EUPLAN

European Platelet Network

2nd EUPLAN Conference

http://www.euplan.eu

Basic and Clinical aspects of platelet research including megakaryocytes

September 24-26th, 2014
Le Bischenberg (France)

PROGRAM

2nd Euplan meeting Workshop

UNIVERSITÉ DE STRASBOURG

September 24th, 2014
EFS Alsace, Strasbourg

Inserm

Workshop on platelet and megakaryocyte biology

12:00-12:50 **Registration and Buffet Lunch**
EFS-Alsace, 10 rue Spielmann

12.50-13.00 **Welcome and introduction**
Participants will be organized in 12 groups of 5-6 people and rotate between the sessions detailed below (15 min per session):

A. Study of platelet adhesion and activation under flow

Pierre Mangin, Elmina Mammadova-Bach

1. **Flow-based assays and microfluidics**
   Elmina Mammadova-Bach, Catherine Bourdon

2. **Measurements of Ca2+ signaling and thrombus volume by confocal microscopy**
   Pierre Mangin, Nicolas Receveur

3. **Cellix flow devices**
   Philippa Timmins

B. Animal models of thrombosis

Beatrice Hechler, François Lanza

1. **$FeCl_3$-injury model**
   Béatrice Hechler, Stéphanie Benoit

2. **Laser-injury model**
   François Lanza

3. **Microscope technology for Intravital research**
   Ben Atkinson

C. Platelet and Megakaryocyte imaging by electron microscopy
Anita Eckly, Harry Heijnen

1. **TEM/IEM/Tomography**
   Harry Heijnen, Fabienne Proamer

2. **FIB-SEM**
   Anita Eckly, Jean-Yves Rinckel

3. **SEM : World Phenom**
   Pierre Forestier

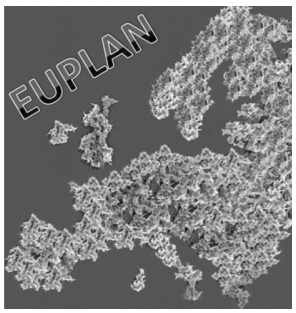
D. Proplatelet formation from murine and human megakaryocytes
Catherine Strassel/Catherine Léon

1. **Proplatelet formation from *in vitro* differentiated human CD34+ and murine Lin$^-$ cells**
   Catherine Strassel, Lea Mallo

2. **Proplatelet formation in bone marrow explant**
   Catherine Léon, Alicia Aguilar

3. **Simultaneous phenotypic and ploidy analysis on live cells by flow cytometry**
   Nathalie Brouard

17:15 **Bus transportation from EFS-Alsace to Le Bischenberg**



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PROGRAM

Welcome message

Welcome to Alsace for this second meeting of the European Platelet Network (EUPLAN) in the congress and training center "Le Bischenberg". We hope that you will enjoy the program which includes 8 keynote lectures, 25 selected oral communications and 71 poster presentations. The first edition in Maastricht was a great success. We hope that this second edition will equally satisfy the audience and further promote exchanges between our laboratories.

The undertaking of the conference would not have been made possible without the generous support of our sponsors. We are very grateful for this, especially as the times are difficult. Companies are less prone to support academic meetings as compared to previous years. Also, too many simultaneous meetings, with more direct medical applications make it difficult to get their preference. Therefore, the companies listed in the abstract book should be warmly acknowledged and thanked.

We would also like to thank the association ARMESA for its strong support of this second EUPLAN meeting and its continuous support to our research. Finally EFS also substantially contributed to the organization and support of the workshops held in Strasbourg and the EUPLAN meeting and must be thanked.

We wish you a very pleasant stay in Alsace.

Christian Gachet on behalf of the local organizing committee.

Wednesday, September 24th, 2014 - evening

From 18.30 Welcome cocktail

SESSION 1

19:30-20:30 **Epidemiology and pharmacology**
Chairmen: Christian Gachet (Strasbourg, France)
Bernhard Nieswandt (Würzburg, Germany)

19:30-20:00 **Keynote Lecture**
Carlo Patrono (Roma, Italy)
Overview on antiplatelet therapy

20:00-20:30 **Keynote Lecture**
Pierre Morange (Marseille, France)
Genetics of incident venous thrombosis

20:30 *Dinner*

Thursday, September 25th, 2014

SESSION 2

08:30-10:30 **Megakaryocytes/thrombopoiesis**
Chairmen: Carlo Balduini (Pavia, Italy)
Catherine Léon (Strasbourg, France)

08:30-09:00 **Keynote Lecture**
Carlo Balduini (Pavia, Italy)
Inherited platelet disorders

ORAL COMMUNICATIONS

09:00-09:15 **OC1**
Satoshi Nishimura (Japan)
Morphological Distinction Unravels Two Modes of Platelet Biogenesis from Bone Marrow Megakaryocytes: Regulated by Cytokine Balances

09:15-09:30 **OC2**
David Stegner (Würzburg, Germany)
Light-sheet fluorescence microscopy enables studies on megakaryopoiesis in intact murine bones

09:30-09:45 **OC3**
Antonija Jurak Begonja (Rijeka, Croatia)
Filamin A stabilizes thrombopoietin receptor surface expression and inhibits its degradation

09:45-10:00 **OC4**
Alexandra Mazharian (LAIR-1) (United Kingdom)
Mice lacking the inhibitory collagen receptor leukocyte-associated immunoglobulin-like receptor-1 exhibit a mild thrombocytosis and hyperactive platelets

10:00 -10:15 **OC5**
Sebastian Dütting (Würzburg, Germany)
Redundant functions of RhoA and Cdc42 in platelet function and biogenesis

10:15-10:30 **OC6**
Salima Benbarche (Strasbourg, France)
IL-21 promotes the in vitro expansion of primary human megakaryocytes

10:30-11:00 *Coffee break*

Thursday, September 25th, 2014

SESSION 3

11:00-12:30 **Inflammation/blood cells interplay**
Chairmen: Martine Jandrot-Perrus (Paris, France)
Steffen Massberg (Munich, Germany)

11:00-11:30 **Keynote Lecture**
Steffen Massberg (Munich, Germany)
The role of platelets and leukocytes in the context of vascular inflammation

ORAL COMMUNICATIONS

11:30-11:45 **OC7**
Béatrice Hechler (Strasbourg, France)
The P2X1 receptor is required for neutrophil extravasation during LPS-induced lethal endotoxemia

11:45-12:00 **OC8**
Waltraud Schrottmaier (Vienna, Austria)
Platelets mediate oxidized low-density lipoprotein-induced monocyte extravasation and foam cell formation

12:00-12:15 **OC9**
Yacine Boulaftali (Chapel Hill, USA)
Platelet Rap1 signaling, mediated by CalDAG-GEFI and $P2Y_{12}$, contributes to atherosclerotic lesion development in mice

12:15-12:30 **OC10**
Angèle Gros (Paris, France)
Platelets limit neutrophil histotoxic activities and prevent bleeding during immune complex-mediated dermatitis by a glycoprotein VI-dependent repairing action

12:30-14:00 *Lunch*

Thursday, September 25th, 2014

SESSION 4

14:00-16:00 **Platelet signaling**
Chairmen: Mauro Torti (Pavia, Italy)
Steve Watson (Birmingham, United Kingdom)

14:00-14:30 **Keynote Lecture**
Steve Watson (Birmingham, United Kingdom)
Overview on platelet signalling

ORAL COMMUNICATIONS

14:30-14:45 **OC11**
Yotis Senis (Birmingham, United Kingdom)
Loss of phosphotyrosine binding in Syk rescues platelet counts and GPVI expression in G6b-B-deficient mice

14:45-15:00 **OC12**
Michael Tomlinson (Birmingham, United Kingdom)
Tetraspanin Tspan18 regulates GPVI-induced platelet activation by interacting with the store-operated Ca^{2+} entry channel Orai1

15:00-15:15 **OC13**
Alyssa Moroi (Birmingham, United Kingdom)
Akt and MAPK promote CLEC-2 mediated platelet activation by inhibition of GSK3α/β

15:15-15:30 **OC14**
Stephanie Jung (Cambridge, United Kingdom)
Internalization of glycoprotein VI (GPVI) in platelets activated by soluble GPVI-specific agonists

15:30-15:45 **OC15**
Zoltan Nagy (Dublin, Ireland)
Regulation of Rac GTPases by cyclic nucleotides in human platelets

15:45-16:00 **OC16**
Marilena Crescente (Reading, United Kingdom)
PDI And ERP57 co-cluster in platelets and their movement is regulated by actin polymerization

16:00-16:30 *Coffee break*
16:30-18:00 **Poster session (from page 51 to page 122)**
19:00 *Concert/Recital*
20:00 *Dinner*

Friday, September 26th, 2014

SESSION 5

08:30-10:30 **Platelet signaling and thrombosis**
Chairmen: Hans Deckmyn (Kortrijk, Belgium)
Johann Heemskerk (Maastricht, The Netherlands)

08:30-09:00 **Keynote Lecture**
Johann Heemskerk (Maastricht, The Netherlands)
Experimental thrombosis: understanding platelet function

ORAL COMMUNICATIONS

09:00-09:15 **OC17**
Lucia Stefanini (Reading, United Kingdom)
The Rap-GAP Rasa3 is a critical negative regulator of platelet activation

09:15-09:30 **OC18**
Sakthivel Vaiyapuri (Reading, United Kingdom)
EphB2 Mediated Contact-dependent and independent Signalling in the Regulation of Thrombosis and Haemostasis

09:30-09:45 **OC19**
Suzanne Korporaal (Utrecht, The Netherlands)
Disturbed glycoprotein VI-mediated signaling in platelets from hypercholesterolemic mice and humans

09:45-10:00 **OC20**
Judith Van Eeuwijk (Würzburg, Germany)
Syk deficiency protects mice from occlusive arterial thrombus formation and ischaemic stroke without inducing intracranial haemorrhage

10:00-10:30 **Keynote Lecture**
Denis Vivien (Caen, France)
Experimental models of ischemic stroke

10:30-11:00 *Coffee break*
11:00-12:30 **Poster session (from page 51 to page 122)**
12:30-14:00 *Lunch*

Friday, September 26th, 2014

SESSION 6

14:00-15:30 **Various aspects of platelet physiology and pharmacology**
Chairmen: Harry Heijnen (Utrecht, The Netherlands)
Michael Tomlinson (Birmingham, UK)

14:00-14:30 **Keynote Lecture**
Harry Heijnen (Utrecht, The Netherlands)
Visions on platelets: A historical overview

ORAL COMMUNICATIONS

14:30-14:45 **OC21**
Kate Lowe (Birmingham, United Kingdom)
Podoplanin and CLEC-2 interactions drive vascular patterning in the developing brain

14:45-15:00 **OC22**
Ejaife Agbani (Bristol, United Kingdom)
Coordinated platelet ballooning and procoagulant membrane spreading is driven by salt and water entry

15:00-15:15 **OC23**
Nadine Mattheij (Maastricht, The Netherlands)
Altered phosphorylation profile of Scott syndrome platelets as revealed by quantitative phosphoproteomics

15:15-15:30 **OC24**
Bernard Payrastre (Toulouse, France)
Ibrutinib treatment affects collagen and von Willebrand Factor-dependent platelet functions

15:30-15:45 **OC25**
Pierre Fontana (Geneva, Switzerland)
Identification of candidate platelet miRNA regulating platelet reactivity in aspirin-treated cardiovascular patients

15:45-16:00 Concluding remarks, next meeting

16:15 Meeting point in the lobby and bus departure to Strasbourg Station/Airport

POSTERS

P1 Osama Alshehri (Birmingham, United Kingdom)
Diesel particles activate platelets through GPVI and CLEC-2

P2 Catherine Angénieux (Strasbourg, France)
RNA in platelets, no more than a relic

P3 Thomas Blair (Bristol, United Kingdom)
Deletion of PI3K p110α results in enhanced platelet priming by distinct growth factors.

P4 Yacine Boulaftali (Chapel Hill, USA)
The calcium-binding protein S100A1 negatively regulates collagen-dependent platelet activation and thrombosis in mice.

P5 Arnaud Dupuis (Strasbourg, France)
Looking for genes responsible for δ-storage pool disease.

P6 Joanna-Marie Howes (Cambridge, United Kingdom)
Matrix metalloproteinase-13 influences thrombus formation under flow conditions

P7 Julia Kral (Vienna, Austria)
Human Cytomegalovirus–Platelet Interaction Triggers Toll-Like Receptor 2–Dependent Proinflammatory and Proangiogenic Responses

P8 Catherine Léon (Strasbourg, France)
Improved megakaryocyte differentiation using 3D hydrogel progenitor culture

P9 Tomas Lindahl (Linkoping, Sweden)
Extraculllar loop II in platelet thrombin receptor PAR4 is important for binding of thrombin.

P10 Thomas Thiele (Greifswald, Germany)
Tolerance of Platelet Concentrates Treated With UVC Light Only for Pathogen Reduction

P11 Amanda Unsworth (Reading, United Kingdom)
Non-genomic effects of nuclear receptors: Different mechanisms of regulation of outside–in signalling in platelets

P12 Jonathan M. Gibbins (Reading, United Kingdom)
Activation of human platelets by Staphylococcus aureus secreted protease staphopain A

P13 Marina Camera (Milano, Italy)
Functionally active tissue factor is expressed during megakaryocyte maturation and is transferred to a subset of platelets

P14 Richard Farndale (Cambridge, United Kingdom)
The recognition of collagen by the VWF A domains

P15 Hendrik Feys (Ghent, Belgium)
Persistent particles in apheresis platelet concentrates

P16 Sue Fox (Nottingham, United Kingdom)
Assessment of platelet function using a P-selectin based Platelet fuction test compared with other commercial tests

P17 Eelo Gitz (Birmingham, United Kingdom)
CLEC-2 expression is maintained on activated human platelets and on platelet microparticles

P18 Elizabeth Haining (Wüersburg, Germany)
Regulation of the platelet collagen receptor GPVI by the tetraspanin Tspan9

P19 Hind Hamzeh-Cognasse (Lyon, France)
Human platelets sense differentially various Staphylococcus aureus exotoxins and adapt subsequent immunomodulatory molecule secretion profiles

P20 Martine Jandrot-Perrus (Paris, France)
Losartan-induced Inhibition of GPVI clustering by collagen and collagen-induced platelet responses is achievable in vitro but not in treated patients.

P21 Friederike Jönsson (Paris, France)
The human IgG receptor FcgRIIA induces allergic and inflammatory reactions

P22 Daria Manganaro (Pavia, Italy)
The interplay between Pyk2 and phosphatidylinositol 3-kinaseß in GPVI signaling

P23 Nadine Mattheij (Maastricht, The Netherlands)
Human platelet STIM1 and ORAI1 are essential in store-operated calcium entry and thrombus formation at venous conditions

P24 Satoshi Nishimura (Japan)
Thrombus Development Processes Dependent on Endothelial Injuries: Visualized by In vivo Two-photon Molecular Imaging

P25 Jean-Luc Reny (Geneva, Switzerland)
Vascular risk levels affect the predictive value of platelet reactivity for the occurrence of major adverse cardiovascular events in patients on clopidogrel: Systematic review and collaborative meta-analysis of individual patient data

P26 Isabella Russo (Turin, Italy)
GLP-1 reduces the activation of PI3K and MAPK pathways and of oxidative stress induced by Sodium Arachidonate in platelets

P27 Mariangela Scavone (Milano, Italy)
Does the inhibition of $P2Y_{12}$ inhibit the production of thromboxane A2 by platelets?

P28 Marco van der Stoep (Lieden, The Netherlands)
Impaired response to collagen of platelets from hyperlipidemic mice

P29 Dmytro Zhernosiekov (Kyiv, Ukraine)
Platelet aggregation: the effect of Lys- and Glu-plasminogen

P30 Nathalie Brouard (Strasbourg, France)
Megakaryocyte Expansion by the Fetal Liver Microenvironment

P31 Ilaria Canobbio (Pavia, Italy)
Activation of human platelets by amyloid ß peptides: essential role of Ca^{2+} and ADP in aggregation and thrombus formation

P32 Benoit Decouture (Paris, France)
Inhibition of MRP4 down regulates platelet activation and prevents pre-clinical arterial thrombosis

P33 Céline Delierneux (Liège, Belgium)
Phosphorothioate-modified bacterial DNA activates platelets and promotes thrombosis through integrin αIIbβ3.

P34 Angèle Gros (Paris, France)
Collagen can selectively trigger a platelet secretory phenotype via glycoprotein VI

P35 Azhar Hafeez (Leicester, United Kingdom)
Reversal of the Effect of Aspirin by Heparin

P36 Samir William Hamaia (Cambridge, United Kingdom)
Regulation of platelets collagen receptor α2β1 integrin and the possibility of intermediate affinity

P37 Stefan Heber (Vienna, Austria)
Endurance training normalizes pro-inflammatory aspects of platelet function in (formerly) sedentary females and also affects platelet proteome

P38 Sangar Osman (Leicester, United Kingdom)
Properties of the membrane invagination pore of the megakaryocyte demarcation membrane system

P39 Natalie Poulter (Birmingham, United Kingdom)
GPVI clustering in platelets imaged by super resolution microscopy

P40 Parvathy Sasikumar (Reading, United Kingdom)
The Chaperone Protein Hsp47: A Novel Platelet Collagen Receptor that Contributes to Thrombosis and Haemostasis

P41 Adrien Geoffrey Antkowiak (Toulouse, France)
Cdc42-dependent F-actin organization is critical for demarcation membrane system structuration and proplatelet emission in megakaryocytes

P42 Ana Carolina Antunes Naime (San Paulo, Brazil)
Effect of pravastatin on the number of circulating platelets in rats treated or not with lipopolyssaccharide

P43 Paul Armstrong (London, United Kingdom)
Characterising cell-type interactions following platelet activation in a novel whole blood assay

P44 Paul Armstrong (London, United Kingdom)
Validation of novel miniaturised whole blood aggregation assay using adhesion molecule knockout mice

P45 Marta Brambilla (Milano, Italy)
Cellular distribution of Tissue Factor within platelet-leukocyte aggregates in human whole blood

P46 Alexandra Bussey (Cambridge, United Kingdom)
Metabolomic response of human platelets to collagen receptor engagement

P47 Isabel Cortegano (Madrid, Spain)
CD45 negative megakaryocytes are in the mouse embryo

P48 Zsolt Fejes (Debrecen, Hungary)
Altered expression of serum, plasma and platelet microRNAs in type 2 diabetes mellitus

P49 Florian Gaertner (Munich, Germany)
Sphingosine 1-phosphate produced by sphingosine kinase 2 intrinsically controls platelet homeostasis and arterial thrombosis in mice

P50 Gisele Goulart (Campinas, Brazil)
Reduction of beta3tyr785 chain phosphorylation of alphallb beta3 integrin takes part on the inhibitory effect of statins in stimulated-platelet adhesion to fibrinogen of high fat-fed rats.

P51 Rebecca Knowles (London, United Kingdom)
The inhibitory effects of nitric oxide and prostaglandin I_2 on platelet aggregation are greatly enhanced by blockade of $P2Y_{12}$ receptors

P52 Maria Elisa Lopes Pires (San Paulo, Brazil)
The increases reactive oxygen species generation by lipopolysaccharide in vivo in rat platelet, via nadph-oxidase activation, involving pkc, sgc and pkg

P53 Stéphane Loyau (Paris, France)
In-vitro imaging of platelet fibrin clot formation and lysis in flow condition

P54 Elmina Mammadova-Bach (Strasbourg, France)
Platelet integrin $\alpha 6\beta 1$ promotes metastatic dissemination

P55 Aastha Mathur (Heidelberg, Germany)
Role of cytoskeleton in platelet activation

P56 Bela Nagy Jr (Debrecen, Hungary)
Comparison of the effects of elective bare metal versus drug-eluting percutaneous coronary stents in stable angina patients

P57 Nadezhda Podoplelova (Moscow, Russia)
Coagulation factors X and Xa bind to procoagulant platelets in a hysteresis manner enabling their retention in thrombi despite rapid flows

P58 Ashwin Radhakrishnan (Nottingham, United Kingdom)
A novel $P2Y_{12}$ P-selectin test as an alternative approach to assessment of platelet function in relation to clinical outcomes in a coronary artery disease population

P59 Ashwin Radhakrishnan (Nottingham, United Kingdom)
Use of VASP assays to assess platelet response to $P2Y_{12}$ antagonists in ACS patients: interesting findings in relation to bleeding

P60 Irina M. Kolesnikova (Moscow, Russia)
Changes in clot properties due to platelet concentrate storage (in vitro study)

P61 Vishal Salunkhe (Amsterdam, The Netherlands)
Understanding the impact of storage and pathogen inactivation treatment on platelet concentrates at the protein level using mass spectrometry analyses

P62 Sonia Severin (Toulouse, France)
ABCA1 deficiency decreases platelet reactivity by reducing positive feedback loop mechanisms and changing the pattern of lipid mediator production

P63 Anna Södergren (Linkoping, Sweden)
Reactive oxygen species enhance generation of subpopulations of procoagulant platelets

P64 Alexander Stainer (Reading, United Kingdom)
The relationship between the metabolites of the dietary flavonoid quercetin and haemostasis, thrombosis and platelet function

P65 Catherine Strassel (Strasbourg, France)
Evolution of the α- and β-tubulin repertoire and post-translational modifications during platelet biogenesis

P66 Tony Walsh (Bristol, United Kingdom)
Elucidating SDF-1α-mediated regulation of platelet function

P67 Stephanie Watson (Birmingham, United Kingdom)
Mechanism of platelet activation by Fucoidan and Dextran

P68 Ozkan Yesim (Ankara, Turkey)
Compounds Having Antiplatelet Activity: 3(2H) pyridazinone

P69 Jean-Luc Reny (Geneva, Switzerland)
International Clopidogrel Pharmacogenomics Consortium Genome Wide Association Study Identifies Novel Variants for Clopidogrel Response

P70 CS Whyte (Aberdeen, United Kingdom)
Platelets support direct and indirect binding of plasminogen facilitating local fibrinolysis

P71 Laura Twomey (Dublin, Ireland)
Investigation of Platelet microRNA as a marker of Epigenetic Drift

ORAL COMMUNICATIONS

OC1

Morphological Distinction Unravels Two Modes of Platelet Biogenesis from Bone Marrow Megakaryocytes: Regulated by Cytokine Balances

Satoshi Nishimura [1,2,3]

[1] *Department of Cardiovascular Medicine, the University of Tokyo, Tokyo JAPAN*

[2] *Translational Systems Biology and Medicine Initiative, the University of Tokyo, Tokyo, Japan*

[3] *Center for Molecular Medicine, Jichi Medical University, Tochigi, Japan*

Introduction: Blood platelets are generated in the bone marrow (BM) from their precursors, megakaryocytes (MK). Although we know that MKs produce platelets throughout life, precisely how platelets are produced in vivo remains uncertain, largely because of the rarity of MKs in the BM and the lack an adequate visualization technique.

Methods: In the present study, we were able to visualize MK dynamics leading to platelet release in living animals at high resolution by two photon microscope. To clearly understand the nature of thrombopoiesis in BM MKs, we optimized an in vivo imaging technique that enabled us to visualize living BM in CAG- enhanced green fluorescent protein (eGFP) and CD41-tdTomato mice.

Results: By visualizing living bone marrow in vivo, we observed that a second thrombopoietic process, rupture-like MK fragmentation in addition to the proplatelet formation in BM of living mice. Short proplatelets predominated in the steady state, but highly elongated proplatelets were apparent when thrombopoietin (TPO) levels were high (e.g., after BM transplantation). Conversely, following blood loss, 5-FU administration, antibody-based platelet depletion or acute inflammation, there was accelerated release of larger platelets from mature MKs mediated by 'rupture'. This type was mediated by the interleukin-1 (IL-1)alpha-type1 IL-1 receptor axis and ERK-dependent MK pre-apoptosis. Moreover, microtubule disorganized contributed to proplatelets in TPO-stimulated MKs but disassembly take place in IL-1alpha-stimulated MKs due to uncoordinated expression of alpha- and beta-tubulin.

Conclusions: These findings support the idea that IL-1alpha acts acutely as a platelet releasing factor, coordinating with TPO to dynamically modulate the cellular programming of MKs that regulates platelet counts.

OC2

Light-sheet fluorescence microscopy enables studies on megakaryopoiesis in intact murine bones

David Stegner[1,2]*, Judith M.M. van Eeuwijk[1,2]*, Jürgen Pinnecker[2], Mike Friedrich[2], Christian Brede[3], Andreas Beilhack[3], Bernhard Nieswandt[1,2] & Katrin G. Heinze[2]

[1] Chair of Vascular Medicine, University Hospital Würzburg, Würzburg, Germany
[2] Rudolf Virchow Center for Experimental Biomedicine, University of Würzburg, Würzburg, Germany
[3] Department of Medicine II and Interdisciplinary Centre for Clinical Research (IZKF), University Hospital Würzburg, Würzburg, Germany

Introduction: Blood platelets are small anucleated cell fragments generated from bone marrow megakaryocytes (MKs), which develop during a largely thrombopoietin-driven process, termed megakaryopoiesis. Previous studies on megakaryopoiesis have been hampered by the necessity to section the bones, potentially resulting in cutting artefacts. Moreover, these sections are intrinsically two-dimensional, which might result in an underestimation of MK-vessel contacts and MK volumes.

Methods: Femora and sterna of C57Bl/6J mice were collected, PFA-fixed and made chemically transparent by a process called clearing, where water is replaced by a chemical substance with a higher refractive index. Samples were antibody-stained for MKs and endothelial cells. Large tissue specimens (up to 1.5 mm) were imaged via light-sheet fluorescence microscopy (LSFM).

Results: We combined and optimised antibody penetration, tissue clearing, and triple-colour illumination to create a method for visualising MKs in the intact bone, at a resolution which enabled us to visualise proplatelets. This approach allowed us to successfully quantify changes in MK volume, localisation and number throughout the bone marrow of the unsectioned sternum. Furthermore, we could distinguish classical MK differentiation stages (megakaryoblast, promegakaryocyte and mature megakaryocyte) by the correlation of the MK volume and the distance to the vessel wall. In addition, thrombocytopenia was induced with a bolus injection of platelet-depleting anti-GPIbα antibodies and the effects on megakaryopoiesis were monitored for several days. Thus, we were able to follow the spatio-temporal responses of the different MK differentiation stages after platelet-depletion.

Conclusions: In conclusion, the methodology presented here complements current *in situ* and *ex vivo* techniques and is beneficial in studying megakaryopoiesis. Moreover, our data underscores the correlation between MK maturation stages and MK localisation.

OC3

Filamin A stabilizes thrombopoietin receptor surface expression and inhibits its degradation

Antonija Jurak Begonja[1,2], Renata Grozovsky[1], Karin M. Hoffmeister[1], Hervé Falet[1], John H. Hartwig[1]

[1] *Translational Medicine Division, Brigham and Women's Hospital, Harvard Medical School, Boston, USA*
[2] *Department of Biotechnology, University of Rijeka, Rijeka, Croatia*

Introduction. Filamin A (FlnA) is a large cytoplasmic protein that crosslinks actin filament networks and links membrane glycoproteins and signaling proteins to the underlying cytoskeleton. We have previously shown that FlnA is critical for platelet production and survival since mice that specifically lack FlnA in their platelets (FlnA loxP-PF4-Cre) have macrothrombocytopenia, due to rapid platelet clearance and an altered megakaryocyte (MK) maturation program. FlnA loxP-PF4-Cre mice have a marked increase in MK numbers in bone marrow and spleen. Duration and amplitude of thrombopoietin receptor (TPOR, c-Mpl) signaling is a highly regulated process that is crucial for MK differentiation.

Methods. We used MKs and platelets from FlnA loxP-PF4-Cre+ mice to study biochemistry of TPOR by flow cytometry, immunofluorescence and Western blot.

Results. FlnA loxP-PF4-Cre mice have increased TPO plasma levels and 35% of their circulating platelets are reticulated indicating increased thrombopoiesis. Here we show that TPOR binds to the actin cytoskeleton in bone marrow derived MKs upon TPO stimulation in a dose dependent manner and is inhibited by actin depolymerizing drug, lantranculin A. Linkage of TPOR to the actin cytoskeleton was dependent on FlnA since TPOR was mostly in soluble, F-actin unbound fraction of the cell in FlnA KO MKs. Immunofluorescence analysis revealed partial colocalization of FlnA and TPOR in WT MKs. Moreover, surface expression of TPOR receptor was markedly decreased in both FlnA KO MK and platelets. Prolonged incubation with TPO downregulated TPOR in FlnA KO platelets. This effect was inhibited by lysosome (bafilomycin) and proteasome (MG132) inhibitors. In addition, culturing of FlnA KO MKs with bafilomycin and MG132 rescued surface TPOR expression to normal levels.

Conclusion. Our results suggest an important role for FlnA in stabilizing TPOR on the cell surface by linking it to the actin cytoskeleton and preventing its lysosomal and proteasomal degradation.

OC4

Mice lacking the inhibitory collagen receptor leukocyte-associated immunoglobulin-like receptor-1 exhibit a mild thrombocytosis and hyperactive platelets

Alexandra Mazharian[1], Steven G. Thomas[1], Marie Lordkipanidzé[1], Danai Bem[1], Linde Meyaard[2], Yotis A. Senis[1] and Steve P. Watson[1]

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[2]Department of Immunology, University Medical Center Utrecht, Utrecht, The Netherlands

Introduction: Inhibition of platelet activation is important for controlling pathological thrombosis and is emerging as an important regulatory pathway in platelet formation. The leukocyte-associated immunoglobulin-like receptor-1 (LAIR-1) is an immunoreceptor tyrosine-based inhibition motif (ITIM)-containing collagen receptor that inhibits signalling via the immunoreceptor tyrosine-based activation motif (ITAM)-containing collagen receptor complex GPVI-FcR γ-chain. It is expressed on hematopoietic cells and immature megakaryocytes, but is not detectable on platelets. Although the inhibitory function of LAIR-1 has been described in immune cells, its physiological role in megakaryocytes and platelet formation has not been explored.

Methods: LAIR-1-deficient mice were generated and analysed for defects in megakaryocyte development, platelet production and function.

Results: LAIR-1-deficient mice had significantly increased platelet counts and normal platelet volumes. Platelet half-life *in vivo* was prolonged, accounting for the mild thrombocytosis. LAIR-1-deficient megakaryocytes develop normally *in vitro*; however, a larger proportion of LAIR-1-deficient megakaryocytes formed proplatelets *in vitro* compared with control megakaryocytes. Interestingly, platelets from LAIR-1-deficient mice exhibited a mild enhancement of platelet aggregation and ATP secretion in response to collagen and the GPVI-specific agonist collagen-related peptide (CRP), despite not expressing LAIR-1. As a result, LAIR-1-deficient platelets formed larger aggregates on a collagen matrix and under arterial shear. Tyrosine phosphorylation of the FcR γ-chain, the activation loop of the tyrosine kinase Syk and its downstream target PLC 2 was increased in CRP-stimulated LAIR-1-deficient platelets.

Conclusions: This study demonstrates that deletion of LAIR-1 in megakaryocytes disrupts the homeostatic balance of megakaryocytes and platelets, shifting the balance to a prothrombotic state, which may predispose to thrombotic complications. This study represents the first demonstration of a cellular phenotype associated with LAIR-1-deficiency and adds to the growing evidence that ITIM-containing receptors are critical regulators of megakaryocyte development, platelet formation and function.

This work was funded by the British Heart Foundation.

OC5

Redundant functions of RhoA and Cdc42 in platelet function and biogenesis

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Introduction: The Rho GTPase family members RhoA and Cdc42 play major roles in cytoskeletal reorganization that occurs during platelet production from megakaryocytes (MK) and their activation at sites of vascular injury. We have shown that a MK-/platelet-specific RhoA-deficiency results in a moderate macrothrombocytopenia and impaired platelet responses downstream of G protein-coupled receptors. On the other hand, mice with a MK-/platelet-specific Cdc42-deficiency also display a moderate thrombocytopenia but increased agonist-induced secretion. Although the signaling pathways downstream of RhoA and Cdc42 partially overlap, possible redundant functions of these Rho GTPases in regulating platelet biogenesis and function have remained elusive.

Methods: Mice with MK-/platelet-specific (PF4-Cre/loxP) double-deficiencies in RhoA and Cdc42 (referred to as *RhoA/Cdc42*$^{-/-}$) were generated by intercrossing the respective single knockout mice. Platelet function was assessed by biochemical methods. Platelet and MK morphology, and proplatelet formation were investigated *in vitro* and *in situ* by confocal and transmission electron microscopy (TEM).

Results: In contrast to single-deficiency of either RhoA or Cdc42, double-deficiency in RhoA and Cdc42 resulted in a severe macrothrombocytopenia with platelet counts below 25% of control mice. The size of the circulating platelets was very heterogeneous and detailed analysis by TEM revealed a highly abnormal ultrastructure concerning shape, vacuoles, paucity in granules, and abnormal distribution of microtubules, which was also observed in bone marrow MKs. RhoA/Cdc42-deficiency was associated with a markedly reduced platelet life span which was attributed to increased platelet clearance, whereas the rate of platelet production following antibody-mediated platelet depletion was comparable between wild-type and *RhoA/Cdc42*$^{-/-}$ mice. Furthermore, *RhoA/Cdc42*$^{-/-}$ mice displayed impaired agonist-induced integrin activation and phosphatidylserine exposure. Notably, these platelet abnormalities resulted in a pronounced hemostatic defect, while unexpectedly, occlusive thrombus formation was largely unaltered.

Conclusions: These results demonstrate for the first time a functional redundancy of RhoA and Cdc42 in platelet function and biogenesis.

OC6

IL-21 promotes the *in vitro* expansion of primary human megakaryocytes

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Introduction: Several inflammatory cytokines influence megakaryopoiesis. Gene profiling studies indicate that *in vitro* differentiated megakaryocytes express the receptor for IL-21 (IL-21R), a cytokine produced during immune responses and implicated in a number of chronic inflammatory diseases. The aim of this study was to assess the role of IL-21 on human megakaryocytes.

Methods: Megakaryocytes were differentiated from peripheral blood CD34+ cells in the presence of thrombopoietin and proliferative cytokines (stem-cell factor, IL-6 and IL-9) for 7 days (phase 1) and then, thrombopoietin alone for 5 days (phase 2). IL-21 was added in phase 1 and/or phase 2. Expression of IL-21R on these megakaryocytes was evaluated by RT-PCR and flow cytometry. Phosphorylation of STAT proteins mediated by IL-21 and thrombopoietin receptors was analyzed by flow cytometry. The effect of IL-21 on the number of *in vitro* differentiated megakaryocytes was evaluated by checking the effects of inhibitors of the JAK3-STAT3 pathway. The phenotypes of the cells and generated platelet were analyzed by flow cytometry. Expression of IL21R on bone marrow megakaryocytes was checked by immunofluorescence.

Results: Experiments demonstrated that IL-21R was expressed on a subpopulation of bone marrow megakaryocytes and progressively, on all *in vitro* differentiated megakaryocytes. IL-21 induced STAT3 phosphorylation in megakaryocytes, while thrombopoietin, preferentially induced STAT5 phosphorylation. Addition of IL-21 increased in a dose dependent manner the number of *in vitro* differentiated megakaryocytes, up to 1.8±0.2 fold when used at 100ng/ml. Inhibitors of JAK3-STAT3 inhibited this effect. The phenotypes of the cells were similar, whether IL-21 was added or not, as judged by the expression of megakaryocytes specific receptors (CD42) and, the ploidy of CD41+ cells. Moreover, platelet production was not altered by the presence of IL-21.

Conclusion: These observations indicate that during immune responses, IL-21 can modulate megakaryopoiesis and platelet counts, suggesting consequences in IL-21-dependent diseases to be investigated.

OC7

The $P2X_1$ receptor is required for neutrophil extravasation during LPS-induced lethal endotoxemia

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Introduction: Extracellular adenosine 5'-triphosphate (ATP) is becoming increasingly recognized as an important regulator of inflammation. However, the known repertoire of P2 receptor subtypes responsible for the pro-inflammatory effects of ATP is sparse. We looked at whether the $P2X_1$ receptor, an ATP-gated cation channel present on platelets, neutrophils and macrophages, participates in the acute systemic inflammation provoked by lipopolysaccharide (LPS).

Methods: LPS (10 mg/kg) was injected intraperitoneally into wild-type (WT) mice and mice deficient for the $P2X_1$ receptor ($P2X_1^{-/-}$ mice). The concentrations of blood cytokines and chemokines, the activation of coagulation, lung histology and survival of mice were evaluated.

Results: Within 5 days following LPS administration, 70% of WT mice vs 15% of $P2X_1^{-/-}$ mice, died (P=0.0003, n=20). As compared to WT mice, $P2X_1^{-/-}$ mice displayed strongly diminished pathological responses, namely lower activation of coagulation reflected by lower TAT concentrations in plasma (35±2 ng/mL in $P2X_1^{-/-}$ mice vs 79±12 ng/mL in WT mice, P=0.0015, n=10), diminished neutrophil accumulation in the lung ($429x10^3$±$41x10^3$ pixels in $P2X_1^{-/-}$ mice vs $660x10^3$±$57x10^3$ pixels in WT mice, P=0.0017, n=6), and reduced tissue damages. $P2X_1$ receptor deficiency was also associated with a marked reduction in blood concentration of main pro-inflammatory cytokines (TNFα, IL6) and chemokines (MIP1α, MIP1β, RANTES), known to be induced by LPS. Interestingly, macrophages and neutrophils isolated from WT and $P2X_1^{-/-}$ mice produced similar levels of cytokines when stimulated with LPS *in vitro*. Intravital microscopy revealed a defect in LPS-induced neutrophil emigration from cremasteric venules into the tissues of $P2X_1^{-/-}$ mice. Using adoptive transfer of immunofluorescently labeled neutrophils from WT and $P2X_1^{-/-}$ mice into WT mice, we could demonstrate that the absence of the $P2X_1$ receptor on neutrophils *per se* was responsible for this defect.

Conclusion: This study reveals a major role of the $P2X_1$ receptor in LPS-induced lethal endotoxemia through its critical involvement in neutrophil emigration from venules.

OC8

Platelets mediate oxidized low-density lipoprotein-induced monocyte extravasation and foam cell formation

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Introduction: Activation of leukocytes by oxidized low-density lipoprotein (oxLDL) is a well-established step in the development of atherosclerosis. OxLDL promotes leukocyte recruitment, adhesion and endothelial transmigration, processes which are also facilitated by activated circulating platelets. Monocytes/macrophages are of particular importance as their unlimited oxLDL uptake results in the formation of characteristic foam cells. However, oxLDL also activates platelets and recent findings associate oxLDL binding by platelets with acute coronary syndrome. Therefore, we aimed to further investigate the interplay between oxLDL, monocytes and platelets in the formation of foam cells.

Methods: Formation of platelet-monocyte aggregates (PMA) in vitro and in vivo as well as lipid uptake in vitro was analyzed by flow cytometry and confocal microscopy. In vitro monocyte transmigration was determined using transwell filters. In vivo extravasation and foam cell formation were assayed in a mouse model of thioglycollate-ellicited sterile peritonitis by subjecting LDLR-/- mice to platelet depletion by R300 anti-CD42b antibody.

Results: Our results show that in response to oxLDL platelets rapidly interact with monocytes via P-selectin to form PMAs in vitro and in vivo. OxLDL-mediated activation of monocyte CD11b was enhanced by platelets in a P-selectin dependent way, indicating that direct cell interaction is required for the promigratory effect of platelets on monocytes. Indeed, platelets enhance oxLDL-mediated transmigration of monocytes across HUVEC-coated transwells in vitro. Further, platelet depletion significantly reduces monocyte extravasation in vivo. Additionally, direct platelet-monocyte interaction via P-selectin increases and accelerates oxLDL binding and uptake in monocytes, pointing to a crucial role of PMAs in foam cell development. In vivo platelet depletion confirms that platelets promote foam cell formation.

Conclusion: We could show that direct platelet-monocyte interaction is critically involved in monocyte extravasation and foam cell formation.

OC9

Platelet Rap1 signaling, mediated by CalDAG-GEFI and P2Y12, contributes to atherosclerotic lesion development in mice

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Background and Objective: The development and progression of atherosclerotic lesions is mediated by various blood cell types, including platelets and neutrophils. We recently identified CalDAG-GEFI (CDGI) as a critical component of integrin-mediated adhesion of platelets and neutrophils at sites of vascular injury. In platelets, signaling by CDGI mediates the early activation of Rap1, while receptor for ADP, P2Y12, the target of the clinically used drug Plavix, is required for sustained Rap1 activation and thrombus stability. In this study, we evaluated lesion formation in atherosclerosis-prone Ldlr$^{-/-}$ mice lacking CDGI and/or P2Y12 in hematopoietic cells.

Methods and Results: Ldlr$^{-/-}$ mice were irradiated and reconstituted with bone-marrow from wild-type (WT), CalDAG-GEFI$^{-/-}$ (CdgI), P2y12$^{-/-}$ (P2y12) or CdgI$^{-/-}$ P2y12$^{-/-}$ (DKO) mice. Reconstituted mice were fed a high-fat diet (21% fat, 0.2% cholesterol) for 12 weeks, ad libitum. Atherosclerotic lesions in the aortic sinus of Ldlr$^{-/-}$;*CdgI*$^{-/-}$ chimeras were 42% smaller than those in Ldlr$^{-/-}$;WT controls (0.18 ±0.02 mm^2 vs 0.31 ± 0.05 mm^2, respectively) (n=13, p<0.001). Lesions in Ldlr$^{-/-}$;*P2y12*$^{-/-}$ chimeras were also significantly smaller than those in controls (0.22 ± 0.10 mm^2 n=13, p<0.05). Lesions in Ldlr$^{-/-}$;DKO were reduced by 48% compared to WT controls (0.16 ± 0.02 mm^2, n=13 p<0.001), but they were not statistically different from Ldlr$^{-/-}$;*CdgI*$^{-/-}$ chimeras. Platelet adhesion and activation on collagen under flow was markedly impaired in Ldlr$^{-/-}$;*CdgI*$^{-/-}$ and Ldlr$^{-/-}$; DKO blood but partially in Ldlr$^{-/-}$;*P2y12*$^{-/-}$ blood. Consistently, firm neutrophil adhesion to collagen-bound platelets was significantly reduced in Ldlr$^{-/-}$;*CdgI*$^{-/-}$ and Ldlr$^{-/-}$; DKO blood, but not in Ldlr$^{-/-}$;*P2y12*$^{-/-}$ blood. Total cholesterol and triglyceride levels were similar among groups.

Conclusions: Our findings reveal a critical role for CDGI and P2Y12, and Rap1-dependent platelet activation, in promoting atherosclerotic lesion development in hypercholesterolemic mice. Further studies are required to determine if the observed protection in Ldlr$^{-/-}$;*CdgI*$^{-/-}$ chimeras is in part due to impaired neutrophil function.

OC10

Collagen can selectively trigger a platelet secretory phenotype via glycoprotein VI

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Introduction: Platelets are most commonly known for their central role in hemostasis and thrombosis but platelets are also central actors of other processes including inflammation, angiogenesis, and wound healing.
Accumulating evidence indicates that these "non classical" functions of platelets do not necessarily rely on their well-known ability to form thrombi upon activation. This suggests the existence of non-thrombotic alternative states of platelets activation.

Aims: We investigated the possibility of alternative states of platelet activation uncoupled from their procoagulant and/or prothrombotic activities.

Methods: Human washed platelets were stimulated by different doses of collagen and thrombin and we analyzed the morphological and functional markers of platelet activation: morphology, aggregation, P-selectin, activation of glycoprotein IIb/IIIa and phosphatidylserine surface expression, procoagulant activity, secretion of soluble granular content and calcium signalling.

Results: We show that collagen at low dose (0.25 µg/mL) selectively triggers a platelet secretory phenotype characterized by the release of dense- and alpha granule-derived soluble factors without causing any of the other major platelet changes that usually accompany thrombus formation. Using a blocking antibody to glycoprotein VI (GPVI), we further show that this response is mediated by GPVI.

Conclusion: Our results show that platelet activation goes beyond the mechanisms leading to thrombus formation and also includes alternative platelet phenotypes that might contribute to their thrombus-independent functions.

OC11

Loss of phosphotyrosine binding in Syk rescues platelet counts and GPVI expression in G6b-B-deficient mice

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Introduction: Mice lacking the immunoreceptor tyrosine-based inhibition motif (ITIM)-containing receptor G6b-B exhibit a complex phenotype that includes severe macrothrombocytopenia, enhanced GPVI shedding, platelet surface immunoglobulins (IGs) and reduced proplatelet formation *in vitro*. We have previously hypothesized that this phenotype is due to tonic activation signalling in megakaryocytes and platelets that is normally supressed by G6b-B. In this study, we tested this hypothesis by using various approaches to rescue the phenotype of G6b knockout (KO) mice.

Methods: Platelet counts, volume, surface receptor expression, aggregation and ATP secretion were measured in G6b KO/SykR41A conditional knockin (KI) mice, G6b/Rag1 double-KO mice, and G6b KO mice treated with the thrombopoietin-mimetic Romiplostim, the Src family kinase inhibitor Dasatinib and intravenous IG (IVIG).

Results: Platelet counts, volume and GPVI expression were restored in G6b KO mice expressing a mutant form of the tyrosine kinase Syk (R41A), which disrupts binding of the N-terminal SH2 domain to phosphotyrosine. In contrast, Dasatinib treatment did not restore platelet counts or GPVI levels in G6b KO mice. Romiplostim treatment dramatically increased megakaryocyte counts in G6b KO mice, and restored platelet counts, GPVI expression and platelet reactivity to the GPVI-specific agonist collagen-related peptide (CRP). IVIG treatment transiently restored platelet counts in G6b KO mice to 50% of control levels. Similarly, ablation of B and T cells, by crossing G6b KO mice with Rag1 KO mice, resulted in a comparable increase in platelet counts, however GPVI levels remained dramatically reduced.

Conclusions: Findings from this study support our hypothesis that tonic signalling via Syk underlies the macrothrombocytopenia and shedding of GPVI in G6b KO mice. Romiplostim bypasses the defect in G6b KO mice by boosting megakaryocyte counts and platelet production, whereas IVIG targets the auto-immune component of the G6b KO phenotype.

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OC12

Tetraspanin Tspan18 regulates GPVI-induced platelet activation by interacting with the store-operated Ca^{2+} entry channel Orai1

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

Methods: Experiments were conducted in cell line models and in platelets from Tspan18-deficient mice.

Results: The previously uncharacterized platelet tetraspanin Tspan18 was found to be unique amongst tetraspanins in its activation of a Ca^{2+}-responsive signalling pathway in cell lines. We hypothesized that Tspan18 might regulate Ca^{2+} and/or GPVI signalling in platelets. To address this we performed the first analyses of Tspan18-deficient mice. Tspan18-deficient platelets were defective in aggregation and secretion via the GPVI-specific agonist collagen-related peptide, but aggregation via thrombin or the CLEC-2 receptor was normal. Furthermore, platelet spreading on collagen-related peptide was impaired, but spreading on fibrinogen was normal. This GPVI-specific phenotype is similar to that previously reported for mice with platelets deficient for the store-operated Ca^{2+} entry channel Orai1. Consistent with a role for Tspan18 in regulating Orai1, store-operated Ca^{2+} entry was impaired in the absence of Tspan18, and Tspan18 specifically interacted with Orai1. Unexpectedly, Tspan18-deficient mice exhibited defective haemostasis, since five-fold more blood was lost than wild-type mice in a tail bleeding assay.

Conclusions: We have characterized a new platelet tetraspanin Tspan18 that positively regulates GPVI signalling via interaction with the store-operated Ca^{2+} entry channel Orai1. Experiments are in progress to determine the mechanism by which Tspan18 regulates Orai1 and the reason for defective haemostasis in the absence of the tetraspanin.

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OC13

Akt and MAPK promote CLEC-2 mediated platelet activation by inhibition of GSK3α/β

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Introduction: Akt and mitogen activated protein kinase (MAPK), p38 and extracellular stimuli responsive kinase (ERK) regulate many processes including inflammation, stress responses, gene transcription, cell proliferation and differentiation, and cell death. In platelets, Akt has been shown to contribute to platelet aggregation and granule secretion by several stimuli, including thrombin, thromboxane A_2 and collagen. However, the role of MAPK in platelet activation has been controversial with reports that MAPK inhibition has no effect or to abolish platelet activation. To investigate whether the C-type lectin receptor (CLEC-2) activates the Akt/PI3K and MAPK pathways and to determine the role of these pathways in platelet activation, human platelets were treated with PI3K/Akt and MAPK inhibitors and platelet activation and phosphorylation of specific signaling molecules were assessed.

Methods: Platelet activation was assessed by aggregometry/ATP secretion and phosphorylation of specific signaling molecules after stimulation was determined by western blotting.

Results: The CLEC-2 specific ligand snake venom toxin, rhodocytin, stimulated phosphorylation of Akt, p38 and ERK with a distinct delay relative to phosphorylation of the proximal signaling protein, Syk. The PI3K inhibitor wortmannin or the protein kinase C inhibitor Ro31-8220 blocked rhodocytin-induced Akt and ERK phosphorylation, respectively, and inhibited platelet aggregation and ATP secretion. Furthermore, specific inhibitors of Akt (MK2206) and ERK (PD0325901) had a similar effect on platelet activation and phosphorylation induced by rhodocytin. GSK3α/β, a known substrate for Akt, was also phosphorylated in response to CLEC-2 activation. The Akt inhibitor MK2206 blocked GSK3α/β phosphorylation, whereas, in the presence of the ERK inhibitor, PD0325901, phosphorylation of GSK3α/β was transient. The GSK3 inhibitor CHIR-99021 recovered the activation of MK2206- and PD0325901-treated platelets.

Conclusion: CLEC2 stimulates activation of Akt/PI3K and MAPK pathways. Activated Akt and ERK contribute to platelet activation by inhibition of GSK3α/β.

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OC14

Internalization of glycoprotein VI (GPVI) in platelets activated by soluble GPVI-specific agonists

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Introduction: Most membrane receptors are internalized upon ligand engagement, possibly to downregulate their function or to transport signals from the periphery to inside of the cell. By flow cytometry, we found that surface GPVI expression is significantly decreased in CRP(collagen-related peptide)- or Cvx(convulxin)-induced platelets, so we investigated the mechanism of this phenomenon.

Results: *(1)* Reduction of surface GPVI expression is specific for GPVI-induced activation because it is dependent on the concentration of GPVI-specific agonists and not reduced in thrombin-stimulated platelets. *(2)* Platelets stimulated with a high Cvx concentration (10 μg/ml) essentially lost all their surface GPVI. This is not due to shedding: extent of shedding is low under the same conditions, and neither the metalloproteinase inhibitor GM6001 nor EDTA prevented the reduction of surface GPVI. *(3)* Confocal microscopy indicated that GPVI was internalized to the center of the cell as assessed with platelets pre-labeled with fluorescent-dye conjugated 204-11 Fab (GPVI-dimer–specific mAb) or 1G5 (antibody against pan GPVI) and then activated with CRP or Cvx. *(4)* Sulfo-NHS-SS-biotin-labeled 1G5 treated with excess Alexa-Fluor-647–streptavidin was reacted with platelets. Confocal microscopy indicated that Cvx- or CRP-activated platelets contained internalized GPVI, and treatment with the membrane-impermeant reducing agent TCEP [tris(2-carboxyethyl)phosphine hydrochloride] did not reduce this internalized fluorescence, but instead reduced the surface fluorescence of both resting and thrombin-activated platelets, supporting GPVI internalization. *(5)* GPVI internalization is reduced by actin antagonists, suggesting that cytoskeletal proteins are involved in this process.

Discussion: Previously, we demonstrated GPVI-dimer clusters are formed on the surface of platelets adhered to immobilized collagen. Platelet binding to a soluble GPVI-ligand, instead, results in internalization of GPVI-ligand complexes. Since actin antagonists inhibit both GPVI clustering and internalization, we will be determining if both processes may in part involve similar mechanisms.

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OC15

Regulation of Rac GTPases by cyclic nucleotides in human platelets

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Introduction: Small G proteins of the Rho family connect extracellular signals with the rapid reorganization of cytoskeleton, thus regulating platelet spreading, secretion, aggregation and eventually thrombus formation. In platelets, the activity of Rac1 has been shown to be inhibited by cyclic nucleotides which mediate the effects of endothelial prostacyclin and nitric oxide. To better understand how endogenous platelet inhibition works our aim is to find molecular targets of protein kinases A (PKA) and G (PKG) that mediate Rac1 inhibition.

Methods

Specific membrane-permeable cyclic nucleotide analogues and upstream activators of cyclic nucleotide pathways were used to activate PKA or PKG. Activity of Rac proteins were investigated by pull-down assays. Phosphorylation of candidate substrates (PKA/PKG consensus sequence -R-R/K-X-S-) was studied by radioactive phosphate incorporation and Phos-tag supplemented SDS-PAGE. Site-directed mutagenesis was utilized to generate phosphorylation site mutants.

Results: We could confirm that PKA and PKG block thrombin-induced Rac1 activation in human platelets. Furthermore, we found that the related proteins Rac2 and RhoG are activated by thrombin and this activation is blocked by PKA/PKG. Rho GTPase activating proteins (RhoGAPs) terminate Rho/Rac signalling and we hypothesized that specific RhoGAPs might mediate the effects of PKA/PKG on Rac proteins. ARHGAP17 was found to be phosphorylated by PKA in vitro and in intact HEK293T cells. We mapped the phosphorylation site to serine 702 of ARHGAP17. ARHGAP17 was also phosphorylated by PKA in intact platelets. By pull-down assay we found that ARHGAP17 interacts with NHERF1 and that this interaction is increased upon PKA activation. PKA activation might also lead to increased total protein levels of ARHGAP17.

Conclusions: These data expands the inhibitory actions of cyclic nucleotides to all Rac subfamily proteins present in human platelets. Rac inhibition might be mediated by ARHGAP17 involving increased protein stability or reorganization of a NHERF1-ARHGAP17 complex. Our findings provide new insights into endogenous platelet inhibition.

OC16

PDI And ERP57 co-cluster in platelets and their movement is regulated by actin polymerization

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Introduction: The thiol isomerases PDI and ERp57 are released to the platelet surface where they contribute to several cellular responses. PDI is present in subcellular structures, not corresponding to α - and δ -granules, and lysosomes. It was, however, shown to co-localise with TLR9 in T-granules, intracellular bodies underlying the plasma membrane. The subcellular localisation of other platelet thiol isomerases or their mechanisms of translocation to the cell surface have not been established.

Hypothesis and Methods: Using spinning disk confocal microscopy we explored whether ERp57 and PDI co- localise in resting and activated platelets and whether their translocation to the platelet surface depends on actin polymerization.

Results: In resting platelets, PDI and ERp57 were organized in punctate structures both on the platelet surface and in the cytoplasm. They did not colocalise with P-selectin consistent with them residing outside α -granules. TLR9 did not colocalise with PDI and ERp57, suggesting their exclusion from T-granules. Partial co-localisation was observed in resting platelets between PDI and ERp57 in granule-like structures (Pearson's correlation coefficient=0.597±0.08, mean±SD). Upon platelet activation, the co-distribution persisted with PDI and ERp57 migrating to the surface (Pearson's correlation coefficient=0.533±0.08, mean±SD). Latrunculin A, an inhibitor of actin polymerization, decreased P-selectin exposure on the platelet surface, as measured by flow cytometry, and agonist-stimulated platelets treated with this agent retained a rounded shape, as shown by tubulin staining. Importantly, latrunculin A also blocked the translocation of PDI and ERp57 from internal granule-like structures to the platelet surface.

Conclusions: In resting platelets PDI and ERp57 are organized, and partially co-localised, in punctuate structures unlikely to represent α- or T-granules. The polymerization of actin during platelet activation exerts a fundamental role in the relocalisation of PDI and ERp57 from these cytoplasmic structures to the platelet surface, thus suggesting that thiol isomerases undergo a bonfide secretory mechanism.

OC17

The Rap-GAP Rasa3 is a critical negative regulator of platelet activation

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Introduction: The small-GTPase Rap1 is a crucial regulator of platelet integrin activation and thrombus formation. Rap1 activity in platelets is controlled by the guanine nucleotide exchange factor CalDAG-GEFI (CDGI) and an unknown regulator operating downstream of the ADP receptor, P2Y12, the target of antithrombotic therapy. We here evaluated the role of the GTPase-activating protein Rasa3 in Rap1-dependent platelet activation and thrombosis.

Methods: Mice with a point mutation in the N-terminus (G125V, *Rasa3^{scat}*) or the C-terminus (H794L, *Rasa3^{hlb}*) of *Rasa3* were analyzed. Platelet counts, lifespan and the percentage of young/reticulated platelets were determined by flow cytometry. The activation state of platelets was evaluated by aggregometry, flow cytometry and Rap1 pull-down assay. The hemostatic response of the mice was monitored *in vivo* upon laser-induced injury of the saphenous vein.

Results: Both *Rasa3^{scat}* and *Rasa3^{hlb}* mice exhibited severe thrombocytopenia (>99% and >95% reduction in platelet count, respectively) due to systemic activation and clearance of platelets from circulation. Concurrent deletion of CDGI significantly prolonged platelet lifespan and increased peripheral platelet counts in Rasa3 mutant mice. Compared to *CDGI-/-* controls, *CDGI-/-Rasa3^{scat}* or *CDGI-/-Rasa3^{hlb}* platelets were hyper-reactive to agonist stimulation and insensitive to inhibitors of P2Y12 or PI3-kinase, both *in vitro* and *in vivo*. Compared to *WT* platelets, however, aIIb3 activation in *CDGI-/-Rasa3^{hlb}* platelets occurred with a delay, confirming that the Ca^{2+}/CalDAG-GEFI/Rap1 signaling module is essential for the very rapid inside-out activation of platelet integrins, even in the absence of functional Rasa3.

Conclusions: Our work (1) indicates that constitutively active Rasa3 ensures that platelets circulate in a quiescent state by restraining CalDAG-GEFI/Rap1 signaling, (2) identifies Rasa3 inactivation as the missing link between P2Y12 engagement and sustained Rap1-dependent platelet activation and (3) provides evidence that clinically successful P2Y12 inhibitors, such as Plavix, destabilize thrombi and protect from thrombosis mainly by preventing Rasa3 inhibition and sustained Rap1 activation.

OC18

EphB2 Mediated Contact-dependent and independent Signalling in the Regulation of Thrombosis and Haemostasis

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Introduction: Eph/ephrin signalling plays important roles in the development of central nervous system and vasculature. The presence of EphA4, EphB1 and ephrinB1 in platelets was reported previously where forced clustering of EphA4 or ephrinB1 resulted in cytoskeletal rearrangements, fibrinogen binding and granule secretion. Further studies have emphasized the role of EphA4 in regulation of integrin αIIbβ3 mediated outside-in signalling. We have recently reported the presence of EphB2 in platelets and established its involvement in regulation of platelet function.

Methods: We assessed the hypothesis that Eph/ephrin signalling is mainly mediated through the cytoplasmic domains [including a kinase domain, sterile alpha motif and PDZ motif] of Eph kinases using a mouse model in which the intracellular region of EphB2 (*EphB2*^{LacZ}) was replaced with β-galactosidase. Use of this mouse model enabled us to explore the importance of EphB2 cytoplasmic signalling in regulating platelet function without interfering with its ligand binding.

Results and Conclusions: The absence of EphB2 cytoplasmic tail resulted in reduced agonist-induced platelet activation, fibrinogen binding, granule secretion and thrombus formation. Integrin αIIbβ3 mediated outside-in signalling was also reduced and associated with a diminished level of myosin binding to integrin β3 tail in *EphB2*^{LacZ} mouse platelets. Moreover, a reduced level of calcium mobilisation and phosphorylation of protein kinase B and phospholipase C 2 in *EphB2*^{LacZ} platelets suggest a potential role in the regulation of phosphoinositide-3 kinase signalling. Reduced levels of fibrinogen binding, granule secretion and platelet spreading observed on individual *EphB2*^{LacZ} platelets suggest a role for EphB2 signalling without the need for cell-cell contact. Conversely, diminished platelet aggregation, clot retraction and thrombus formation in *EphB2*^{LacZ} platelets point towards the importance of EphB2 mediated intracellular signalling in a contact-dependent manner when platelet-platelet contacts occur. In conclusion, this study implicates the EphB2 cytoplasmic region in mediating contact-dependent and independent signalling in platelets.

OC19

Disturbed glycoprotein VI-mediated signaling in platelets from hypercholesterolemic mice and humans

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Introduction: High plasma HDL-cholesterol (HDL-C) levels are associated with platelet hyperreactivity in mice and humans. We aimed to investigate platelet responsiveness under conditions of elevated levels of plasma LDL-C.

Methods: Experiments were performed with platelets from LDL receptor-deficient ($LDLr^{-/-}$) mice, a commonly used hypercholesterolemic mouse model, and their wildtype (WT) littermates. To verify the relevance for human physiology, platelets from familial hypercholesterolemia (FH) patients with reduced LDLr functionality due to a defect in the *ldlr* gene, were studied before and after statin treatment.

Results: Elevated LDL-C levels in $LDLr^{-/-}$ mice were associated with lower platelet counts. ADP-induced activation of platelets from $LDLr^{-/-}$ mice, measured as the presence of active integrin $\alpha_{IIb}\beta_3$ and surface-expressed P-selectin, was increased compared to platelets from WT littermates. Surprisingly, GPVI-mediated $\alpha_{IIb}\beta_3$ activation and surface expression of P-selectin was reduced. ADP also enhanced fibrinogen binding to and P-selectin expression on human FH platelets as compared to control platelets. In contrast, CRP-XL was less effective in activating FH platelets, resulting in reduced GPVI-mediated tyrosine phosphorylation, diminished fibrinogen binding, lower P-selectin expression, and a reduced ability to adhere to collagen under conditions of flow. GPVI-mediated platelet signaling is dependent on its translocation to lipid rafts. Förster Resonance Energy Transfer (FRET) by time-gated fluorescence lifetime imaging microscopy (FLIM) analysis revealed that although slightly more GPVI resided in rafts of unstimulated FH platelets, significant translocation of GPVI into lipid rafts upon stimulation by collagen was only observed in control platelets. Statin treatment normalized GPVI translocation to lipid rafts as well as platelet function in response to collagen.

Conclusion: Hypercholesterolemia due to elevated plasma LDL-C levels impairs translocation of GPVI to lipid rafts, thereby diminishing the platelet response to collagen. These findings reflect an important protective mechanism to prevent early platelet activation in hypercholesterolemic mice and humans.

OC20

Syk deficiency protects mice from occlusive arterial thrombus formation and ischaemic stroke without inducing intracranial haemorrhage

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Introduction: Spleen tyrosine kinase (Syk) phosphorylates key adapter proteins and phospholipase Cγ isoforms. Syk is highly expressed in haematopoietic cells and present in the immunoreceptor tyrosine-based activation motif (ITAM) and hemITAM signalling pathways mediating signal transduction by glycoprotein (GP) VI and the C-type lectin-like receptor 2 (CLEC-2). To assess whether Syk might be a potential therapeutic target to prevent arterial thrombosis and infarct progression, its function in haemostasis, thrombosis and thrombo-inflammatory brain infarction in $Syk^{fl/fl,\ Pf4\text{-}Cre}$ mice was analysed.

Methods: Platelet function *in vitro* was analysed by flow cytometry, aggregometry, clot retraction and a spreading assay. Thrombus formation *ex vivo* was determined by a flow adhesion assay. Tail bleeding times were measured and mice were subjected to two different models of arterial thrombosis. In addition, $Syk^{fl/fl,\ Pf4\text{-}Cre}$ mice were subjected to an experimental model of stroke.

Results: $Syk^{fl/fl,\ Pf4\text{-}Cre}$ platelets spread normally, whereas *ex vivo* thrombus formation on collagen was abolished. Tail bleeding times in $Syk^{fl/fl,\ Pf4\text{-}Cre}$ mice were significantly increased, but the defect was not as dramatic as the severe haemostatic defect seen in GPVI/CLEC-2 double-deficient mice. Interestingly, Syk deficiency protects from occlusive thrombus formation, but replacing Syk with Zap70 ($Syk^{Zap70/Zap70}$ mice), which is normally not expressed in platelets and has a lower intrinsic kinase activity, restored arterial thrombus formation. In addition, $Syk^{fl/fl,\ Pf4\text{-}Cre}$ mice were protected from stroke without signs of cerebral haemorrhage.

Conclusions: Syk is required for arterial thrombosis and infarct progression in ischaemic stroke. Importantly, Syk deficiency did not lead to intracranial haemorrhage in the ischaemic brain despite prolonged tail bleeding times. Remarkably, the minimal (hem)ITAM signalling observed in $Syk^{Zap70/Zap70}$ platelets is sufficient for arterial thrombus formation *in vivo*. These findings indicate, that pharmacological inhibition of Syk has to provide a complete block of Syk activity in order to prevent thrombo-inflammation.

OC21

Podoplanin and CLEC-2 interactions drive vascular patterning in the developing brain

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Introduction: Mice with a constitutive or platelet-specific deletion of the C-type lectin-like receptor, CLEC-2, exhibit haemorrhaging in the developing nervous system in mid-gestation. We sought to test the hypothesis that the development of cerebral haemorrhages is mediated by the loss of CLEC-2 activation by its only known endogenous ligand, podoplanin, which is expressed at high level on the neural tube early in development.

Methods and Results: We used 3-dimensional ultramicroscopy to characterise the developing vasculature and sites of haemorrhaging in CLEC-2-deficient and podoplanin deficient mice. To induce deletion of podoplanin at the two egg stage we generated a podoplanin$^{fl/fl}$ mouse crossed to a PGK.Cre. Aberrant vascular patterning was observed in both mouse models at embryonic (E) day 10.5 of development and preceded haemorrhaging throughout the fore-, mid- and hind-brain, which was first observed at E11.5. Immunofluorescence and electron microscopy revealed defective pericyte recruitment and misconnections between the endothelium of developing blood vessels and surrounding pericytes and neuro-epithelial cells. Altered vascular development and haemorrhaging were present following deletion of podoplanin on neural progenitors using a Nestin.Cre8 transgenic mouse. Haemorrhaging was observed in 100% of embryos deficient in the platelet integrin subunit glycoprotein IIb (αIIb) and in 2/12 embryos deficient in neurobeachin-like-2 (NBEAL-2), which lack platelet α-granules.

Conclusions: We propose a novel role for podoplanin on the developing neuro-epithelium in which it interacts with CLEC-2-expressing platelets to guide the maturation and integrity of the developing vasculature and prevent haemorrhaging.

OC22

Coordinated platelet ballooning and procoagulant membrane spreading is driven by salt and water entry

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Introduction: Platelets activated by contact with collagen transform into balloon-like structures as part of the haemostatic response.

Methods: We utilised high resolution live-cell imaging and correlative light-electron microscopy in tandem with pharmacological and physiological manipulations.

Results: The study provides spatio-temporal evidence of the morphological transformation of human platelets into phosphatidylserine-exposing balloon-like structures and demonstrates for the first time, that it can be associated with procoagulant membrane spreading in real time. The data reveal that (i) balloons, but not smaller reversible membrane protrusions or blebs, are procoagulant; (ii) although blebbing and ballooning are progressive phases of procoagulant membrane transformation, ballooning is mechanistically distinct from membrane blebbing; (iii) whereas the hydrostatic pressure required for bleb formation is fluid entry-independent, the rapid expansion and sustained phases of ballooning are driven by external osmotic pressure and can be promoted by diminishing actomyosin contraction; (iv) ballooning involves dramatic disruption to the platelet microtubule cytoskeleton and increased membrane permeability; (v) ballooning is consequent upon Na^+/Cl^- influx and water entry via putative aquaporin-1 water channels.

Conclusion: Coordinated platelet ballooning and procoagulant membrane spreading is a highly regulated platelets response, which serves to amplify clot formation at wound sites.

OC23

Altered phosphorylation profile of Scott syndrome platelets as revealed by quantitative phosphoproteomics

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Introduction: The Scott syndrome is a rare bleeding disorder, associated with a mutation in anoctamin-6. Blood cells from Scott patients are defective in Ca^{2+}-induced phospholipid scrambling and Ca^{2+}-induced Cl^- conductance. When stimulated with strong agonists, Scott platelets fail to expose phosphatidylserine (PS) and form membrane blebs, pointing to differences in membrane structure. We hypothesized that these altered responses are due to changes in protein phosphorylation.

Methods: Washed platelets from healthy control donors and a Scott patient were activated with the Ca^{2+}-mobilizing agonists convulxin/thrombin or ionomycin. Phosphorylation samples was quantitatively compared for >2200 phospho-sites and >970 proteins.

Results: Stimulation of control platelets by convulxin/thrombin or ionomycin resulted in a major fraction of cells exposing PS, having inactivated adhesive receptors and a blebbing structure. Scott platelets stimulated by convulxin/thrombin or ionomycin gave negligible PS exposure and bleb formation. Surprisingly, aggregation responses upon strong platelet activation occurred in Scott platelets which were lacking in control platelets.
In control platelets, thrombin stimulation changed 26% of the phosphorylation sites while convulxin/thrombin or ionomycin stimulation induced more drastic changes in phosphorylation sites (56% and 57%, respectively). At baseline and after thrombin stimulation, a high overlap of 99.3% was observed between the phosphorylation profiles of Scott platelets and control platelets. Ca^{2+}-mobilizing agonists reduced the overlap in phosphorylation pattern to 81%. Strongly activated Scott platelets showed more frequently increased (16.5-16.7%) than decreased (2.7-2.9%) phosphorylation. The top 50 most altered phosphorylated proteins of activated Scott platelets were assigned to the following function/structure classes: actin-myosin cytoskeleton (20%), platelet adhesion (22%), signaling/adapter proteins (24%), receptor-linked cytoskeleton (8%), and microtubule cytoskeleton (8%).

Conclusion: These data reveal major alterations in the phosphorylation pattern of PS-exposing stimulated platelets. In Scott syndrome platelets, the inability to PS exposure and membrane blebbing is accompanied by changes in phosphorylation pattern, linked to cytoskeleton integrity and platelet adhesion.

OC24

Ibrutinib treatment affects collagen and von Willebrand Factor-dependent platelet functions

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Introduction: The oral Bruton's tyrosine kinase inhibitor, ibrutinib, has recently demonstrated high efficiency in patients with relapsed B-cell malignancies. Occurrence of bleeding events has been reported in up to 50% of ibrutinib-treated patients. Most events were of grade 1-2 (spontaneous bruising or petechiae) but, in 5% of patients, they were of grade 3 or higher after trauma.

Methods: To characterize the bleeding events in ibrutinib-treated patients we have investigated the effect of this drug on platelet functions in vitro, and ex vivo in fourteen patients by using a series of complementary methods including platelet aggregometry, platelet adhesion under flow and flow cytometry to monitor Btk phosphorylation.

Results: We demonstrate that ibrutinib has no effect on thrombin or ADP-induced platelet aggregation but inhibits platelet signaling and functions downstream of the collagen receptor GPVI. The drug also efficiently affects platelet adhesion on von Willebrand Factor under arterial flow. Occurrence of bleeding events in treated patients correlates with a decrease in platelet aggregation to collagen in platelet-rich plasma and a strong reduction of adhesion onto von Willebrand Factor. Addition of 50% untreated platelets is sufficient to efficiently reverse the effects of ibrutinib, and platelet functions recover following treatment interruption as physiological platelet renewal occurs.

Conclusion: These data have important clinical implications and provide a basis for haemostasis management during ibrutinib treatment.

OC25

Identification of candidate platelet miRNA regulating platelet reactivity in aspirin-treated cardiovascular patients

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Introduction: Platelet reactivity (PR) in cardiovascular (CV) patients is variable between individuals and modulates clinical outcome. However, the determinants of platelet reactivity are largely unknown. Integration of data derived from high-throughput omics technologies may yield novel insights into the molecular mechanisms that govern platelet reactivity.

Methods: PR was assessed in 110 CV patients treated with aspirin 100mg/d by aggregometry using several agonists. CV patients with extreme high or low PR were selected for further analysis. Quantitative proteomic of platelets and platelet sub-cellular fractions as well as transcriptomic analysis were integrated with a network biology approach. This allowed the identification of gene products differentially expressed in patients with extreme PR phenotypes. Since recent data showed that circulating microRNAs (miRNAs) are associated with PR, we then specifically assessed the level of miRNAs that were differentially expressed in these two groups of patients (Nanostring technology). The predicted targets of these miRNAs were mapped onto the network to identify the most relevant genes.

Results: We first constructed a network gathering platelet proteomic and transcriptomic data associated to extreme PR phenotypes. It yielded a network of 99 nodes and 309 edges, representing platelet activation pathways. Five miRNAs were identified as differentially expressed and associated with platelet reactivity. Among these 5 miRNAs, 2 are predicted to have an identical gene target whose gene expression is up-regulated in patients with extreme high PR compared to patients with extreme low PR.

Conclusions: The integration of several 'omics datasets allowed the identification of candidate genes that may be associated with the modulation of platelet reactivity in aspirin-treated cardiovascular patients.

POSTER

POSTER

P1

Diesel particles activate platelets through GPVI and CLEC-2

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Introduction: We are exposed to a number of pollutants on a daily basis. A major contributor to these pollutants is diesel exhaust. Diesel exhaust contains both gasses and nano-particulate matter, referred to as diesel exhaust particles (DEP). Such particles have been shown to cross the lung epithelium and enter the blood stream and have been proposed to cause inflammation and to be cytotoxic. Several studies have shown that DEP induce aggregation of washed platelets. Our aim was to understand the mechanism of platelet activation.

Methods: We have monitored aggregation and protein phosphorylation in human and mouse washed platelets, and used transgenic mouse platelets and transfected cell lines to investigate the mechanism of platelet activation by DEP.

Results: DEP stimulated aggregation of human and mouse platelets, predominantly via the GPVI pathway, but with CLEC-2 playing a minor role as shown by measurement of receptor phosphorylation and use of CLEC-2 and GPVI-deficient platelets. Further, DEP achieved aggregation solely through Syk and Src tyrosine kinases. Nevertheless, DEP stimulated protein phosphorylation in platelets double-deficient in GPVI and CLEC-2. DEP was unable to stimulate CLEC-2 or GPVI in transfected DT40 cells.

Conclusions: This study demonstrates that DEP activate platelets through the GPVI and/or CLEC-2 independent of direct receptor binding.

P2

RNA in platelets, no more than a relic

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Introduction: The proposal that platelets harbor a functional translation system suggests their possible involvement in delayed cellular and immunological responses. However, this view needs to be supported by a better knowledge of their RNA, mainly present in newly generated reticulated platelets. So, we characterized the amount, integrity and lifespan of RNA molecules in reticulated platelets.

Methods: Diphtheria toxin was administrated to transgenic mice specifically expressing its receptor in megakaryocytes, inducing a profound thrombocytopenia, followed by a rebound of the platelet count. Platelet RNA platelets was analyzed by flow cytometry after thiazole orange (TO) staining, by immunofluorescence using anti-rRNA Y10b monoclonal antibody, transmission electron microscopy (TEM) and, on a Bioanalyzer 2100 instrument.

Results: Four administrations of diphtheria toxin induced the deprivation of late stage bone marrow megakaryocytes and a severe thrombocytopenia (<10.000/µl). Three days later, platelet count started recovering (>250 000/µl), >80% of platelets being TO+, versus <10% in control mice. TEM revealed that these platelets contain rough endoplasmic reticulum and Golgi organelles and occasionally, nuclei remnants.
Flow cytometry showed that the RNA content of reticulated platelets (i) is stable in platelets incubated *ex vivo* at room temperature over 48h, but decreases within a few hours (ii) at 37°C and (iii) *in vivo*. Immunofluorescence microscopy indicated the disappearance of 75% rRNA in platelets incubated 6 hours at 37°C. RNA extraction and quality analysis confirmed the decrease of total RNA and rRNA. Calculations indicate that the mean RNA content in one reticulated platelet exceeds 20fg, compared to less than 1 pg in non-reticulated platelets.

Conclusions: Based on these results and published data, the mean content of platelets is less than 200 ribosomes and 100 mRNA molecules, with mRNAs such as IL1β mRNA present in one platelet out of 1000. Thus, platelets do not seem to harbor a biologically relevant translation machinery.

P3

Deletion of PI3K p110α results in enhanced platelet priming by distinct growth factors

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Introduction: Platelet hyperactivity is a contributing factor in the pathogenesis of cardiovascular disease, and can be induced by elevated levels of circulating peptide hormones, such as the insulin-like growth factors (IGF-1 and IGF-2), insulin and thrombopoietin (TPO). These hormones are primers that cannot induce platelet activation independently, but can enhance platelet functional responses to physiological stimuli in a phosphoinositide 3-kinase (PI3K)-dependent manner.

Methods: We explored the role of the PI3K p110α isoform in primer-mediated enhancement of platelet function using a platelet-specific p110α knockout murine model.

Results: We demonstrate that agonist-stimulated signalling to Akt/PKB as well as integrin $\alpha_{IIb}\beta_3$ activation, α-granule secretion, aggregation and thrombus-formation over collagen are unaffected by p110α deletion. Furthermore, the platelet primers insulin, IGF-1, IGF-2 and TPO induced significant enhancements in integrin αIIbβ3 activation, α-granule secretion and *ex vivo* thrombus formation in both wild-type and p110α KO platelets. Deletion of p110α however, resulted in platelet primers having a greater effect when compared to wild-type responses. Interestingly, pharmacological inhibition (PIK-75), but not genetic deletion of p110α, blocked primer-mediated increases in integrin activation and α-granule secretion. Additionally, the p110β inhibitor TGX-221 was able to block primer-mediated enhancements in p110α KO platelets, suggesting that p110β drives priming in the absence of p110α.

Conclusion: Together these data demonstrate that (i) p110α deletion results in enhanced platelet priming, suggesting that p110α negatively regulates the priming process and (ii) in the absence of p110α, p110β is able to drive increases in platelet activation by primers.

P4

The calcium-binding protein S100A1 negatively regulates collagen-dependent platelet activation and thrombosis in mice

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Introduction: S100A1 is a member of the S100 family of calcium-binding proteins. S100A1 controls Ca2+ dynamics in cardiomyocytes and plays an important role in heart failure. S100A1 is also expressed in mouse platelets, but its role in platelet biology has not been investigated.

Methods and Results: Platelet activation in response to threshold levels of convulxin, a specific agonist for the collagen receptor GPVI, showed significantly increased activation of αIIbβ3 integrin and α-granule release in S100A1-deficient (SKO) platelets compared with wild-type (WT) platelets. Consistently, SKO platelets also showed a more robust aggregation response to convulxin and collagen. In contrast, SKO platelets responded normally to stimulation with PAR4 receptor-activating peptide or ADP. Adhesion of SKO platelets to collagen under flow conditions was not significantly different to that of WT platelets. However, we observed a ~3-fold increase in phosphatidylserine positive SKO platelets bound to the collagen surface. We also observed increased coated platelet formation and more sustained calcium transients in SKO platelets compared to controls. The increased reactivity of SKO platelets to GPVI agonists is explained by a ~1.5-fold increase in GPVI receptors expressed on the surface of these cells. A similar increase in GPVI expression was also found in bone marrow-derived megakaryocytes. When subjected to the $FeCl_3$ carotid artery thrombosis model, the time to vessel occlusion was significantly shorter in SKO mice compared to WT controls. In a tail bleeding assay, SKO mice exhibit a shorter time to occlusion compared to WT mice and in a presence of a P2Y12 inhibitor, ticagrelor, they show less blood loss than ticagrelor-treated WT mice.

Conclusions: We here identify S100A1 as a negative regulator of GPVI expression and collagen-dependent platelet activation and thrombosis in mice. Our studies further suggest that the regulation of GPVI expression by S100A1 occurs at the level of the maturing megakaryocyte.

POSTER

P5

Looking for genes responsible for δ-storage pool disease

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Introduction: Delta (δ)-storage pool diseases (δSPD) are platelet disorders due to deficiency in dense granules and are responsible for heterogeneous primary haemostasis defects. Syndromic forms are caused by anomalies in cellular vesicular trafficking and are accompanied by oculocutaneous albinism and immunodeficiency. A list of genes involved in syndromic forms has been identified such as LYST, HPS1-6, DTNBP1 or BLOC1S. But the genetic causes of the most frequent non syndromic forms are still unknown.

Patients/Methods: In the present study, we identified a family of 6 members (2 parents, 4 children) with 3 children among 4 suffering from non syndromic δ-SPD. One of them (propositus) experienced a dramatic skull accident treated by multiple platelet transfusions and FVIIa administration. We examined platelet functions by light transmission aggregometry, granular content by HPLC and ELISA and platelet morphology by transmission electronic microscopy (TEM). To single out the gene(s) responsible for the disease we performed a full exome sequencing in each family member.

Results/discussion: The propositus exhibited a mild defect of ADP-induced aggregation. Other agonists did not show any aggregation abnormality. The nucleotide content in ADP was dramatically decreased (80%) in the propositus and 2 other children. The serotonin content was decreased in the propositus exclusively who was treated with an antidepressant drug (SSRI) since his skull trauma. TEM did not reveal any defect in the number and ultrastructure of the dense granules. From these data we diagnosed δ-storage pool disorder in 3 children out of 4. With the hypothesis of a recessive transmission, the whole exome sequencing did not yet result in a clear compound of heterozygous mutations in a single gene in the 3 children. A nucleotide transporter remains a major hypothesis to explain the defect but it has still to be identified. Twenty potential candidate genes involved in vesicular biogenesis are currently evaluated.

P6

Matrix metalloproteinase-13 influences thrombus formation under flow conditions

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Background: Following atherosclerotic plaque rupture, the exposure of flowing blood to collagen-rich surfaces triggers platelet activation and aggregation. The engagement of GPIb-IX-V/GPVI by VWF and collagen is pivotal to thrombus formation on the exposed endothelium. MMP-13 is a collagenolytic metalloproteinase that is up–regulated in the vulnerable plaque and released upon rupture.

Aim: Here we aim to determine the effects of MMP-13 on platelet receptor function.

Methods: Solid phase binding assays, receptor-specific compounds and blocking single-chain variable fragment antibodies (scFvs) were used to determine the interaction of MMP-13 with platelet receptors. The degradation of recombinant human platelet receptors by MMP-13 was assessed by SDS-PAGE and corresponding assays performed *in vitro*. Aggregation experiments were used to elucidate the effect of MMP-13 and catalytically inactive MMP-13(E204A) on washed platelet activation in response to CRP-XL, A23187, thrombin and fibrillar Type I collagen. The effects of MMP-13 on platelet adhesion, rolling and thrombus size on fibrillar Type I collagen were analysed under flow conditions in whole blood.

Conclusions: Both latent and active forms of MMP-13 bind to washed platelets via GPVI and αIIbβ3 and can be inhibited by the scFvs and RGD peptides respectively. Although MMP-13 is able to cleave rhGPVI, no evidence was found for sheddase activity *in vitro*. These interactions, while resulting in some degree of platelet shape change, do not impede the activation of platelets or a significant inhibition of aggregation in response to any agonist tested. Pre-incubation of active MMP-13 and MMP-13(E204A) with whole blood however resulted in a differential reduction in surface coverage and volume of the thrombi formed on fibrillar collagen. Pre-treatment of whole blood with MMP-13 resulted in a significant decrease in thrombus height and volume.

P7

Human Cytomegalovirus–Platelet Interaction Triggers Toll-Like Receptor 2–Dependent Proinflammatory and Proangiogenic Responses

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Introduction: Platelets are discoid anucleated blood cells that fulfill a central function in hemostasis. Additionally, the expression of TLR1/2, TLR4 and TLR9 on platelets allows direct interactions with pathogens and although only a small proportion of platelets were positive for TLRs, they play an important role in modulating immune responses. Human cytomegalovirus (CMV) is endemic, but remains subclinical in an immunecompetent host and is associated with atherosclerosis. As exogenous stimulation of myeloid TLR2 promotes atherosclerotic plaque progression and platelets also have a well-known pathophysiological function in atherosclerosis, we were interested if CMV can interact with platelets and therefore may promote atherosclerotic development.

Methods: To investigate CMV-platelet interactions flow cytometry was used to define platelet activation, platelet-leukocyte interactions and platelet receptor for CMV. To clarify in vivo relevance of CMV-platelet interaction, murine platelets were treated ex vivo with murine CMV (MCMV) and platelet-leukocyte aggregate formation and neutrophil recruitment were determined in a model of thioglycollate induced peritonitis.

Results: We could show that CMV is bound by platelet TLR2, leading to platelet activation, indicated by increased CD62P expression. Interestingly, we found an increase in CD62P expression of 15%, although approximately 2% of platelets are TLR2 positive. Hence, we investigated feedback signaling and could show that the supernatant of MCMV treated platelets could activate platelets even in the absence of TLR2. We identified the ADP-feedback loop to be responsible for this TLR2-independent platelet activation. Investigating platelet-neutrophil interactions in response to HCMV, we could show that CMV triggered platelet-neutrophil aggregate formation and that platelets potentiated CMV induced neutrophil activation. Platelets exposed to MCMV ex vivo slightly enhanced platelet-leukocyte aggregate formation and neutrophil extravastion in vivo.

Conclusion: Our results give evidence that CMV activates platelets via TLR2, leading to platelet-leukocyte aggregate formation. Thus CMV likely promotes leukocyte extravasation, which may play a role in atherosclerosis.

P8

Improved megakaryocyte differentiation using 3D hydrogel progenitor culture

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Introduction: Platelets are produced by differentiated megakaryocytes (MKs) following extension of proplatelets. In liquid culture, MK differentiation remains highly inefficient to produce platelets. We hypothesized that MK differentiation could be improved by mimicking the 3D architecture and/or the physical constraints encountered by progenitors in the bone marrow (BM).

Methods: Mouse Lin- progenitors were grown for 5 days, either in liquid medium or in methylcellulose (MC) hydrogel and analyzed for differentiation/maturation markers (proplatelet formation, ploidy, organization of the demarcation membrane system (DMS)).

Results: Using electron microscopy, we observed that MKs from liquid culture have a less developed/organized DMS compared to BM. In addition, *Myh9*-/- MKs lacking acto-myosin-based intracellular tension exhibit a dramatic abnormal morphology *in situ*, which is not reproduced in liquid culture, indicating that *in vitro* culture lacks some critical aspects of the microenvironment. We used MC hydrogel to develop a 3D culture and introduce controlled and homogeneous mechanical constraints. At a concentration of 2%, MC behaves as a solid gel at 37°C, with stiffness equivalent to that in BM. We showed that 2% was the optimal concentration for MKs to extend proplatelets (40% MK-forming proplatelets compared to 28% in liquid) while MC concentrations of 2.5% and above decreased the proportion of MK-forming proplatelets to less than 5%. MC 2% increased mean MK ploidy (N=36.5±3.8 vs. 28.1±2.1), and increased DMS surface/organization, resembling *in situ* MK, suggesting higher maturation. To evaluate whether mechanical constraints are involved, we differentiated *Myh9*-/- progenitors within MC gel. Under such conditions, myosin-deficient MKs, which are unable to adapt their intracellular tension to the stiffness of the extracellular medium, acquired the abnormal invasive morphology similar to that in BM.

Conclusions: Altogether, we show that an MC-based 3D-culture, by mimicking mechanical and/or physical features of the BM, contributes to a more favorable *in vitro* environment for MK differentiation.

POSTER

P9

Extraculllar loop II in platelet thrombin receptor PAR4 is important for binding of thrombin

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Thrombin is a potent platelet agonist, acting via protease-activated receptors (PARs) 1 and 4. The two receptors have distinct and complementary roles in platelet biology. A PAR1 inhibitor has failed in a clinical trial. PAR4 may be a new target for antithrombotic drugs. To study of PAR4 activation and interaction with thrombin we made mutants substituting aspartic residues in the extracellular loop II (ECLII) of PAR4 as they could interact with the exosite II of thrombin and possible the new N-terminal after thrombin cleavage. We utilized a proven system in yeast, based in a signal of LacZ reporter gene connected to a recombinant GPCR that can be stimulated[1] in our case, by thrombin, the natural ligand of PAR4.
Human PAR4 cloning, directed mutagenesis and expression was successful in yeast. PAR4 was detected by flow cytometry and WB There was a dose-dependent response of the Lac Z assay by thrombin. Our preliminary results showed a clear decrease in this functional assay when using PAR4 mutants affecting different aspartic residues of the ECLII as we expected, because potential interactions of their negative charges with the positively charged exosite II would be erased. We confirmed these results by detection of molecular events once PAR4 was activated (on wild type and mutants). One approach was the detection of PAR4 cleavage (by Western Blot) and the other approach was the release of G protein (assessed by immunoprecipitation with PAR4 as bait and G protein detected), i.e. the intensity of the bands depends on how much PAR4 is activated and this was less in the mutations of interest.

1. Dowell SJ, Brown AJ. Yeast assays for G protein-coupled receptors. Methods Mol Biol 2009;552:213-29.

P10

Tolerance of Platelet Concentrates Treated With UVC Light Only for Pathogen Reduction

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Background: UVC-pathogen reduction for platelet concentrates (PCs) operates with UVC-light alone (254 nm) without addition of any photosensitizers. This Phase-1study was designed to evaluate platelet increments and safety in healthy volunteers after transfusion of autologous UVC-treated PCs.

Methods: Eleven volunteers underwent two single (series 1 and 2) and one double apheresis procedures (series 3). An interval of two weeks was between each series. PCs were treated with UVC light (0.2 J/cm²), stored for two days and retransfused in a dose-escalation scheme (1/8 to ½ of a PC unit in series 1; one complete PC in series 2; two PCs in series 3). Safety and tolerability of transfusions were assessed by recording platelet count, fibrinogen, activated partial thromboplastin time, prothrombin time and D-dimer, standard hematology, temperature, pulse, blood pressure and clinical chemistry parameters. The recipients were tested for platelet-specific antibodies by flow cytometry, monoclonal antibody-specific immobilization of platelet antigens assay and ELISA. As parameters for in vivo-platelet viability corrected count increments (CCIs) were evaluated in series 2 and 3.

Results: Transfusions showed no signs of intolerance**.** In Series 2, the mean 1h-CCI was 18.92 (95% CI: 24.4-64.9), while in Series 3 the mean 1h-CCIs were 16.5 (95% CI: 4.7-28.4) after the first and 13.65 (95%CI: 1.53-25.77) after the second transfusion. The CCI results did not significantly differ between series 2 and 3.

Conclusion: Repeated transfusions of UVC-treated autologous PCs were well tolerated and did not induce specific antibody responses in healthy volunteers. The CCIs suggest a good viability of transfused UVC-treated platelets.

POSTER

P11

Non-genomic effects of nuclear receptors: Different mechanisms of regulation of outside–in signalling in platelets

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Introduction: A number of nuclear receptors, which are members of the nuclear hormone superfamily have been identified in human platelets, including the peroxisome proliferator-activated receptor- (PPAR), Liver X receptors (LXRs) and the Farnesoid- X receptor/Bile acid receptor (FXR/BAR). Several of these have been shown to have inhibitory non-genomic effects on GPVI-stimulated inside-out signaling and platelet activation. Outside-in signaling also plays a critical role in platelet function, and normal haemostasis. As several components of the αIIbβ3 and GPVI signalling pathways are shared, in particular tyrosine kinase signalling, we have investigated whether the nuclear receptors are involved in the regulation of outside-in signalling

Methods: The effect of the PPAR , LXR and FxR agonists (15d-PGJ_2, Gw3665 and Gw4064 respectively) on platelet adhesion, spreading and clot retraction was studied using human washed platelets.

Results: Platelets treated with each of the three nuclear receptor agonists, 15d-PGJ_2, Gw3665 or Gw4064 showed a significant inhibition of platelet adhesion to fibrinogen, platelet spreading on fibrinogen and clot retraction when compared to vehicle treated controls. Interestingly, phosphorylation of key αIIbβ3 signalling pathway components Focal adhesion kinase (FAK) and Phospholipase C gamma (PLC) were only reduced in the PPAR agonist treated fibrinogen activated platelets and not LXR or FXR agonist treated samples, indicating distinct modes of action for the different receptors.

Conclusions: This work provides further evidence for non-genomic roles for the nuclear receptors in platelets, and specifically identifies inhibitory but distinct regulatory roles for the PPAR , LXR and FXR agonists in the regulation of outside-in signalling. Better understanding of these differences could lead to the development of specifically targeted therapies.

POSTER

P12

Activation of human platelets by *Staphylococcus aureus* secreted protease staphopain A

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Introduction: *Staphylococcus aureus* is a major human pathogen capable of causing life threatening bacteremia that results in an increased risk of myocardial infarction, stroke and is also the leading aetiological agent of infective endocarditis (IE). The pathogen can induce the activation of platelets, resulting in aggregation and thrombus formation, which are regarded as important steps in the pathogenesis of IE. Staphopain A is a cysteine protease that is secreted by *S. aureus.*

Methods: Using light transmission aggregometry, supernatant from a staphopain A deficient *S. aureus* mutant was shown to be unable to activate human platelets when compared to the parental strain. Platelet aggregation assays were used to determine the effect of staphopain A on platelets and specific inhibitors and antagonists were used to determine which platelet receptors may be responsible. Platelet functional assays measuring thrombus formation were used to illustrate the effects of staphopain A on platelets. Flow cytometry was used to confirm that staphopain A activated platelets by measuring integrin $\alpha_{IIb}\beta_3$ activation and P-selectin exposure.

Results: In this study, we demonstrate that staphopain A induces the aggregation of human platelets. The presence of staphopain A in the supernatant of *S. aureus* confers an ability to activate platelets, in addition to the actions of alpha haemolysin also released by the pathogen. Staphopain A platelet agonist activity was inhibited by addition of the specific inhibitor staphostatin A, implicating its protease activity in the agonism. Concentrations of staphopain A that were insufficient to induce platelet aggregation caused increased platelet binding to collagen. Using specific antagonists, protease-activated receptors 1 and 4 were shown to be responsible for mediating staphopain A induced activation of platelets.

Conclusions: Protease activating receptor-1 and -4 are receptors for staphopain A. Staphopain A mediated activation of protease activating receptors represents a novel axis in the human host-*S. aureus* interaction.

POSTER

P13

Functionally active tissue factor is expressed during megakaryocyte maturation and is transferred to a subset of platelets

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Background: Tissue factor (TF), the main activator of the blood coagulation cascade, has been shown to be expressed also by platelets. Although both megakaryocytes and platelets express TF mRNA and platelets have been shown to make *de novo* protein synthesis, the main mechanism claimed to be responsible for the presence of TF within platelets is through the uptake of TF positive microparticles.

Aim: To assess whether human megakaryocytes synthesize TF and transfer it to platelets.

Methods: In order to avoid the cross-talk with circulating microparticles, we took advantage from an *in vitro* cultured megakaryoblastic cell line (Meg-01) able to differentiate into megakaryocytes releasing platelet-like particles (Meg-platelets) upon treatment with valproic acid (2mM). TF expression and activity were evaluated by different approaches in human megakaryoblasts, megakaryocytes and platelets. To confirm that TF expression documented in Meg-01 was not a feature of the transformed cell line we analysed TF expression in human $CD34^{pos}$-derived megakaryocytes ($CD34^{pos}$-MK) and in their released platelets ($CD34^{pos}$-platelets).

Results: Real-time PCR experiments showed a 3 fold higher expression of TF mRNA in Meg-megakaryocytes compared to Meg-megakaryoblasts. Immunoassays showed that also TF protein was significantly more abundant in Meg-megakaryocytes than in Meg-megakaryoblasts (90±15 and 50±10 pg/mg cell protein, respectively) and this was paralleled by a significantly higher thrombin generation capacity in Meg-megakaryocytes compared to Meg-megakaryoblasts. Flow cytometry analysis showed that TF was present in the cytoplasm of 40% of Meg-platelets where it was functionally active and blocked by an anti-TF antibody. Both TF pre-mRNA and mRNA were detected in Meg-platelets and all these data were confirmed in $CD34^{pos}$-MK and $CD34^{pos}$-platelets.

Conclusions: Functionally active TF is expressed in human megakaryoblasts, megakaryocytes and in their shedded platelets. These data indicate that platelets express TF independently of the interaction with other cells.

P14

The recognition of collagen by the VWF A domains

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Introduction: von Willebrand factor (VWF) has a crucial role in capturing platelets by subendothelial collagens that become exposed upon vascular injury or plaque rupture. The VWF A domains are central to this process, A3 binding to a known site in collagen, Lisman et al, Blood, **108:**3753-3756 (2006), whilst A1 binds to platelet Glycoprotein Iba. Although a growing strand of literature reports that A1 may also bind collagen, this interaction remains ill-defined.

Methods: We applied recombinant VWF A1 and A3 domains, expressed as either GST-fusion or His-tagged proteins, to the collagen Toolkits, libraries of synthetic triple-helical peptides, and measuring binding using a colorimetric static adhesion assay.

Results: As before, A3 bound to a single peptide in Toolkit III (representing the full COL domain of collagen III). Using Toolkit II, A3 bound the corresponding, conserved, site in collagen II. Unexpectedly, a second peptide in Toolkit II proved strongly A3-reactive, at a site conserved in collagen I but absent from collagen III. A1 domain was found to bind several conserved sites in both Toolkits, mapping its collagen-binding activity for the first time.

Conclusions: VWF can interact with collagen in two distinct ways, through both its A1 and A3 domains. The known A3 site in collagen II is not fully conserved in collagen I, being degenerate between its two a-chains, neither of which individually can bind A3: binding activity would require one specific interchain registration of collagen I. However, the new A3-binding site is conserved in both collagens I and II, and the novel A1 sites are conserved across all three collagens. We conclude that both the main fibrillar collagens of the vessel wall, I and III, can bind VWF via both its A1 and A3 domains. The relationships between these interactions remains to be established.

P15

Persistent particles in apheresis platelet concentrates

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Introduction: Thrombopheresis allows donation of platelets in real-time. Aggregates often appear during donation but are generally transient. Sometimes aggregates persist throughout storage, causing discard of the product. This study aimed to determine factors that contribute to persistent aggregate formation.

Methods: Donor characteristics, donation metadata and platelet variables from donations by Trima Accel® (TerumoBCT) were investigated by retrospective logbook analysis and laboratory measurements, respectively. Donations with persistent particles (PP) were compared to unpaired particle-free donations (PF).

Results: A dataset collected in 2012 found no statistical differences in donor-related variables (n=55) for gender, height, weight, hematocrit, total blood volume and donor platelet count. However, the same cohort revealed significantly higher platelet yields in PP (6.2±1.5 vs 5.5±1.4(x10^{11}), *P*=.02). This was confirmed in a subsequent independent cohort (2013, n=183) again revealing higher yields (6.0±1.5 vs 5.5±1.4(x10^{11}), *P*=.002), donated volumes (514±127 vs 462±123mL, *P*<.001) and donor platelet counts (286±50 vs. 268±49(x10^{3})/µL, *P*<.001). Platelet concentrates available on day six post donation were analyzed further in the lab. The pH was significantly lower in PP concentrates (7.18±0.16 vs 7.36±0.17, *P*<.001, n=15) which was caused by higher lactic acid concentrations (11.3±3.4 vs. 8.6±2.7mM, P=.02). Flow cytometry showed no differences in GPIb, phosphatidylserine exposure or PAC1 binding. However, PP platelets expressed significantly more P-selectin (P=.02, n=15) indicating increased alpha-degranulation. Platelet agglutination was significantly higher at low but not high-dose ristocetin (P=.02, n=15), while collagen or thrombin analogue induced similar aggregation. Subsequent von Willebrand factor ristocetin cofactor activity and -collagen binding measurements in the concentrate supernatants indicated no differences. Nonetheless, significantly lower fibrinogen levels were found in PP concentrates (2.8±0.6 vs. 3.7±1.2mg/mL, P=.04, n=13) which may indicate consumption during platelet cross-linking in PP concentrates.

Conclusions: Increases in P-selectin, lactic acid and decreased fibrinogen levels point to elevated platelet activation in concentrates (from donors) with slightly higher platelet counts.

P16

Assessment of platelet function using a P-selectin based Platelet function test compared with other commercial tests

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Background: We have assessed a range of platelet function testing methods alongside a remote P-selectin test for monitoring the effectiveness of antiplatelet agents in acute coronary syndromes. Patients were on therapy for at least 1 month following their thrombotic event. The tests focus on assessment of the effectiveness of treatment with both aspirin and $P2Y_{12}$ antagonists. P-selectin test kits (Platelet Solutions Ltd) are simple to use and may be used remotely in a variety of locations. Following activation P-selectin test samples are treated with PAMFix, a patented fixing solution and sent to a central laboratory for analysis by flow cytometry.

Methods: We compared the P-selectin aspirin and $P2Y_{12}$ tests with three aspirin and four $P2Y_{12}$ commercial tests including PRP aggregometry (PRP), Multiplate (MP), VerifyNow (VN) and Biocytex VASP (VASP). Citrate-anticoagulated blood samples were obtained from patients following treatment with either aspirin plus clopidogrel (n=145) or aspirin plus the more potent $P2Y_{12}$ antagonist, prasugrel (n=74).
A set of PAMFix treated P-selectin samples were reanalysed after storage for up to 28 days, to demonstrate the stability of fixed P-selectin samples. Optimal cut-off values for each of the tests were investigated.

Results: All of the $P2Y_{12}$ tests used in this study demonstrated variability in the effectiveness of clopidogrel to inhibit platelet function while prasugrel treatment resulted in greater inhibition and less variability in responses. The aspirin tests used consistently identified a small cluster of four to six patients as being either non-responders or non-compliant while all other patients demonstrated inhibition by aspirin with all of the tests.

Conclusions: The P-selectin tests, with their potential for remote platelet testing, were at least as effective in determining the inhibitory effects of antiplatelet therapies as other tests but may be analysed later. Fixed P-selectin samples remained stable even after storage for 28 days.

P17

CLEC-2 expression is maintained on activated human platelets and on platelet microparticles

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Introduction: The C-type lectin-like receptor CLEC-2 mediates powerful platelet activation through a hem-Immunoreceptor Tyrosine-based Activation Motif (ITAM). CLEC-2 initiates a Src and Syk dependent signalling cascade which is closely related to that of the two platelet ITAM receptors, glycoprotein (GP) VI and FcgRIIa. Interestingly, activation of either of the ITAM receptors induces shedding of GPVI and proteolysis of the ITAM domain in FcgRIIa.

Methods: We have generated monoclonal antibodies (mAbs) against human CLEC-2 and used these to measure CLEC-2 expression on resting and stimulated platelets and on other hematopoietic cells.

Results: We show that CLEC-2 is restricted to platelets with an average copy number of ~2000 per cell and that activation of CLEC-2 induces proteolytic cleavage of GPVI and FcgRIIa but not of itself. We further show that CLEC-2 along with GPVI is expressed on $CD41^+$ microparticles in platelet-rich plasma, which are derived from megakaryocytes in healthy donors, whereas microparticles derived from activated platelets only express CLEC-2. Patients with rheumatoid arthritis, an inflammatory disease associated with increased microparticle production, had raised plasma levels of microparticles that expressed CLEC-2 but not GPVI.

Conclusions: CLEC-2, unlike platelet ITAM receptors, is not regulated by proteolysis and, together with GPVI, can be used to monitor platelet-derived microparticles.

P18

Regulation of the platelet collagen receptor GPVI by the tetraspanin Tspan9

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Background: The platelet-activating collagen receptor GPVI recognises collagen as a dimer and initiates signalling via its associated Fc receptor -chain. GPVI-deficient mice are protected from arterial thrombosis but have no major bleeding complications, suggesting GPVI as a target for anti-thrombotic therapy. The tetraspanins comprise 33 transmembrane proteins in humans with at least 10 expressed in platelets. Advanced imaging techniques, such as single particle tracking, have shown that tetraspanins regulate the lateral mobility of specific membrane proteins. We have previously identified GPVI as a tetraspanin-associated protein, but it is unclear how this contributes to GPVI function. We have also previously identified Tspan9 as a relatively platelet specific tetraspanin, but have not addressed Tspan9 function.

Aims: To functionally characterise Tspan9-deficient platelets and to investigate whether Tspan9 affects GPVI lateral mobility.

Methods: Tspan9-deficient platelets were functionally assessed by aggregometry. Subsequent live cell imaging using single particle tracking and TIRF microscopy measured GPVI membrane dynamics in wildtype and Tspan9-deficient platelets .

Results: Tspan9-deficient platelets had normal surface expression of major surface proteins, including GPVI. However, aggregation studies highlighted a specific delay in response to GPVI agonists. Given the function of other tetraspanins this suggested Tspan9 may influence GPVI lateral mobility. Therefore single particle tracking was employed to assess the membrane dynamics of GPVI. In wild-type platelets GPVI molecules followed trajectories that had elements of both Brownian and confined motion. In Tspan9-deficient platelets, however, GPVI molecules displayed an increase in confined motion. This increase in confinement resulted in a significant decrease in the median diffusion coefficient of GPVI in Tspan9-deficient platelets.

Conclusions: The uncharacterised tetraspanin Tspan9 is important for fine-tuning GPVI function in platelets, by regulating GPVI membrane dynamics. Additionally, for the first time, the membrane dynamics of a platelet receptor have been studied at the single molecule level.

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POSTER

P19

Human platelets sense differentially various *Staphylococcus aureus* exotoxins and adapt subsequent immunomodulatory molecule secretion profiles

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POSTER

Introduction: Sepsis is accompanied by several biological phenomena including acute generalized inflammation. Platelets are involved in the pathophysiology of sepsis, as deduced from the frequent occurrence of thrombocytopenia in sepsis, although the mechanisms for platelet depletion are not completely elucidated. Platelets react to bacterial components and secrete copious amounts of proinflammatory molecules. *Staphylococcus aureus* is one of the most frequent bacteria causing sepsis in Europe. *S. aureus* and its components can bind to and activate platelets, leading to their aggregation.

Methods: To investigate the effects of *S. aureus* exotoxins on the capacity of platelets to release inflammatory cytokines/chemokines. Platelets were exposed to prolonged contact with staphylococcal exotoxins α-and -hemolysin, Panton-Valentine leukocidin and toxic shock syndrome toxin-1 to investigate whether exposure induces the release of soluble immunomodulator factors, such as soluble CD62P, soluble CD40L, regulated on activation, normal T cell expressed and secreted (RANTES) and stromal cell-derived factor-1α (SDF-1α).

Results: Profiles of secreted immunomodulators appeared dependent on the staphylococcal exotoxin used for stimulation, with differential release of sCD62P, sCD40L and RANTES by platelets and. SDF-1α release was not elicited by staphylococcal toxins. Stimulation of platelets with combinations of exotoxins used at suboptimal concentrations resulted in contrasting releases of sCD40L and RANTES compared with each exotoxin alone.

Conclusions: The results suggest that platelets discriminate between staphylococcal toxins and adapt their inflammatory response to perceived specific "danger" signals. This is of particular importance in the context the inflammatory phase of sepsis.

P20

Losartan-induced Inhibition of GPVI clustering by collagen and collagen-induced platelet responses is achievable *in vitro* but not in treated patients

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Introduction: Pharmacological agents capable of inhibiting platelet activation by collagen are of potential therapeutic interest. Glycoprotein VI (GPVI) interaction with collagen triggers platelet activation that is reinforced by ADP and thromboxane A2. Losartan is an angiotensin II (Ang II) type I receptor (AT1R) antagonist proposed to have an antiplatelet activity via the inhibition of both the thromboxane A2 receptor (TP) and GPVI. Our aim was to clarify the mechanism of action of losartan on platelets *in vitro* and to establish if a therapeutic administration of losartan impacts platelet functions

Methods: *In vitro*: platelet aggregation, P-selectin exposure, GPVI dimerization; platelet adhesion and thrombus formation in flow conditions; binding of FITC-collagen to platelets, binding of GPVI-Fc to collagen, GPVI clustering analysis using the Duolink technology and the 9E18 mAb specific for GPVI dimers; Patients suffered from Marfan syndrome and the assay was designed to determine the efficacy of losartan on aortic dilatation (ClinicalTrials.gov Identifier: NCT00763893). Platelet responses were analyzed *ex vivo* in a double blind study.

Results: The IC50 of losartan inhibited on collagen- and U46619-induced platelet aggregation and P-selectin exposure were of 2.9 and 18 $\mu g.mL^{-1}$ indicating that low doses of losartan inhibit collagen-induced platelet activation independently of its effect on TP. Losartan did not inhibit the binding of GPVI to collagen but reduced the binding of collagen to platelets by a mechanism shown to involve the inhibition of GPVI clustering. However, the antiplatelet effect of losartan was without clinical relevance since no statistical differences were observed between losartan-treated (n=25) and non-treated (n=30) patients in terms of collagen and U46617-induced platelet activation.

Conclusions: These data indicate that therapeutic plasma concentrations of losartan are not sufficient to achieve a measurable antiplatelet effect but also provide the proof of concept that inhibiting collagen-induced GPVI clustering would achieve antithrombotic efficacy.

P21

The human IgG receptor FcγRIIA induces allergic and inflammatory reactions

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Introduction: IgG antibodies and their receptors, FcγRs, contribute to various inflammatory and autoimmune conditions. Much less is known about their active participation in allergic reactions and especially anaphylaxis. On the contrary it is generally considered that IgG antibodies negatively regulate IgE-induced allergies. We could recently demonstrate that a murine model of allergic shock (anaphylaxis) depended primarily on IgG- and FcγR-mediated activation of neutrophils. FcγRIIA is an activating low-affinity IgG receptor unique to humans. It is the most widely expressed IgG receptor in humans, present on all myeloid cells. We therefore hypothesized that FcγRIIA could contribute to inflammatory and allergic reactions in humans.

Methods: To explore this hypothesis we developed a unique model of genetically engineered mice rendered deficient for endogenous FcR and transgenic for hFcγRIIA (mFcR^{KO}/hFcγRIIA^{tg}). IgG immune complexes were administered intravenously (i.v.) or intradermally (i.d.), and systemic or cutaneous anaphylaxis was assessed by monitoring body temperature or Evan's Blue extravasation, respectively. Airway inflammation was induced by intranasal instillation of anti-BSA antiserum and concomitant i.v. injection of BSA.

Results: We could show that hFcγRIIA was sufficient to induce local and systemic anaphylactic reactions in mice. hFcγRIIA-triggered activation of dermal mast cells for was required for cutaneous reactions, whereas activation of monocytes and neutrophils contributed to systemic anaphylactic reactions. hFcγRIIA-triggered activation of alveolar macrophages was sufficient to induce massive neutrophil recruitment and respiratory distress in a model of airway inflammation.

Conclusions: Our results demonstrate that anaphylactic and inflammatory reactions can be induced via hFcγRIIA in mFcR^{KO}/hFcγRIIA^{tg} mice. They suggest that FcγRIIA can contribute to these reactions in humans.

P22

The interplay between Pyk2 and phosphatidylinositol 3-kinaseβ in GPVI signaling

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Introduction: Phosphatidylinositol 3-Kinaseβ (PI3Kβ) plays a predominant role in platelet activation triggered by integrin outside-in signaling or GPVI engagement. We have previously demonstrated that the tyrosine kinase Pyk2 mediates PI3Kβ activation downstream integrins αIIbβ3 and α2β1, possibly through the phosphorylation and association of the adaptor protein c-Cbl. In this study, we investigated the functional correlation between Pyk2 and PI3Kβ upon GPVI ligation.

Methods: Platelets from Pyk2 knockout mice (Pyk2 KO) or from mice expressing a catalytically inactive form of PI3Kβ (PI3KβKD) were stimulated with collagen related peptide (CRP). Phosphorylation of Akt, Pyk2 or c-Cbl was determined by immunoblotting.

Results: Analysis of CRP-induced Akt phosphorylation on both Ser473 and Thr308 confirmed that PI3Kβ is the only PI3K isoform stimulated downstream of GPVI. In contrast to what previously observed upon integrin engagements by immobilized collagen or fibrinogen, Akt phosphorylation by GPVI stimulation occurred normally in Pyk2-deficient platelets. Comparable results were also obtained when ligation of GPVI was induced by platelet adhesion to immobilized CRP. CRP induced the time-dependent phosphorylation of c-Cbl, which occurred normally in the absence of Pyk2, but failed to promote association of c-Cbl with p85 regulatory subunit. By contrast, GPVI-induced Pyk2 activation, monitored as autophosphorylation on Tyr402, was completely abrogated in PI3KβKD platelets, and strongly inhibited by Src kinases and phospholipase C inhibitors, as well as by BAPTA-AM. The absence of PI3Kβ activity also completely prevented GPVI-induced phosphorylation of pleckstrin and reduced intracellular Ca2+ elevation, which occur downstream of phospholipase C activation.

Conclusions: These results outline important differences in the regulation of PI3Kβ by GPVI stimulation versus integrin engagement. In particular, activation of PI3Kβ by GPVI is not promoted by the tyrosine kinase Pyk2; rather, PI3Kβ lies upstream Pyk2, through regulation of phospholipase C activity.

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POSTER

P23

Human platelet STIM1 and ORAI1 are essential in store-operated calcium entry and thrombus formation at venous conditions

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Introduction: Patients with deleterious mutations in the *ORAI1* gene, encoding for a store-regulated Ca^{2+} channel (SOCC), or in the *STIM1* gene, encoding for stromal interaction molecule 1 suffer from severe immunodeficiency and autoimmunity, and often require transplantation. In mice, full deficiency in platelet Orai1 or STIM1 prolongs tail bleeding, and results in impaired arterial thrombus formation and platelet-dependent procoagulant activity. However, in man the functional consequences of defects in these proteins are not well established.

Methods: Blood was obtained from an Orai1-patient (homozygous R91W mutation), before and after bone marrow transplantation, as well as from the parents. In addition, blood was obtained from a STIM1-patient (heterozygous R429C mutation). Platelet calcium fluxes were determined. Platelet activation properties were analyzed by flow cytometry. Thrombus formation was determined in whole blood perfused over collagen at high or low shear rates (1600 or 150 s^{-1}, respectively).

Results: Carriers of the Orai1 mutation (also after transplantation), but not the STIM1 mutation, were slightly thrombocytopenic. In platelets from these subjects, TG/Ca^{2+}-induced calcium responses were abolished (Orai1 patient before transplantation), reduced (father, STIM1-patient) or normal (mother, Orai1-patient after transplantation). Calcium responses evoked by convulxin/Ca^{2+} followed a similar pattern. Flow cytometry indicated no alterations in agonist-induced integrin $\alpha_{IIb}\beta_3$ activation or P-selectin expression. Whole-blood perfusion studies demonstrated, for all patients, a small to moderate reduction in thrombus formation that was however more prominent at low shear rate than at high shear rate. Markedly, platelet procoagulant activity was diminished with blood from the patients but not from the parents.

Conclusion: The reduced SOCC activity, linked to genetic mutations in *ORAI1* or *STIM1*, is accompanied by a mild reduction in platelet count, moderate impairment of thrombus formation at low or high shear rate, and reduced platelet procoagulant activity. These moderate changes in platelet count and function are compatible with normal hemostasis.

POSTER

P24

Thrombus Development Processes Dependent on Endothelial Injuries: Visualized by In vivo Two-photon Molecular Imaging

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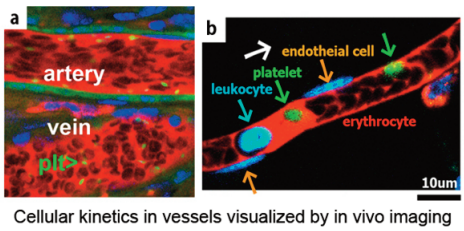
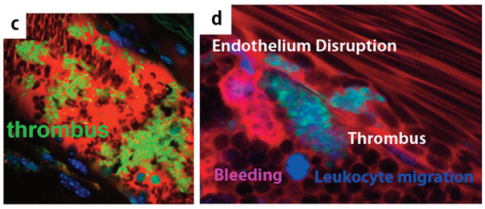
Aim: The thrombotic cellular mechanisms associated with cardiovascular events remains unclear, largely because of an inability to visualize thrombus formation.

Methods: Thus, we developed in vivo imaging technique based on single- and multi-photon microscopy to revealed the multicellular processes during thrombus development (Figure a,b). We visualized the cell dynamics including single platelet behavior, and assessed dynamic cellular interplay in two thrombosis models.

Results: First, we visualized that rapidly developing thrombi composed of discoid platelets without EC disruption was triggered by ROS photochemically induced by moderate power laser irradiation (Figure c). In this model, thrombus consisted by discoid platelet aggregations without leukocyte recruitment. And we found the adapter protein (Lnk) is vital for the stabilization of developing thrombus in this model.
The second model is, thrombus with EC disruption. High power laser induced EC erosion and extravasations of circulating leukocytes with thrombus development. Inflammatory cytokine, adhesion molecules dynamically control these two processes. (Figure d)
As for the thrombus formation with EC disruption, chemokine expressions in endothelium and leukocyte (especially neutrophils) recruitment played a significant role in these processes. Leukocyte was immediately recruited into the subendothelial layers with bleeding and hemostatic reactions. TLR4 signaling also contributed to these steps, and pretreatmet of LPS markedly enhanced these steps.
Gr-1 (anti-Ly6G and 6C) antibody treatment decreased the number of recruited leukocyte and thrombus development, indicating the contribution of granulocyte. Thrombus included calcium activated cores and deformed platelets, and immigrated leukocyte also showed the increase of intracellular calcium.
In sum, using our imaging system can be a powerful tool to analyze thrombus formation and evaluate the therapeutic strategies.



Cellular kinetics in vessels visualized by in vivo imaging



Thrombus formation without endothelium disruption

Thrombus formation with endothelium disruption

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P25

Vascular risk levels affect the predictive value of platelet reactivity for the occurrence of major adverse cardiovascular events in patients on clopidogrel: Systematic review and collaborative meta-analysis of individual patient data

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Background: Prior studies have shown an association between high on-clopidogrel platelet reactivity (PR) and the risk of major adverse cardiovascular events (MACE). However, large intervention trials on PR-tailored treatments have been neutral. We assessed the clinical relevance of PR in predicting MACE according to patients' cardiovascular risk levels.

Methods: We undertook a systematic review and meta-analysis of individual patient data on MACE outcomes (acute coronary syndromes (ACS), ischemic strokes, and vascular deaths) in relation to PR and its interaction with cardiovascular risk levels. PR was determined using ADP-induced light transmission aggregometry with a primary concentration of 20µM ADP.

Results: Thirteen prospective cohort studies totalled 6,478 clopidogrel-treated patients who experienced 421 MACE (6.5%) during a median follow-up of 12 months. The risk of MACE associated with PR increased differentially according to the number of risk factors present (age>75 years, ACS at inclusion, diabetes, and hypertension; interaction p=0.04): no association to PR in low-risk patients (no risk factor) (p=0.48); 3.2 times greater risk of MACE in high PR (> 60%) intermediate-risk patients (one risk factor) (p=0.001); 2.9 and 3.7 times greater risk of MACE in medium PR (41%–60%) and high PR (>60%) high-risk patients (≥2 risk factors) (p=0.0004 and 0.0003, respectively). These interactions followed the same trends in studies using 5µM ADP.

Conclusion: The magnitude of the association between PR and MACE risk is strongly dependant on the level of cardiovascular risk faced by patients on clopidogrel suggesting that PR-tailored strategies may be most effective in higher-risk patients.

P26

GLP-1 reduces the activation of PI3K and MAPK pathways and of oxidative stress induced by Sodium Arachidonate in platelets

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Background and aims: Glucagon-like peptide-1 (GLP-1), an incretin hormone secreted by intestine after meal, exerts important metabolic effects justifying the GLP-1-based therapy in diabetes and acts also on cardiovascular system: its influence on platelets, however, is unknown.
Aim of this study is to investigate the GLP-1 influence on the arachidonic acid-induced activation of the signalling pathways PI3-Kinase (PI3K) and MAP-Kinase (MAPK) and of oxidative stress in platelets.

Materials and Methods: In washed platelets from 24 healthy subjects (M/F 13/11; age 25.6±5.9 years, BMI 22.5±2.4 kg/m^2) we measured the influence of a 15 min pre-incubation with the native form GLP-1(7-36)(100nmol/l) on the effects of Na-arachidonate (NaA) (0.5 mmol/l) on: i) phosphorylation of Akt and Erk-1/2, molecules of the PI3K and MAPK pathways, respectively (WB); ii) ROS production (DCF-DA assay). Experiments were repeated in the presence the Erk-1/2 inhibitor U0126 (40 micromol/l) and the GLP-1 receptor (GLP-1R) antagonist exendin (9-39) (100 nmol/l). Results: GLP-1 reduced platelet signalling induced by NaA. In particular: i) the fold increase on basal values with NaA alone and Na+GLP-1 (7-36) was 11.2±2.1, 3.1±0.8 respectively ($p<0.0001$ vs NaA alone) for pAKT, and 14.6±2.5, 3.9±1.0 respectively ($p<0.0001$ vs NaA alone) for pERK-1/2; ii) the fold increase on basal values of ROS with NaA alone and NaA+GLP-1 (7-36) was 8.2±1.1, 5.6±2.0, respectively ($p=0.001$vs NaA alone); iii) the Erk-1/2 inhibitor U0126 reduced the NaA-induced activation of ROS ($p<0.0005$ vs NaA alone). In the presence of GLP-1R antagonist exendin (9-39) the effects of GLP-1 were not modified.

Conclusions: In human platelets, GLP-1, independently of GLP-1R, reduces the NaA-induced activation of PI3K and MAPK pathways and of oxidative stress. Because MAPK activation is involved in the NaA-induced increase of oxidative stress, the inhibiting effects of GLP-1 on MAPK activation can account for its ability to attenuate the NaA-induced increase of oxidative stress.

P27

Does the inhibition of $P2Y_{12}$ inhibit the production of thromboxane A_2 by platelets?

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Introduction. Patients with acute coronary syndromes (ACS) are treated with aspirin, which inhibits thromboxane A_2 (TxA_2) production, and an antagonist of $P2Y_{12}$. It has been surmised that ACS patients may be treated with $P2Y_{12}$ antagonists only, based on the observation that these drugs inhibit the platelet production of TxA_2. However, this contrasts with the demonstration that platelets congenitally deficient of $P2Y_{12}$ normally synthesize TxA_2. Aim. To test whether the reported inhibitory effect of $P2Y_{12}$ antagonists on TxA_2 production is due to off-target effects, or secondary to inhibition of platelet aggregation (PA).

Methods. Serum TxB_2 was measured in patients with severe $P2Y_{12}$ deficiency and healthy subjects in presence/absence of high concentrations of $P2Y_{12}$ antagonists, and in 20 patients treated with prasugrel or placebo for 14 days in a randomized, double-blind, cross-over study. TxB_2 levels were also measured in normal citrate-PRP or hirudin-PRP stimulated by collagen or arachidonic acid in presence/absence of $P2Y_{12}$ antagonists, of a $P2Y_1$ antagonist (MRS2500) and of a $P2Y_{12}$ antagonist plus epinephrine, under stirring and non-stirring conditions (PA does not occur without stirring). Moreover, we studied the in vitro effects aspirin and of a $P2Y_{12}$ antagonist alone and in combination on PA and TxB_2 production by platelets stimulated by collagen (0.5-10µg/mL) +/- epinephrine.

Results. $P2Y_{12}$ antagonists did not decrease serum TxB_2 levels *in vitro* and *ex vivo*. Under stirring conditions, they inhibited PA and TxB_2 production; under no stirring conditions (no PA occurred) they did no inhibit TxB_2 production. Similar results were obtained with MRS2500. Epinephrine restored PA and TxB_2 production in the presence of $P2Y_{12}$ antagonists. Aspirin plus a $P2Y_{12}$ antagonist inhibited PA and TxB_2 production induced by high concentrations of collagen +/- epinephrine better than either drug alone.

Conclusions. There is no pharmacological evidence that ACS patients may be safely treated with $P2Y_{12}$ antagonists only.

P28

Impaired response to collagen of platelets from hyperlipidemic mice

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Introduction: Platelets are key players in atherothrombosis and their responsiveness is modulated by lipoproteins. We showed that elevated plasma HDL-cholesterol due to HDL receptor dysfunction induces platelet hyperreactivity in mice and humans. The aim of this study was to investigate platelet responsiveness under conditions of elevated plasma (V)LDL-cholesterol.

Methods: Experiments were performed with platelets from hyperlipidemic apolipoprotein E-deficient (apoE KO) mice with extremely elevated plasma (V)LDL-cholesterol (plasma total cholesterol (TC) level = 396±67 mg/dL), their wildtype (WT) littermates (32±13 mg/dL), or apoE KO mice transplanted with WT bone marrow (BM) (46±9 mg/dL). Surface expression of active integrin $\alpha_{IIb}\beta_3$ and P-selectin upon stimulation with collagen-related peptide (CRP-XL) or PAR-4 peptide was assessed by flow cytometry.

Results: ApoE deficiency significantly reduced platelet counts (1024±77x10^9 platelets/L vs 1131±188x10^9 WT platelets/L, $p<0.05$). Bone marrow histology did not show a difference in megakaryocyte number, indicating that the reduced platelet count may be caused by increased platelet clearance. While the response to PAR-4 peptide was unaltered, activation of integrin $\alpha_{IIb}\beta_3$ in response to CRP-XL was significantly reduced for platelets from apoE KO mice (-32% vs WT, $p<0.05$). Similarly, the amount of surface-expressed P-selectin was 5.7-fold lower after CRP-XL stimulation ($p<0.05$). Normalization of plasma TC by reintroducing apoE in hematopoietic cells by WT bone marrow transplantation restored CRP-XL-induced $\alpha_{IIb}\beta_3$ activation (+126%, $p<0.05$ vs apoE KO BM-transplanted mice). An increased cholesterol content in platelets from apoE KO mice may be responsible for the differences observed, by changing the organization of lipid rafts, to which the collagen receptor GPVI localizes after stimulation, or by activating the cellular cholesterol sensor LXR, which is known to affect GPVI signaling.

Conclusions: *In vivo*, collagen becomes exposed after vessel wall injury and activates platelets. Hence, our findings may reflect a protective mechanism to prevent early platelet activation upon vessel wall injury under hyperlipidemic conditions.

P29

Platelet aggregation: the effect of Lys- and Glu-plasminogen

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Introduction. Platelets possess a central role in coagulation but can also be considered as the place for plasmin generation. Components of plasminogen/plasmin system bind to platelet surface and affect platelet function. The aim of this work is to investigate the effect of Lys- and Glu-plasminogen on platelet aggregation.

Methods. Human platelets were isolated from blood of healthy volunteers. Glu-plasminogen was purified from fresh donor plasma and Lys-plasminogen was isolated from Cohn fraction II-III with the use of affinity chromatography. Mini-plasminogen, kringles K1-3 and K4 were obtained by elastase hydrolyses of plasminogen. Fragment K5 was obtained by the limited proteolysis of mini-plasminogen with pepsin. All plasminogen preparations had no spontaneous plasmin activity. Activated platelets were monitored by optic aggregometry.

Results. Exogenous Lys-plasminogen (1.2 μM) but not Glu-plasminogen inhibited thrombin- and collagen-induced aggregation of washed platelets. The level of aggregation in both cases decreased at least in two times. Inhibitory effect of Lys-plasminogen was observed during the second wave of aggregation. -Aminocaproic acid (1mM) abolished inhibitory effect of Lys-plasminogen. Aprotinin made no influence on the inhibitory effect. Plasminogen kringles (K1-3, K4 and K5) abolished inhibitory effect of Lys-plasminogen on platelet aggregation at concentrations 0.12-1.2 μM. Kringle 5 was more effective than K1-3 and K4.

Conclusions. Inhibitory action of Lys-plasminogen is related with the participation of lysine binding sites of proenzyme molecule. Serine protease domain of plasminogen does not take part into realization of the mentioned inhibitory effect. We suggested that Lys-plasminogen interacts with proteins exposed on the platelet surface and leads to the disturbance of protein-protein interaction which is the necessary for efficient platelet aggregation. It is possible that Lys-plasminogen makes influence on platelet signal transduction as it was shown that thrombin-induced washed platelets preincubated with Lys-plasminogen did not reveal the cytoskeleton rearrengement which was observed in case of control activated platelets.

P30

Megakaryocyte Expansion by the Fetal Liver Microenvironment

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Introduction: While there is an increasing interest on the specific microenvironment (the niche) regulating the maintenance and proliferation of hematopoietic stem cells HSC little is known about the microenvironment regulating megakaryopoiesis, in particular the cellular elements involved. The fetal liver is the site of a major expansion of HSC. Hence we hypothesized that the fetal liver could be the source of specific determinants capable of supporting the expansion of megakaryocytes (MK) and their progenitors. The present study focuses on defining the cellular components of the fetal liver that promote the production of MK from HSC.

Methods: Using a combination of cell surface markers, we identified from the mouse fetal liver at day 13.5 that can produce adherent layers and will be referred to as stromal cells. We tested in serum free conditions, with minimal addition of cytokines their capacity to support the production of MK from purified bone marrow HSC (SLAM phenotype).

Results: In the co-cultures with stromal layers derived from CD45-TER119-CD31-CD51+VCAM-1+PDGFRα-cells (V+P-), we observed the production of a large number of megakaryocytes identified by the expression of CD41 and CD42c. In contrast, very few megakaryocytes were produced in co-culture with other fetal liver derived stromal layers. While the proportion of MK produced in absence of stromal layers was similar, their number was dramatically increased (up to 1000 fold) in the co-culture with the V+P-derived stromal layers. The megakaryocytes produced were then sub-cultured under conditions allowing full maturation where they efficiently produced proplatelet extensions.

Conclusions: We propose that the V+P-stromal cell population represents an essential component of the fetal microenvironment supporting the engagement toward the megakaryocytic lineage and, more importantly, the expansion of MK progenitors.
Further characterization of these stromal cells and the factors they produce may guide the development of improved methods for the in vitro production of platelets.

POSTER

P31

Activation of human platelets by amyloid β peptides: essential role of Ca^{2+} and ADP in aggregation and thrombus formation

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Introduction. Alzheimer's disease is associated with the accumulation of amyloid β (Aβ) peptides in the brain. Besides their cytotoxic effect on neurons, Aβ peptides are thought to be responsible for the atherothrombotic complications associated with Alzheimer's disease, which are collectively known as cerebrovascular disease. In this study, we have investigated the effect of Aβ peptides on human platelet signal transduction and function.

Methods. Aβ25-35 which retains the toxicity of the entire peptide was used to stimulate human platelets and platelet activation, adhesion and thrombus formation were evaluated.

Results. We discovered that the 25-35 domain of Aβ peptides induces an increase in platelet intracellular Ca^{2+} which stimulates α- and dense granules secretion and leads to the release of the secondary agonist ADP. Released ADP acts in an autocrine manner as a stimulant for critical signaling pathways leading to the activation of platelets, which include the activation of the protein kinases Syk, protein kinase C, Akt, and mitogen-activated protein kinases. Ca^{2+}-dependent release of ADP is also responsible for the activation of the small GTPase Rap1b and the fibrinogen receptor integrin αIIbβ3, which leads to increased platelet aggregation and increased thrombus formation in human whole blood.

Conclusion. Our discoveries complement existing understanding of cerebrovascular dementia and suggest that Aβ peptides can induce vascular complications of Alzheimer's disease by stimulating ADP release by platelets in a Ca^{2+}-dependent manner, which leads to undesired blood coagulation and thrombus formation in brain microvasculature.

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Inhibition of MRP4 down regulates platelet activation and prevents pre-clinical arterial thrombosis

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Introduction: Any mechanism involved either on platelet location of cyclic nucleotides or ADP may interfere with platelet activity. MRP4, a transporter of cyclic nucleotides and of ADP, is located on dense granule membrane in platelets and is a good candidate to control these mechanisms.

Methods: The total knock-out MPR4-/-mouse model, originally derived in the laboratory of J. Schuetz (St. Jude Hosp, TN, USA) on a FVB background, was compared to FVB wild-type (WT) mice. *In vitro* tests included transmission electron microscopy (MET), whole blood platelet adhesion in flow conditions onto a fibrillar collagen matrix, washed platelet aggregation and CD62-P expression, and cAMP location. *In vivo* tests performed were bleeding time (tail cut) and carotid arterial thrombosis (15% FeCl3 patch).

Results: MET showed no major difference in platelet structure between WT and MRP4-/-. MRP4-/- platelet aggregates on collagen under flow were reduced in number and size compared to WT. MRP4-/- platelet aggregation was impaired in the presence of low agonist concentrations (50µM PAR4-ap, 2µM ADP), but not with high concentrations, known to induce an activation poorly dependent of secreted ADP (100 µM PAR4-ap, 10 µM ADP). In line, CD62-P expression on MRP4-/- platelets was delayed compared to WT (PAR4-ap). Finally, despite an equivalent total cAMP level, the maximum cAMP release from MRP4-/- dense granule was halved, suggesting an altered location. ADP location and PKA signaling are being investigated.
In vivo, the platelet function defect resulted in a prolonged bleeding time in MRP4-/- mice vs WT, with a greater volume of elapsed blood. Conversely, MRP4-/- were protected against arterial thrombosis, experiments showing a significant longer time to occlusion compared to WT.

Conclusion: These results showing an impaired platelet function in vivo and in vitro in MRP4-/- mice support previous studies, and suggest MRP4 as a new target for an antiplatelet agent.

POSTER

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Phosphorothioate-modified bacterial DNA activates platelets and promotes thrombosis through integrin $\alpha_{IIb}\beta_3$

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Background: In addition to their role in hemostasis and thrombosis, platelets contribute to protect the host against bacterial infection. Platelets express major receptors of the innate immune system. They express Toll-like receptor 9 (TLR9), responsible for selective recognition of unmethylated CpG-bearing bacterial DNA by immune cells. However, whether platelets recognize and respond to bacterial DNA is currently unknown.

Methods: We incubated human or mouse platelets with different phosphorothioate-modified oligodeoxynucleotides bearing unmethylated CpG motifs (PT-CpG ODN), PT-non-CpG ODN, or with non-modified CpG-ODN. PT-CpG ODN are commonly used as bacterial DNA mimetics that activate TLR9. DNA phosphorothioation is another natural feature of DNA from diverse bacteria. We performed platelet aggregometry, flow cytometric binding and platelet activation assays, signal transduction analyses, and blood clotting tests. Thrombus formation was also analyzed by intravital microscopy in mouse microcirculation upon intravenous injection of ODN.

Results: We found that PT-ODN potently activate and cause aggregation of human and mouse platelets. Non-modified CpG ODN had no effect. Platelets deficient for TLR9 or MyD88 responded normally to any PT-CpG-ODN tested, excluding a role for the TLR pathway in these platelet responses. PT-ODN binds to platelet surface, leading to fibrinogen binding to $\alpha_{IIb}\beta_3$ and granule release. Subsequently, PT-ODN induces Syk phosphorylation and accelerates clot retraction via activated $\alpha_{IIb}\beta_3$ outside-in signaling. PT-ODN binds less efficiently to Glanzmann's thrombasthenia platelets expressing very low levels of $\alpha_{IIb}\beta_3$ than to normal platelets, resulting in impaired PT-ODN-induced granule release. Furthermore, clotting time was significantly shortened in the presence of PT-ODN. In vivo, PT-ODN interacts with platelets adhered to injured endothelia and facilitate thrombus growth.

Conclusion: Phosphorothioate-bearing bacterial DNA activates platelets and promotes thrombosis via $\alpha_{IIb}\beta_3$, independently of TLR9. Platelets could thus be endowed with an important role in host defense through their ability to discriminate self from pathogenic DNA.

POSTER

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Collagen can selectively trigger a platelet secretory phenotype via glycoprotein VI

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Introduction: Platelets are most commonly known for their central role in hemostasis and thrombosis but platelets are also central actors of other processes including inflammation, angiogenesis, and wound healing.
Accumulating evidence indicates that these "non classical" functions of platelets do not necessarily rely on their well-known ability to form thrombi upon activation. This suggests the existence of non-thrombotic alternative states of platelets activation.

Aims: We investigated the possibility of alternative states of platelet activation uncoupled from their procoagulant and/or prothrombotic activities.

Methods: Human washed platelets were stimulated by different doses of collagen and thrombin and we analyzed the morphological and functional markers of platelet activation: morphology, aggregation, P-selectin, activation of glycoprotein IIb/IIIa and phosphatidylserine surface expression, procoagulant activity, secretion of soluble granular content and calcium signalling.

Results: We show that collagen at low dose (0.25 µg/mL) selectively triggers a platelet secretory phenotype characterized by the release of dense- and alpha granule-derived soluble factors without causing any of the other major platelet changes that usually accompany thrombus formation. Using a blocking antibody to glycoprotein VI (GPVI), we further show that this response is mediated by GPVI.

Conclusion: Our results show that platelet activation goes beyond the mechanisms leading to thrombus formation and also includes alternative platelet phenotypes that might contribute to their thrombus-independent functions.

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Reversal of the Effect of Aspirin by Heparin

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Background: Aspirin and heparin are used together in surgery to reduce the risk of thrombosis. We have previously shown that, in fully aspirinated patients, unfractionated heparin (UH) increases the responsiveness of platelets to agonists including arachidonic acid (AA) and ADP. This effect occurs immediately after heparin infusion, is transient (aspirin inhibition returns within a few hours without further treatment), and was associated with a rise of the 12-lipoxygenase (12-LOX) AA metabolite, 12HETE, but not of the cyco-oxygenase-1 (COX-1) metabolite, thromboxane A2. Here we investigated possible mechanisms of this transient rise in platelet response after heparin in patients undergoing open cardiac bypass surgery (CABG).

Methods: Blood samples were collected from 21 CABG patients immediately before and 3 minutes after receiving 10,000U UH. Aggregation was measured in PRP in response to AA ($2x10^{-3}$M) and ADP ($3.2x10^{-6}$M), with and without inhibitors of COX-1 (aspirin; $3x10^{-4}$M), the thromboxane receptor (SQ 29548; $1x10^{-6}$M), 12-LOX (esculetin; $2x10^{-5}$M and $5x10^{-5}$M) and platelet activating factor (PAF; WEB2086; $3.5x10^{-5}$M).

Results: All patients showed a significant rise in platelet aggregation in response to AA (~3-fold; p=0.0005) and ADP (1.3fold; p=0.0055). The increases to AA were partially inhibited by additional aspirin and SQ 29548 (~30%; p<0.02), unaffected by the PAF-antagonist, but completely blocked by $5x10^{-5}$M Esculetin. The increased response to ADP was only inhibited by esculetin. Addition of heparin to PRP showed that the increase in aggregation in response to ADP could be replicated *in vitro*, however the rise in response to AA was only seen *in vivo*. Mixing experiments showed that the effect of heparin on the AA response was seen in the patients' platelets rather than in their plasma.

Conclusions: Platelets become responsive to AA after heparin administration despite effective Aspirin inhibition. This is mediated primarily by metabolites of AA generated through the 12-lipoxygenase pathway.

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Regulation of platelets collagen receptor α2β1 integrin and the possibility of intermediate affinity

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Background: Collagen-binding integrins (α1β1, α2β1, α10β1 and α11β1) form a class of I domain-containing integrin. They differ structurally from leucocyte integrins ($\alpha_M\beta_2$, $\alpha_L\beta_2$, $\alpha_X\beta_2$, and $\alpha_D\beta_2$) by the presence of a small helix of about 5 amino acids named the c-helix. Integrin α2β1 is a surface-expressed heterodimer, important in adhesion of platelets to damaged blood vessel wall, in the normal function of endothelium, and in the migration and development of vascular smooth muscle cells. Molecular dynamic simulations showed that both αL and αM I domains can adopt 3 conformations where the β6-α7 loop moves successively between three ratchet positions, from closed to intermediate, and then to open. In a different study, two functional active conformations of α2β1 were identified on the platelet surface using a conformation-sensitive antibody.

Aim: To explore the collagen binding activity of the human and rat collagen-binding integrins, to investigate possible intermediate affinity states.

Methods: We designed a series of single and double amino acid substitutions in helix 7 and the c-helix. Site-directed mutagenesis was carried out in the I domain expressing vectors. Collagen II and III triple-helical Toolkit were used as adhesive substrates in solid-phase binding assays.

Results: The data showed that α2 I domain is the only collagen binding I domain that can adopt an intermediate affinity.

Conclusions: The structure of a mutant that adopts intermediate affinity is equivalent to a closed conformation, as found in the crystal structure of the wild-type I domain, but with some differences around the MIDAS that must be responsible for its enhanced binding activity.

POSTER

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Endurance training normalizes pro-inflammatory aspects of platelet function in (formerly) sedentary females and also affects platelet proteome

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Introduction: Low cardiorespiratory fitness represents an important risk factor for the development of atherosclerosis. Recent findings show that (activated) platelets play a key role in atherogenesis. Interestingly, endurance training (ET) performed with (formerly) sedentary volunteers was shown to modulate platelet function to some extent. However, it is currently unclear how these effects relate to platelet function observed in volunteers of average or superior fitness.

Methods: Platelet function, platelet proteome and cardiorespiratory fitness were analyzed in healthy, young, non-smoking women during follicular phase. Sedentary volunteers were studied before and after ET (running 3x / week, 2 menstrual cycles). Additionally, volunteers of average fitness (AF) and endurance athletes (EA) were studied at one occasion. Cardiorespiratory fitness indicated by maximal oxygen consumption (VO_2max) was quantified by an incremental treadmill exercise test; platelet activation state and platelet reactivity (surface expression of CD62P and CD40L, formation of platelet leukocyte aggregates (PLA), intraplatelet ROS formation) were assessed by flow cytometry, platelet proteome by 2D-DIGE.

Results: Overall, there was a negative correlation between VO_2max and basal expression of platelet CD62P as well as agonist-induced expression of CD62P, CD40L and ROS formation ($p<0.01$ each). Agonist-induced expression of CD62P/CD40L and ROS-formation were significantly decreased after ET (before vs after ET) and thereby aligned with the respective values of the AF and EA group. While the number of PLA did not differ between groups, data obtained from a subset of volunteers indicate that the observed modulation of platelet function is associated with significant changes in platelet proteome (i.e., myosin regulatory light chain 2, phosphoglucomutase, integrin alpha-IIb).

Conclusion: A sedentary lifestyle favors a pro-inflammatory platelet phenotype. ET of moderate intensity and duration normalizes platelet function, but high levels of cardiorespiratory fitness show no further benefit for platelet function.

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P38

Properties of the membrane invagination pore of the megakaryocyte demarcation membrane system

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Introduction: The surface-connected demarcation membrane system (DMS) of the megakaryocyte (MK) serves as a membrane reservoir for platelet formation. In this study we have evaluated the physical properties of the connection between the extracellular environment and the DMS using electron microscopy and live cell imaging.

Methods: Freshly isolated femoral or tibial marrow rodent MKs were exposed to impermeant extracellular fluorescent indicators (HPTS, Oregon green 488 BAPTA-1 (OGB1), FITC-dextrans between 4000 and $2x10^6$ daltons (Da), and quantum dots) and imaged by confocal microscopy. For electron microscopy (EM), intact marrow or dispersed marrow cells were fixed and processed for standard high resolution scanning EM (FEGSEM or Verios XHR SEM) or serial block face EM (Gatan 3-view).

Results: The lumen of the DMS within live megakaryocytes was rapidly stained by small molecular weight indicators (HPTS,OGB1) and dextrans up to 110 kDa while larger dextrans were excluded. From dynamic light scattering measurements of the dextrans and reported hydrated diameters of quantum dots we estimate that the DMS rejects molecules or particles with a diameter of $\geq\approx$12.5 nm. In SEM studies we resolved membrane invagination pores (MIPs), the majority of which were constant in size across a range of MKs of varying cellular diameter. Furthermore, constrictions at the entrance of the DMS were observed at high resolution within serial block face EM.

Conclusion: Imaging of small molecular weight dyes by confocal fluorescence microscopy provides a straightforward approach to quantify the DMS in living MKs. This approach, in combination with fluorescent indicators of different size and SEM studies, provides evidence for a discrete entry pore that can reject large adhesion molecules (eg. fibrinogen) from the DMS. Current work is assessing whether similar pores exist on the surface of the platelet OCS and whether MIPs are regulated during thrombopoiesis and platelet activation.

POSTER

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GPVI clustering in platelets imaged by super resolution microscopy

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Introduction: GPVI is the main signalling receptor for collagen and is expressed on platelets as a monomer and dimer. Once bound to collagen, GPVI signals through the ITAM of FcR , triggering formation of the LAT-based signalosome and platelet activation. However, accurate localization of signalling components by light microscopy is difficult due to platelet size and constraints imposed by the wavelength of light. Super resolution microscopy combined with specific antibodies against GPVI has enabled visualization of GPVI clusters at unprecedented resolution. Direct stochastic optical reconstruction microscopy (dSTORM) permits visualization of single molecules at resolutions of ~20nm and allows quantitative comparisons between experimental platelet treatments. We used dSTORM to visualize and quantitatively compare GPVI clustering in platelets spread on collagen.

Methods: GPVI was localized using pan-GPVI and GPVI dimer-specific antibodies and dSTORM in human fixed platelets spread for 30min on fibrous collagen or cross-linked collagen-related peptide (CRP). Cluster analysis was used to quantitate GPVI clustering on these agonists.

Results: GPVI clusters are highly organized along fibrous collagen and contain dimeric GPVI. In contrast, GPVI clusters are distributed over the platelet surface on CRP. There are twice as many receptors in clusters formed on collagen than those formed on CRP and the receptors are closer together (nearest neighbour distance in clusters: ~7nm and 11nm respectively). Sub-maximal concentrations of NEM induced shedding of GPVI and, as expected, reduced the number of GPVI molecules in a cluster.

Conclusions: dSTORM is a powerful technique for visualizing platelet proteins and allows quantitative comparisons. GPVI clusters to a greater extent on fibrous collagen than on CRP and these changes can be detected with cluster analysis algorithms. We aim to extend this work to 2-colour imaging to build up a picture of the receptors and signalling components at the platelet surface.

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The Chaperone Protein Hsp47: A Novel Platelet Collagen Receptor that Contributes to Thrombosis and Haemostasis

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Introduction: Heat Shock Protein 47 (Hsp47) is a collagen binding protein that is normally localised within the secretory system of collagen producing cells, functioning as a chaperone for procollagen. However, studies have shown they can be present on surface of some cells. We have previously demonstrated that platelets contains Hsp47 and surface levels increased upon platelet activation. Since platelet function is dependent on tethering to collagen exposed in the arterial wall upon injury, using selective inhibitors and platelet Hsp47 deficient mice we investigated whether Hsp47 functions as platelet receptor for collagen.

Methods: Immunofluorescence and Western blotting was performed on resting human and mouse platelets using specific Hsp47 antibodies. The role of Hsp47 on platelet function *in vitro* was established using platelet aggregation and thrombus formation assays using Hsp47 deficient mouse platelets, and Hsp47 inhibitors on human platelets. The significance of platelet Hsp47 *in vivo* was studied by intravital microscopy to measure thrombosis using laser injury model.

Results: The presence of Hsp47 on platelet surface and megakaryocytes was confirmed by immunofluorescence and western blotting. An inhibitory polyclonal antibody against Hsp47 attenuated human platelet aggregation, and similarly Hsp47 deficient mouse platelets showed reduced platelet aggregation. Thrombus formation *in vivo* using laser injury model in platelet Hsp47 deficient mouse, resulted in a 40% reduction in thrombus size compared to controls. The contribution of Hsp47 was further explored *ex vivo*, when whole blood from platelet Hsp47 deficient mouse was perfused over collagen and observed approximately 50% reduction in thrombus size. Similar levels of inhibition of thrombus formation was observed using human blood flowed over collagen under arterial flow conditions with Hsp47 inhibitors in the presence and absence of $\alpha IIb\beta 3$ antagonists.

Conclusions: Hsp47 on the platelet surface functions as an adhesion receptor for extracellular collagens that are exposed at sites of blood vessel injury.

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Cdc42-dependent F-actin organization is critical for demarcation membrane system structuration and proplatelet emission in megakaryocytes

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Introduction: Release of platelets into the blood stream results from the maturation of megakaryocytes (MKs), which involves structuration of the internal demarcation membrane system (DMS). Megakaryopoiesis is driven by an intense remodeling of membranes and cytoskeleton prior to extension of proplatelets (PPTs) through bone marrow sinusoids. F-actin dynamics is recognized as the driving force of endomembrane trafficking in various cell types. However, its implication in DMS formation has never been thoroughly assessed.

Methods: By using complementary models (murine primary MKs and human megakaryoblasts) and 3D confocal imaging, we unraveled a functional relationship between F-actin and DMS structuration.

Results: Interestingly, microtubules were not involved in this process. The Rac1 and Cdc42 GTPases were reported to participate to PPTs elongation by acting on microtubules. We found that Cdc42 was also involved in earlier steps by driving actin-dependent DMS structuration and partitioning of nuclei and actin/DMS territories. FRET demonstrated that active Cdc42 was associated with endomembranes dynamics and culminated at sites of strong F-actin polymerization from which PPTs were protruding.

Conclusion: Our data shed new light on the molecular mechanisms of terminal DMS maturation by providing a functional link between Cdc42 and actin dynamics that is mandatory for proper PPTs and platelets production.

P42

Effect of pravastat in the number of circulating platelets in rats treated or not with lipopolyssaccharide

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Introduction: The septic responses include thrombocytopenia and alteration of platelets activation. Statins, have been successfully used in the treatment of inflammatory diseases. In the present study we decided to investigate the effect of pravastatin in platelets of experimental sepsis induced by LPS.

Methods: Wistars rats were treated with saline or pravastatin (20 mg/kg, gavage once daily for 7 days) (Ethical Committee 3290-1). On the sixth day, the rats in both groups received saline or LPS (1 mg / kg, i.p.) and after 48h the arterial blood was collected. Plasmatic TNF-α and thrombopoietin concentrations were measured by ELISA. The number of megakaryocytes was determined by histology. Platelet aggregation was induced by ADP (1-10 mM).

Results: Platelet aggregation induced by ADP was significantly reduced by this statin. Increased TNF-α concentration in LPS-treated rats was reduced by pre-treatment with pravastatin. LPS reduced 6.8 fold the number of circulating platelets, which was accompanied by increased numbers of megakaryocytes and reduced thrombopoietin concentration. Pre-treatment with pravastatin restored the values of thrombopoietin and megakaryocytes, but did not prevent the drop in circulating platelets. ADP-induced platelet aggregation was inhibited by LPS and restored by pravastatin.

Conclusion: Therefore, our results show that administration of pravastatin in healthy animals, in addition to lead thrombocytopenia, it promotes an increase in TNF-a levels, which could result in tissue injury in long-term. Pravastatin restores platelet aggregation and improves the inflammatory condition of LPS-injected rats. However, this statin does not prevent sharp drop in the number of circulating platelets, an important marker for evaluating the severity of sepsis.

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Characterising cell-type interactions following platelet activation in a novel whole blood assay

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Introduction: Platelet-platelet interactions are central to the processes of haemostasis, while platelet-neutrophil and platelet-monocyte interactions have been linked to a range of cardiovascular disease states. Study of these interactions is technically challenging, particularly in animal or small volume clinical samples. Here we couple our miniaturised whole blood stimulation assay with flow cytometric imaging (ImagestreamX, Amnis) to visualise such interactions.

Methods: In brief, blood was collected C57bl6 mice or the antecubital vein of healthy volunteers into hirudin. Aliquots of blood (35µl) were then stimulated in wells of half-area 96-well plates, with or without agonist (collagen, PAR-4 activating peptide, or U46619) and mixed (37°C, 5min). At the end of this period blood was incubated with fluorochrome-conjugated antibodies. Samples were then analysed using the ImagestreamX providing flow cytometric size and fluorescence measurements and images (x60 objective) simultaneously captured of antigen-positive events.

Results: Stimulation of whole blood with agonists caused concentration-dependent reductions in the population of single platelets that were associated with the identifiable appearance of a range of physiological phenomena including platelet micro-aggregates, platelet aggregates, and platelet-leukocyte interactions Furthermore, p-selectin and active-confirmation GPIIb/IIIa could be observed at the interface between platelets, and cells.

Conclusion: This approach, using un-lysed whole blood, easily permits this visualisation and characterisation of cell-type interactions using low volume human or animal samples. Importantly, the low volume permits the simultaneous testing of a wide range of stimulatory conditions. Furthermore, utilisation of a broader panel of antibodies and protein or lipid dyes will further expand the achievable detail and information.

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Validation of novel miniaturised whole blood aggregation assay using adhesion molecule knockout mice

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Introduction: Current haemostatic assays are less suitable for use with mice as they require relatively large volumes of blood. In response, we have developed a miniaturised approach to whole blood aggregometry with quantification by flow cytometric analysis. The whole blood nature of this assay incorporates not only platelet-platelet but also platelet-leukocyte interactions. We therefore sought to validate our assay in the adhesion molecule knockout mice, JAM-A and ICAM-1.

Methods: In brief, whole blood (35 l) anticoagulated with hirudin was stimulated under mixing with increasing concentrations of collagen, PAR4 agonist (AYPGKF amide) or the thromboxane mimetic U46619. Single platelet counts were then determined by flow cytometry and platelet loss, normalised to vehicle stimulation, calculated for comparison.

Results: Compared to wild-type mice, JAM-A KO mice displayed a trend to increased platelet loss in response to collagen and PAR4 stimulation. This trend was absent in response to U46619. In contrast, ICAM-1 KO mice displayed almost identical platelet reactivity to wild type mice when stimulated by collagen or PAR4 amide. However, a significant but surmountable inhibition to U46619 was observed.

Conclusion: JAM-A, expressed on platelets, has recently been shown to suppress thrombosis via action on GPIIb/IIIa outside-in signalling and would concur with the observed trend to increased platelet loss in response to the collagen and PAR-4 agonist. ICAM-1, expressed in blood on leukocytes and not platelets, is known to interact with CD18; expression of which on platelets has been shown to be increased following platelet activation. As such, decreased loss of single platelets following stimulation with sub-maximal concentrations of U46619 may reflect a decrease in platelet-leukocyte interaction; potentially reflecting inflammation mediated interactions. Whilst preliminary, these results support the use of this assay as a viable screening tool for the phenotyping of genetically modified mice.

P45

Cellular distribution of Tissue Factor within platelet-leukocyte aggregates in human whole blood

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Introduction: Activated platelets may adhere to leukocytes forming circulating aggregates (PLA) which could express Tissue Factor (TF), the main activator of coagulation cascade. Both leukocytes and platelets can be a source of circulating TF but no data are available on the localization of TF on platelet or leukocyte in PLA.

Aims: In this study we evaluate the potential for the Amnis FlowSight imaging flow cytometer to discriminate the cellular distribution of TF within PLA.

Methods: Platelet-, leukocyte- and PLA-associated TF was assessed by imaging flow cytometry. 100µL of whole blood, collected from healthy volunteers, were stimulated with 10µM ADP and incubated with saturating concentration of the following mouse anti human monoclonal antibodies: CD45-APCH7 (to identify leukocyte populations), CD61- PerCp (to identify platelet) and TF-FITC. Hoechst stain was performed in order to visualize nuclei.

Results: Monocytes, lymphocytes and granulocytes were discriminated according to a physical parameter (side scatter) and CD45 positivity. PLA within these populations were identified by CD61 positivity. The percentage of monocyte-platelet aggregates was 42.4% within monocyte population, that of lymphocyte-platelet aggregates was 3.2% within lymphocyte population and the percentage of granulocytes-platelet aggregates account for the 28% of the granulocyte population. TF positive aggregates were 38% within the monocyte population and 27% in the granulocyte one. Single cell analysis, performed on high resolution images of each cell in flow, indicates that TF expression within aggregates formed upon ADP stimulation was localized exclusively on platelets. Of note, not all platelets adhering to monocytes or granulocytes were TF positive.

Conclusions: Imaging flow cytometry allowed us to discriminate the cellular distribution of TF indicating that TF within PLA, upon ADP stimulation, is expressed only by platelets. This approach could be therefore of interest to investigate the role of platelet associated TF in pathological conditions.

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Metabolomic response of human platelets to collagen receptor engagement

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Introduction: Platelet interactions with collagen receptor agonists, including CRP-XL, are largely well characterised at a cellular level however little is known about the implications of collagen receptor engagement on the platelet lipidome. In the past lipids were seen as biomolecules with little use other than providing structural integrity, though increasingly evidence is emerging for lipids fulfilling essential roles in varied cellular signaling pathways, e.g. phospholipase C/Calcium release pathway. This can be through individual lipid species acting as direct signaling molecules, or by flux in lipid levels throughout the cell. In recent years, the advancement in mass spectrometry tools such as LC-MS/MS has enabled great progress in the field of lipidomics, however this approach is yet to be broadly applied to platelet studies. Our aim is to apply a mass spectrometry method to investigating the effects of collagen receptor agonists including CRP-XL, GFOGER-XL and fibrillar type I collagen, on the global platelet lipidome. The use of specific direct agonists known to stimulate different receptor axis allows clarification of individual signaling pathways and the response of lipid species.

Methods: Total lipid extraction by the Folch method (2:1 Chloroform/Methanol) followed by LC/MS analysis on Xevo G2 qtof spectrometer.

Conclusions: All collagenous ligands used perturbed the platelet lipidome to some extent, appearing to cause flux in varied species from the common lipid classes, glycerophospholipids, glycerolipids and fatty acids. This includes platelet stimulation via Integrin $\alpha 2\beta 1$ which may suggest a greater role for this receptor in platelet activation, having previously been considered predominantly as an adhesion receptor.

P47

CD45 negative megakaryocytes are in the mouse embryo

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Introduction: Recent studies have established the importance of platelets and megakaryocytes (MKs), from which platelets are generated, not only in coagulation processes, but in others such as in wound healing, cancer, vasculogenesis, and tissue regeneration and innate immunity responses. MKs, a rare population in the adult bone marrow, are rather abundant cells in the liver during mouse embryo development and have recently been implicated in the establishment of the hepatoblast progenitors in the fetal liver (FL) through cell to cell contacts and involving also soluble factors, in particular VEGF.

Methods: Mice were mated overnight, and the day the vaginal plug was detected was considered day 0.5 of gestation (E0.5). Pregnant mice were sacrificed, the embryos were obtained and the desired tissues were obtained from them. By using flow cytometry, cell purification, semisolid and liquid cultures, and genetic analyses, we have addressed the cell differentiation stages of Mks in the mouse embryo liver and the topographic location of MKs in the E11.5 liver.

Results: The cells of the MK lineage present in the developing mouse embryo liver represent 5-9% of total cells in this organ at E11.5. The phenotypic traits of embryo-derived megakaryocytes, from E9.5-E11.5 identify them as $CD41^{++}CD9^{++}CD42c^{+}$. At difference to BM and E15.5 liver MKs, E9.5/E11.5 MKs are $CD45^{-}CD49f^{++}KDR^{+}$. FL MKs display several immature traits in comparison with those present concurrently in the yolk sac, suggesting in-situ differentiation. The staining with CD45, CD41 and CD42c allows the identification of cell subsets containing MK precursors, showing that both $CD45^{-}CD41^{+}$ and $CD45^{+}CD41^{+}$ produce MKs that develop proplatelets in vitro through independent pathways.

Conclusions: The data presented here show that in FL $CD45^{-}CD41^{+}$ and $CD45^{+}CD41^{+}$ progenitors are able to directly produce proplatelet-bearing MKs, that remain CD45- until E15.5, a feature that is not found in later embryos nor in those from BM.

P48

Altered expression of serum, plasma and platelet microRNAs in type 2 diabetes mellitus

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Introduction: Increased level of platelet activation has been thoroughly studied in type 2 diabetes mellitus (DM2) that results in atherosclerosis and thrombotic complications. MicroRNAs (miRNAs) are the key regulators of numerous physiological processes, but these non-coding RNAs also play a critical role in the development of human diseases. In this study, we aimed to measure and compare the expression of several miRNAs in three different types of samples of DM2 patients and healthy controls.

Methods: Samples were simultaneously drawn from DM subjects and normal volunteers. Platelet miRNAs were isolated from leukocyte-depleted platelets, while circulating miRNAs were obtained from serum and plasma samples. For leukocyte depletion we used CD45+ magnetic beads, and the level of leukocyte contamination was examined by RT-qPCR analyzing CD45 mRNA content. The profiling of some selected miRNAs (miR223-3p, miR126-3p, miR26b-5p, miR10a-5p, miR424-5p, miR24-3p) was performed by UPL-based RT-qPCR (Roche). The reference gene RNU43 was used for the normalization of miRNA expression results. Beside other clinical parameters, platelet P-selectin was analyzed by flow cytometry, and von Willebrand factor antigen (vWFAg) levels were measured by immunoturbidimetry.

Results: All miRNAs were substantially expressed in platelets as well as in the serum and plasma except for miR10a-5p displaying a very low quantity extracellularly. There was a significantly decreased expression of circulating miR223-3p, miR126-3p and miR26b-5p in both serum and plasma samples of DM2 subjects ($p<0.001$) compared to normal counterparts. Enhanced platelet P-selectin positivity (7.1±2.5% vs. 1.1±0.4%) in diabetics showed a negative association with decreased miR26b-5p expression. In addition, miR424-5p and miR24-3p were also downregulated in DM2, which correlated with the elevated vWFAg levels in this disease.

Conclusions: Abnormal platelet and endothelial function in such metabolic disorders may be explained by the analysis of selected target miRNAs that probably contribute to the altered expression of some proteins.

P49

Sphingosine 1-phosphate produced by sphingosine kinase 2 intrinsically controls platelet homeostasis and arterial thrombosis in mice

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Introduction: Platelets are known to play a crucial role in hemostasis. Their activation at sites of vascular injury prevents life threatening blood loss and promotes wound healing. Sphingosine kinases 1 and 2 (Sphk) catalyze the conversion of sphingosine (Sph) to the bioactive metabolite sphingosine 1-phosphate (S1P). Although platelets express both Sphk1 and 2 and are able to secrete S1P upon activation, little is known about a potential intrinsic effect of S1P production on platelets.

Objective: To investigate the role of Sphk1 and 2 in the regulation of platelet function.

Methods and Results: We found a 100-fold reduction in intracellular S1P levels in platelets derived from Sphk2$^{-/-}$ compared to Sphk1$^{-/-}$ or wildtype (WT) mice, as analyzed by mass spectrometry. Moreover Sphk2$^{-/-}$ platelets failed to secrete S1P upon stimulation. Interestingly, the release of arachidonic acid (AA) metabolites in response to stimulation by thrombin was significantly reduced in Sphk2$^{-/-}$ as compared to WT platelets. In addition, blood from Sphk2 deficient mice showed decreased aggregation after protease-activated receptor 4 (PAR4) and ADP stimulation in vitro, as assessed by whole blood impedance aggregometry. Finally, Sphk2 knock-out mice showed significantly reduced arterial thrombus formation in response to ferric chloride injury compared to WT mice.

Conclusions: We demonstrate here that Sphk2 is the major isoform responsible for the generation of S1P in platelets, and plays a pivotal intrinsic role in the control of platelet activation. Correspondingly, Sphk2-deficient mice are protected from arterial thrombosis after vascular injury. Targeting this pathway therefore could present a new strategy to prevent or treat thrombosis.

P50

Reduction of beta3^{tyr785} chain phosphorylation of alphaIIb beta3 integrin takes part on the inhibitory effect of statins in stimulated-platelet adhesion to fibrinogen of high fat-fed rats

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Besides reduction of serum cholesterol, statins have pleiotropic effects. There are works showing that statins inhibits platelet aggregation through reduction of calcium mobilization and TXA2 synthesis, however, the studies about their effect on platelet adhesion are scarce. Therefore, in the present work we decided to study the effects *in vitro* of pravastatin and rosuvastatin on platelet adhesion to fibrinogen of high fat-fed rats. The present study was approved by the Committee for Ethics in Animal Research (State University of Campinas – UNICAMP, Brazil). Rats received high fat diet for 16 weeks and after that the blood was collected. Washed platelet adhesion was evaluated using fibrinogen-coated 96-well microtiter plates. Platelets were maintained in the plate for 30 min, in absence or presence of statins. After that, the plate was washed and adherent platelets were incubated with the acid phosphatase substrate for 1h. The plate was read by a microplate reader set at 405nm. Pravastatin and rosuvastatin inhibited 30% non-stimulated-platelet adhesion, but nearly abolished ADP- or thrombin-stimulated platelet adhesion. The effect of these statins on stimulated-platelet adhesion was accompanied by significant reduction of beta3^{tyr785} chain phosphorylation but not at Tyr773 residue. Reduction of beta3 phosphorylation was not observed on non-stimulated-platelet. Therefore, the present results show that inhibition of stimulated-platelet adhesion to fibrinogen of high fat-fed rats is mediated by reduction of Tyr785 residue phosphorylation of beta3 chain of alphaIIb beta3 integrin.

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POSTER

P51

The inhibitory effects of nitric oxide and prostaglandin I_2 on platelet aggregation are greatly enhanced by blockade of $P2Y_{12}$ receptors

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Introduction: Whilst circulating, platelet quiescence is sustained by constant exposure to two endothelial derived inhibitors, nitric oxide (NO) and prostacyclin (PGI_2). Upon activation, platelets produce secondary mediators that override this inhibitory tone, notably ADP. $P2Y_{12}$ receptor antagonists block pro-aggregatory ADP and are prescribed with aspirin as dual antiplatelet therapy (DAPT). $P2Y_{12}$ receptor antagonists also potentiate the inhibitory effects of PGI_2 and enhance the antiplatelet potency of NO. Thus, the *in vivo* synergy between NO and PGI_2 may amplify the anti-thrombotic effects of $P2Y_{12}$ receptor antagonists, suggesting that for individual patients the level of endothelial function may be key in determining anti-platelet drug efficacy. Here, we characterise the interplay on platelet function of PGI_2, NO and $P2Y_{12}$ blockade.

Methods: Eight healthy male volunteers received aspirin (75mg) and prasugrel (10mg) for 7 days. Platelet responses to TRAP-6 (25 M) or collagen (4 and 10 g/ml) in the presence of PGI_2 (1nM), NO (100nM), PGI_2+NO or vehicle were assessed by LTA and lumi-aggregometry. Experiments were replicated *in vitro* utilising aspirin (30 M) and prasugrel active metabolite (PAM; 6 M, 3 M and 1.5 M) representing maximal and partial $P2Y_{12}$ blockade.

Results: *Ex vivo,* prior to DAPT, PGI_2, NO, or PGI_2+NO had little effect upon platelet aggregation. TRAP-6 (25 M): vehicle, 74±3%; PGI_2+NO, 66±3%. Following DAPT, PGI_2 or NO alone caused minor inhibition. TRAP-6: vehicle, 57±4%; PGI_2, 47±6%; NO 49±6%. Conversely, the combination of PGI_2+NO produced strong inhibitory effects, 19±6% ($p<0.05$).
In vitro, with maximal $P2Y_{12}$ inhibition (PAM, 6 M) aggregation to TRAP-6 (25 M) was reduced: vehicle, 56±5; PGI_2, 30±12; NO, 46±7; PGI_2+NO, 15±8. However, with partial inhibition (PAM, 3 M) only PGI_2+NO inhibited aggregation: vehicle, 67±6; PGI_2, 56±9; NO, 62±5; PGI_2 + NO, 33±15 ($p<0.05$).

Conclusion: Assessment of endothelial function in patients undergoing *ex vivo* platelet function testing may help bridge the disconnect between results of platelet testing and thrombotic patient outcomes.

POSTER

P52

The increases reactive oxygen species generation by lipopolysaccharide in vivo in rat platelet, via NADPH-oxydase activation, involving PKC, sGC and PKG

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Introduction: The septic responses increased production of reactive oxygen species (ROS) can be reproduced by LPS in experimental animals. The PKC has been involved the ROS production by NADPH oxidase. Works reported that the NO-cGMP-PKG may participate in the activation of this enzyme. In the present study, we aimed investigate the role of PKC in modulating of ROS formation by NADPH oxidase from LPS-treated rats.

Methods: Wistar rats were injected i.p. with saline or LPS (1 mg/kg), and at 48h thereafter arterial blood was collected (Ethical Committee-2097-1). ROS production in platelets was measured by flow cytometry DCFH-DA (5 μM). The activation of NADPH-oxidase was analyzed through immunobloting.

Results: The ROS production in platelets from LPS-treated rats was 4.5-fold higher ($P<0.05$) than in saline-injected rats. The increase of ROS generation in platelets of LPS-treated rats was accompanied by the increased phosphorylation of p47-phox subunit of NADPH oxidase. Incubation of platelets with PKC inhibitor GF109203X (10μM) decreased about 26% the release of ROS of LPS-treated rats. Furthermore, GF109203X reduced about 2 times the phosphorylation of Ser 345 residue of p47 phox in LPS-treated rats. The release of ROS in platelets of LPS-treated rats decreased in about 35% after incubation with inhibitor of guanylyl cyclase ODQ (10 μM) and inhibitor of PKG Rp-8-Br (25 μM).

Conclusion: PKC is involved in the activation of NADPH oxidase and consequently on the increase of ROS formation in platelets from rats injected with LPS 48h afterwards. The sGC, PKG and PKC modulate the release of ROS in platelets of LPS-treated rats.

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POSTER

P53

In-vitro imaging of platelet fibrin clot formation and lysis in flow condition

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Introduction: Modulating thrombus formation and lysis is a major concern in the search of efficient and safe treatments fighting thrombosis. Numerous methodologies are available to analyze clot formation but much less allow studying clot lysis particularly in flow conditions.

Aim: We propose here an original methodology to evaluate clot formation and lysis in whole blood under arterial flow rate.

Methods: A thrombogenic surface is produced by coating flow chambers (Vena8 Fluo+, Cellix) with a mix of fibrillar collagen and tissue factor (Innovin, Dade). After adding a fluorescent antibody to platelets and Alexafluor 647-conjugated human fibrinogen to citrated blood, the blood is recalcified and platelet adhesion and clot formation are monitored with an inverted microscope (Zeiss) using a 20X objective. Data are analyzed using the "Zen" software, the first step consisting in following thrombus formation and growth at a flow rate of 1500 s^{-1}. The duration of this step is set so that the produced clots do not significantly modify the flow. The surface occupied by the thrombi is estimated by taking successive pictures along the channel. In a second step and after a brief wash, fibrinolysis is induced by flowing rt-PA-supplemented citrated blood and analyzed by focusing on a large fibrin(ogen)-rich clot during 8 minutes. Real time changes in mean fluorescence of fibrin(ogen)-rich clots are measured during the flow. In addition, specific fibrin labelling can be performed at the end of the run by using a specific monoclonal antibody.

Results and Conclusions: A therapeutic concentration of rt-PA (20µg.mL^{-1}) induces a very significant decrease in clot fluorescence (over 50%) indicating that the method allows monitoring of fibrinolysis in flow conditions. This in vitro global method opens the possibility for simultaneous analysis of the capacity of a given blood sample to form platelet-fibrin thrombi and the susceptibility of these thrombi to lysis.

P54

Platelet integrin α6β1 promotes metastatic dissemination

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Introduction: Cancer progression is a multistep process, where tumor cells acquire properties that enable their survival, proliferation and invasion, finally leading to dissemination and establishment of metastasis, which represents the major cause of cancer-related death. Platelets encounter tumor cells during their transit through the bloodstream and at the distant sites of metastases. They are known to support metastasis by adhering to circulating tumor cells and providing shielding from the immune system responses, and actively induce a motile and invasive phenotype in cancer cells. Clinical data suggest a reduced risk of metastasis under treatment with aspirin administered at an anti-platelet dose, further supporting a role of platelets in metastasis.
Several platelet adhesion receptors were described to participate in metastatic dissemination, but the role of β_1 integrins, including $\alpha_6\beta_1$, the main receptor for laminins, is unknown.

Methods/Results: We examined the role of platelet integrin $\alpha_6\beta_1$ in tumor cell metastasis by using a knock-out strategy restricted to megakaryocyte lineage. In this study, using experimental and spontaneous metastasis models of melanoma and breast cancer (injection of B16F10 and E0771 cells in syngeneic hosts) we demonstrate that platelet integrin $\alpha_6\beta_1$ highly favors and accelerates the metastatic process. Antibody-mediated blockade of integrin $\alpha_6\beta_1$ in human platelets inhibited their adhesion to melanoma and breast cancer cells. Similar results were observed with murine platelets lacking integrin $\alpha_6\beta_1$, suggesting that the effect of this integrin is to ensure platelet/tumor cell interaction.

Conclusion: In summary, our studies show that platelet integrin $\alpha_6\beta_1$ contributes to metastatic process by mediating communication between the platelets and tumor cells. Platelet integrin $\alpha_6\beta_1$ could represent a novel therapeutic target to prevent metastasis.

P55

Role of cytoskeleton in platelet activation

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Introduction: Platelet activation and adhesion followed by aggregation, is the first response to block an injured blood vessel, a process involving major morphological changes. The drastic changes in morphology are coupled with characteristic modifications in the cytoskeletal organization that involves interplay of actin and microtubules. Our aim is to have a mechanistic understanding of cytoskeletal remodeling during platelet activation.

Methods:
We have devised strategies to characterize organization of microtubules in resting platelets and their structural reorganization during activation. With electron tomography we obtain detailed structure of microtubule organization in platelet marginal band at different stages of activation. We can also access dynamics of the process by performing high-resolution live cell microscopy on primary platelets from transgenic GFP-tubulin mice. In combination with mathematical modeling and computer simulations, we can discern forces exerted on and by the cytoskeletal structures.

Results: With 3D-tomographic reconstruction of platelets, we have obtained precise quantitative data describing structure of the marginal band in terms of number, length and polarity of microtubules. The total length of polymerized microtubules in resting platelets is found to be 101.8 10.9 αm. We are also able to observe coiling of marginal band during activation in live platelets and reproduce this behavior in simulations.

Conclusions: The amount of polymerized tubulin in resting platelets seems to be regulated which could control its mechanical properties. With the live imaging, we find coiling of marginal band to be reversible. In addition, simulations suggest that actin contraction is enough to cause coiling of marginal band.

For a deeper understanding of these mechanisms, we will analyze the role of system components, for instance, various molecular motors, cross linkers, etc. Information regarding morphological transitions and knowledge of their driving mechanisms will have high impact on our understanding of platelet physiology in healthy and disease states.

P56

Comparison of the effects of elective bare metal versus drug-eluting percutaneous coronary stents in stable angina patients

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Introduction: Drug-eluting stents (DES) have been introduced for coronary stenting especially when a second implantation is needed after bare metal stenting (BMS), or in the presence of other susceptible conditions for thrombotic complications such as diabetes mellitus. There is a growing body of evidence with DES as a reliable tool; however, direct effects of these stents on platelet and endothelial function have not been fully investigated.

Methods: Forty-nine stable angina patients were studied who underwent elective coronary stent implantation: 28 with BMS, 21 received DES. Plasma samples were obtained i) prior to stenting, ii) after procedure within 12 hours, and iii) after a 1-month period with dual anti-platelet medication. In these time points, activation of platelets was monitored via sCD40L levels with surface P-selectin by flow cytometry and that of leukocytes by analyzing sICAM-1, respectively. Levels of sVCAM-1, sE-selectin, sP-selectin and von Willebrand factor antigen (vWFAg) were measured to evaluate the degree of endothelial injury caused by the procedure. Response to aspirin/clopidogrel treatment was also tested.

Results: Stenting produced increasing levels of soluble fibrin monomers during the study period in both groups but caused no stent thrombosis until the clinical end point of 6 months. Elevation in concentrations of early (sVCAM-1, vWFAg) and late (sE-selectin, sP-selectin) activation markers of coronary endothelium was more prominent after BMS especially in those with abnormal cTnT values compared to patients with DES. Increased platelet activation was sustained regardless of stent type. Notably, there were 6 BMS patients displaying restenosis with significantly increased sCD40L (645±358 vs. 355±177 pg/mL) and sICAM-1 levels (228±55 vs. 186±51 ng/mL) after 1 month compared to those without complications. However, impaired response to anti-platelet drugs did not affect significantly the alteration in these markers.

Conclusions: DES implantation resulted in an attenuated elevation of different cellular activation markers compared to BMS.

P57

Coagulation factors X and Xa bind to procoagulant platelets in a hysteresis manner enabling their retention in thrombi despite rapid flows

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Introduction: All major reactions of blood coagulation proceed on the negatively charged phospholipid membranes, usually provided by activated platelets, which increases their rates by orders of magnitude. Systematic kinetic studies for the binding of a major coagulation enzyme e factor Xa and its predecessor factor X to either phospholipids or activated platelet subpopulations are lacking.

Methods: The binding of coagulation factors to negatively-charged phospholipid membranes was studied with an Accuri C6 cytometer (BD Biosciences, USA) and an Axio Observer Z1 confocal microscope (Carl Zeiss, Jena, Germany) using fluorescein-labeled FX or FXa. Fluorescence intensity was converted to a mean number of molecules per platelet or per phospholipid vesicle using special calibration beads. Computer simulations of fXa binding to a single platelet or to platelet aggregate were carried out using the Virtual Cell environment (http://vcell.org).

Results: The fXa and fX preferentially binds to the membranes of PS-positive platelets (Apparent k_d= 623±110 nM for fX and k_d = 65±22 nM for fXa). Data from dissociation experiments shown the concentration of the bound factor Xa decreases to a value several times higher than the equilibrium one, after diluting 20 times with buffer A with calcium.

Thus the curve of association-dissociation fX/fXa have a hysteresis -like behavior. This phenomenon is observed on the activated platelets and on the phospholipid vesicles, therefore, it can't be explained by the presence of a specific proteins receptor. By mathematical modeling , we have shown that this behavior can only be explained by trimerization of factors.

This mathematical model predicted that the phenomenon of oligomerization may be important for to keep factors on the surface thrombi. Our experiments confirmed the prediction model. Thus, we can assume that the trimerization factors ensures their fixing to the surface of a thrombus under the flow.

P58

A novel $P2Y_{12}$ P-selectin test as an alternative approach to assessment of platelet function in relation to clinical outcomes in a coronary artery disease population

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Introduction: A novel $P2Y_{12}$ P-selectin test detects high on-treatment platelet reactivity (HTPR) in patients with acute coronary syndrome (ACS), and this is associated with subsequent cardiovascular events. Here we evaluated P-selectin testing in stable post-ACS patients and compared the results with other platelet function tests (PFT). We also assessed the association of HTPR with adverse clinical outcomes during and beyond dual antiplatelet therapy (DAPT).

Methods: 94 patients were recruited at one month following ACS. All were established on DAPT for 1 month prior to testing (clopidogrel and aspirin n=66, prasugrel and aspirin n=28) and continued for a further 11 months. Platelet reactivity was assessed using the dual $P2Y_{12}$/Aspirin P-selectin kit (Platelet Solutions Ltd), light transmission aggregometry (LTA), VerifyNow, Biocytex and Multiplate. Adverse ischaemic events were identified by retrospective case-note review. The follow up period was from 19-24 months.

Results: The $P2Y_{12}$ P-selectin test showed variable inhibition of platelet reactivity, similar to all other PFTs. Practically all low responders were among patients receiving clopidogrel. During the 12 months of DAPT, only 1 event occurred and this patient showed a poor response to both aspirin and clopidogrel on all PFTs. Between 12-24 months, when patients were on aspirin monotherapy, a further 6 events occurred. All of these patients were originally taking clopidogrel. They had significantly higher mean platelet reactivity while on DAPT assessed by the $P2Y_{12}$ P-selectin test and all other PFTs apart from LTA.

Conclusions: The small number of events in our cohort may reflect the improved management of ACS. Patients with HTPR demonstrated increased risk of future ischaemic events both during and after treatment with DAPT. The increase in events after cessation of clopidogrel raises questions about whether a 12-month period of treatment is sufficient for these patients.

P59

Use of VASP assays to assess platelet response to $P2Y_{12}$ antagonists in ACS patients: interesting findings in relation to bleeding

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Introduction: Flow cytometric measurement of VASP phosphorylation (VASP-P) is the most specific method of testing the platelet response to $P2Y_{12}$ antagonists. Low response is associated with re-thrombosis and high response may be associated with bleeding.

Aims: To compare platelet reactivity in ACS patients using two approaches, VASPFix and Biocytex.

Methods: The study included 196 patients who were on antiplatelet therapy for at least 1 month prior to testing (clopidogrel and aspirin n=121, prasugrel and aspirin n=75). VASP-P was measured using the established Biocytex assay and also a simplified and more sensitive assay using VASPFix, a solution that lyses, captures and fluorescently labels VASP-P in one step. In 47 patients bleeding symptoms were evaluated using the BARC definition.

Results: Platelet reactivity indices (PRI) for both assays were variable in different patients and there was only moderate agreement in identifying poor responders to $P2Y_{12}$ antagonists (kappa=0.512, CI 0.394 to 0.630) with higher number of non-responders for Biocytex (n=82/196) as compared to VASPFix (n=50/196). This is in accordance with our previously published evidence that Biocytex underestimates platelet inhibition with $P2Y_{12}$ antagonist through stimulation of the G_i-coupled EP3 receptor. The majority of non-responders according to both tests were among patients taking clopidogrel (77 and 45, respectively).
Bleeding symptoms were seen in 13/47 patients, with BARC severity score 2 for 3/47, score 1 for 10/47 and score 0 for 34/47 (no bleeding). PRI values were inversely related to bleeding score using both VASPFix and Biocytex. More patients receiving prasugrel had bleeding events (4/18 and 3/18 with BARC score 1 and 2, respectively) than patients receiving clopidogrel (6/29 with BARC score 1).

Conclusions: VASPFix provides a simplified method for measuring VASP-P and shows fairly good agreement with the Biocytex assay, although PRI values for Biocytex were higher.

P60

Changes in clot properties due to platelet concentrate storage (in vitro study)

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Introduction. Despite platelets properties varies during storage, transfused platelets are included in blood clot in the same way as well as native. We assume that after transfusion the prevalence of platelets with changed activity lead to worse quality of blood clot in vivo. The aim was the in vitro study of platelet-dependent clot properties that are formed from some stored platelet concentrates (PCs).

Methods. Twenty-six PCs in autologous plasma and 24 PCs in platelet additive solution SSP+ (MacoPharma, France) (70 vol.%) were analized by thromboelastography with and without platelets activation, and by standard aggregometry. The testing were carried out at the day of proceeding, after 24 hours, and at 3rd and 5th days of storage. We used Trima Accel (Terumo BCT, USA) for the proceeding of platelets apheresis.

Results. We found that clot demonstrated gradual reduction of elasticity and deformability starting from second day to fifth day in stored PCs suspended in autologous plasma. From the third storage day platelets lost their meaning for clot properties.
The platelets apheresis with next re-suspending in SSP+ solution leads to the depression for platelets aggregability. Actived platelets had no impact to clot properties during full storage time. Total decline of clot quality including low elasticity and impaired deformability were found starting from 3rd storage day compared to the day of proceeding.

Conclusions. We assume that such properties of clot as both elasticity and deformability are forming in PCs at the day of proceeding. Further clot changes observed in PCs does not depend directly from platelets aggregability because clot forming are under other influences. The last are determined mainly by the coagulation what was no included in this study. Also obtained results confirms the 5th-days storage as a benefit independent from PCs proceeding method.

P61

Understanding the impact of storage and pathogen inactivation treatment on platelet concentrates at the protein level using mass spectrometry analyses

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Introduction: Riboflavin-UVB (Mirasol) pathogen reduction treatment (PRT) involves addition of riboflavin to the platelet concentrates (PCs) followed by UV-B light irradiation. This modifies guanine residues within nucleic acid of pathogens and prevents their replication. However, the use of PRT on PCs is still under question because it also leads to a certain level of platelet activation, characterized by spontaneously aggregation and degranulation during storage, which compromises platelet responsive capacity to stimuli. This study investigates the impact of storage and PRT on PCs at the protein level using mass spectrometry analyses.

Methods: Untreated or PRT PCs were collected at different time points through storage (Day 2, 5 and 8) and whole platelet lysates were subject to mass spectrometry (MS) analysis using one-dimensional gel electrophoresis and liquid chromatography (LC) coupled to tandem MS. Peptide and protein identification were analyzed using Sequest (XCorr Only) and X! Tandem. Bioinformatics and statistical analysis was used to semi-quantitatively analyse the kinetics of identified proteins through storage and after PRT.

Results: Overall, we have identified a total of ~ 2000 proteins in our platelet lysates. We have analysed the kinetics of identified proteins through storage and upon PRT in PCs, relative to the proteome of fresh platelets, and we have categorised them in different subgroups based on the parameters “storage time” and/or “PRT”. Granule and degranulation-related proteins and kinases were generally affected. We have performed Gene ontology (GO) analysis and were able to link proteomic changes due to these variables with already described platelet functions due to storage or PRT, such as hyperactivation, reduced spreading or altered microaggregation.

Conclusions: This study has enabled us to better understand the alterations of the platelet proteome and how those changes are linked to platelet dysfunction previously described in PCs during storage or after PRT.

P62

ABCA1 deficiency decreases platelet reactivity by reducing positive feedback loop mechanisms and changing the pattern of lipid mediator production

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POSTER

Introduction – The ATP-binding cassette transporter ABCA1 is required for the conversion of apoA-1 to high density lipoprotein (HDL) and its defect causes Tangier disease, a rare disorder characterized by an absence of HDL and accumulation of cholesterol in peripheral tissues. The role of ABCA1 in platelet functions remains unclear and there are controversies concerning its implication in processes as fundamental as platelet granule maturation, phosphatidylserine exposure and control of platelet membrane lipid composition.

Methods and results – We studied the impact of ABCA1 deficiency on platelet responses in a mouse model and a Tangier patient. We show that platelets from ABCA1-deficient mice have a slight increase in size and exhibit aggregation defects in response to low concentrations of thrombin and collagen. They have a reduction in positive feedback loop mechanisms including dense granule secretion and thromboxane A2 (TXA2) production, and elevated levels of end-products of docosahexaenoic acid lipoxygenation known to inhibit platelet aggregation. Similar functional defects were observed in platelets from a Tangier patient with decreased production of TXA2 and elevated levels of inhibitory platelet docosahexaenoic acid derivatives. Conversely, ABCA1 and HDL deficiency did not affect platelet cholesterol and major phospholipid composition,αgranule morphology or calcium-induced phosphatidylserine exposure.

Conclusions – Absence of ABCA1 and low HDL level do not change significantly platelet major lipid composition but induce the emergence of protective mechanisms reducing platelet reactivity by decreasing positive feedback loops efficiency and changing the profile of eicosanoid and other bioactive lipids mediators.

P63

Reactive oxygen species enhance generation of subpopulations of procoagulant platelets

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Introduction: We have observed that strongly activated platelets will fragment into microparticles exposing procoagulant phosphatidylserine (PS). However, only a distinct subpopulation of smaller platelets, also characterized by downregulation of the fibrinogen receptor GPIIb/IIIa from its active state, will expose PS. In this study we investigated the influence of reactive oxygen species (ROS) on platelet activation and the formation of platelet subpopulations.

Methods: Platelets were activated with high dose collagen-related peptide (CRP) or CRP+PAR1+PAR4-activating peptides in the presence of ROS donors (H_2O_2 or 2,3-dimetoxy-1,4-naphthoquinone; DMNQ) or ROS scavengers (N-acetyl-cysteine; NAC and Superoxide dismutase; SOD). Platelets were analyzed by flow cytometry for size (defined as normal, smaller or fragments), expression of PS (binding of annexin V), and active GPIIb/IIIa (binding of PAC-1).

Results: Platelets activated by CRP formed subpopulations of smaller platelets and fragments (approx. 10+5% of total population), both highly positive for PS and with low grade fibrinogen receptor activation (PAC-1 binding). With CRP+PAR-APs, this increased to approx. 35+15%. The activation-induced fragmentation of platelets increased in the presence of ROS donors, while especially fragmentation decreased in the presence of ROS scavengers. In general, PS exposure in the different subpopulations increased upon activation in the presence of ROS donors and decreased in the presence of scavengers. GPIIb/IIIa activation was reduced by ROS donors in platelet subpopulations while it varied depending on the ROS scavenger used.

Conclusions: ROS donors increase fragmentation and exposure of PS by activated platelets, while ROS inhibitors were able to reduce fragmentation and exposure of PS, indicating that the processes are oxidation-dependent. However, the regulation of GPIIb/IIIa activation seems to be more complex. Further investigations will reveal more details about the role of ROS in the generation of platelet subpopulations.

P64

The relationship between the metabolites of the dietary flavonoid quercetin and haemostasis, thrombosis and platelet function

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Introduction: Flavonoids are plant secondary metabolites that have beneficial effects on cardiovascular health. One notable flavonoid due to its anti-platelet properties is quercetin (3,3′,4′,5,7-pentahydroxyflavone), found in foods such as apples and onions. Upon consumption, quercetin is metabolized in the small intestine and liver, yielding methylated, sulphated and glucuronidated metabolites; their anti-platelet effects and relevance at concentrations achieved *in-vivo* is not understood. A long-term goal of my PhD is to develop an understanding of the effects of these main metabolites, both individually and in combination, on platelet function and signaling, and building a model of this, allowing *in-silico* experimentation to predict the identities of metabolites that function *in vivo*.

Methods: Platelet aggregation was performed by light transmission aggregometry. Fibrinogen-binding and P-Selectin exposure were measured by flow cytometry.

Results: Consistent with previous studies, quercetin inhibited collagen-stimulated (5 g/ml) platelet aggregation in a dose-dependent manner; significant inhibition was observed as low as 3μM (lower than previously reported). Above 6μM, aggregation was inhibited >90%. *In-vivo*, numerous agonists are present at lower concentrations than are typically used in *in-vitro* assays. Concentrations of agonists that on their own are subthreshold for aggregation, when added together may synergise and stimulate substantial functional responses. The ability and potency of quercetin to inhibit fibrinogen-binding to platelets stimulated by this synergistic response between thrombin and CRP (Collagen-Related Peptide) was therefore explored. Quercetin inhibited significantly fibrinogen-binding at concentrations as low as 500nM. This was associated with substantial inhibition of fibrinogen binding and a-granule secretion. Fibrinogen-binding and P-Selectin exposure stimulated by 0.1μg/ml and 1μg/ml CRP alone was also significantly inhibited by quercetin, at concentrations as low as 500nM.

Conclusions: Quercetin inhibits platelet function at concentrations lower concentrations than previously recognised and within potential physiological concentrations. Development of this work will allow elucidation and modelling of the effects of quercetin's main metabolites.

WC: 298

POSTER

P65

Evolution of the α- and β-tubulin repertoire and post-translational modifications during platelet biogenesis

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Introduction: Platelet biogenesis from fully mature megakaryocytes involves profound reorganization of microtubules (MTs) into a sub membranous coil supporting platelets typical discoid shape. Formation of this unique MT cytoskeleton could be generated by the incorporation of specialized isotypes of α- or β-tubulins or by post-translational modifications (PTMs) of tubulin, but nothing is known about their identity and time course during platelet biogenesis.

Aim: Our goal was to establish an exhaustive list of platelet isotypes and their evolution and to perform a comprehensive analysis of tubulin-modifying enzymes involved in PTMs during platelet formation.

Methods: MTs purified from human platelets were analyzed by 2D electrophoresis followed by nanoLC-MS/MS. Megakaryocytes (MKs) were differentiated in culture from CD34+ cells for 12 days. Evolution of the tubulin isotypes was studied using a combination of semi-quantitative RT-PCR and proteomic analyses. In parallel, PTMs were followed by Q-PCR analysis of the modifying enzymes.

Results: All the known α- and β- isotypes were detected in purified platelet MTs by MS analysis, with an enrichment in α4A-and α8-tubulins and an expected prevalence of β1-tubulin. These data were confirmed in semi-quantitative RT-PCR of platelet RNA. Transcripts for 4A-, α8- and β1-isotypes were not detected during the first days (0-7) of MK differentiation, contrary to β6-, β5- and α1c- transcripts, but appeared during the late stages of maturation (from days 7-12). Concerning PTMs, progressive increases in transcripts for glutamylation (TTLL4, 5,-7), glycylation (TTLL3), and deacylation (HDAC6) enzymes were observed along MK differentiation indicating possible regulation of MTs organization by these PTMs.

Conclusion: This study provides important information on the evolution of the tubulin isotype repertoire and PTMs leading to platelet formation. This should allow to identify specific αβ combinations and PTM-regulated recruitment of partners improving our knowledge on the mechanisms of MT assembly during platelet biogenesis.

P66

Elucidating SDF-1α-mediated regulation of platelet function

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Introduction: The chemokine, stromal cell-derived factor (SDF)-1α, has been previously shown to induce platelet activation. SDF-1α was presumed to signal exclusively via the CXCR4 receptor. However, subsequent studies identified CXCR7 as a higher affinity receptor for SDF-1α. Both receptors are expressed on the platelet surface, but their physiological significance with respect to thrombosis/haemostasis in response to primary platelet agonists and SDF-1α is poorly understood. The aim of this study was to broaden our mechanistic understanding of SDF-1α-mediated regulation of platelet function, and in particular, to explore potential autocrine/paracrine roles for SDF-1α and the signalling mechanisms facilitating these responses.

Methods: Washed platelets and platelet rich plasma (PRP) from healthy human donors were pre-treated with various inhibitors; Indomethacin (COX1/2), AMD3100 (CXCR4), blocking antibody 11G8 (CXCR7) - used in the presence of IV.3 Fc RIIA blocking antibody. Following stimulation with different agonists (collagen, CRP, SDF-1α), platelet activation was monitored for aggregation, dense granule secretion and ELISAs for thromboxane (TX)A2 and SDF-1α.

Results: Inhibition of CXCR4, but not CXCR7, reduced collagen-induced platelet aggregation, ATP secretion and TXA2 generation implying a possible autocrine/paracrine role for platelet-derived SDF-1α. Subsequent ELISA analysis confirmed a time-dependent release (1-5 min) of SDF-1α with collagen stimulation. SDF-1α could induce platelet aggregation in PRP, but not in washed platelets. However, co- stimulation of washed platelets with SDF-1α and threshold concentrations of collagen elicited enhanced aggregation responses in a dose-dependent manner, which was substantially attenuated by CXCR4 inhibition, while blocking CXCR7 had no apparent effect. Furthermore, this potentiating effect of SDF-1α was TXA2-dependent, which aligns with experiments in PRP demonstrating a key role for TXA2 downstream of integrin $\alpha_{IIb}\beta_3$, following SDF-1α stimulation.

Conclusion: SDF-1a enhances collagen-mediated TXA2 generation and platelet activation by activating CXCR4. In contrast, CXCR7 does not appear to be involved, and its role in platelet function requires further investigation.

POSTER

P67

Mechanism of platelet activation by Fucoidan and Dextran

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Introduction: A diverse range of non-endogenous stimuli, including oxidised proteins and nanoparticles, have been found to induce platelet activation and aggregation via the collagen receptor complex, the GPVI-FcR γ-chain. Fucoidan, a sulphated polysaccharide, is believed to cause activation via the Podoplanin receptor, CLEC-2, whilst the receptor for dextran (a complex branched polysaccharide) remains unidentified. This study aims to further clarify the pathway of activation by fucoidan and dextran, and to describe associated receptors.

Methods: Fucoidan and dextran induced aggregation and activation of human and mouse model platelets was measured using light transmission aggregometry and Western blotting, and direct activation of GPVI and CLEC-2 receptors via an NFAT assay and surface plasmon resonance.

Results: Fucoidan stimulated aggregation of human and mouse platelets predominantly via the CLEC-2 pathway, but also, to a lesser extent, via the GPVI pathway. Further, fucoidan achieved aggregation solely through Syk and Src tyrosine kinases. Interestingly, fucoidan stimulated protein phosphorylation in GPVI/CLEC-2 deficient platelets. In human platelets, dextran-induced aggregation was mediated through a Src kinase pathway, independent of Syk, whereas in mouse platelets, activation was dependent on Src and Syk tyrosine kinases, and also CLEC-2. Neither fucoidan nor dextran stimulated CLEC-2 or GPVI transfected DT40 cells or bound directly to either of the recombinant receptors.

Conclusions: This study demonstrates that fucoidan and dextran have the common ability to activate the CLEC-2 receptor and, to a lesser extent, the GPVI-FcR γ-chain complex in the absence of direct binding to either receptor. While both polysaccharides can activate other, unidentified Src kinase coupled receptors, only dextran causes aggregation via this mechanism (in human platelets). Given the absence of direct binding, it is likely that activation is mediated by alterations in receptor location or interactions of the stimuli with the membrane bilayer.

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P68

Compounds Having Antiplatelet Activity: 3(2*H*) pyridazinone

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Introduction: Enhanced platelet activation plays a key role in the initiation and development atherothrombotic cardiovascular disease and in the prothrombotic complication of other diseases. Many antiplatelet agents such as cyclooxygenase inhibitors, ADP P2Y12 receptor antagonists, GPIIb/IIIa antagonists and phospodiesterase inhibitors have been reported as a standart care for those at high risk. In spite of increasing clinical use and clear benefits, week inhibition of platelet function, slow onset of action and increased bleeding risk upon utilization of these drugs limit success currently available antiplatelet therapy. The search for more potent and safer platelet aggregation inhibitors targeting specific signaling pathways is ongoing and promising approach for prevention and treatment of atherothrombosis.

Methods: We have synthesized and performed biological evaluation of a small set of potential antiplatelet 4,6-disubstituted 3(2*H*)-pyridazinone derivatives containing N -benzyliden-acetohydrazide moiety at position 2. The structures of these compounds were confirmed by IR, ^{1}H NMR, and MS data. Antiaggregatory activities against arachidonic acid-induced platelet aggregation were measured by turbidimetric method of Born.

Results: Synthesized new 18 compounds showed 100% platelet aggregation inhibitor activity in 30-100 μM range against 700μM arachidonic acid-induced platelet aggregation.
Conclusions: These pyridazinone derivatives may possess potential in the desing of more potent compounds for antiplatelet therapy.

POSTER

P69

International Clopidogrel Pharmacogenomics Consortium Genome Wide Association Study Identifies Novel Variants for Clopidogrel Response

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Introduction: Clopidogrel (Plavix) is a commonly used antiplatelet medication in coronary artery disease patients to prevent recurrent thrombotic events. It is a pro-drug that requires activation by CYP2C19, and common loss of function variants of *CYP2C19* (e.g., *CYP2C19**2) are associated with decreased antiplatelet responsiveness phenotype but explained only a marginal part of this trait. Heritability estimates are high and suggest that there are additional genetic determinants of clopidogrel response.

Methods: To identify these variants, the International Clopidogrel Pharmacogenomics Consortium (ICPC) amassed clopidogrel response phenotypes (namely on-treatment ADP-induced platelet reactivity and cardiovascular outcomes) and DNA from more than 6,000 clopidogrel-treated patients. A GWAS (Omni Express with exome coverage) was conducted in 2,721 Caucasian patients.

Results: We observed strong association between on-treatment platelet reactivity phenotypes and the *CYP2C19* locus on chromosome 10 (best SNP rs1926711; $p=6.25x10^{-32}$). After adjustment for the known *CYP2C19**2 loss of function variant (rs4244285; $r^2=0.963$ with rs1926711), there remained residual association just upstream of *CYP2C19* (e.g., rs1998591; $p=1.66x10^{-05}$) suggesting other variants in this region as determinants of clopidogrel response. Other loci nominally associated with platelet response to clopidogrel ($p<10^{-5}$) included rs12651351, intergenic between *FHDC1* and *TRIM2*; rs10505836 in *PLEKHA5*; and rs2295306 in *DHRS1*.

Conclusion: While we conclude that *CYP2C19*2* remains a major genome-wide determinant of clopidogrel response, other loci are likely to exist, but play a lesser role. Replication studies, now in progress, will be required to affirm potentially novel loci for clopidogrel response.

P70

Platelets support direct and indirect binding of plasminogen facilitating local fibrinolysis

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Introduction: The interaction of plasminogen with platelets is not clearly defined but may result from an indirect association with fibrin(ogen). Coated platelets are known to express high levels of phosphatidylserine (PS) on their membrane and show increased affinity for fibrinogen. Here we examine platelet-associated plasminogen and determine its function during lysis of thrombi under flow.

Methods & Results: Endogenous surface plasminogen, detected by flow cytometry, was enhanced on thrombin + convulxin (CVX) stimulated platelets compared to unstimulated platelets (72.3 ± 5.5 % vs. 26.5 ± 6.4 %). Binding of exogenous plasminogen-DyLight 633 (DL633) was augmented by thrombin + CVX stimulation of platelets (89.4 ± 3.1 % vs. unstimulated 11.5 ± 7.4 %) and to a lesser extent by TRAP6 + CVX activation (50.5 ± 16.2 %), suggesting a role for fibrin. Plasminogen-DL633 and PS, detected using FITC-annexin V, were detected on thrombin-collagen stimulated platelets by fluorescent confocal microscopy. Plasminogen was localized in distinct 'caps' on PS-positive platelets. Plasmin activity on the surface of stimulated platelets was analyzed using a fluorogenic substrate ± plasminogen activator (PA). Endogenous plasmin activity detected on thrombin + CVX stimulated platelets was enhanced by addition of PA. Plasminogen binding was examined under flow by perfusing recalcified citrated whole blood (1000 s^{-1}) over slides coated with collagen + tissue factor. Platelets labelled with DIOC6 and/or fluorescently labelled fibrinogen (OG488 or Alexa fluor 647) were incorporated during thrombus formation. Plasminogen-DL633 primarily localized with fibrin(ogen) bound to the platelet surface, with hirudin significantly abrogating the effect. Lysis was visualized by incorporating PAs during thrombus formation.

Conclusions: A functional pool of plasminogen is exposed directly on the surface of platelets exposed to strong agonists. A secondary pool of plasminogen associates indirectly with the platelet surface primarily via fibrin(ogen). It is fibrin-bound plasminogen that predominantly mediates thrombus lysis under flow.

POSTER

P71

Investigation of Platelet microRNA as a marker of Epigenetic Drift

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Introduction: Cardiovascular Disease (CVD) is a name given to a group of disorders of the heart and blood vessels. CVD is the leading cause of mortality in the world and the prevailing cause of death in Ireland. Most CVDs may be prevented by addressing modifiable risk factors some of which include obesity, hypertension and physical inactivity. The involvement of platelet dysfunction in cardiovascular related diseases has become increasingly more recognised. Damaged blood vessels as a consequence of smoking, high blood pressure, obesity and physical inactivity eventually develop plaques which line the blood vessel.
Abnormalities in platelet reactivity can cause them to adhere to the atherosclerotic plaque in an uncontrolled manner participating in the formation of a thrombus.
Platelet reactivity was profiled in Irish subjects (N=100) to determine the relationship between platelet function, platelet count, and various physiological parameters including BMI, Waist circumference, body fat %, visceral fat %, physical activity levels/sedentary lifestyle. A preliminary microRNA profile of fibrinogen activated platelets was performed to profile miRNA in activated vs quiescent platelets.

Methods: Anthropometric Measurements: Weight, body fat % and visceral fat % measured by Bioelectrical Impedance Analysis using the TANITA device. Blood pressure, BMI and waist circumference were calculated.
Whole blood counts: Platelet count, RBC, WBC and haematocrit measured using the Sysmex xs1000-i blood counter.
Platelet function: Adhesion and aggregation were examined using the Impact R cone and plate machine which tests platelet function in whole blood under physiological conditions.
miRNA profile: Platelets from a healthy male were allowed to adhere to a fibrinogen (20ug/ml) coated surface to encourage adhesion and activation. miRNA analysis was performed on platelet RNA using real time PCR.

Results: Subjects of an overweight category (higher BMI, waist circumference, sedentary lifestyle) displayed higher SC and AS (platelet function) values than normal weight.
Majority of miRNA down-regulated (mIR-26a & miR-222) or undetected upon fibrinogen adhesion - platelet specific miRNA

Conclusions: Platelet screen: Increased reactivity of platelets in subjects with increased body fat %, BMI and waist circumference possibly due to production of

unhealthy platelets due to sedentary lifestyle.
miRNA profile: Considerable number of miRNA not detected after activation possibly due to release of platelet microparticles. microRNA profile of platelets is altered upon adhesion to fibrinogen.
Exercise intervention and miRNA profile will be performed on outliers from platelet function to evaluate if exercise can epigenetically (through miRNA biomarkers) reprogram platelets to a healthier state.

GENERAL INFORMATION

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A certificate of attendance will be issued to all registered participants upon request at the Secretariat Desk at the end of the Meeting.

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