can label human platelets without impacting normal behaviour. This will enable fusogenic liposomes to be used as a method of delivering compounds into human platelets to interrogate both known and novel molecular mechanisms *in vitro*, and in real time.

PC52. Regulation of thrombus formation by the extracellular matrix in ruptured and eroded plaques

Amelia Drysdale,[1] Ryan Riley,[1] Dr Amanda Unsworth,[1] Dr Stephen White,[1] Dr Sarah Jones,[1]

[1] *Manchester Metropolitan University*

E-mail: amelia.drysdale@stu.mmu.ac.uk

Background: Atherothrombosis is a leading cause of mortality worldwide, and occurs following the rupture or erosion of an atherosclerotic plaque. Plaque components activate circulating platelets, initiating thrombosis. Recent studies have shown that components of the extracellular matrix differ in plaque rupture and erosion.

Aims: The purpose of this study was to investigate platelet responses to matrix proteins present in ruptured and eroded plaques.

Methods: Recombinant protein coatings representing the matrices relevant in plaque rupture and erosion were assessed under flow conditions and using flow cytometry.

Results: The main findings revealed differences in platelet adhesion and thrombus formation on the rupture and erosion matrices ($p<0.05$). Both collagen I and collagen III were observed to be the main drivers of thrombosis in each matrix respectively. Addition of biglycan or decorin to collagen I in the rupture model reduced thrombi number and area coverage under flow. Additionally, both matrix proteins only weakly increased platelet activation, and decorin was observed to reduce platelet fibrinogen binding. In contrast, adding versican or hyaluronan to collagen III in the erosion model increased thrombi number and area coverage. Versican, however, was observed to markedly reduce fibrinogen binding and P-Selectin exposure ($p<0.05$), suppressing platelet aggregation but not adhesion.

Conclusions: Differences in platelet responses to vascular proteoglycans may account for variations in thrombus formation observed following plaque rupture and erosion. Developing in vitro models that more closely represent atherothrombosis will enable assessment of the efficacy of both existing and novel antithrombotic drugs, informing a more personalised approach to treatment.

PC53. Alternating current (AC) susceptometry as a novel platform to monitor human blood clotting using magnetic nanoparticles

Alan GS Harper[1, 2], Antonio Santana-Otero[3], Daniel Ortega[3,4,5], Neil D Telling[2], David Cabrera[2]

[1] *School of Medicine, Keele University, Newcastle-under-Lyme, Staffordshire. ST5 5BG, UK.*

[2] *School of Pharmacy and Bioengineering, Keele University, Guy Hilton Research Centre, Thornburrow Drive, Hartshill, Stoke-on-Trent, ST4 7QB, UK*

[3] *iMdea Nanociencia, Campus Universitario de Cantoblanco. C/Faraday, 9, 28049, Madrid, Spain.*

[4] *Condensed Matter Physics department, Faculty of Sciences, Campus Universitario Río San Pedro s/n. 11510 Puerto Real (Cádiz) Spain*

[5] *Institute of Research and Innovation in Biomedical Sciences of the Province of Cádiz (INiBICA), University of Cádiz, 11002 Cádiz, Spain*

E-mail: d.c.cabrera@keele.ac.uk

Background: AC susceptometers measure an intrinsic property of magnetic materials called complex magnetic susceptibility. Susceptometry can reveal variations in mobility and agglomeration of particulate magnetic materials suspended in biological tissues such as human blood. By labelling human blood with magnetic nanoparticles, this technique could be exploited to monitor blood coagulation, as well as clot compaction and internal viscosity properties of clots in real-time.

Aims: To assess whether AC susceptometry can monitor in vitro blood clotting processes in real-time.

Methods: AC susceptibility measurements were performed in citrated human whole blood or poor platelet plasma (PPP) containing iron oxide nanoparticles (IONPs). After recalcifying and stimulation with adenosine diphosphate or thrombin, measurements in blood/PPP samples were run consecutively either in intervals of 10 minutes during a 90-minute recording over a broad-frequency range (60 Hz to 400 Hz), or acquiring discrete measurements at 100 Hz every 2 minutes for 30 minutes.

Results: An evident correlation between susceptibility and blood/PPP clotting over time was observed in initial experiments. Broad-frequency measurements revealed a shift in the peak of susceptibility to lower frequencies, which indicates an immobilisation and aggregation of IONPs within the forming clots. Discrete-point measurements showed a decrease in the magnetic susceptibility over time, consistent with a diminished mobility and aggregation of IONPs, and reduction in clot volume over time.

Conclusions: AC susceptometry could be used as a novel platform to better monitor changes in the physical structure of blood clots. This could result in novel susceptibility-based assays for diagnosis of bleeding disorders.

PC54. Potential opportunity to repurpose cancer drugs in clinical trial as a novel anti-platelet therapy.

Alistair J Marsden[1], David Riley[2], Francisco Rivero[2], Pedro Beltran-Alvarez[1]

[1]*Department of Biomedical Sciences, University of Hull, United Kingdom*
[2]*CAM, Hull York Medical School, University of Hull, United Kingdom*

E-mail: a.marsden-2014@hull.ac.uk

Background: Post-translational modification (PTM) of proteins helps to regulate protein function which in platelets can modulate the ability to aggregate and form a thrombus. Drugs targeting specific PTMs are currently undergoing clinical trials in the context of various disease state. By investigating the off-target effects of a class of these drugs we have the novel opportunity to repurpose a current drug therapy to use as a novel antiplatelet agent.

Aims: Establish the effect of a class of PTM inhibitor cancer drugs on platelets and the possibility to repurpose these as a novel antiplatelet agent.

Methods: Platelets were treated with the drug AJM001 to examine its effects on a range of functions incuding aggregation, spreading, secretion and integrin activation. Biochemistry techniques including IP and mass spectrometry were also used in an attempt to identify the mechanism.

Results: Treatment with AJM001 functionally inhibited aggregation over a period of 4h in the mid micro-molar range. Flow cytometry analysis of platelets incubated with an IC50 dose showed a 10% down-regulation in $\alpha_{IIb}\beta_3$ and GP1b as well as a significant reduction in active $\alpha_{IIb}\beta_3$. A proteomics data mining approach used to establish potential targets for PTM inhibitors analysing publicly available MSMS data (ProteomeXchange) has found components of the $\alpha_{IIb}\beta_3$ and GP1b signalling pathways to be modified at novel sites.

Conclusions: This novel method of inhibiting PTMs has proved to be an exciting new method of inhibiting platelet function *ex vivo*. Work so far suggests a potential mechanism involving $\alpha_{IIb}\beta_3$ however further study is still required.

PC55. Analysis of platelet populations following platelet activation and priming using dimensionality reduction and automated clustering tools

Alicia Veninga[1], Constance C.F.M.J. Baaten[1,2], Bibian M.E. Tullemans[1], Ilaria De Simone[1,3], Marijke J.E. Kuijpers[1,4], Johan W.M. Heemskerk[1], Paola E.J. van der Meijden[1,4]

[1] *Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, The Netherlands*
[2] *Institute for Molecular Cardiovascular Research (IMCAR), RWTH Aachen University, University Hospital Aachen, Germany*
[3] *Institute for Cardiovascular and Metabolic Research, University of Reading, Reading, UK*
[4] *Thrombosis Expertise Center, Heart and Vascular Center, Maastricht University Medical Center, Maastricht, The Netherlands*

E-mail: a.veninga@maastrichtuniversity.nl

Background: Platelets from healthy donors display heterogeneity in responsiveness to agonists. The activation treshold of platelets is controlled by multiple bioactive molecules, acting as negative or positive priming substances.

Aims: To analyse different platelet populations after exposure to agonists and primers in multicolour flow cytometry data using a workflow of dimensionality reduction and automated clustering tools.

Methods: Washed platelets were stimulated with CRP-XL, 2-MeSADP, or TRAP6 and labelled with anti-CD62P (Psel) and PAC1, plus anti-TLT1 and anti-CD42a mAbs. Negative primer adenosine or positive primer succinate were added before activation. Data were analysed using tSNE and FlowSOM in FlowJo V10 software.

Results: Platelet stimulation with all agonists resulted in a small single $PAC1^-/Psel^+$ population and larger single $PAC1^+/Psel^-$ population, while the double positive population increased with higher agonist doses. Addition of adenosine or succinate, respectively, decreased or increased the $PAC1^+/Psel^+$ population, while leaving the single positive populations unchanged. Automated analysis of multicolour flow cytometry data revealed that with maximum stimulation of CRP-XL or TRAP6; 75% of events were positive for all markers ($GPIX^+/PAC1^+/Psel^+/TLT1^+$), 14% had only activated integrins ($GPIX^+/PAC1^+/Psel^-/TLT1^-$), and 6-8% were inactive ($GPIX^+/PAC1^-/Psel^-/TLT1^-$). For ADP, the population distribution was 24%, 38%, and 35%, respectively. Strikingly, secretion-only platelet populations constituted <1% of all events.

Conclusions: The agonist type was determinative for the fractions of single positive platelets, while the stimulus strength and the presence of primers mainly changed the populations of double positive/negative platelets. Furthermore, clustering analysis confirmed that the stimulated platelets first activate their integrins before secreting their a-granules.

PC56. Smartphone-based detection of platelet activation in microfluidic strips

Daniel Hodge[1], Gemma Little[1], Alexander Edwards[2], Chris Jones[1]

[1]*School of Biological Sciences, University of Reading*

[2]*School of Pharmacy, University of Reading*

E-mail: d.j.hodge@pgr.reading.ac.uk

Background: To enable platelet function analysis in a wider range of settings, we used microfluidic technology coupled with standard smart-phone imaging, to develop a 'dip-stick' platelet function analyser capable of assessing platelet response to a range of agonists and anti-platelet drugs.

Aims: We aimed to demonstrate the device's capability of detecting platelet activation via ADP and thrombin stimulation in a reproducible manner.

Methods: 10-capillary Micro-Capillary Film (MCF) was coated internally with a hydrophilic polymer (PVOH) then loaded with ADP or thrombin. These capillaries were dipped into whole citrated blood for 30 seconds. Formation of platelet aggregates impeded capillary rise, which was imaged with a smart-phone camera. Height of capillary rise was then analysed in ImageJ.

Results: Capillaries coated with thrombin or ADP showed a concentration dependant reduction in capillary rise. Optimisation of the coating and loading procedures has allowed a highly reproducible test, with an average intra-strip CV value below 8%, and an average inter-strip CV value below 3%, with no significant variance across strip batches, indicating a robust manufacturing procedure. In strips loaded with ADP, mean inhibition of capillary rise was 16.1%, upon treatment of blood with cangrelor this decreased to 5.1% ($p<0.05$), indicating a reduction in platelet aggregation.

Conclusions: We have developed a proof-of-principle 'dip-stick' platelet function analyser with reproducible capillary rise, demonstrable dose-dependent reduction in capillary rise in the presence of platelet agonists which can be reversed by pathway specific inhibition of platelet function. This demonstrates the potential utility of this device as a viable platelet assay.

PC57. Development of a 3D tissue engineered neointimal model as an alternative to animal models of human atherosclerosis.

Jassim Echrish[1,2], Ying Yang[1], and Alan G.S. Harper[2]

[1]School of Pharmacy and Bioengineering
[2]School of Medicine, Keele University.

Email: j.h.j.echrish@keele.ac.uk

Background: Ischemic heart disease is the leading cause of death worldwide. Most cases are caused by atherosclerosis. Erosion of this plaque triggers unwanted blood clotting which blocks the blood supply to the heart, triggering heart attacks. This process is currently principally studied in animal models.

Aim: To create a tissue-engineered neointima as an alternative to current animal models and assess its effect on coagulation of human blood plasma.

Methods: THP-1 derived foam cells were cultured within collagen hydrogels and triggered to differentiate into foam cells by treatment with lipopolysaccharide, IFN-γ, and oxidised low-density lipoprotein. Prothrombin times were measured to assess the procoagulant activity of the tissue-engineered neointimal constructs using platelet poor plasma prepared from blood of healthy medication free volunteers. Tissue factor activity was measured using inactive factor VII and the fluorescent substrate SN-17.

Results: This novel *in vitro* neo-intima tissue can trigger rapid coagulation of human plasma due to the presence of significant tissue factor activity. Prothrombin times were 87.5 ± 8.4s, 158.3 ± 12.7, and 376.6 ± 28.1 for gels containing foams cell, M1 cells or no additional cells respectively ($n = 6$; $P < 0.05$). Collagen hydrogels containing THP-1 derived foam cells samples were found to have significantly greater tissue factor activity compared to cell-free hydrogels or M1 macrophage-containing hydrogels.

Conclusion: Our tissue-engineered neointimal model can recreate the pro-thrombotic potential of human atherosclerotic plaques. This could form the basis for a novel *in vitro* 3D model of human atherosclerosis to replace current animal models.

Posters from our Sponsors and The Platelet Society

PC58 – Stago UK

PC59 – Izon Science

PC60 – Platelet Society Summer School

Posters from our Oral Presenters

PC61 – Elisabetta Liverani - Sex-related variations in the response of anti-platelet drug therapies targeting purinergic signaling pathways in sepsis

PC62 – Jacob Ranjbar - Tissue-engineered human arteries replicate primary and secondary haemostatic functions seen in vivo: An alternative to mouse thrombosis models?

PC63 – Anadi Krishnan - Platelet transcriptome yields progressive and predictive markers for subtype-specific risk in myeloproliferative neoplasms

PC64 – Adela Constantinescu-Bercu - The importance of the GPIba intracellular tail in VWF-mediated platelet signalling events

PC65 – Daniel Howard - Generating large numbers of in vitro platelets from Human induced stem cell derived Megakaryocytes via a platelet extruding bioreactor.