BRITISH SOCIETY FOR HAEMOSTASIS & THROMBOSIS

UK PLATELET GROUP

Anticoagulation*in*Practice

Joint 2016 BSHT, AiP & UK Platelet Group Annual Meeting

Royal Armouries, Leeds

9th-11th November 2016

Welcome to the BSHT, AiP & UK Platelet Group Annual Meeting 2016

We would like to welcome you all to the first Haemostasis and Thrombosis meeting at the Royal Armouries, Leeds. Following on from the success of last year's joint meeting between Anticoagulation in Practice (AiP), and the British Society for Haemostasis and Thrombosis (BSHT) we are happy to be holding the second joint meeting between these societies. This year, we also welcome the UK Platelet group, who have had several successful joint meetings with the BSHT. We think we are appealing to the widest possible audience with interests in all things to do with bleeding and clotting, both basic science and clinical. We are expecting well over 100 delegates comprising laboratory scientists, nurses, doctors and pharmacists. On Wednesday and Friday there will be just single sessions. On Thursday, there will be parallel sessions.

Highlights include the opening session in which **Saskia Middeldorp** (University of Amsterdam, NL) *will speak on "Anticoagulant treatment of women with recurrent miscarriage."* and the presentations by the emerging research fellows. On Thursday we welcome a variety of invited international speakers including **Johan Heemskerk** (University of Maastricht, NL), **Alisa Wolberg** (UNC, Chapel Hill, USA) and **David Stegner** (University of Würzburg, Germany).

We hope that you enjoy the format of this meeting and we would be grateful for any feedback you have as to how this could be improved for future conferences. We would also value feedback on what you think has worked well. We hope that bringing together such a diverse group of professionals will provide an opportunity to develop new insights and perhaps new friendships. The social highlights are the poster and wine session on Wednesday evening and the Conference Dinner on Thursday.

On behalf of the organising committee we would like to acknowledge the hard work of everyone involved, in particular Amy Partleton from AiP and Jean Machin from BSHT: we hope you have a good conference.

Robert Ariëns	*Steve Watson*	*Nicky Fleming*
BSHT	***UK Platelet Group***	***AiP***

Programme

UK PLATELET GROUP

AnticoagulationinPractice

Wednesday pm, 9th November

12.00-13.20 Registration **(Churchill Suite)** & Lunch

13.20-13.30 Welcome & Introduction (Robert Ariëns)

13.30-15.15 **Plenary #1 & Oral Communications #1 (Bury Theatre)**
(Chairs – Henry Watson & Sue Pavord)

13.30 **Saskia Middeldorp** (University of Amsterdam, NL)
"Anticoagulant treatment of women with recurrent miscarriage."

14.15 **Mary Underwood** (Imperial College London)
ADAMTS13 antigen levels and IgG subclasses in patients with acquired immune TTP

14.30 **Thea Chandler** (University of Leeds)
Fibrin γ′ cross-linking by FXIII V34L: FXIII V34L normalises clustering of γ′ fibres

14.45 **Keziah Austin** (UCLH)
Venous thromboembolism in ambulatory patients with pancreatic, endometrial and colorectal cancer, and association with khorana score.

15.00 **Catherine Bagot** (Glasgow Royal Infirmary)
Laboratory markers of thrombotic risk are present very early in the 1st trimester of pregnancy

15.15-15.35 Tea & Coffee

15.35-18.00 **Plenary #2 & Emerging Fellows Session (Bury Theatre)**
(Chairs – Jim Crawley & Paul Harrison)

15.35 **Mike Laffan** (Imperial College London)
"Type 1 VWD, low VWF and changes with age - what do we tell patients?"

16.20 **Rens de Groot** (Imperial College London)
"ADAMTS13 and VWF: To cut a long molecule short"

16.45 **Tadbir Bariana** (UCL)
"NGS to identify novel variants in platelet and bleeding disorders"

17.10 **Alexandra Mazharian** (University of Birmingham)
"MAPKs and DUSPs: the Yin and Yang of platelet production"

17.35 **Ejaife Agbani** (University of Bristol)
"Platelets and coagulation: Controlling thrombosis by fluid entry"

18.00-19.30 Poster Viewing, Drinks Reception **(Royal Armouries Hall)**

Thursday am, 10th November

8.45-10.45 **Oral Communications #2 & Plenary #3 (Bury Theatre)**
(Chairs – Jon Gibbins & Yotis Senis)

8.45 **Marie-Blanche Onselaer** (University of Birmingham)
Binding of the D-dimer fragment of fibrin to GPVI

9.00 **Julie Rayes** (University of Birmingham)
CLEC-2 deficient platelets modulate the inflammatory response following toxin mediated sepsis in mice

9.15 **Anna Jongen** (University of Southampton)
Investigating platelet functional heterogeneity using droplet microfluidics

9.30 **Malou Zuidscherwoude** (University of Birmingham)
Platelet aggregation visualized by lightsheet microscopy

9.45 **Maryam Ahmed Aldossary** (University of Sheffield)
Use of whole exome sequencing to investigate patients with unexplained platelet bleeding disorders: Identification and characterization of a novel FLI1 variant in a patient with a dense granule secretion defect

10.00 **Johan Heemskerk** (University of Maastricht, NL)
"Integration of humoral and cellular blood components in thrombus formation."

10.45-11.15 Tea & Coffee

11.15-12.45 **Scientists in Training Symposium (Bury Theatre)**
(Chairs – Josefin Ahnström & Alastair Poole)

11.15 **Adela Constantinescu-Bercu** (Imperial College London)
VWF-mediated platelet 'priming' potentiates novel leukocyte interactions under flow

11.30 **Zeki Ilkan** (University of Leicester)
Role for the mechanosensitive ion channel Piezo1 in human platelet shear-dependent calcium entry and thrombus formation

11.45 **Victoria Ruiz De Miguel** (Imperial College London)
The effect of Thioredoxin-1 on VWF function

12.00 **Anastasis Petri** (Imperial College London)
The role of ADAMTS13 exosites in VWF recognition and proteolysis

12.15 **Gael Morrow** (University of Aberdeen)
Fibrinolytic proteins are retained on the surface of phosphatidylserine exposing platelets in a protruding 'cap'

12.30 **A Sabban** (University of Leeds)
Characterisation of the erythrocyte binding site on fibrinogen

12.45-14.00 BSHT General Meeting - Members only **(Bury Theatre)**
Lunch (Royal Armouries Hall)

13.00-14.00 **Bayer Symposium (Wellington Suite)**
"From large-scale study to larger-scale practice: What does the real world evidence for the non-VKA oral anticoagulant tell us?"

Thursday pm, 10th November

14.00-16.00 **Plenary #4 & Oral Communications #3 (Bury Theatre)**
(Chairs – Robert Ariëns & Nikki Mutch)

14.00 **Alisa Wolberg** (UNC, Chapel Hill, USA)
"Role of RBC and FXIII in VTE."

14.45 **Salvatore Santamaria** (Imperial College London)
Molecular basis of FV/protein S enhancement of the inhibition of FXa by TFPI

15.00 **Claire Whyte** (University of Aberdeen)
Opposing effects of polyphosphate in modulation of tPA and uPA-mediated fibrinolysis

15.15 **Magdalena Gierula** (Imperial College London)
Activated FV in synergy with protein S enhances APC association to phospholipids

15.30 **Cedric Duval** (University of Leeds)
Cross-linking of α2-AP into fibrin by FXIII: Evidence for a second FXIII cross-linking site

15.45 **Clare Wilson** (University of Leeds)
Elucidating the fibrinogen-FXIII binding site by surface plasmon resonance

16.00-16.30 Tea & Coffee

16.30-17.15 **Plenary #5 (Bury Theatre)**
(Chairs – Helen Philippou & Khalid Naseem)

16.30 **David Stegner** (University of Würzburg, Germany)
"Light sheet microscopy of thrombus formation."

19.00-22.30 Reception and Conference Dinner
(War Gallery, Royal Armouries Leeds)

Thursday am, 10th November (Wellington Suite)

8.30	Tea & Coffee
8.45	Welcome and Introduction
9.00	**Mrs Patricia Apenteng** *"VTEC - an observational study to determine the incidence of VTE among care home residents"*
9.30	**Prof David Fitzmaurice** *"Anticoagulation treatment patterns in atrial fibrillation, data from the GARFIELD registry"*
10.00	**Mr Andi Orlowski** *"AF service transformation using NHS (big) data"*
10.35	**Dr Nicky Fleming** *"Innovation; the future of personalised medicine - where are we now with genomics and anticoagulation?"*
10.45	Break & Exhibition
11.15	**Mrs Eve Knight & Mrs Diane Eaton** *"Ask the patient!"*
12.00	**Expert panel** Case studies
12.45	Lunch & Exhibition
14.00	**Dr Matt Fay** "New technologies for AF screening"
14.45	**Dr Rhona MacLean** "Improving patient safety around new orals"
15.30	**Dr Will Lester** "Complex anticoagulation issues"
16.00	Close - Ellen Murray Bursary Award
19.00	Reception & Conference Dinner **(War Gallery, Royal Armouries Leeds)**

Friday am, 11th November

8.30-10.15 **Oral Communications #4 & Plenary #6 (Bury Theatre)**
(Chairs – Anne Goodeve & Chris Gardiner)

8.30 **Paraskevi Untiveros** (University of Aberdeen)
Thrombi from anaemic patients are resistant to fibrinolysis

8.45 **Fraser Macrae** (University of Leeds)
(Patho)Physiological fibrinogen γ′ levels have functional effects on clot structure

9.00 **Cedric Duval** (University of Leeds)
Murine FXIII-A Val34 and Leu34 variants present similar functional characteristics as that of their human counterparts

9.15 **Scott Dos Santos** (University of Surrey)
Local coagulopathy in the skin lesions of Buruli ulcer patients: Enhanced fibrin deposition around individual vessels is associated with thrombomodulin depletion, but not platelet adherence

9.30 **Neil Morgan** (University of Birmingham)
"Identification of novel mutations in thrombocytopenia."

10.15-10.30 Tea & Coffee

(Please note there is a Remembrance Day Service in the Royal Armouries Leeds that will prevent access to/from the lecture theatre from 10-35am until 11-30am)

10.30-12.45 **Plenary #7 & Oral Communications #5 (Bury Theatre)**
(Chairs – Catherine Bagot & Rhona Maclean)

10.30 **David Fitzmaurice** (University of Warwick)
"Learning from history, planning for the future"

11.15 **Tom McKinnon** (Imperial College London)
The importance of free thiols for proper VWF function under shear stress

11.30 **Tom McKinnon** (Imperial College London)
The role of N-linked glycosylation and sialic acid in modulating ADAMTS13 proteolytic activity

11.45 **R. Al Ghaithi** (University of Birmingham)
Evaluation of the total thrombus-formation system (T-TAS): Application to human and mouse blood analysis

12.00 **Federico Zerbinato** (University of Aberdeen)
Platelet-derived microparticles as biomarkers of brain microvascular ageing and dementia

12.15 **Deepa Arachchillage** (Imperial College London)
Efficacy and safety of prothrombin complex concentrate in patients undergoing major cardiac surgery

12.30 **Polly Bowman** (University of Leeds)
Clot structure is affected by granulocyte clone size and Eculizumab in patients with paroxysmal nocturnal haemoglobinuria

12.45-13.00 Concluding Words & Farewell (Robert Ariëns)

13.00 Lunch & Depart

The venue: Set in a modern waterfront development, close to Leeds city centre, New Dock is home to the Royal Armouries Museum and NEW DOCK Hall providing an idyllic setting for this year's meeting. and 850 Hotel bedrooms within 5 minutes' walk.

Registration and information desk: The desk will be open from 08:30 on both days for registration, please ask a member of the conference team if you require assistance at any other time.

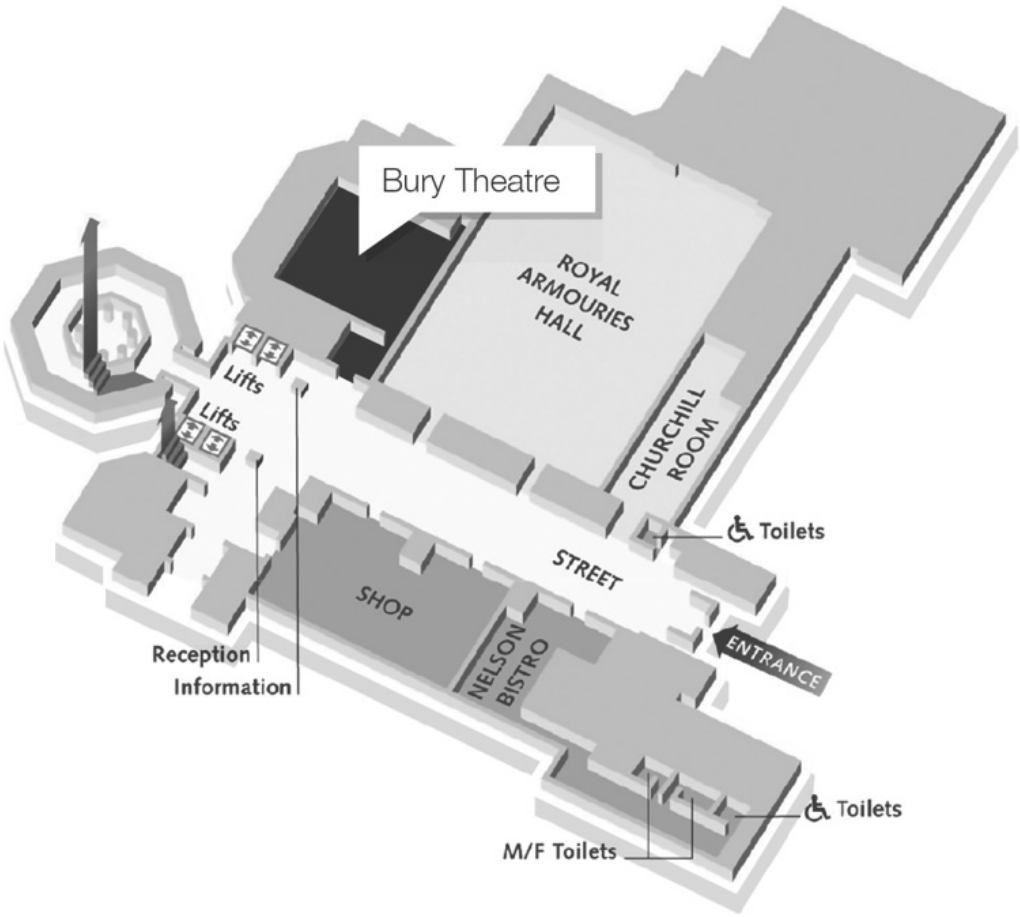
Parking: On site there is a multi-storey car park for 1,650 vehicles

Wi-fi access: Free wireless internet is available throughout the centre, this can be accessed by entering your e-mail address. Each login will provide access for 4 hours, after which you will need to login again. Please ask centre staff if you have any problems setting this up.

Conference rooms and dining arrangements: Please refer to the floor plan of the conference centre below, which gives details of the rooms being used and how to reach them. The programme lists the location of all of the talks and sessions.

Conference Dinner and Drinks Reception: Our drinks reception the conference dinner will take place at the War Gallerry at the Royal Armouries. Please speak to Amy Partleton or Jean Machin should you wish to purchase a ticket for the conference dinner in the evening of Thursday 10th November

Certificates of attendance: Certificates of Attendance can be requested from Jean Machin and Amy Partleton.



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The BSHT, AiP and UK Platelet Group are indebted to our sponsors who, through their support, make this meeting possible. Please do take time to visit their stands

Abstracts
Wednesday afternoon

14.15

ADAMTS13 ANTIGEN LEVELS AND IgG SUBCLASSES IN PATIENTS WITH ACQUIRED IMMUNE THROMBOTIC THROMBOCYTOPENIC PURPURA Mary Underwood[1], Mari Thomas[2], Marie Scully[2], James Crawley[1] [1]Centre for Haematology, Imperial College London, Hammersmith Hospital Campus, Du Cane Road, London W12 0NN, UK. [2]Department of Haematology University College Hospital, London NW1 2BU, UK

B**ackground** Patients with a deficiency in ADAMTS13 develop thrombotic thrombocytopenic purpura (TTP), which is associated with life-threatening microvascular thrombosis. Acquired, immune mediated TTP (iTTP) accounts for ~95% of cases, characterised by the development of IgG autoantibodies. We recently found in a group of 92 iTTP patients that the inhibitory effect of anti-ADAMTS13 IgG was insufficient to account for severe ADAMTS13 deficiency in patients. Rather, as ADAMTS13 antigen was severely reduced in 92% of these patients (<25%), ADAMTS13 depletion is likely a major pathogenic mechanism in iTTP. The effector function of IgG antibodies is associated with their IgG subclass, with IgG1 and IgG3 associated with an increased ability to promote phagocytosis compared to IgG2 and IgG4. **Aims** To investigate the relationship between IgG subclass and ADAMTS13 antigen levels in patients with iTTP. **Methods** The IgG subclass of iTTP patient antibodies against full length ADAMTS13 and ADAMTS13 domain fragments was measured using novel ELISAs. Domain specificity and IgG subclass data were correlated with ADAMTS13 antigen levels at presentation.**Results** Our results revealed that iTTP patients with lower ADAMTS13 antigen levels (<10%) had higher proportions of anti-ADAMTS13 IgG1 and 3 (>50%). In contrast, in those patients with higher ADAMTS13 antigen levels (>10%) the proportion of anti-ADAMTS13 IgG2 and IgG4 levels were higher (>50%). **Summary/conclusions** Our preliminary results suggest that higher ADAMTS13 antigen levels are associated with higher IgG4 proportions, which has little/no ability to promote antigen clearance. We also found that lower ADAMTS13 antigen levels are associated with higher proportions of anti-ADAMTS13 IgG1 and IgG3. These subclasses have an increased ability to promote phagocytosis and, therefore, antigen clearance. Given the marked reduced ADAMTS13 antigen levels in iTTP patients, anti-ADAMTS13 IgG1 and IgG3 subclasses may be particularly pathogenic and an important marker of ADAMTS13 antigen clearance.

14.30

FIBRIN γ' CROSS-LINKING BY FXIII VAL34LEU: VAL34LEU NORMALISES CLUSTERING OF γ' FIBRES

Chandler T., Duval C., McPherson H.R., Ariëns R.A.S. Thrombosis and Tissue Repair Group; Division of Cardiovascular and Diabetes Research; Leeds Institute of Cardiovascular And Metabolic Medicine; School of Medicine; University of Leeds; UK

Background Fibrinogen and FXIII variants alter the structure of the clot. Val34Leu is a FXIII polymorphism that increases FXIII activation rates and the density of the clot, but paradoxically decreases cardiovascular risk. Fibrinogen γ' is a splice variant that interferes with fibrin polymerisation, produces fibre clusters within the network, and increases cardiovascular risk. The combined effects of these variants is unknown and of interest, particularly in view of the close interaction between fibrinogen γ' and FXIII.
Aims The aim of this project was to investigate how combinations of γ' fibrinogen and FXIII Val34Leu impact clot structure and function, and whether interactions with γ' may contribute to Val34Leu's reported cardioprotective effect.
Methods Recombinant Val34 and Leu34 FXIII variants were expressed in E.coli. Fibrinogen γA/γA and γA/γ' were purified from plasma, and recombinant fibrinogen γ'/γ' was expressed in CHO cells. Purity and degradation was confirmed through SDS-PAGE. FXIII activation rates were analysed by pentylamine-biotin incorporation and clot formation by turbidity. Clot structure was investigated through Confocal Microscopy (CM) and Scanning Electron Microscopy (SEM).
Results FXIII activation was increased by 21-25% for 34Leu in comparison to 34Val. With the addition of γ' (γA/γ' and γ'/γ'), the activation of FXIII decreased in a dose-dependent manner. Incorporation of γ' into the fibrin network resulted in clustering of fibrils, visualised with CM and SEM, which increased from heterodimer (γ'/γA) to

homodimer (γ′/γ′). Comparison across FXIII clots showed normalisation of γ′ clot structure and reduced clustering of γ′ fibres in the presence of 34Leu FXIII.

Conclusions The effects of γ′ and Val34Leu on FXIII activation by thrombin appear independent and additive. The normalisation of γ′ fibrin fibre clustering by 34Leu FXIII may provide a plausible mechanism to explain a cardioprotective effect of this FXIII variant.

Acknowledgements: T Chandler was supported by a Summer Research Studentship from the BSHT

14.45

NOVEL RGD PEPTIDOMIMETIC COMPOUNDS FOR ANTITHROMBOTIC DRUG DISCOVERY Maria Demetriou, Isobel Ford, Monica Piras, Ian Fleming, Matteo Zanda. Institute of Medical Sciences, School of Medicine, Medical Sciences and Nutrition, University of Aberdeen, Scotland

Background Synthetic Arg-Gly-Asp (RGD)-mimetic compounds may offer potential for selective modification of thrombotic and haemostatic mechanisms. Altering structure and conformation leads to differential affinities and specificities for receptors.

Aims To test two novel synthetic compounds for effects on various aspects of platelet prothrombotic activity.

Methods Compounds were prepared *via* a convergent synthetic approach. Building blocks mimicking the R (Arginine) and D (Aspartate) amino acids were equipped with an azide and an alkyne function, respectively, and then assembled *via* copper catalyzed cyclo-addition to give the triazole based RGD peptidomimetic scaffold. Two compounds with increased binding affinity for $alpha_{IIb}$-$beta_3$ compared with $alpha_v$-$beta_3$, were selected for functional testing in blood from healthy donors not taking antiplatelet drugs. Four techniques were used: Light transmission aggregometry (LTA); whole blood impedance aggregometry on Multiplate analyser; fibrinogen binding and P-selectin expression by flow cytometry; thromboelastometry on ROTEM with Extem and Fibtem assays.

Results Compound ZMPZAT36 had a more potent effect on each platelet function than ZMPZAT56 in all experiments. Inhibition of ADP-stimulated aggregation was achieved after incubation for 1 min, although incubation for 15 min was allowed for equilibration in most experiments. Dose-response curves were established. At a final concentration of 22μM, ZMPZAT36 inhibited ADP-induced platelet aggregation in PRP by 80%, and in whole blood by 90%. ZMPZAT56 induced at least 45% inhibition at 44μM. Fibrinogen binding to platelets was inhibited by lower concentrations of ZMPZAT36 –50% inhibition at 0.5μM. Inhibition of P-selectin expression was more variable among donors. The ZMPZAT36 molecule also prolonged clotting time and decreased maximum clot firmness, whereas ZMPZAT56 was associated with slightly accelerated clotting.

Summary/Conclusions Further work is required to relate these differential functional activities to structural changes. Optimisation of conformation is required to result in the ideal interaction of the molecules with the target receptor and subsequent modification of platelet function.

15.00

LABORATORY MARKERS OF THROMBOTIC RISK ARE PRESENT VERY EARLY IN THE FIRST TRIMESTER OF PREGNANCY Catherine N. Bagot[1], Emma Leishman[1], Christopher C Onyiaodike[2], Fiona Jordan[2], Dilys J Freeman[2]. [1] Department of Haematology, Glasgow Royal Infirmary, UK; [2] Institute of Cardiovascular and Medical Sciences, University of Glasgow, UK

Background Pregnant women are at increased risk of venous thrombosis compared to their non pregnant counterparts. Epidemiological data suggests that hypercoagulability begins in the first trimester but it is unknown exactly how early in pregnancy this develops.

Aims How early in pregnancy do laboratory features of hypercoagulability develop?

Methods Plasma samples were taken just prior to conception and five times in early pregnancy, up to Day 59 gestation, from 22 women undergoing natural cycle *in vitro* fertilization, who subsequently gave birth at term following a normal pregnancy. Thrombin generation (Calibrated Automated Thrombography method, using 1pM tissue factor + thrombomodulin), free Protein S, Ddimer, Fibrinogen and factor VIII were measured. To counter inter-individual variability, change in coagulation factor levels between the pre-pregnant and pregnant state was measured over time.

Results The mean change in peak thrombin, Endogenous Thrombin Potential (ETP) and Velocity Index (VI) significantly increased from pre-pregnancy to 32 days gestation (p=0.0006, 0.0017, 0.0034 respectively) and persisted to Day

59 gestation ($p<0.0001$ for all). Fibrinogen and free Protein S demonstrated a significant increase and decrease respectively from pre-pregnancy to 32 days gestation ($p<0.0001$, $p=0.0096$), persisting to Day 59 gestation. Ddimer and VIII significantly increased from pre-pregnancy levels by 59 days gestation ($p<0.0001$ for both parameters). All 4 coagulation factors correlated significantly with ETP, peak and velocity index (Protein S negative correlation [$p<0.0001$]; VIII, fibrinogen, Ddimer positive correlation [$p<0.001$ for all]).

Summary/Conclusions Our work is the first to demonstrate that the prothrombotic state of pregnancy develops very early in the first trimester. Well recognized features of hypercoagulability in pregnancy (a decrease in Protein S and an increase in VIII, fibrinogen and Ddimer) correlated strongly with thrombin generation providing further evidence that thrombin generation is a marker of the prothrombotic state. Pregnant women at high thrombotic risk should commence thromboprophylaxis as early as possible in pregnancy.

Thursday morning

8.45

BINDING OF THE D-DIMER FRAGMENT OF FIBRIN TO GPVI

Marie-Blanche Onselaer[1], Clare Wilson[2], Jeanette L.C. Miller[3], Stephanie K. Watson[1], Helen Philippou[2], Andrew B. Herr[3], Robert A.S. Ariëns[2] and Steve P. Watson[1]. [1]Centre for Cardiovascular Sciences, Institute for Biomedical Research, College of Medical and Dental Sciences, University of Birmingham, Birmingham, UK. [2]Thrombosis and Tissue Repair Group. Institute of Cardiovascular and Metabolic Medicine. University of Leeds, Leeds, UK. [3]Division of Immunobiology, Center for Systems Immunology, & Division of Infectious Diseases, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA.

Background: We demonstrated recently that fibrin, but not fibrinogen, binds to and activates the platelet collagen receptor glycoprotein VI (GPVI). Thus, GPVI plays a dual role in haemostasis mediating initiation of adhesion and aggregation through collagen and propagation of aggregation through fibrin.
Aim: The aim of this study is to map the site of interaction between GPVI and fibrin.
Methods: Binding of GPVI to fibrin(ogen) and their fragments was measured by an adapted version of a soluble GPVI ELISA and by Surface Plasmon Resonance (2). Platelet spreading from mice was imaged on a Zeiss Axiovert 200M microscope. Human platelet aggregation and secretion was analysed with Chrono-log 700.
Results: We have shown that mice platelets spread on fibrin but not on fibrinogen. To map the binding site we used fragments of fibrin derived from isoforms of fibrinogen and on fibrin-derived fragment. Spreading was observed on fibrin derived from fibrinogen γA/γA, γA/γ' or γ'/γ' isoforms, and on D-dimer, but not on D- or E-fragments. Spreading induced by D-dimer was abolished in GPVI-deficient mouse platelets. D-dimer inhibited aggregation induced by collagen with a threshold effect at 10 µg/ml. Using SPR, we demonstrate that fibrin binds selectively to monomeric over dimeric GPVI with nanomolar affinity.
Conclusions: The present results localise the site of interaction of GPVI to fibrin D-dimer but not to the fibrinogen D-fragment, suggesting the key binding site for GPVI lies within a cryptic site exposed upon conformational change that occurs during conversion of fibrinogen to cross-linked fibrin. Physiologically, the high levels of D-dimer observed in disease may impair platelet activation by collagen and give rise to a mild bleeding diathesis. The observation that fibrin binds selectively to monomeric GPVI is in contrast to collagen which binds selectively to dimeric GPVI and suggests the involvement of distinct conformations of the Ig-like receptor.

9.00

CLEC-2 DEFICIENT PLATELETS MODULATE THE INFLAMMATORY RESPONSE FOLLOWING TOXIN-MEDIATED SEPSIS IN MICE Julie Rayes, Surasak Wichaiyo, Sian Lax, Steve P. Watson
Centre for Cardiovascular Sciences, Institute of Biomedical Research, The Medical School, University of Birmingham, Birmingham B152TT, UK.

Background: Platelets play multiple roles during infection and inflammation. Most notably they regulate the inflammatory reaction through cross-talk with immune cells and the endothelium, as well as secreting inflammatory mediators and chemokines. However, excessive platelet activation can lead to thrombosis and organ failure in patients with septic shock, with platelet depletion increasing mortality. The molecular mechanism of how this balance is controlled however remains unclear.
The platelet ITAM receptors, CLEC-2 and GPVI, have been shown to modulate bleeding and the inflammatory response in mouse models of immune complex-mediated inflammation.
Aim: The aim of this study is to investigate the role of the ITAM receptors using a mouse model of LPS-mediated endotoxemia.
Methods: Wild-type (WT) mice and platelet specific CLEC-2-deficient mice (CLEC-$2^{fl/fl}$PF4.Cre) were intraperitoneally injected with LPS 10mg/Kg for 8 hours. Liver and kidney function were assessed by measuring alanine transaminase (ALT), creatinine and blood urea nitrogen (BUN) in the serum. Clinical severity was assessed by our in-house welfare score.
Results: In WT mice, LPS-induced endotoxemia resulted in a mild decrease in liver function as measured by an increase in serum ALT levels 8 hours post injection compared to unchallenged mice. No significant change in kidney filtration was observed as measured by serum creatinine and BUN. Kidney filtration and liver function were decreased in CLEC-$2^{fl/fl}$PF4.Cre mice compared to

unchallenged mice as measured by creatinine, BUN and ALT. The decrease in liver and kidney function were associated with an increase in leukocyte infiltration in the liver and fibrin generation in the vessels of both tissues, as assessed histologically. Moreover, CLEC-$2^{fl/fl}$PF4.Cre mice presented with a pronounced increase in clinical severity 8 hours following LPS injection.

Conclusion: CLEC-2 expressed on platelets plays a protective role in mouse models of LPS-mediated inflammation. CLEC-2 deletion alters liver and kidney function by increasing leukocytes infiltration and tissue damage.

This work was supported by the British Heart Foundation

9.15

INVESTIGATING PLATELET FUNCTIONAL HETEROGENEITY USING DROPLET MICROFLUIDICS

Maaike Sybilla Anna Jongen[1], Nicola Englyst[2], Jonathan James West[1]. [1]Institute for Life Sciences, University of Southampton, Room 2111, Building 85, Highfield Campus, Southampton, SO17 1BJ. [2]Institute of Developmental Sciences, University of Southampton, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD

Background Platelets have long since been known to be heterogeneous in size, volume and density. Functional heterogeneity has been suggested in several studies, however, there are no methods currently available that can study platelets on a single cell level. Research on a single cell level is needed to study pure intrinsic heterogeneity without the influence of adjacent cells. Such a method should provide a high throughput way of studying platelets in isolation, to be able to detect potentially rare phenotypes, without interfering with normal platelet function. To this effect, this study implements a droplet microfluidics approach to study single platelet functionality.

Aims To develop a methodology for high throughput single platelet function analysis.

Methods The response of single platelets to an agonist is investigated by adding the agonist during encapsulation in droplets. After incubation within the droplets, the platelets are extracted from the droplets into fixative and analysed by flow cytometry. PAC-1 and anti-CD62P antibodies are used to detect platelet activation, while anti-CD42b is used to confirm platelet identity.

Results Platelets are individually encapsulated in monodisperse (CV of 1-4%) water-in-oil droplets with a mean diameter of 30 µm. Droplets are produced with a throughput of 4 kHz, with droplets containing a single platelet produced at a rate of 0.25 kHz (following a Poisson distribution). No platelet activation is observed that originates from microfluidic transport through the device, but can be observed with stimulating concentrations of the agonist convulxin. With this approach an intrinsic variation in the platelet response to convulxin is observed, that is unrelated to platelet size.

Conclusion A high throughput approach based on single platelet analysis has been developed to study intrinsic platelet functional heterogeneity in response to agonists.

9.30

PLATELET AGGREGATION VISUALIZED BY LIGHTSHEET MICROSCOPY

Malou Zuidscherwoude[1], Benjamin T. Atkinson[2], Steven G. Thomas[1] and Steve P. Watson[1]. 1. University of Birmingham, Institute of Cardiovascular Sciences, College of Medical and Dental Sciences, Edgbaston, Birmingham B15 2TT, UK. 2. Intelligent Imaging Innovations, Inc. Denver, CO 80216, USA.

Background: Recent studies have shown that platelet aggregates are heterogeneous structures comprised of regions with platelets at different stages of activation. However, current microscopy techniques are limited in allowing us to visualise individual platelets within these regions. Advances in lightsheet microscopy, which provides high spatial and temporal resolution combined with low photo-toxicity, allow us to study dynamic changes in the structure of platelet aggregates to understand how these different regions contribute to aggregate stability.

Aims: To visualize the platelet aggregate architecture using dual-view inverted selective plane illumination microscopy (diSPIM), which allows for imaging with isotropic resolution, and to compare this to existing widefield techniques.

Methods: Human washed platelets were stained with lipid dye $DiIC_{18}(5)$ (DiD). Platelet aggregates were formed on collagen in a Born aggregometer at 1200 rpm and imaged via diSPIM. The aggregates were visualized using two perpendicular lightsheets and the final image was reconstructed in 3D using information from both angles. The poor axial resolution in each angle was compensated for by the better lateral resolution

from the other angle, resulting in isotropic resolution. The ability of diSPIM to visualise differences in aggregate structure was determined by treating platelets with cytochalasin D which modifies platelet appearance in the aggregate.

Results: With diSPIM we were able to visualize individual platelets within all regions of the aggregate with a significant increase in resolution in both X, Y and Z, compared to conventional widefield microscopy. Untreated platelets were densely packed within aggregates, however, treatment of platelets with cytochalasin D resulted in the formation of loose aggregates with platelets only attached to the collagen fibres. Current work is focusing on imaging platelet aggregates formed under flow to establish the temporal resolution of the system.

Summary/Conclusions: diSPIM is a promising tool for studying the structure and heterogeneity of platelets within aggregates in 4D.

9.45

USE OF WHOLE EXOME SEQUENCING TO INVESTIGATE PATIENTS WITH UNEXPLAINED PLATELET BLEEDING DISORDERS; IDENTIFICATION AND CHARACTERIZATION OF A NOVEL *FLI1* VARIANT IN A PATIENT WITH A DENSE GRANULE SECRETION DEFECT

Maryam Ahmed Aldossary[1], Vincenzo C. Leo[1], Mike Makris[1], Neil V. Morgan[2], Martina E. Daly[1]
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Background Inherited qualitative and/or quantitative platelet abnormalities are associated with mucocutaneous bleeding symptoms. Diagnosis is based on the phenotypic and clinical features of affected patients and the genetic defects remain unknown in most cases. We have investigated the use of whole exome sequencing (WES) to identify candidate gene defects in patients with a history of mucocutaneous bleeding whose platelets demonstrated defects in agonist-induced dense granule secretion.

Aims To investigate the hypothesis that platelet dense granule secretion defects are due to inheritance of one or more alterations in genes involved in platelet granule biogenesis and secretion through the characterisation of candidate disease-causing variants identified in patients with platelet secretion defects.

Methods WES was undertaken for 22 index cases enrolled in the UK Genotyping and Phenotyping of Platelets (GAPP) study, with a history of excessive bleeding and reduced dense granule secretion, and a range of computational bioinformatic tools was used to predict the pathogenicity of candidate defects identified.

Results WES identified a median of 24,705 (range: 11,768-27,715) sequence variants per index case, with a median of 99 (range: 46-175) variants, affecting a total of 1,476 genes, being predicted to be pathogenic per index case. A novel *FLI1* alteration, predicting a p.Arg340Cys substitution in the transcription factor FLI1, was investigated further. Reporter gene studies showed that the R340C substitution led to a loss of transcriptional activity of FLI1 ($p<0.0001$). The R340C variant also demonstrated reduced nuclear accumulation in human embryonic kidney cells.

Summary WES has highlighted the genetic complexity of platelet bleeding disorders and identified plausible candidate gene defects for further investigation. Our findings suggest that the p.Arg340Cys substitution in FLI1 causes a loss of function of FLI1, a transcription factor that is essential for megakaryopoiesis. Further work will be required to explain the reduced platelet granule secretion associated with this defect.

Scientists in training symposium. Thursday

11.15

VWF-MEDIATED PLATELET 'PRIMING' POTENTIATES NOVEL LEUKOCYTE INTERACTIONS UNDER FLOW Adela Constantinescu-Bercu, Isabelle I Salles-Crawley, Kevin J Woollard, James TB Crawley. Imperial College London, Hammersmith Hospital Campus, Du Cane Road, London

Background. Platelet-leukocyte interactions are important in certain pathophysiological conditions, including atherosclerosis, infection and deep vein thrombosis. Previously characterised interactions between these cells (e.g. via P-selectin-PSGL-1, GPIb-$\alpha_M\beta_2$ or CD40-CD40L interactions) require the platelets and/or leukocytes to be fully activated. However, more recent studies have suggested that von Willebrand Factor (VWF)-bound platelets may be capable of binding leukocytes *in vivo*. We hypothesised that the VWF A1 domain-GPIbα interaction 'primes' platelets under flow and promotes leukocyte binding.

Methods. His-tagged VWF A1 domain (A1) and an A1 domain mutant (Y1271C/C1272R) with enhanced GPIbα affinity (termed A1*) were purified and captured onto flow channels. Fluorescently-labelled whole blood or plasma-free blood was perfused over these surfaces at defined shear rates ($100s^{-1}$ to $1000s^{-1}$). Platelet binding and leukocyte binding were monitored in real time and quantified.

Results. At $1000s^{-1}$, platelets rolled ~10-fold faster on VWF A1 than on A1*, reflecting the 10-fold higher affinity of A1* for GPIbα. Capture of platelets under flow through the A1/A1*-GPIbα interaction 'primed' platelets by inducing rapid transient releases of intracellular Ca^{2+}, and activation of $\alpha_{IIb}\beta_3$ that promoted platelet aggregation, but did not cause P-selectin exposure or granule release. Aggregation was inhibited by Src kinase inhibition, $\alpha_{IIb}\beta_3$ blockade or in plasma-free blood (i.e. in the absence of fibrinogen). VWF A1/A1*-'primed' platelets captured leukocytes under low shear conditions ($50s^{-1}$). Leukocyte spreading was also observed, implying that these leukocyte-platelet interactions could lead to leukocyte polarisation/activation. Leukocyte binding was not influenced by P-selectin blockade, but was completely inhibited using $\alpha_{IIb}\beta_3$ blockers, potentially suggesting a role of 'outside-in' signalling. However, this contention was discounted as leukocyte binding to 'primed' platelets was increased 3- to 5-fold in the absence of fibrinogen (plasma-free blood). Addition of fibrinogen to plasma-free blood induced a concentration-dependent decrease in leukocyte binding. These results may suggest that leukocytes and fibrinogen compete for binding 'primed' platelets, possibly via a direct interaction with activated $\alpha_{IIb}\beta_3$.

Summary/Conclusions. A1-GPIbα under flow 'primes' platelets in the absence of other physiological agonists, leading to the activation of $\alpha_{IIb}\beta_3$ and their cross-talk with leukocytes. Our results suggest a potentially novel interaction between platelets and leukocytes involving activated $\alpha_{IIb}\beta_3$.

11.30

ROLE FOR THE MECHANOSENSITIVE ION CHANNEL PIEZO1 IN HUMAN PLATELET SHEAR-DEPENDENT CALCIUM ENTRY AND THROMBUS FORMATION Zeki Ilkan[1]; Joy R. Wright[1,2]; Alison H. Goodall[2]; Chris I. Jones[3]; Martyn P. Mahaut-Smith[1] [1]Department of Molecular and Cell Biology and [2]Department of Cardiovascular Sciences, University of Leicester, Leicester, UK; [3]School of Biological Sciences, University of Reading, Reading, UK

Background Increases in intracellular Ca^{2+} ($[Ca^{2+}]_i$) are essential for platelet function. Despite the established role of shear stress in haemostasis and thrombosis, it remains unknown whether mechanosensitive (MS) Ca^{2+}-permeable ion channels contribute to platelet activation.

Aims To characterise and investigate the role of MS cation channels in platelets.

Methods Confocal microscopy was used to study $[Ca^{2+}]_i$ responses in single platelets and Meg-01 cells loaded with Fluo-3. Cells attached to coverslips via poly-D-lysine or PECAM1 antibody were exposed to saline at arterial shear rates in flow chambers. MS channel function was explored using the MS channel blocker GsMTx-4 and the Piezo1 agonist Yoda1. Thrombus formation on collagen was studied using 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$)-stained platelets in a flow chamber and aggregometry was assessed by light transmission. The effect of GsMTx-4 and Yoda1 on various Ca^{2+} entry pathways was also examined using Fura-2-loaded

platelet suspensions. Piezo1 expression was assessed by qRT-PCR and Western blotting.

Results Arterial shear stress at 1002.6s^{-1} induced an increase in $[Ca^{2+}]_i$ in the form of a single response (Meg-01: F/F_0 from 1 to 1.10±0.03, n=53, P<0.05) or an enhanced frequency of Ca^{2+} transients (platelets: F/F_0 integral for 4min (F/F_0.4min) from 0.36±0.09 to 1.92±0.27, n=6, P<0.0001). 2.5μM GsMTx-4 inhibited these responses: F/F_0 and F/F_0 integral were reduced to 1.02±0.04 in Meg-01 (n=45, P<0.0001) and 0.61±0.14 in platelets (n=3, P<0.001), respectively. Moreover, 25μM Yoda1 potentiated Ca^{2+} transients in platelets both in presence (by ~1.7-fold) and absence (by ~3-fold) of shear stress (n=4, P<0.01). GsMTx-4 pre-treatment reduced thrombus volume by ~50%, however, did not inhibit collagen-induced aggregation. These GsMTx-4-sensitive shear-evoked responses were independent of P2X1, Orai1 and TRPC6. Piezo1 mRNA and protein were detected in both Meg-01s and platelets.

Summary/Conclusions We provide evidence that functional MS Piezo1 channels are expressed in platelets and can contribute to Ca^{2+} signalling and thrombus formation.

This work was funded by a Medical Research Council Doctoral Training Award and the British Heart Foundation (PG/11/56).

11.45

THE EFFECT OF THIOREDOXIN-1 ON VON WILLEBRAND FACTOR FUNCTION Victoria Ruiz De Miguel, Mike M Laffan, Tom AJ McKinnon. Dept of Haematology, Imperial College London

Background Von Willebrand Factor contains a significant number of cysteine residues and some of these have been shown to be in the free-thiol form in the circulating molecule. However it is not understood how and where these residues become unpaired. Thioredoxin-1 (TRX) is a ubiquitously expressed enzyme that catalyses the formation and reduction of disulphide bonds. We hypothesised that TRX may be responsible for the formation of free-thiols in VWF

Aims To determine the effect of TRX on the free-thiol content of VWF.

Methods VWF was purified from Haemate P by gel filtration. Plasma was obtained from healthy donors. Recombinant TRX was activated with thioredoxin reductase and NADPH before use. PX12 was used to inhibit specific TRX function. Static collagen binding, multimers gel analysis and GPIb binding assays and free-thiol analysis were performed using standard methods. Assessment of VWF function under shear stress was performed in a flow chamber assay

Results TRX treatment of VWF induced the formation of new free-thiols in a concentration and time dependent manner. The effect was specific to TRX since it was inhibited by PX-12. Moreover, TRX addition to plasma resulted in an increase in VWF free thiols. Functional assays demonstrated a loss of collagen binding and GPIb binding function. Multimer analysis showed a loss of high molecule weight multimers with higher concentrations of TRX, however despite collagen binding being affected at low concentrations of TRX, the multimeric pattern was not affected sufficiently to cause this. Mass spectroscopy analysis of TRX treated VWF highlighted the formation of new thiols in the D4 and C-domains of VWF and significantly highlighted C1142 (responsible for multimers formation) and C1872 (forms part of the A3 disulphide loop) as being unpaired. Consistently, TRX treated VWF perfused over collagen demonstrated reduced platelet capture and the addition of TRX to whole blood before perfusion significantly reduced thrombus formation.

Summary/Conclusions TRX can specifically attack the disulphide bonds responsible for multimer formation and maintaining the structural integrity of the A3 collagen binding domain and thus may therefore be a novel regulator of VWF function at sites of injury

12.00

THE ROLE OF ADAMTS13 EXOSITES IN VWF RECOGNITION AND PROTEOLYSIS Anastasis Petri, Rens de Groot, Yaoxian Xu, James Crawley. Faculty of Medicine, Department of Medicine, Centre for Haematology, Imperial College London, London, UK

Background ADAMTS13 cleaves VWF with unprecedented specificity, which has been attributed to multiple exosite interactions between ADAMTS13 spacer, cysteine-rich (Cys), disintegrin-like (Dis) and metalloprotease (MP) domains and complementary binding sites in the VWF A2 domain. Although the identity of the interacting exosites is known, their precise contribution in the proteolysis of VWF has not been characterised.

Aims To characterise how different ADAMTS13 exosite interactions coordinate VWF proteolysis and dictate enzyme specificity and activity.
Methods VWF96, a VWF A2 domain fragment (VWF1573-1668) was expressed and purified. VWF96 mutants, containing specific mutations/deletions that ablate each exosite interaction individually, were also generated. VWF96, VWF96-MP (L1603N), VWF96-Dis (DIKRD1614/6/7/8/1622AQEET), VWF96-Cys (IWAILI1642/4/7/9/1650/1QYSQQQ), VWF96-Spacer (LVL1664/5/6TNQ), VWF87ΔSpacer (VWF1573-1659) were used as substrates in activity and plate binding assays with ADAMTS13. A novel ELISA assay was developed to monitor the kinetics of proteolysis.
Results VWF96 was proteolysed efficiently (k_{cat}/K_m=14x10^{5}M^{-1}s^{-1}). C-terminal deletion of the spacer domain exosite (VWF87ΔSpacer) resulted in 18-fold reduced proteolysis. Mutating just 3 hydrophobic residues, LVL1664/5/6TNQ, in the spacer binding region (VWF96-Spacer) resulted in the same (18-fold) reduction in proteolysis. Mutation of the Cys domain binding site (VWF96-Cys) caused a ~40-fold reduction in proteolysis. The impaired proteolysis of VWF96-Cys, VWF96-Spacer and VWF87ΔSpacer was primarily manifest through reduced binding affinity. Proteolysis of VWF96-MP was ~150-fold reduced, despite the normal/near normal binding affinity to ADAMTS13. The greatest reduction (~400-fold) in proteolysis was measured with the Dis domain binding site mutant (VWF96-Dis). The moderately reduced binding of ADAMTS13 to VWF96-Dis could not account for the ~400-fold reduction in proteolysis.
Summary/Conclusions For the first time, we demonstrate that the ADAMTS13 spacer exosite involves interaction with VWF L1664, V1665, L1666. Disrupting the spacer and Cys exosites compromises ADAMTS13 binding and results in 18- to 40-fold reductions in proteolysis. The ~150-fold effect of mutating the P3 residue may be explained by its proximity to the scissile bond. The ADAMTS13 Dis exosite interaction is the most important in VWF proteolysis, which probably facilitates scissile bond access to the active site.

1**2.15**

FIBRINOLYTIC PROTEINS ARE RETAINED ON THE SURFACE OF PHOSPHATIDYLSERINE EXPOSING PLATELETS IN A PROTRUDING 'CAP' GB Morrow, CS Whyte, NJ Mutch. School of Medicine, Medical Sciences and Nutrition, Institute of Medical Sciences, University of Aberdeen, Aberdeen, UK.

Background: Sub-populations of platelets form upon activation; phosphatidylserine (PS)-exposing platelets are pro-coagulant and balloon-shaped, while aggregating platelets are PS-negative with a 'spread' morphology. A protruding 'cap' is present on PS-exposing platelets containing fibrinogen, factor XIII and plasminogen. Upon activation, platelets release their α-granules containing a plethora of pro-coagulant and fibrinolytic proteins.
Aim: To analyse the exposure of platelet-derived proteins and binding of fibrinolytic proteins on PS-exposing platelets.
Methods: Platelets activated with collagen or convulxin (CVX) ± thrombin or thrombin receptor activator peptide 6 (TRAP-6) were analysed using confocal microscopy and flow cytometry. Fluorescently-labelled fibrinogen, plasminogen, factor XII (FXII) or histidine rich glycoprotein (HRG) were added during stimulation and PS-positive platelets detected by Annexin V (AF647). Alternatively, fluorescently-labelled antibodies were used to detect platelet-derived plasminogen activator inhibitor-1 (PAI-1), HRG, α_2-antiplasmin (α_2AP), plasminogen, vitronectin, fibrinogen and $\alpha IIb\beta_3$. In some cases, platelets were pre-treated with Gly-Pro-Arg-Pro (GPRP), to inhibit fibrin polymerization, or tirofiban, to block $\alpha IIb\beta_3$.
Results: PAI-1, HRG and PS expression were significantly increased on CVX ± TRAP-6 stimulated platelets compared to unstimulated platelets. Maximal exposure was observed with CVX and thrombin stimulation, suggesting a requirement for fibrin to localise these proteins. PAI-1 was present in a protruding 'cap' on the surface of PS-exposing platelets, which was attenuated by inhibiting fibrin polymerisation or blocking $\alpha IIb\beta_3$. PAI-1 co-localised in the 'cap' with platelet-derived vitronectin, HRG, α_2AP, plasminogen, fibrinogen and the $\alpha IIb\beta_3$ receptor. Exogenously added FXII, plasminogen and fibrinogen were also visualised within the same protruding 'caps'.
Summary: PS-positive platelets accumulate pro- and anti-fibrinolytic proteins in a protruding 'cap' via a fibrin-dependant mechanism. Many of these proteins are released from the α-granules, however, some exogenously added fibrinolytic proteins also associate with the 'caps'. The balance of the pro- and anti-fibrinolytic proteins in these 'caps' may regulate fibrinolysis in platelet-rich areas of thrombi.

1**2.35**

CHARACTERISATION OF THE ERYTHROCYTE BINDING SITE ON FIBRINOGEN Sabban A.[1], Duval C.[1], Guedes A.F.[2], McPherson H.R.[1], Saha S.[1], Ajjan R.[1], Santos N.C.[2], Ariëns R.A.S.[1]1 [1] Thrombosis and Tissue Repair Group; Division of Cardiovascular and Diabetes Research; Leeds Institute of Cardiovascular And Metabolic Medicine; University of Leeds; UK [2] Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, Lisbon, Portugal

Background Recent data suggest a new role for red blood cells (RBCs) in haemostasis and thrombosis, driven through interactions of RBCs with the clot. RBCs can produce polyhedrocytes in the clot, RBC retention in the clot is dependent on FXIII, and fibrinogen has previously been reported to bind directly to RBCs via $\alpha_V\beta_3$. However, the binding site for RBCs on fibrinogen is currently unknown.

Aims The aim of this study is to investigate the role of the fibrinogen α-chain $R_{95}G_{96}D_{97}$ sequence, which is located in the coiled coil region of the protein, in the binding of fibrinogen with RBCs and to study the effect of mutations on the binding.

Methods Three mutations in and near the RGD sequence were produced: R95Q, D97N and F98I. These mutations were stably transfected into βγ-CHO cells, expressed in roller bottles and purified. The recombinant proteins were analysed by SDS-PAGE, CD-spectra and turbidity for their basic structure/function compared to wild type. A plate RBC-fibrinogen binding assay was developed to study the binding between RBCs and fibrinogen.

Results SDS-PAGE analysis showed high integrity and purity of the proteins, but the mobility of the α-chains appeared slightly affected, indicating some differences in polypeptide folding for all mutations. CD spectra confirmed minor differences in α-helical content for each mutation. Turbidity analysis showed reduced maximum absorbency for each compared to the wild type, indicative of thinner fibres. RBC binding was increased for F98I, and reduced for D97N but not R95Q. RBC binding was inhibited by anti-α_V IgG, but not by non-immune IgG.

Summary/Conclusions Our present data confirm the specific binding of RBCs to fibrinogen. The results further indicate a role for the fibrinogen $\alpha R_{95}G_{96}D_{97}$ sequence in RBC binding, and indicate that binding involves the interaction of fibrinogen with an α_V receptor.

Thursday afternoon

14.45

MOLECULAR BASIS OF FV/PROTEIN S ENHANCEMENT OF THE INHIBITION OF FXA BY TFPI Salvatore Santamaria[1], Natalia Reglińska-Matveyev[1], Magdalena Gierula[1], Rodney M. Camire[2,3], James T.B. Crawley[1], David A. Lane[1] and Josefin Ahnström[1] [1]Centre for Haematology, Faculty of Medicine, Imperial College London, London, UK; [2]Division of Hematology, Department of Pediatrics, The University of Pennsylvania, Perelman School of Medicine, Philadelphia USA; [3]The Center for Cell and Molecular Therapeutics, The Children's Hospital of Philadelphia, Philadelphia, USA.

Background. Tissue factor pathway inhibitor (TFPI) exerts an important anticoagulant role by inhibiting factor (F) Xa. We recently reported that FV and protein S synergistically enhance TFPI-mediated inhibition of free FXa. One issue that might confound interpretation of these results is the proteolytic susceptibility of FV which might alter the interpretation of inhibition experiments.
Aims. To determine the molecular basis for FV enhancement of TFPI inhibition of free FXa and prothrombinase, and to define any role of protein S.
Methods. The enhancement of TFPI mediated by protein S and FV was tested in pure component FXa inhibition assays and prothrombinase assays.
Results. We prepared a thrombin/FXa-resistant FV variant, FV R709Q/R1018Q/R1545Q ($FV^{\Delta IIa}$). This variant caused a 12-fold enhancement of TFPI inhibition of FXa amidolytic activity in the presence of protein S (K_i from 2.71 to 0.23nM), whereas no significant enhancement was observed in the presence of $FV^{\Delta IIa}$ alone. This enhancement is similar to that by wild-type FV together with protein S. In contrast, thrombin-activated FV (FVa) and a recombinant B-domain deleted FV variant, failed to enhance TFPI, both in the presence and absence of protein S. Using TFPI and protein S variants we found that the synergistic enhancement of TFPI-mediated FXa inhibition by protein S and FV is dependent on a direct protein S/TFPI interaction, but independent of the TFPI C-terminal tail basic region. To clarify the physiological importance of this synergism, we performed experiments that analysed TFPI-mediated inhibition of prothrombin activation by FXa. In these studies, protein S enhanced TFPI inhibition in the presence of $FV^{\Delta IIa}$ and wild-type FV (but not FVa), 11.4- and 13.6-fold, respectively.
Summary/Conclusions. Collectively, these results point to an important anticoagulant role of FV (but not FVa) that regulates the initiation of coagulation prior to assembly of FXa and FVa into the prothrombinase complex.

15.00

OPPOSING EFFECTS OF POLYPHOSPHATE IN MODULATION OF TPA AND UPA-MEDIATED FIBRINOLYSIS CS Whyte, NJ Mutch. School of Medicine, Medical Sciences and Nutrition, Institute of Medical Sciences, University of Aberdeen, Aberdeen, UK.

Background Polyphosphate (polyP), a biomolecule released from platelet dense granules, binds to fibrin and alters its network structure thereby attenuating tPA-mediated fibrinolysis.
Aims To investigate the influence of polyP on uPA-mediated fibrinolysis.
Methods Fibrin clots containing fibrinogen, glu- or lys-plasminogen, tPA or uPA ± polyP were clotted with thrombin and $CaCl_2$. Fibrinolysis was monitored by change in absorbance and plasmin generation quantified using a fluorogenic substrate. Real-time fibrinolysis of clots containing fibrinogen (9% was DyLight 488-labelled) and plasminogen (20% was DyLight 633-labelled) was visualised by confocal microscopy. Binding of proteins to biotinylated-polyP was quantified using streptavidin-coated plates and appropriate chromogenic substrates or a plasminogen-HRP antibody
Results PolyP significantly accelerates the rate of uPA-mediated fibrinolysis and plasmin generation (1.6-fold). In contrast, tPA-mediated fibrinolysis and plasmin generation is attenuated by polyP. Modulation of uPA-mediated lysis was dependant on polymer concentration and length. Real-time analysis of fibrinolysis demonstrated that polyP enhances uPA-mediated and delays tPA-mediated lysis. PolyP binds both glu- and lys-plasminogen and co-localises in fibrin dense areas of the clot. Increasing concentrations of glu- or lys-plasminogen (0 – 1 μM) enhance the rate of uPA-mediated fibrinolysis and plasmin generation. At high concentrations of plasminogen polyP was no longer able to stimulate uPA-mediated plasmin generation. In contrast, a similar attenuation of tPA-mediated lysis by polyP was observed at all

plasminogen concentrations. PolyP bound with significantly higher affinity to uPA (K_d 9.8 nM) than to tPA (K_d 245.3 nM). Interestingly, polyP shortened the lag time in uPA-mediated plasmin generation from glu-plasminogen, suggesting it potentially enhances conversion to lys-plasminogen which is subsequently more readily activated to plasmin.

Summary/Conclusions PolyP binds to fibrin thereby attenuating its cofactor function in tPA-mediated plasminogen activation. In contrast, polyP binds with high affinity to uPA and enhances plasmin generation and fibrinolysis potentially by a template based mechanism.

15.15

ACTIVATED FACTOR V IN SYNERGY WITH PROTEIN S ENHANCES APC ASSOCIATION TO PHOSPHOLIPIDS Magdalena Gierula, Isabelle I. Salles-Crawley, Santamaria S, James T.B. Crawley, David A. Lane and Josefin Ahnström. Centre for Haematology, Hammersmith Hospital Campus, Imperial College London, London, UK

Background Activated factor V (FVa) can be proteolytically inactivated by activated protein C (APC). Inactivation of FVa depends upon APC binding to phospholipids and is greatly enhanced by protein S. In functional assays, protein S residues Gla36 and Asp95 are essential for its APC co-factor function. FVa, APC and protein S likely form a tri-molecular complex on negatively-charged phospholipid surfaces. However, the formation of this complex, and the relative roles of FVa and protein S in the complex assembly, have not yet been investigated.

Aims To investigate the mechanism underlying APC-mediated FVa inactivation by studying the assembly of the APC/protein S/FVa tri-molecular complex on phospholipid surfaces, and the influence of protein S residues Gla36 and Asp95 in complex formation.

Methods Association of active-site-labelled (FITC) APC to phospholipid coated beads was analysed by flow cytometry in the presence and absence of protein S and/or FVa.

Results APC (0-150nM) by itself bound to phospholipids with low affinity (K_d ~3μM), (Sum et al. Blood, 2003). Addition of protein S (100nM) strongly enhanced APC binding (K_d ~140nM). Whereas FVa (25nM) alone did not influence association of APC to phospholipids, when added in combination with protein S, APC-phospholipid association was further enhanced by ~ 2.8-fold (K_d ~50nM). The synergistic enhancement by FVa and protein S confirms the formation of an APC/protein S/FVa complex. The enhancements of APC association to phospholipids by the protein S Gla36Ala and Asp95Ala were severely reduced both in the presence and absence of FVa compared to wild-type protein S, suggesting a direct involvement of these residues in complex formation.

Summary/Conclusions FVa, together with protein S, synergistically enhance the association of APC to phospholipid membranes, confirming the formation of an APC/protein S/FVa tri-molecular complex. Protein S residues Gla36 and Asp95 are essential for the formation of this complex.

15.30

CROSS-LINKING OF α_2-AP INTO FIBRIN BY FXIII: EVIDENCE FOR A SECOND FXIII CROSS-LINKING SITE Duval C.[1], McPherson H.R.[1], Bridge K.I. [1], Domingues M.M. [1], Ajjan R. [1], Ridger V.C. [2], Connell S.D.A.[3], Philippou H. [1], Ariëns R.A.S. [1]. [1]Thrombosis and Tissue Repair Group; Division of Cardiovascular and Diabetes Research; Leeds Institute of Cardiovascular and Metabolic Medicine; University of Leeds; UK. [2]Department of Infection, Immunity & Cardiovascular Disease (IICD); Faculty of Medicine, Dentistry and Health; University of Sheffield; UK

[3] Molecular and Nanoscale Physics Group; School of Physics and Astronomy; University of Leeds; UK

Background The fibrin clot is subject to mechanical (blood flow) and proteolytic (dissolution of the clot by plasmin; fibrinolysis) challenges. A key regulator of fibrinolysis is α_2-antiplasmin (α_2-AP), which is cross-linked to the fibrin α-chain by Factor XIII (FXIII), at residue Lys303.

Aims The aim of this study was to generate a fibrinogen mutant lacking the cross-linking site for α_2-AP, and investigate the contribution of α_2-AP cross-linking to the inhibition of fibrinolysis.

Methods The fibrinogen α-chain was mutated by site-directed mutagenesis (αK303R) and transfected into β- and γ-chains containing CHO cells for expression, followed by IF-1 affinity chromatography purification. The effect of the αK303R and WT fibrinogens on clot formation and lysis in the absence and presence of α_2-AP, and on α_2-AP incorporation to fibrin were investigated.

Results In the absence of α_2-AP, turbidity and lysis profiles were similar for both αK303R and WT fibrinogens, in both absence (MaxOD 0.696±0.022

and 0.713±0.024, $^1/_2$lysis 32.2±0.8min and 32.3±0.8min, respectively) and presence (MaxOD 0.840±0.016 and 0.840±0.006, $^1/_2$lysis 36.7±1.5min and 37.8±0.5min, respectively) of FXIII, indicating that this mutation intrinsically does not affect fibrin polymerisation and lysis. When α_2-AP was added, the difference in time to half-lysis between presence and absence of FXIII was significantly reduced (-38.8%, $p<0.05$) for fibrinogen αK303R (33.5±4.5 min) compared to WT (54.7±3.3min). Although the mutation of the α_2-AP cross-linking site to fibrin reduced the effect of α_2-AP on fibrinolysis, this was not completely abolished. Furthermore, analysis of α_2-AP incorporation into fibrin showed decreased cross-linking by 42.7±4.8% ($p<0.05$) for fibrinogen αK303R compared to WT, indicating the presence of a possible secondary cross-linking site for α_2-AP in fibrin.

Summary/Conclusions These data indicate that fibrinogen likely possesses a second FXIII cross-linking site for α_2-AP. Further studies are required to identify this potential second site and investigate its physiological relevance.

15.45

ELUCIDATING THE FIBRINOGEN-FXIII BINDING SITE BY SURFACE PLASMON RESONANCE Clare Wilson[1], James R. Byrnes[2], Alisa S. Wolberg[2] and Helen Philippou[1] [1]Division of Cardiovascular and Diabetes Research, Leeds Institute of Cardiovascular and Metabolic Medicine, Leeds Institute for Genetics, Health, and Therapeutics, Multidisciplinary Cardiovascular Research Centre, University of Leeds, Leeds, LS2 9NL, UK
[2]Department of Pathology and Laboratory Medicine and McAllister Heart Institute, University of North Carolina at Chapel Hill, Chapel Hill, USA.

Background Inactive $FXIIIA_2B_2$ circulates in plasma, associated with fibrinogen. Upon coagulation, thrombin cleaves a 37 residue activation peptide from the A subunit, and in the presence of calcium the inhibitory B subunits dissociate, permitting $FXIIIA_2$* (or FXIIIa) to mediate ε-N-(γ-glutamyl)-lysine cross-linking between fibrin γ and α chains, enhancing the tensile strength of the clot and retention of erythrocytes. The fibrinogen residues that mediate binding to $FXIIIA_2B_2$ have not been identified. Historically, the primary interaction site was speculated to occur at the γ' splice variant.

Aims The aims of this study were to identify the residues where the $FXIIIA_2B_2$ interaction takes place on fibrinogen, and which subunit of FXIII is responsible for fibrinogen binding.

Methods A COOH-V chip was installed into a SensiQ Pioneer platform and $FXIIIA_2B_2$ or $FXIIIB_2$ was immobilised to the chip surface using amine coupling to an appropriate RU according to the R_{MAX} equation. Fibrinogen variants: γAγA; γ'γ'; Aα251 (αC truncation) and $\gamma^{390\text{-}396A}$ (residues 390-396 of the γ-chain mutated to alanine) were titrated into the flow chamber up to a final concentration of 50nM or 1μM. kinetic interactions were measured and affinity calculated using Qdat software.

Results Fibrinogen isoforms: γAγA, γ'γ' and Aα251 all bound with high affinity to $FXIIIA_2B_2$, with affinities of: 3.8 +/- 2.4nM; 10.4 +/- 11nM and 71 +/- 16.2nM respectively. Notably, fibrinogen γ390-396A did not bind to $FXIIIA_2B_2$. The same isoforms also bound to $FXIIIB_2$ with affinities of: 0.4 +/- 0.3nM; 53.0 +/- 75.1nM and 58.6 +/- 26.5nM respectively. Notably, fibrinogen $\gamma^{390\text{-}396A}$ did not bind to $FXIIIA_2B_2$ or B_2.

Summary/Conclusions These data show that the primary interaction site between fibrinogen and FXIII lies between the residues of 390-396 on the γ chain of fibrinogen, and this interaction occurs via the FXIIIB2 subunit. Sequence alignment of γ390-396 reveals that these residues are well conserved between mammalian species.

Friday morning

8.30

THROMBI FROM ANAEMIC PATIENTS ARE RESISTANT TO FIBRINOLYSIS P Untiveros, M Greaves, HG Watson, NJ Mutch School of Medicine, Medical Sciences and Nutrition, Institute of Medical Sciences, Foresterhill, Aberdeen, AB25 2ZD

Background The role of red blood cells (RBC) in coagulation and thrombus formation has been considered insignificant, yet their sheer abundance in blood means that they are a dominant element of a thrombus. Patients with anaemia are at increased risk of thrombosis, however the mechanisms by which RBCs influence this outcome remain unclear.

Aims To examine the influence of haematocrit (HCT) on coagulation parameters, clot firmness and resistance of thrombi to fibrinolysis in anaemic patients.

Methods Whole blood drawn from patients with myeloproliferative disease with and without anaemia and autoimmune haemolytic anaemia. Coagulation parameters were assessed using thromboelastography (ROTEM). Chandler thrombi were formed under continuous flow for 90 min from reconstituted blood in the presence of FITC-labelled fibrinogen. Lysis was induced by bathing thrombi in 1 µg/ml tPA and samples taken at 30 min intervals.

Results Results from anaemic myeloproliferative patients show that thrombi are longer in length (n=6/7 p<0.001) and lyse slower (n=6/7 p<0.05) than non-anaemic myeloproliferative patients. Serial samples were taken from patients with autoimmune haemolytic anaemia from the time of initial diagnosis or relapse through to recovery. As haematocrit increased thrombi decrease in length and show increased susceptibility to lysis (HCT≤0.3 vs HCT >0.30, n=3 p<0.05). Thromboelastography revealed shorter clot formation time with a sharper alpha angle and higher maximum clot firmness in lower haematocrit samples (HCT≤0.3 vs HCT >0.30, n=3 p<0.05).

Summary/Conclusions Anaemia has a dramatic impact on thrombus formation and stability, with low haematocrit enhancing clot formation, resulting in thrombi with increased firmness and resistance to fibrinolysis. These data in anaemic patients mirror our results of *ex-vivo* manipulation of the haematocrit in healthy volunteers. These differences in clot formation, stiffness and fibrinolytic resistance of thrombi formed at low haematocrit may contribute to the increased risk of thrombosis in patients with anaemia.

8.45

(PATHO)PHYSIOLOGICAL FIBRINOGEN γ' LEVELS HAVE FUNCTIONAL EFFECTS ON CLOT STRUCTURE Fraser Macrae[1], Tittu Thomas[1], Helen Philippou[1], Julian Scott[1], Marlien Pieters[2], Robert Ariëns[1]. [1] Thrombosis and Tissue Repair Group; Division of Cardiovascular and Diabetes Research; The Leeds Institute of Cardiovascular and Metabolic Medicine Multidisciplinary Cardiovascular Research Centre; University of Leeds; UK. [2] Centre of Excellence for Nutrition, North-West University, Potchefstroom, North West, South Africa.

Background Fibrinogen γ' differs from the γA chain at its C-terminus with a unique 20-amino acid extension. In vitro studies show that fibrinogen γ' influences clot structure, however, the effects of fibrinogen γ' at (patho)physiological levels are poorly understood.

Aims Investigate the effects of high and low fibrinogen γ' plasma levels on clot structure.

Methods Fibrinogen and γ' levels were measured by Clauss method and specific ELISA respectively in patients with AAA. 33 high γ' (median(range): 27.4%(22.2-40.0)) and 41 low γ' (3.9%(1.4-6.2)) patient samples were selected. Purified γA/γ' and γA/γA fibrinogen were combined (total fibrinogen 0.5mg/ml), at increasing γA/γ' ratios (5%, 10%, 40%). Fibrinogen depleted plasma was repleted with the same fibrinogen ratios (total fibrinogen 1.5mg/ml). Clots from patient samples and purified fibrinogen were analyzed by laser scanning confocal microscopy. Patient, purified and repleted plasma samples were analyzed by turbidity and lysis assays.

Results In patient plasma, high γ' (37%) resulted in higher fiber counts (37.3 fibers/100µm) compared to low γ' (4%)(34.3 fibers/100 µm)(*P=0.0126*). This was supported by clots made with purified fibrinogens, with increased fiber count (10.3 fibers/100µm) in high γ'(40%) compared to low γ' (3%)(6.0 fibers/100µm)(*P=0.002*). Patients with high γ' presented with a lower Max OD (0.2390 ± 0.005) and a lower maximum lysis rate than low γ' (0.2802 ± 0.0086)(*P=0.0002*), suggesting thinner fibers and smaller pores. This was reflected in

purified and fibrinogen repleted plasma with high γ' (40%) having a lower Max OD than the low γ' (3%).

Summary/Conclusions High γ' levels in patients resulted in increased fiber count as well as decreased Max OD and lysis rate. These findings were corroborated in both purified systems and in fibrinogen repleted plasma. These data show that fibrinogen γ' influences clot structure and function at plasma levels as observed in patients with vascular diseases.

9.00

MURINE FXIII-A VAL34 AND LEU34 VARIANTS PRESENT SIMILAR FUNCTIONAL CHARACTERISTICS AS THAT OF THEIR HUMAN COUNTERPARTS Duval C., MacLachlan E., Ariëns R.A.S. Thrombosis and Tissue Repair Group; Division of Cardiovascular and Diabetes Research; Leeds Institute of Cardiovascular And Metabolic Medicine; University of Leeds; UK

Background FXIII-A subunit SNP Val34Leu has been shown to affect activation rates by thrombin, clot structure and clot stability, and is associated with decreased risk cardiovascular risk. Contrary to humans, laboratory mice exclusively have FXIII 43Leu. In order to develop murine models of Val34Leu, it is important to establish whether the 34Leu to Val mutation in murine FXIII behaves similarly as their human counterpart.

Aims The aim of this study was to produce murine recombinant Val34 and Leu34 variants in order to investigate effects on FXIII activation, clot structure and fibrinolysis in an all-murine model.

Methods Wild-type (Leu34) and mutated (Val34) murine FXIII were expressed in *E.coli*. FXIII activation rate was measured using a pentylamine-biotin incorporation assay. Turbidity and Lysis assays measured the effects of mFXIII-A variants on clot formation and fibrinolysis. Confocal microscopy and electron microscopy were used to visualise clot structure, and determine clot fibre density. Purified mFXIII-A, and murine $FXIII^{-/-}$ plasma reconstituted with mFXIII-A, were used for all assays.

Results Results are shown as 1) data from purified and 2) reconstituted plasma systems. mFXIII-A Leu34 showed higher activation rate than Val34 (1.95- and 1.67-fold). Val34 variant decreased maximal absorbency (1.15- and 1.13- fold), which was further decreased by Leu34 (1.39- and 1.33-fold). Time to half-lysis was increased by Val34 (1.17- and 1.82-fold), and further increased by Leu34 (1.29- and 2.55-fold). Clot fibre density was increased by Val34 (1.42- and 1.35-fold) and further increased by Leu34 (2.10- and 2.19-fold). These data clearly indicate that murine FXIII-A Val34 and Leu34 have similar functional characteristics as that of human FXIII-A variants.

Summary/Conclusions Our data show that murine FXIII-A Val34 and Leu34 variants could be used in an all-murine model of thrombosis to further investigate the mechanisms by which this SNP influences risk of MI, CAD and VTE.

9.15

LOCAL COAGULOPATHY IN THE SKIN LESIONS OF BURULI ULCER PATIENTS: ENHANCED FIBRIN DEPOSITION AROUND INDIVIDUAL VESSELS IS ASSOCIATED WITH THROMBOMODULIN DEPLETION BUT NOT PLATELET ADHERENCE. Scott J Dos Santos[1], F. Javier Salguero-Bodes[2], Marie-Thérèse Ruf[3], Gerd Pluschke[3] and Rachel E Simmonds[1] [1]Department of Microbial Sciences, School of Bioscience and Medicine, University of Surrey, Guildford, GU2 7TE, [2]School of Veterinary Medicine, University of Surrey, Guildford, GU2 7AL, [3]Swiss Tropical and Public Health Institute, Department of Medical Parasitology and Infection Biology, 4002 Basel, Switzerland.

Background Buruli ulcer (BU) is a neglected tropical disease caused by infection with *Mycobacterium ulcerans*. The lesions are caused by the exotoxin, mycolactone, triggering coagulative necrosis within subcutaneous tissue. We recently established that BU pathogenesis involves mycolactone-dependent depletion of thrombomodulin (TM) from endothelial cells *in vitro*. Furthermore, fibrin could be commonly detected in BU patient skin and TM staining of vasculature was poor. However, our interpretation was limited due to skin heterogeneity and the complex pathology.

Aims Using serial sections of BU patient samples, and a sequential staining strategy, we assessed the effect of TM depletion on individual vessels in non-necrotic regions of skin. This unbiased approach allowed us to correlate changes to endothelial TM with fibrin deposition and other haemostatic markers, such as platelets (CD61).

Methods Staining of 5µm-thick serial sections of 8 BU patient 4mm skin lesion biopsies was performed in the following order: H&E, Fibrin, TM, CD61, CD51, SMA, Ziehl-Neelsen. Immunostained

slides were counter-stained with haematoxylin and analysed using NIS Elements (Nikon). Non-necrotic regions were initially identified on H&E stained sections and all vessels within the chosen regions were tracked then categorised according to biomarker expression. The intensity of fibrin staining within 20µm of each vessel was also quantified.

Results A total of 962 unique vessels were analysed. Staining patterns were complex, however, a decrease in TM-stained vessels was found in 7/8 patients, and 75.3% of unique vessels were TM-negative. Fibrin staining was significantly higher around the TM-negative vs. positive vessels ($p < 0.0001$). Surprisingly CD61 staining was very rare within BU patient biopsies, despite the extensive fibrin deposition in some samples.

Conclusions This study supports the hypothesis that TM depletion occurs prior to tissue necrosis. However, the lack of platelets suggests the fibrin observed is not a result of homeostatic wound healing; rather pathogenic fibrin deposition.

11.15

THE IMPORTANCE OF FREE THIOLS FOR PROPER VON WILLEBRAND FACTOR FUNCTION UNDER SHEAR STRESS O'Brien H[1], Shapiro S[1], Zhang F[2], Henne P[1], Doerr A[1], Laffan MA[1] and McKinnon TAJ[1]
[1]Dept of Haematology, Imperial College London. [2]Dept of Mechanical Engineering and mechanics, Lehigh University, USA

Background Von Willebrand Factor is a multimeric protein critical for platelet capture under high shear stress. Several studies have shown that Von Willebrand Factor contains a number of free thiols that may mediate its function. While this has been investigated using a plate and cone viscometer, no studies have addressed the role of VWF free-thiols under conditions of acute shear stress.

Aims To determine the effect of VWF free-thiols on function under physiological and pathological shear stress

Methods VWF was purified from Haemate P by gel filtration or recombinantly expressed in HEK293T cells. Free-thiols were blocked with N-ethylmaleimide (NEM) or Maleimide-PEO2-biotin. Flow assays were performed using plasma free blood supplemented with VWF and perfused over collagen or VWF coated surfaces. Analysis of the VWF-collagen interaction was performed using atomic force microscopy (AFM).

Results NEM blockade of VWF free-thiols reduced VWF mediated platelet capture to collagen in a shear dependent manner; with no effect at low shear and platelet capture virtually abolished at high shear (above $3000s^{-1}$). The effect was mediated by free-thiols in the C-terminal domains and was not due to an effect on platelet capture, since the function of a multimeric VWF construct lacking the C-domains was not affected by NEM blockade. Under extreme pathological shear and in flow chambers mimicking stenotic vessels, the formation of thick VWF fibres on collagen was inhibited by blocking VWF thiols. Interestingly some free-thiols in VWF were lost after collagen binding and AFM measurements demonstrated that the strength of the VWF-collagen bond was significantly reduced with NEM-VWF Moreover, NEM-VWF failed to effectively incorporate into long VWF fibres formed independently of collagen and platelets. Finally, time course analysis of shearing VWF showed the appearance and then disappearance of free-thiols, however no new thiols appeared when NEM-VWF was sheared.

Summary/Conclusions Free-thiols residues in VWF are essential for proper collagen binding under shear stress, possibly by mediating a conformational change in the VWF molecule.

11.30

THE ROLE OF N-LINKED GLYCOSYLATION AND SIALIC ACID IN MODULATING ADAMTS13 PROTEOLYTIC ACTIVITY Nowak AA, O'Brien H, Henne P, Doerr A, Laffan MA and McKinnon TAJ
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Background ADAMTS13 controls the multimeric size of Von Willebrand Factor (VWF). Recent work has demonstrated that the C-terminal CUB domains of ADAMTS13 interact with the Spacer domain and maintain ADAMTS13 in a closed conformation and flexible regions with the TSP2-8 domains also contribute to this. Upon binding to the D4 domain of VWF, ADAMTS13 undergoes conformational activation, enhancing its ability to cleave VWF. Interestingly ADAMTS13 contains 10 N-linked glycan (NLG) chains, with four sites present in the TSP2 through CUB domains. We hypothesized that these will contribute to ADAMTS13 function.

Aims To investigate the role of ADAMTS13 N-linked glycans on proteolytic activity.

Methods Recombinant ADAMTS13 (rAD13), truncated ADAMTS13 variants and NLG mutants were expressed in HEK293T cells. Enzymatic glycan modification was performed by neuraminidase and PNGase F digestion.

Proteolytic activity of ADAMST13 was determined using FRETS-73 substrate or full length (FL) VWF under both static and shear stress conditions. Binding of the Spacer and CUB domains was performed by immunoprecipitation.

Results Removal of terminal silaic acid residues (Neu-AD13) or entire N-linked glycan chains (PNG-AD13) reduced ADAMTS13 function against FRETS-73 and FL-VWF under shear stress. Using constructs spanning the MDTCS and MD domains and activating ADAMTS13 with VWF-D4CK we demonstrated this was due to loss of silaic acid from the glycans in the Metalloprotease domain and an effect of NLGs in the TSP2 through CUB domains. Mutation of the N-linked glycan sites in the MDTCS domains reduced or abolished protein expression. However N707Q, N828Q, N1235Q and N1354Q (TSP 2, 4 CUB1 and 2 domains respectively) expressed normally. Interestingly, N707Q and N828Q demonstrated reduced activity towards FRETS-73 but normal activity under flow conditions. In contrast, N1235Q and N1354Q had enhanced activity towards FRETS-73 and under shear stress. Immunoprecipitation experiments confirmed that loss of N-linked glycans in the CUB domains significantly reduced the interaction with the Spacer domain.

Summary/Conclusions N-linked glycosylation is a critical determinant of ADAMTS13 function.

11.45

EVALUATION OF THE TOTAL THROMBUS-FORMATION SYSTEM (T-TAS): APPLICATION TO HUMAN AND MOUSE BLOOD ANALYSIS

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Background: The Total Thrombus-formation Analyser System(T-TAS) is a whole blood flow chamber system for the measurement of in vitro thrombus formation under different shear stress conditions.

Aims: To evaluate the utility of the T-TAS for the measurement of thrombus formation within human and mouse blood.

Methods: T-TAS microchips(collagen,PL chip; collagen plus tissue thromboplastin,AR chip) were used to analyse platelet or fibrin-rich thrombus formation respectively. Healthy normal controls, wildtype(WT) and CD148 conditional knockout(KO) mice(*Pf4Cre^{+};CD148$^{fl/fl}$*) and blood incubated with tricagrelor and rivaroxaban were tested.

Results: Thrombus growth rate(N=22,mins and secs) increased with shear either within PL (04:40±1.11,3:25±0.43 and 3:12±0.48[1000,1500 and 2000s^{-1} respectively]) or AR chips(3:55±0.42 and 1:49±0.19[333s^{-1} and 833s^{-1} respectively]). Area under the curve(AUC) on the PL chip was also shorter at 1000s^{-1} than at 1500/2000s^{-1}(260±51.7,317± 55.4 and 301±66.2 respectively). In contrast no difference in the AUC between 333s^{-1}-833s^{-1} were observed in the AR chip 1593±122 and 1591±158, respectively. The intra-assay CV for a single normal blood sample(n=10) tested in the PL chip(1000s^{-1}) were T_{10}14.1%,T_{60}16.7%,$T_{10\text{-}60}$22.8% and AUC_{10}24.4%. In contrast AR Chips(333s^{-1}) were T_{10} 9.03%,T_{80}8.64%,$T_{10\text{-}80}$23.8% and AUC_{30}5.1%. AR chip thrombus formation was inhibited with the factor Xa inhibitor rivaroxaban(1µM), but not with the $P2Y_{12}$ inhibitor ticagrelor(10µM). PL chip thrombus formation was totally inhibited with either drug. The onset(T_{10}) of thrombus formation in WT mice(N = 4) was shorter on both chips when compared to humans e.g.PL chip(1000s^{-1})T_{10} were 02:02±00:23 and 03:30±0:45 respectively). CD148 KO mice(n=5) exhibited no measurable thrombus formation in PL chips with a delayed onset of thrombus formation in AR chips, correlating with findings from other in vitro and in vivo assays.

Conclusion: T-TAS measures in vitro thrombus formation under differing shear rates within 2 different chips and can be used for monitoring antithrombotic therapy in patients. It can also be applied to investigating thrombus formation in genetically altered mouse models.

12.00

PLATELET-DERIVED MICROPARTICLES AS BIOMARKERS OF BRAIN MICROVASCULAR AGEING AND DEMENTIA

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Background Vascular dysfunction and cerebrovascular damage are involved in the pathophysiology of vascular dementia and Alzheimer's disease. White-matter hyperintensities (WMH), detected by magnetic resonance imaging (MRI), are biomarkers of cerebral small vessel disease, and predict cognitive decline, dementia and death. The prothrombotic nature of microparticles (MP) could make them less costly biomarkers for microvascular changes.

Aims To measure platelet and endothelial MP in elderly people with normal or mildly impaired cognitive function, in relation to WMH.

Methods Participants aged 79-80 with available data from previous MRI brain scans were recruited from the Aberdeen Birth Cohort of 1936. Current cognitive function tests (Montreal Cognitive Assessment MoCA) were performed and blood samples obtained from 19 subjects (10M) with Scheltens' Score >20 indicating increased WMH (Group A) and 20 subjects (11M) with Scheltens' Score <20 (Group B). Participants with diagnosed dementia, or MoCA score below 20 on day of study, were excluded. MP were analysed in platelet-free plasma using multicolour flow cytometry (BD LSR Fortessa). Platelet-derived MP (PMP) were identified by size and single CD41b+ or multiple-staining (CD41b/CD42b/CD61/CD62P). Endothelial-derived MP (EMP) were identified as CD105+/CD62E+/CD45-. Counts per microlitre were established using counting beads. Platelet responsiveness to ADP and arachidonic acid was measured by Multiplate impedance aggregometry.

Results Total numbers of PMP and CD42b-positive PMP were significantly higher in Group A - the higher Scheltens' score group (CD41+/CD61+PMP 1400.47±713.80/microliter, mean±sd) vs Group B (953.46±277.06/microlitre); $p<0.02$). EMP were not significantly different. ADP-stimulated platelet aggregation was significantly higher in Group A vs Group B ($p<0.02$). PMP were not significantly associated with impaired cognitive function, but EMP count was lower.

Summary/Conclusions Increased plasma PMP and platelet responsiveness were associated with increased WMH in elderly people without severe cognitive decline. PMP may be useful biomarkers for early degenerative changes in brain microvasculature, and potential targets for dementia prevention.

12.15
EFFICACY AND SAFETY OF PROTHROMBIN COMPLEX CONCENTRATE IN PATIENTS UNDERGOING MAJOR CARDIAC SURGERY

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Background Administration of coagulation factors after cardiac surgery may be a strategy for reducing risk of bleeding and transfusion. Use of fresh frozen plasma (FFP) is restricted by the competing problem of heart failure. Prothrombin complex concentrate (PCC) can provide an equivalent amount of coagulation factors in a much smaller volume but cardiac surgery patients may also have an increased risk of thrombosis.

Aims To investigate whether the use of PCC is safe and effective compared with FFP to treat coagulopathy in patients undergoing major cardiac surgery.

Methods 170 consecutive adult patients who underwent major cardiac surgery between January 2015 and December 2015 were studied: 87 received PCC (mean age= 56 years) and 83 received FFP (mean age = 58 years) to control coagulopathy. Those receiving both PCC and FFP were excluded. Blood loss within first 12hrs and 24hrs from the end of operation, total use of blood and platelet transfusion, incidence of thrombosis, acute kidney injury and 30-day mortality were compared.

Results There was no significant difference in bleeding at 12hrs from end of operation in the two groups (p=0.25): median and 95%CI were 825mL [926-1317] and 787mL [804-1067] for PCC and FFP respectively. However, blood loss within 24hrs was significantly higher in patients who received PCC: 1575mL [1658-2263] versus 1213mL [1244-1641] (P=0.0034). Aspirin continuation had no significant effect in either group. The use of blood (P=.001) and platelets (P=0.03) was significantly higher in the FFP group. Heart failure was increased with FFP (9.6% vs 3.5%, p=0.002). There was no difference in thrombotic events (1.1%), 30-day mortality (4%) or incidence of acute kidney injury in the two groups.

Conclusions
PCC may be effective in reducing circulatory overload when treating cardiac surgery patients with coagulopathy. The increased bleeding may reflect selection bias or overcautious use due to concern regarding thrombosis.

12.30

CLOT STRUCTURE IS AFFECTED BY GRANULOCYTE CLONE SIZE AND ECULIZUMAB IN PATIENTS WITH PAROXYSMAL NOCTURNAL HAEMOGLOBINURIA

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Background Paroxysmal nocturnal haemoglobinuria (PNH) is a haematopoietic stem cell disorder, which leads to extensive complement-driven intravascular haemolysis and various complications, the most devastating of which is thrombosis. Previous studies demonstrated that patients with thrombosis form denser, thinner-fibred fibrin clots that incur greater resistance to fibrinolysis. We aimed to study whether similar changes to clot architecture may contribute to thrombosis in PNH. Furthermore, inhibition of complement factor C5 by eculizumab reduces thromboembolic events in PNH, indicating that complement activation plays a role in the pathophysiology of thrombosis.
Aims To characterise the fibrin clot structure in patients with varying PNH clone sizes and determine the effect of eculizumab on clot structure.
Methods Thirty-five patients from the PNH National Service in Leeds were recruited into the study and grouped according to PNH granulocyte clone size; small (less than 50%, n=15), and large (greater than 50%, n=20). Plasma samples were obtained and *ex vivo* fibrin clot structure and fibrinolysis rates were analysed by permeation, turbidimetry and confocal microscopy.
Results Maximum absorbency was significantly lower in patients with large clones (0.32±0.09) than small clones (0.39±0.10), *P=0.039.* Twelve of the fourteen patients on eculizumab had large clones. Analysis of results based upon treatment showed patients on eculizumab had a significantly lower maximum absorbency and fibre count/100µm than patients receiving no treatment; (0.30±0.09 versus 0.40±0.09, *P=0.0286*) and (18.47±4.42 versus 23.9±4.21, *P=0.006*) respectively.
Summary/Conclusions Patients with large PNH clones form clots composed of thinner fibrin fibres than those with small clones. Patients treated with eculizumab show a lower average fibre thickness and a less dense clot structure, a potential antithrombotic mechanism of the drug. However, it was not possible to determine the exact effects of clone size on clot structure independent of treatment due to the overlap of patients with large clones also receiving eculizumab.

Posters

P01

MODULATION OF RETICULOCYTES AND LEUKOCYTES ADHESION BY OMEGA-3 (N-3) FATTY ACIDS IN SICKLE CELL DISEASE

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Background Vaso-occlusive crises are the devastating hallmark of sickle cell disease. The paradigm shift in understanding this chronic inflammatory status revealed the central role of adhesiveness of Sickle reticulocytes and leukocytes in triggering crises. Omega-3(n-3) polyunsaturated fatty acids have well established anti-inflammatory and anti-adhesive roles. Interestingly, N-3 fatty acids are profoundly reduced in membranes of blood cells in SCD proportionally to the degree of anaemia.

Aims This study aimed to investigate the potential anti-adhesive properties of N-3 fatty acids in ameliorating SCD crises. By evaluating Vascular Adhesion Molecule (VCAM -1), which demonstrated the most significant increment in SCD during both steady state as well as crises compared to other adhesion molecules and correlate with HB level inversely

Methods Thirty SCD patients (HB SS) were supplemented with two to three capsules of N-3 fatty acids (277.8 mg docosahexaenoic (DHA) and 39.0mg eicosapentaenoic (EPA)) according to age for one year. N-3 fatty acids-treated patients were matched by age, gender and socioeconomic status to placebo- supplemented SCD patients and Twenty-four healthy controls (HB AA) from their siblings. Serum levels of soluble VCAM-1 were analysed by Quantitative Indirect ELISA in duplicates.

Results The ameliorative effect of N-3 fatty acids on frequency of vaso-occlusive crises and need for blood transfusion was re-observed. Haemoglobin level improved significantly by N-3 fatty acid supplementation. Mann - Whitney U test showed significant reduction in the level of sVCAM-1 in SCD patients treated with N-3 fatty acid compared to unsupplemented patients with P value of 0.001.

Summary/Conclusions N-3 fatty acids are promising dietary modifiers of adhesiveness and inflammation in SCD. Further studies on gene expression and membrane levels of adhesion molecules to be carried out.

P02

EVALUATION OF THE APPROPRIATENESS OF D-DIMER TESTING IN THE A&E DEPARTMENT AT MANCHESTER ROYAL INFIRMARY

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Background D-dimer tests are frequently requested in A&E to investigate venous thromboembolism. Trust guidelines suggest D-dimer testing be done based on the Wells score, but testing is frequently requested as part of a triage process. Given the cost and clinical implications of testing, it is important to ensure that D-Dimer testing is appropriately indicated.

Aims Firstly, we wanted to establish whether D-dimer tests were clinically indicated. Secondly, we wished to investigate the subsequent management of patients in the context of the results.

Methods We studied a sample of 93 patients who had a D-dimer test during their A&E attendance, between May and June 2015. To determine if testing was appropriate, we analysed data regarding clinical presentation and Well's score documentation.

Results Only 26% of patients had a documented Wells score. We found that 55% of all tests ordered were inappropriate. Of these, 18% had testing despite a low Wells score, the rest were deemed inappropriate based on review of the clinical presentation. Of those that had a documented Well's score, 58% were tested appropriately. 26 patients had imaging to investigate for thrombosis, 50% of these had a raised D-dimer and 35% had a high risk Well's score. VTE was confirmed in only 3 of these patients. 21 imaging investigations (4 CTPA, 5 V/Q and 12 Doppler USS) were requested despite a low d-dimer and/or Wells score, 3 of these tests confirmed a VTE, however all 3 had high Wells scores.

Summary/Conclusions In conclusion, over half of D-dimer testing done in our A&E department is inappropriate. Consequently, patients are often subject to unnecessary imaging investigations.

The cost implications of this are also significant, at £3720 per month. To address this, we suggest careful consideration of each test ordered. This should involve using a Wells score to ensure relevant indication for testing.

P03

ROLE FOR P2X1 RECEPTORS IN FCγRIIA INDUCED CALCIUM ENTRY IN HUMAN PLATELETS Zeki Ilkan[1]; Lorenza Francescut[1]; Stephanie Watson[2]; Steve P. Watson[2]; Martyn P. Mahaut-Smith[1]. [1]Department of Molecular and Cell Biology, University of Leicester, Leicester, UK [2]Centre for Cardiovascular Sciences, Institute of Biomedical Research, University of Birmingham, Birmingham, UK

Background In addition to their role in haemostasis, platelets contribute to immune responses through Toll-like receptors (TLR) and FcγRIIa. ATP-gated P2X1 receptors amplify platelet signalling during activation of several receptor types, particularly the tyrosine kinase-coupled receptors GPVI and TLR1/2. However, the contribution of P2X1 receptors to events downstream of FcγRIIa receptors, which activate Src family tyrosine kinases, is unknown.

Aims To investigate the role of P2X1 receptors during activation of FcγRIIa in platelets.

Methods FcγRIIa receptor activation was achieved by cross-linking the anti-FcγRIIa monoclonal antibody (mAb) IV.3 (1μg/mL) with anti-mouse IgG $F(ab')_2$ (3.8-30μg/mL), in presence of 0.32U/mL apyrase. Intracellular Ca^{2+} ($[Ca^{2+}]_i$), aggregation and ATP release were studied in washed human platelets. P2X1 receptor activity was desensitised by pre-addition of 600nM α,β-meATP or exclusion of apyrase, or inhibited with 1μM NF449. Bacterial stimulation was performed using *S.sanguinis* at an optical density of 1.6 at 600nm.

Results FcγRIIa activation caused a concentration-dependent elevation in $[Ca^{2+}]_i$ (at IgG $F(ab')_2$ 30μg/mL $\Delta[Ca^{2+}]_i$ = 441±33nM, n=3). The responses were inhibited by NF449 (to 303±32nM, P<0.01), α,β-meATP pre-addition (to 291±40nM, P<0.05), or apyrase exclusion (to 308±43nM, P<0.05), indicating a significant P2X1 receptor contribution. Further inhibition occurred in Ca^{2+}-free saline (to 192±31nM, P<0.01), thus additional Ca^{2+} entry pathways are opened by FcγRIIa activation. $[Ca^{2+}]_i$ increases to FcγRIIa were still observed in the presence of 100μM spermine NONOate (a NO donor; 189±35Nm vs 308±91nM, p>0.05) and 500nM PGI_2 (86±10nM vs 426±81nM, p<0.01), which both completely abolish thrombin-evoked Ca^{2+} signals. Bacteria and mAb-induced FcγRIIa aggregation were also inhibited by P2X1 receptor desensitisation (by ~35% and 55%, respectively).

Summary/Conclusions P2X1 receptors contribute to FcγRIIa receptor-evoked Ca^{2+} signalling and aggregation. The relative contribution of different Ca^{2+} entry pathways to FcγRIIa-evoked signals in the presence of endothelium-derived platelet inhibitors, which may allow immune responses in the intact circulation, is currently being investigated.

P04

FULL CYCLE AUDIT OF VENOUS THROMBOEMBOLISM (VTE) PROPHYLAXIS IN A TERTIARY BURNS UNIT Alexander Yao,[1] Jack JP O'Sullivan,[2] Amy Ireson[b] and Sanjay Varma.[1]. [1]Newcastle Upon Tyne Hospitals Foundation Trust. [2]Newcastle University Medical School, Framlington Place, Newcastle upon Tyne, NE2 4HH

Background Venous thromboembolism (VTE) is a significant cause of death in hospitalised patients, causing at least 6,500 deaths in the UK in 2005. Burn-injured patients are at particular risk due to their potential multiple transfusions, long operations and significant haemostatic disturbance.

Aims This study aimed to audit VTE prophylaxis within a ward of an NHS Trust against NICE guidelines.

Methods A full cycle audit was performed on a tertiary burns unit by way of retrospective collection of patient data from electronic records, including: completion of VTE assessment, prescription of thrombo-embolic deterrent (TED) stockings and chemical prophylaxis (type and dose), and contraindications to VTE prophylaxis. Changes were implemented included a VTE prophylaxis admission checklist and prompting posters for staff. A second cycle was then performed 3 months later which included all eligible patients admitted since the first cycle.

Results 63 and 25 patients participated in each cycle respectively. Prescription of chemical prophylaxis increased from 64% to 100% from the first to second cycle in patients for whom it was indicated. Prescription of TED stockings increased from 19% to 36%. VTE assessment slightly decreased from 63 to 58%.

Summary/Conclusions The implementation of a VTE prophylaxis admission checklist and a visual cue prompting were associated with improvement in the prescribing of indicated prophylaxis. These simple, inexpensive practices are easily transferable to other departments, in the interest of reduction of VTE deaths.

P05

VENOUS THROMBOEMBOLISM IN AMBULATORY PATIENTS WITH PANCREATIC, ENDOMETRIAL AND COLORECTAL CANCER, AND ASSOCIATION WITH KHORANA SCORE Austin K,[1] Borrowman J,[1] Scully M,[1,2] Thomas MR[1,2]. [1]Department of Haematology, University College London Hospitals. [2]Cardiometabolic BRC, UCLH, London,

Background Venous thromboembolism(VTE) causes significant morbidity and mortality in patients with malignancy. Patients with cancer are primarily managed as outpatients and current BCSH guidelines do not recommend routine thromboprophylaxis unless at high-risk. A score developed by Khorana *et al*(2008) is suggested for VTE risk stratification.

Aims To investigate VTE incidence in outpatients not on anticoagulants diagnosed with pancreatic, endometrial or colorectal cancer at a tertiary cancer centre, and role of the Khorana score in risk assessment.

Methods Retrospective data collection from electronic patient records for one year following diagnosis.

Results 122 pancreatic cancer patients diagnosed December 2009-2014 (n=24 missing data). 26/98 developed non-hospital-associated thrombosis in the first year of diagnosis (1 surgical HAT excluded), with 30 individual clots (6=lower limb LL, 4=upper limb UL including 3 PICC-associated, 5=PE, 14=abdominal, 1=IVC; 13/26 patients asymptomatic, 10/26 VTE at diagnosis). 197 endometrial cancer patients diagnosed December 2012-2014 (n=33 missing data). 9/164 developed non-HAT VTE (3 surgical HAT excluded), with 10 individual clots(1=LL, 2=UL both PICC-associated, 7=PE; 3/9 asymptomatic, 3/9 VTE at diagnosis). 232 colorectal cancer patients diagnosed December 2012-2014 (n=23 missing data). 21/209 developed non-HAT VTE (4=LL, 5=UL, all PICC-associated, 9=PE, 2=portal vein, 1=internal jugular; 9/21 asymptomatic; 0 at diagnosis). 46/87 pancreatic, 21/157 endometrial and 3/209 colorectal patients had a high-risk Khorana score(≥3). 41/87 pancreatic, 136/157 endometrial and 206/209 colorectal patients had low-risk Khorana score<3. A high Khorana score was associated with a higher 1 year VTE rate in endometrial [high-risk 3/21 VTE(14.3%) vs. low-risk 3/136 (2.2%),p=0.007]; but not pancreatic [high-risk 8/46 VTE(17.4%) vs. low-risk 7/41(17.1%),p=0.97] or colorectal cancer [high-risk 0/3 VTE(0%) vs. low-risk 21/206(10.2%),p=0.83].

Summary/Conclusions VTE is a significant burden in pancreatic (27%), endometrial (5.6%) and colorectal(10%) cancers. Khorana score predicted VTE risk in endometrial, but not pancreatic or colorectal cancer. Given the high VTE rate in these malignancies, further studies of routine thromboprophylaxis and risk stratification are warranted.

P06

'SURFING THE *PHOME*': EXTENDING AND PROBING THE DRUGGABLE PLATELET PROTEOME Osama I Elhakeem[1], Robert Hoehndorf[2], George V. Gkoutos[3], Mona Al-Shahrani[2], Paul N. Schofield[1] & Gavin E Jarvis[1]. [1]Department of Physiology, Development and Neuroscience, University of Cambridge, UK; [2]King Abdullah University of Science and Technology, Saudi Arabia; [3]College of Medical and Dental Sciences, Institute of Cancer and Genomic Sciences, Centre for Computational Biology, University of Birmingham, UK.

Background Drug discovery often occurs serendipitously. A "*PHOME*" is a virtual drugome comprising known pharmacological actions in a defined proteome. Integration of very large public datasets ("Big Data") into interaction networks enables prediction of the consequences of interrupting such networks using small molecule modulators of protein action.

Aims We used empirical *in vitro* platelet testing to evaluate the effectiveness of a bioinformatics algorithm to predict unknown platelet actions among known compounds.

Methods 1. Drug Selection: We created an algorithm to predict novel drug/target interactions with effects on platelets. A platelet protein-protein interaction (PPI) network was constructed using the Gene Expression Atlas and STRING database. Drug/target annotations were from STITCH (Search Tool for InTeracting CHemicals database). STITCH includes drug indications, and gene function annotations were from the Gene Ontology (GO) database. We scored drug effects in the Platelet-PPI-Network to

identify enriched GO categories for each drug. Lower *P* values (Wilcoxon rank sum test) predicted effects on (i) collagen binding, (ii) platelet activation, (iii) platelet aggregation. Five compounds were randomly selected from all compounds with *P*=0 (P0 drugs) for each function, and five with *P*=1 (P1 drugs). 2. Drug Evaluation: The effect of these drugs on collagen-induced aggregation in washed human platelets was evaluated. Drugs (and two controls) were randomly assigned to 12 groups. The operator was blinded to the assignation. Drugs were tested at 100 µM.

Results Preliminary analysis indicates that 4/5 P0 drugs attenuated platelet aggregation. Only four of the five P1 drugs were tested. Of these, 2 had no effect and the other 2 abolished the response.

Summary/conclusions These data suggest that the algorithm may preferentially identify drugs that alter platelet function. False positives (1/5) may reflect an insufficiently diverse testing regime. False negatives (2/4) are of greater biological/bioinformatic interest. Scrutiny of database entries may suggest novel hypotheses about platelet function and enable algorithm optimisation.

P07

THE EFFECTS OF ANTIDEPRESSANTS ON PLATELET FUNCTION

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Background Selective serotonin reuptake inhibitors (SSRIs) block the platelet serotonin transporter (SERT) and can attenuate platelet function. However, the effect of anti-depressant SSRI treatment on clinical cardiovascular disease is ambiguous. There is also a lack of *in vitro* data on the mechanism(s) by which SSRIs influence platelet function.

Aims To compare the anti-platelet effects of common antidepressant drugs with their previously published potencies for blocking serotonin (5-HT) uptake by SERT.

Methods Washed platelets were prepared from citrated blood collected from healthy human volunteers. The effects of four SSRIs (citalopram (CIT), paroxetine (PAR), fluoxetine (FLU), sertraline (SER)), a serotonin-noradrenaline reuptake inhibitor (milnacipran (MIL)) and a tricyclic antidepressant (imipramine (IMI)) on aggregation (turbidimetric aggregometry), ATP release (HPLC method) and calcium mobilisation (fura-2/AM fluorescence) were determined.

Results Each compound inhibited aggregation, ATP release and calcium mobilisation. Preliminary orders of potency (pIC_{50} values) are as follows:
Aggregation (n=2): FLU (5.64) > PAR (5.20) > SER (5.02) > IMI (5.02) > CIT (4.70) > MIL (3.96). ATP release (n=2): SER (5.38) > PAR (5.32) > FLU (5.28) > IMI (5.12) > CIT (4.68) > MIL (3.96). Calcium (n=3): SER (4.91) > PAR (4.80) > FLU (4.71) > IMI (4.61) > CIT (4.17) > MIL (3.33).
We calculated Pearson correlation coefficients (*r*) [bootstrap 95% confidence intervals] for these values and the following previously reported potencies for SERT inhibition.
5-HT uptake (pKi): PAR (9.08) > SER (8.48) > CIT (8.05) > FLU (7.70) = IMI (7.70) > MIL (6.82).
Aggregation: $r = 0.57$ [-0.60, 1.00]; ATP release: $r = 0.76$ [-0.01, 0.98]; Calcium: $r = 0.78$ [0.10, 0.99].

Summary/conclusions Weak correlations and substantially differing potencies between SERT and platelet inhibition suggest that antidepressants inhibit platelet function via SERT-independent mechanisms.

P08

REGIONAL REVIEW OF MATERNAL THROMBOCYTOPENIA AND NEONATAL OUTCOME

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Background Maternal thrombocytopenia is common, affecting about 8% of all pregnancies, which, in addition to maternal concerns, may affect neonatal platelet count at birth, depending on the cause. In immune thrombocytopenia (ITP), IgG antibodies cross the placenta and may cause neonatal thrombocytopenia; similarly in inherited thrombocytopenias there may be low neonatal platelet counts. Studies show no clear correlation between maternal and neonatal levels with considerable variation in reported percentage of neonates with thrombocytopenia.

Methods We reviewed regional maternal thrombocytopenia data to assess neonatal outcome. Databases were used to identify women presenting to the antenatal units with platelets $<100\times10^9$/l, or known ITP. The nadir of platelet count in each pregnancy was recorded with the respective neonate's platelet count and health outcome.

Results Results were available for 142 maternal-neonatal pairs with maternal age range 17-42

(median 30). The top three diagnoses included: Gestational Thrombocytopenia (GT), n=45, 32%, ITP (n=59, 41.5%) and possible ITP (n=13, 9%). Others included Fanconi anaemia (n=1), Hypoplastic anaemia (n=1), Malaria (n=1), Herediary platelet disorders (n=5), Systemic Lupus Eryrthematosis (n=1), Thrombocytopenia with absent radii (n=2), unknown cause (n=13). The nadir of maternal platelet range was 2-98x10^9/l (median=72), neonatal platelet nadir ranged from 12-580x10^9/l (median=222). Only 10 neonates had platelet <50x10^9/l, all born to ITP mothers. 17 (11.9%) neonates had platelet <100x10^9/l: 15 were of ITP mothers, 1 of mother with hereditary platelet disorder and 1 of mother with hypoplastic anaemia. There were 2 recorded inter-uterine deaths at third trimesters without cause-establishing post mortem. 1 was conceived via IVF with maternal Graves ' disease and one with maternal ITP.

Discussion conclusions Our regional preliminary data did not show any association between maternal thrombocytopenia and neonatal platelet count at birth. 49 (34.5%) of the women had platelet count <80x10^9/l requiring careful planning prior to delivery with ITP and GT being the commonest diagnosis of maternal thrombocytopenia.

P09

CONTRASTING EFFECTS OF NEGATIVELY CHARGED POLYMERS ON PLASMIN-MEDIATED TAFI ACTIVATION G. Lanning, C.S. Whyte, N.J. Mutch
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Background Activated thrombin activatable fibrinolysis inhibitor (TAFIa) down-regulates fibrinolysis by removing lysine residues form fibrin that are critical for the binding of tPA and plasminogen. TAFIa is activated by either thrombin or plasmin in the presence of cofactors. The anionic glycosaminoglycan, unfractionated heparin, has previously been shown to accelerate plasmin-mediated activation of TAFI. Polyphosphate (polyP), another anionic biomolecule, is released from platelet dense granules and regulates haemostasis at several different levels.

Aims To investigate whether polyP acts as a cofactor in plasmin-mediated activation of TAFI.

Methods Binding of polyP to plasmin and TAFI was investigated by coupling polyP to polymethacrylate beads using cross-linking reagent 1-ethyl-3-[3-(dimethylamino) propyl]-carbodiimide. PolyP-beads were incubated with plasmin or TAFI in a column based assay followed by Western blotting with antibodies to plasmin(ogen) and TAFI respectively. Activation of TAFI by plasmin ± unfractionated heparin or polyP, was analysed by Western blotting. TAFIa function was studied in a plasma (30 %) based clot lysis assay. Clotting was initiated with batroxobin and $CaCl_2$ ± carboxypetidase inhibitor (CPI) and/or hirudin to exclude thrombin-mediated TAFI activation. Lysis was monitored as change in absorbance over time.

Results Heparin enhanced plasmin-mediated TAFI activation in line with previous reports. Heparin significantly delayed fibrinolysis, an effect that was reversed by inhibiting TAFI with CPI. Down-regulation of fibrinolysis with heparin was still observed in the presence of hirudin to exclude effects of thrombin-mediated TAFI activation. In contrast, polyP had no discernible effects on plasmin-mediated TAFI activation. In agreement with the lack of functional effects neither TAFI nor plasmin were found to bind to immobilized polyP.

Summary Heparin significantly enhances the rate of plasmin-mediated TAFI activation thereby down-regulating fibrinolysis. Despite the similar nature of these polymers in terms of structure and anionic charge, polyP was unable to act as a cofactor for plasmin-mediated TAFI activation.

P10

NVESTIGATION OF THE RECOMBINANTLY EXPRESSED HUMAN FXII FIBRONECTIN TYPE II DOMAIN INTERACTION WITH ZN^{2+} IONS AND gC1Qr Bubacarr Kaira[1], Jonas Emsley[1,] Centre for Biomolecular Science, School of Pharmacy, University of Nottingham, NG7 2RD, UK

Background Factor XII, also known as Hageman factor, is an 80-kDa-plasma serine protease, implicated in blood coagulation, inflammation and complement activation. It is autoactivated by anionic surfaces with subsequent activation by kallikrein. Prekallikrein and FXII can reciprocally activate each other, producing kallikrein and FXIIa respectively. FXII autoactivation has been associated with Zn^{2+} binding and the binding sites have been mapped to be within the heavy chain of FXII, in which the FXII Fibronectin type II (FXII FnII) domain has been demonstrated to bind to Zn^{2+}

(Schousboe I., Røjkjæ, R., 1997). Furthermore, Zn^{2+} ions binding to FXII have been shown to facilitate binding to complement receptor, gC1qR. FXII has been implicated in pathological thrombosis, hereditary angioedema type III, coronary heart disease, sepsis and diabetes (Stavrou E., *et al*, 2010) and has been considered to be an ideal target for drug design to reduce venous thromboembolism or arterial thrombosis (Gailani, D and Renne, T).

Aims The aim of this study is to ascertain the structural mechanism of Zn^{2+} binding to FXII FnII domain and the role Zn^{2+} has in FXII FnII-gC1qR complex formation.

Methods Methods include expression in Drosophila insect expression system (DES), isolating complexes using Size Exclusion Chromatography and Surface Plasmon Resonance and X-ray crystallography to understand the interaction of the complexes.

Results We have determined the human FXII FnII domain structure at high resolution bound to three Zn^{2+} ions using X-ray crystallography. FXII FnII binds to gC1qR in the presence of zinc.

Summary/Conclusions Our data show FXII FnII domain 3D crystal structure bound to three Zn^{2+} ions. Our SPR and gel filtration data show complex formation of FXII full length (not shown) and FXII FnII bound to gC1qR in a zinc dependent manner.

P11

NON-GENOMIC EFFECTS OF THE PREGNANE X RECEPTOR INHIBIT PLATELET FUNCTION G.D. Flora, K.A. Sahli, A.J. Unsworth, A.R. Stainer, L.M. Holbrook, T. Sage, J.M. Gibbins. Institute for Cardiovascular and Metabolic Research, University of Reading, Reading, UK

Background: Platelets, despite being enucleated, express nuclear receptors that are capable of regulating platelet activity. The pregnane X receptor (PXR) is a nuclear receptor that is involved in the detoxification of xenobiotic compounds. Recently, the presence of PXR was reported in the human vasculature and its ligands were proposed to exhibit anti-atherosclerotic effects.

Aims: To explore the presence of PXR in human platelets and evaluate the role of its ligands in regulating platelet function.

Methods: Western blotting, immunoprecipitation and immunocytochemistry were performed to examine the expression of PXR. Platelet aggregation and dense granule secretion were studied on washed platelets with and without pre-treatment with PXR ligands (rifampicin or SR12813). The extent of fibrinogen binding and α-granule secretion was analysed using flow cytometry. Calcium mobilisation was measured by spectrofluorimetry in FURA-2AM loaded platelets and integrin $\alpha_{IIb}\beta_3$ outside-in signaling was studied by measuring clot retraction. The effects of PXR ligands on thrombus formation *in vitro* were tested in blood, under arterial flow conditions, in $DiOC_6$ loaded platelets.

Results: The expression and localisation of PXR in human platelets was confirmed using western blotting, immunoprecipitation and immunocytochemistry. Platelets incubated with rifampicin or SR12813 (10, 20, 50 and 100μM) inhibited collagen or thrombin-mediated platelet aggregation and dense granule secretion. PXR ligands attenuated CRP-XL or thrombin stimulated fibrinogen binding and P-selectin exposure, indicating a reduction in integrin $\alpha_{IIb}\beta_3$ up-regulation and α-granule secretion, respectively. Calcium mobilisation and clot retraction were also inhibited upon treatment with either of the ligands. Lastly, significant reduction in *in vitro* thrombus formation was observed with both the PXR ligands, demonstrating their potential role in regulating haemostasis and thrombosis.

Conclusions: PXR exist in human platelets and its ligands inhibit platelet activation, integrin $\alpha_{IIb}\beta_3$ outside-in signaling and thrombus formation *in vitro*. Future work will determine the mechanisms through which PXR ligands function in platelets.

P12

DEVELOPMENT OF CHANDLER LOOP FOR EXAMINING THROMBUS FORMATION Giedo Elamin, Judith Holloway, Phil Williamson, John Holloway, Nicola Englyst. Human Development & Health, Faculty of Medicine, Institute of Developmental Science, Southampton General Hospital (MP887), Tremona Road, Southampton, SO16 6YD, UK

Background

Haemostasis is the physiological process that serves to maintain the integrity of the circulatory system and requires a delicate balance between procoagulant activity and anticoagulant activity. Any significant disruption to this system may lead to severe complications such as bleeding or thrombosis.

Microvesicles (MVs) are small plasma membrane fragments (0.1-1μm) released into the circulation

by blood and vascular cells after physical stress or stimuli.
MVs promote coagulant activity and are also involved in anticoagulant pathways, due to the proteins and lipids they carry. Despite this, we do not know whether MVs are involved in thrombus formation or breakdown.
Aims: To develop a method to allow examination of thrombus formation
Methods: The Chandler Loop method forms a thrombus similar to *in vivo* thrombi. Whole blood was collected from healthy donors into trisodium citrate. Blood was re-calcified with 0.25M $CaCl_2$ and placed in the tubing before rotation for 90 minutes at 30rpm. Each thrombus was then measured in terms of weight and length, recording head and tail sections.
Results: The CL produced consistently sized and shaped thrombi consisting of head and tail regions of 3±0.5mm and 7± 0.5mm length and 1mg±0.3mg and 3mg± 0.2mg weight respectively.
Summary/Conclusions: We have developed and optimised a system for investigating thrombus formation and will now use this to research where MVs locate within the thrombus, and to study clot formation and dissolution kinetics.

P13

AN INVESTIGATION OF CALCIUM SIGNALLING DURING INTERACTIONS BETWEEN PLATELETS AND A CELL LINE OF MEGAKARYOCYTIC/ ERYTHROLEUKEMIC ORIGIN Tayyaba Iftikhar, Catherine Vial & Martyn P. Mahaut-Smith. Department of Molecular and Cell Biology, University of Leicester, Leicester, UK.

Background: Several studies have described a role for platelets in cancer metastasis, however the underlying mechanisms are unclear. Tumour cells are known to release various platelet agonists and in turn, activated platelets aggregate and adhere to cancer cells providing protection from immune surveillance.
Aims: To investigate the communication between human platelets and the human megakaryocytic/erythroleukemic cell line (HEL) at the level of the ubiquitous second messenger, intracellular Ca2+ ([Ca2+]i).
Methods: $[Ca^{2+}]_i$ responses were measured in stirred suspensions of fura-2-loaded HEL cells and platelets using dual excitation spectrofluorimetry. Measurements were made from each cell type individually and in a mixed population in which only one cell type was loaded with fura-2. Extracellular ATP was measured using a luciferin-luciferase luminescence assay. P2 receptor expression profile in HEL cells was determined using quantitative PCR.
Results: Thrombin evoked a transient increase in $[Ca^{2+}]_i$ in both platelets and HEL cells when studied individually. Following co-incubation, the HEL cell response was significantly potentiated (2.2 ± 0.23 fold, n=6) in the presence of platelets. This potentiation was not affected by the P2X1 antagonist NF449 at 1μM, but was blocked by higher concentrations of NF449 (10μM), which have been reported to also inhibit P2Y1 receptors. The TLR1/2 receptor agonist PAM_3CSK_4, caused a $[Ca^{2+}]_i$ increase in HEL cells only when co-incubated with platelets; this interaction can be explained by release of adenosine nucleotides from platelets downstream of TLR1/2 (detected by luciferin-luciferase) and a robust P2Y receptor response of HEL cells. HEL cells expressed multiple P2 receptor subtypes (P2X4>P2Y11>>P2X1> P2X7>P2X6>P2Y12>P2Y13> P2Y2).
Conclusions: These experiments demonstrate clear synergistic interactions between platelets and a myeloid cancer cell line at the level of Ca^{2+} signalling. Ongoing experiments are investigating the precise mechanisms, which likely involve ADP and/or ATP release and signalling via P2 receptors.

P14

AFFIMERS FOR MODULATION OF FIBRINOLYSIS: A POTENTIAL THERAPEUTIC TOOL? K Kearney[1], Phoenix F[1], Oxley N[1], Tiede C[2], Smith K[1], Tomlinson D[2], Ajjan RA[1]. 1. Division of Cardiovascular & Diabetes Research, Leeds Institute of Cardiovascular and Metabolic Medicine (LICAMM), University of Leeds, Leeds. 2. Biomedical Health Research Centre, Astbury Building, University of Leeds, Leeds.

Background Fibrin clot structure and resistance to lysis can determine predisposition to thrombotic as well as bleeding events. Therefore, modulating fibrin clot properties may have future therapeutic implications. We hypothesised that fibrinogen-bound peptides represent a tool to manipulate fibrin network characteristics and have the potential to identify novel therapeutic targets for the management of thrombotic and bleeding disorders.
Aims To modulate fibrin clot properties using fibrinogen-bound peptides.
Methods A phage display system was used to screen a large library ($n=10^{10}$) of random 9AA

conformational peptides (termed Affimers) for fibrinogen binding. The effects of fibrinogen-binding Affimers on fibrin clot structure and lysis were assessed by turbidimetric assays and the clots ere subsequently visualised using confocal microscopy.

Results Following phage panning, three fibrinogen-binding Affimers with distinct sequences were identified that had an effect on clot formation and clot lysis. Two (F5 and B9) prolonged plasma clot lysis time from 42.2±11.8 mins (mean±SD) to 234.4±7.4 and 102.7±14.0 mins, respectively ($p<0.01$ for both). Affimer B3 reduced maximum absorbance in a plasma system from 0.2488±0.0167 to 0.0038±0.0011 (OD at 340nm), and appeared to prevent clot formation. Affimer F5 had no significant effect on clot maximum absorbance, while B9 decreased maximum absorbance from 0.2488±0.0167 to 0.0876±0.0113. F5 showed no significant changes in clot structure when assessed by confocal microscopy, and retained its prolongation of clot lysis in whole blood ROTEM, reducing maximum lysis from 100% at 2 hours to 22.3±6.1%. These data indicate that Affimers can modulate fibrin clot formation and lysis with and without maintaining physiological clot structure.

Summary/Conclusions Using a novel approach, we identified Affimers that increase resistance to fibrinolysis, which may help to develop new therapeutic agents for bleeding disorders. Further work is currently ongoing to isolate Affimers that enhance fibrin clot lysis to help in the management of thrombotic conditions.

P15

THE PRESENCE OF CANCER CELLS IN BLOOD LEADS TO INCREASED PLATELET ACTIVATION – CRUCIAL ROLE OF GPIIB/IIIA IN PLATELET-CANCER CELLS INTERACTIONS Karolina Siewiera[1], Hassan Kassassir[1], Joanna Hertel[2], Cezary Watala[1]
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[2]Faculty of Biology and Environmental Protection, Department of Medical Biophysics, University of Lodz, Pomorska 141/143, 90-236 Lodz, Poland.

BackgroundCurrent epidemiological data suggest that cardiovascular complications considerably contribute to increased mortality of cancer patients. This phenomenon may associate with the activation of platelets through the contact with cancer cells.

Aims Our aim was to determine the impact of mouse 4T1 breast tumor cells on blood platelet activation and to evaluate the role of blood platelet receptors in platelet-cancer cell interactions.

Methods Blood obtained from inferior aorta of female Balb/C mice was incubated with mouse 4T1 cultured cells or PBS (control). Activation of platelets was evaluated based on the FC determination of the expressions of P-selectin, the active form of $\alpha_{IIb}\beta_3$, the binding of von Willebrand factor and fibrinogen. The aggregates of platelets with cancer cells were measured after the incubation of blood with 4T1 cells stained with CellTracker. The percent fraction of CellTracker–positive cells within the CD41/61-positive cells (platelets) was estimated. MoAbs against GPIIb/IIIa and GPIb receptors were used to inhibit interactions between cancer cells and blood platelets.

Results Activation of platelets was significantly increased in the presence of 4T1 cells, as evidenced by higher surface expression of P-selectin ($p<0.001$), elevated activation of $\alpha_{IIb}\beta_3$ receptor ($p<0.001$) and elevated binding of vWF ($p<0.001$) and fibrinogen ($p<0.001$) to the surface of blood platelets. Moreover, the number of platelets-cancer cell aggregates were significantly lower after the inhibition of GPIIb/IIIa receptors on the surface of blood platelets ($p<0.05$), but not after the inhibition of GPIb.

Summary/conclusions The 4T1 tumor cells enhance activation of platelets and lead to the formation of platelet-cancer cell aggregates. This aggregation at least in part may be blocked by the GPIIb/IIIa inhibitors, indicating that it is possible to modulate this interaction using some inhibitors of platelet receptors.

P13

THE EP3 AGONIST SULPROSTONE ENHANCES PLATELET ADHESION AND THROMBUS FORMATION UNDER HIGH RATE FLOW CONDITIONS. Talar M.[1], Nagy M.[2], Przygodzki T.[1], Heemskerk J.W.M.[2] and Watala C.[1]
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Background Blood platelet reactivity is regulated by numerous factors released by endothelium.

Among them, the products of cyclooxygenases constitute a very intriguing group of compounds. This is due to the fact that they exert opposing effects on blood platelets. Effects of two of them: thromboxane A_2 and prostaglandin I_2 are well defined. More complex are the effects of another product of COX - PGE_2. There are four EP receptors for PGE_2 on platelet membranes. The binding of PGE_2 to EP_1, EP_2, and EP_4 receptors inhibits the platelet reactivity, while the stimulation of EP_3 activates platelets.

Aims In our present study, we investigated whether the activation of EP_3 receptor enhances blood platelet adhesion and activation at low, high and very high shear rates.

Methods Formation of thrombi under flow conditions: Influence of Sulprostone on blood platelet adhesion to collagen and vWF coated coverslips was checked in-vitro with utilising Maastricht flow chambers. During perfusion of platelets, three different shear conditions: 150 s^{-1}, 1000 s^{-1}, 1600 s^{-1} were tested. Blood platelets were labelled with $DiOC_6$ and Alexa Fluor®647 dyes. Adherent platelets in tested surfaces were observed with using inverted EVOS microscope. Semi-automatic counting of adherent platelets was performed with the use of Fiji software. Measuring of cytosolic [Ca^{2+}] level: In suspensions of fura-2-loaded platelets the changes in [Ca^{2+}] were measured in a thermostated cuvette chamber using a QuantaMaster™ PTI fluorometer. Platelet agonists: TRAP, 2Me-ADP, and CRP were used to trigger Ca^{2+} efflux in platelets.

Results Sulprostone significantly activated blood platelets and caused enhanced adhesion of platelets to collagen and vWF coated surfaces. Sulprostone markedly increased responses to tested agonists.

Summary/Conclusions Our study shows that activation of EP3 receptors in platelets aggravate thrombus formations at high flow conditions. Sulprostone at low doses acts through the activation of $G_{i\alpha}$-coupled receptors.

P17

ACTIVATION OF CIRCULATING PLATELET BECOMES INCREASED IN CANCER DEVELOPMENT – CRUCIAL ROLE OF PLATELET-CANCER CELL INTERACTIONS IN THE MOUSE MODEL OF METASTASIS IN BREAST CANCER

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Background Platelets may interact with cancer cells flowing in a bloodstream, thus providing a scaffold for cancer metastasis.

Aims We aimed at estimating the activation/reactivity of platelets, and their clumping with cancer cells monitored during 5-week tumor progression in the mouse model of breast cancer.

Methods Mouse model of breast cancer was developed by the orthotopic injection of 4T1 cancer cells into mammary fad pads in the 8-week Balb/C female mice. Blood was obtained from inferior aorta immediately after cancer cell injection and after 2-3-4-5 weeks of cancer development (n=8 per time group). Activation of circulating platelets and platelet reactivity in response to ADP (5 or 20 μM) or thrombin (0.025 or 0.25 U/ml) was evaluated with flow cytometry (the expressions of membrane antigens: P-selectin, αIIbβ3, active complex, and the binding of vWf and fibrinogen). Platelet-cancer cell aggregates were determined based on the surface expressions of both CD24 and CD44 (cancer cells) within the population of the CD41/61-gated cells (platelets).

Results Both platelet activation and the fractions of platelet-cancer cell aggregates increased along with the tumor progression. At low agonist concentrations platelets from mice with a breast cancer demonstrated hyperreactivity at the advanced stage of tumor development (5 weeks), with regard to all the tested markers of platelet activation. Otherwise, platelet hyporeactivity was revealed for higher concentrations of agonists in mice with a cancer. **Summary/conclusions** Primed circulating platelets become more sensitive to subthreshold stimuli at more advanced stages of tumor development, which may explain the occurrence of haemostatic disturbances observed in oncologic patients. The formation of platelet-cancer cell aggregates augments upon cancer progression, thus suggesting that platelets may be crucial factors responsible for spreading of cancer cells and facilitating metastasis.

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AGONISTS OF ADENOSINE RECEPTORS REDUCE REACTIVITY OF PLATELETS FROM GERIATRIC VOLUNTEERS UNDER STATIC AND FLOW CONDITIONS

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Background Several studies focused on the modulation of blood platelet reactivity by agonizing of platelet adenosine receptors (AR).

Aims We aimed at estimating of the *in vitro* platelet reactivity inhibition with the agonists of A1 (ENBA (*N*-Bicyclo[2,2,1]hept-2-yl-5'-chloro-5'-deoxyadenosine)), A2 (FAA (2-phenylamino-adenosine)) and A2A (CGS 21680 (4-[2-[[6-Amino-9-(*N*-ethyl-β-D-ribofuranuronamidosyl)-9*H*-purin-2 yl]amino]ethyl] benzenepropanoic acid hydrochloride)) ARs in platelets from geriatric volunteers.

Methods Blood was obtained from healthy volunteers (*n* = 14, M/F = 8/6, mean age 64 ± 1.2), not taking any drugs affecting platelet reactivity. To test platelet reactivity under static conditions in the presence of AR agonists (1, 10 and 100 μM) we used impedance aggregometry and flow cytometry of platelets stimulated with arachidonic acid (0.5 mM), collagen (1 or 20 μg/ml) or ADP (10 or 20 μM) (10 min incubation, 37°C). VenaFlux system (Cellix, Ireland) was used for measurements under flow conditions.

Results The agonization of platelets with A2A agonist, CGS21680 (1, 10 or 100 μM) significantly inhibited platelet aggregation in response to collagen. CGS21680 (10 or 100 μM) significantly reduced ADP-induced platelet aggregation. FAA blocked platelet aggregation in response to arachidonate (at 1 or 100 μM) or collagen (at 10 or 100 μM). ENBA effectively reduced ADP-dependent platelet aggregation at all tested concentrations. Both AR agonists significantly reduced the expressions of P-selectin and the active form of fibrinogen receptor in platelets activated by arachidonate, ADP or collagen. Under flow conditions FAA decreased the formation of platelet aggregates on collagen by 50%.

Summary/conclusions Stimulation of platelet ARs reduces platelet reactivity in the agonist-dependent manner, both under static and flow conditions. For some AR agonists the inhibition occurred merely at high concentrations. Hence, some chemical modifications of the investigated agonists may be the solution allowing to reduce the agonist concentration.

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ENHANCED ADHESION OF BLOOD PLATELETS TO MESENTERIC VESSELS IN MICE WITH STREPTOZOTOCIN-INDUCED DIABETES IS DEPENDENT ON GPIBα.

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Background It is known that platelets and leukocytes roll on and adhere to activated endothelium. Such an effect has been shown both *in vitro* and *in vivo* in various animal models of endothelial dysfunction. Up to date there is however no direct evidence for enhanced interactions of platelets with intact endothelium *in vivo* in animal model of diabetes.

Aims Based on these considerations we have decided to test whether such a phenomenon occurs in one of the most commonly used murine models of human type 1 diabetes – streptozotocin-induced hyperglycemia. We were also interested to verify the role of two platelet receptors, which are mostly involved in this process, namely GPIbα and GPIIb/IIIa.

Methods Intravital fluorescence microscopy was used to visualize and quantify platelet interaction with endothelium in mesenteric vessels. To inhibit GPIIb/IIIa– and GPIbα-dependent interactions the Fab fragments of respective blocking antibodies were injected into a tail vein. The extents of basal activation and reactivity of blood platelets were analyzed with the use of flow cytometry. Histochemistry of mesenteric vascular bed was applied to evaluate the morphology of vascular wall and endothelium.

Results Blood platelets in STZ mice were adhering more often to vascular wall than in non-diabetic animals: 11.9 (6.4; 32.8) plt/min/mm^2 (median [IQR]) *vs*. 2.7 (1.3; 6.4) plt/min/mm^2 . The injection of anti-GPIbα antibodies decreased the number of

adhering platelets from 65.9 (24.7; 109.4) plt/min/mm^2 (median [IQR]) in STZ mice treated with isotype antibodies to 2.3 (1.6; 4.2) plt/min/mm^2 in mice treated with blocking antibodies. The effect of GPIIb/IIIa blocking was not significant. Histochemistry did not reveal substantial changes in endothelial morphology.
Summary/Conclusions In mice with 1-month STZ diabetes platelets are more prone to interact with vascular wall than in non-diabetic animals. These interaction is dependent on platelet GPIbα.

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CRYSTAL STRUCTURES OF PREKALLIKREIN HEAVY CHAIN BINDING TO ITS LIGAND PEPTIDES

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Background Plasma prekallikrein (PPK) plays a role in blood coagulation contact system and kinin-kallikrein system.
Aims To determine crystal structures of PPK and its complexes with binding ligands; and their binding constants so that we can understand the physiological function of PPK at molecular levels.
Methods Analytical size exclusion chromatography, protein crystallography technology and surface plasmon resonance (SPR) spectroscopy were used to determine the PPK-HK complex stoichiometry, to solve PPK and its complex structures, and to determine the binding constants respectively.
Results PPK and its heavy chain open reading frames were amplified from cDNA and cloned into insect cell expression vector pMT-PURO for expression into S2 cells. Full length PPK with an active site mutation and PPK heavy chain (PKHC) were expressed and purified by using ion exchange, affinity and size exclusion chromatography. Purified PPK in complex with HK produced an elution peak corresponding to molecular weight of a dimer HK and a monomeric PPK on an analytical superdex 200 10/30 column. PKHC and its complexes with a range of binding ligand peptides were crystallised successfully and their structures were solved. The structure reveals that peptide long derived from cognate ligand HK binds apple 2 domain of PPK as well as apple 1 domain. The peptide forms two hydrogen bonds on apple 1 and five hydrogen bonds and one salt bridge on apple 2 domain. The peptide binding constants from SPR study were 43.2 nM for PPK and 28.2 nM for PKHC. An alanine mutant to remove the hydrogen bonds and salt bridge from both apple 1 and 2 led to abolish the binding; an alanine mutant to remove the hydrogen bonds and salt bridge from apple 2 caused the significant reduction in binding which confirms that the peptide binding to PPK is tight and specific.
Summary/Conclusions HK peptide binds PPK in low nM range and the crystal structure reveals the peptide binds apple 1 and 2 domains of PPK.

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A NOVEL TRANSCRIPTIONAL MECHANISM FOR THE REGULATION OF THROMBOMODULIN EXPRESSION IN ENDOTHELIAL CELLS

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Thrombomodulin (TM) is constitutively expressed on endothelial cells (EC) in all vascular beds and has a major anti-coagulant and anti-inflammatory role on the EC surface, therefore playing a key role in maintaining vascular homeostasis. Little is known about the mechanisms that drive constitutive, homeostatic TM expression in EC. The ETS transcription factor ERG is a central mediator of EC function, by promoting angiogenesis, vascular stability and endothelial homeostasis. Using RNA interference (siRNA) to inhibit ERG expression in human umbilical vein (HUV)EC, we found a significant reduction in TM protein and mRNA expression compared to control siRNA ($p<0.01$). Preliminary data suggest that ERG mediates the upregulation of TM expression in HUVEC treated with the endothelial-specific growth factor angiopoietin-1(Ang-1). Interestingly, ERG seems to be also important in mediating the reduction in TM mRNA levels induced by the inflammatory cytokine tumour necrosis factor-α (TNFα), when cells treated with ERG-RNAi are compared with controls. TM dysregulation is implicated in multiple inflammatory and ischaemic pathologies, making it clinically important to understand the regulatory mechanisms of its expression.