19-21 SEPTEMBER 2018
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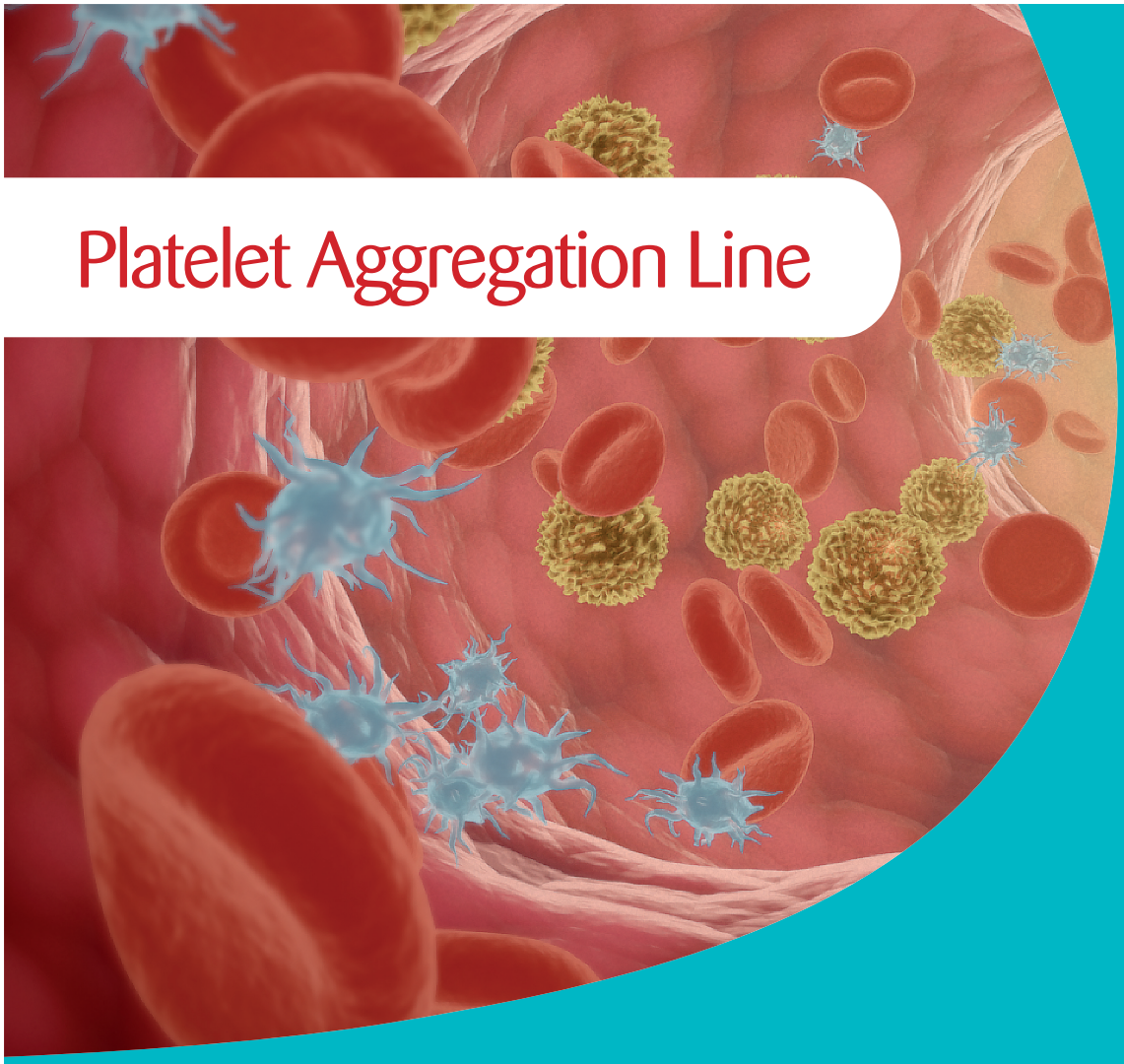

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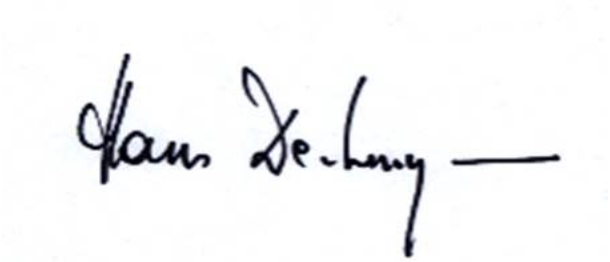
Words of welcome

A warm welcome to the fourth meeting of the European Platelet Network (EUPLAN) in the Concertgebouw in Bruges, a city whose history made it great, resulting in a well-deserved classification as a Unesco World Heritage site. We hope that you will enjoy the program where we did a special effort to highlight platelet functions in and beyond their role in thrombosis and haemostasis. In total the program includes 10 keynote lectures, 24 selected oral communications and over 80 poster presentations. We hope that this fourth edition, as the previous editions in Maastricht, Le Bischenberg and Bad Homburg, will provide the audience with new and challenging insights, generate new ideas and further promote exchanges between our laboratories.

The undertaking of the conference has been made possible by the generous support of our company and institutional sponsors (see further) and of the city of Bruges. We are very grateful for this. Please, visit their displays during the meeting.

We hope that you have an interesting and very pleasant stay in Bruges.



Hans Deckmyn on behalf of the local organizing committee.

EUPLAN scientific committee

EUPLAN 2018 scientific committee

Hans Deckmyn
Karen Vanhoorelbeke
Simon De Meyer
Kathleen Freson
Cécile Oury
François Mullier

EUPLAN international scientific committee

Austria	Ivo Volf
Belgium	Hans Deckmyn
Finland	Riitta Lassila
France	Christian Gachet
	William Vainchenker
Germany	Bernhard Nieswandt
	Peter Presek
Italy	Mauro Torti
	Ilaria Canobbio
Sweden	Tomas Lindahl
Switzerland	Pierre Fontana
	Jean-Luc Reny
The Netherlands	Johan Heemskerk
United Kingdom	Steve Watson

Organized by The Laboratory for Thrombosis Research
www.kuleuven-kulak.be/irf/thrombosis

Event organizer
www.eland.be

General information

Venue
Concertgebouw Brugge
www.concertgebouw.be
Lecture Hall: Kamermuziekzaal
Catering and poster sessions: FORUM 6/7

The city of Bruges www.Brugge.be

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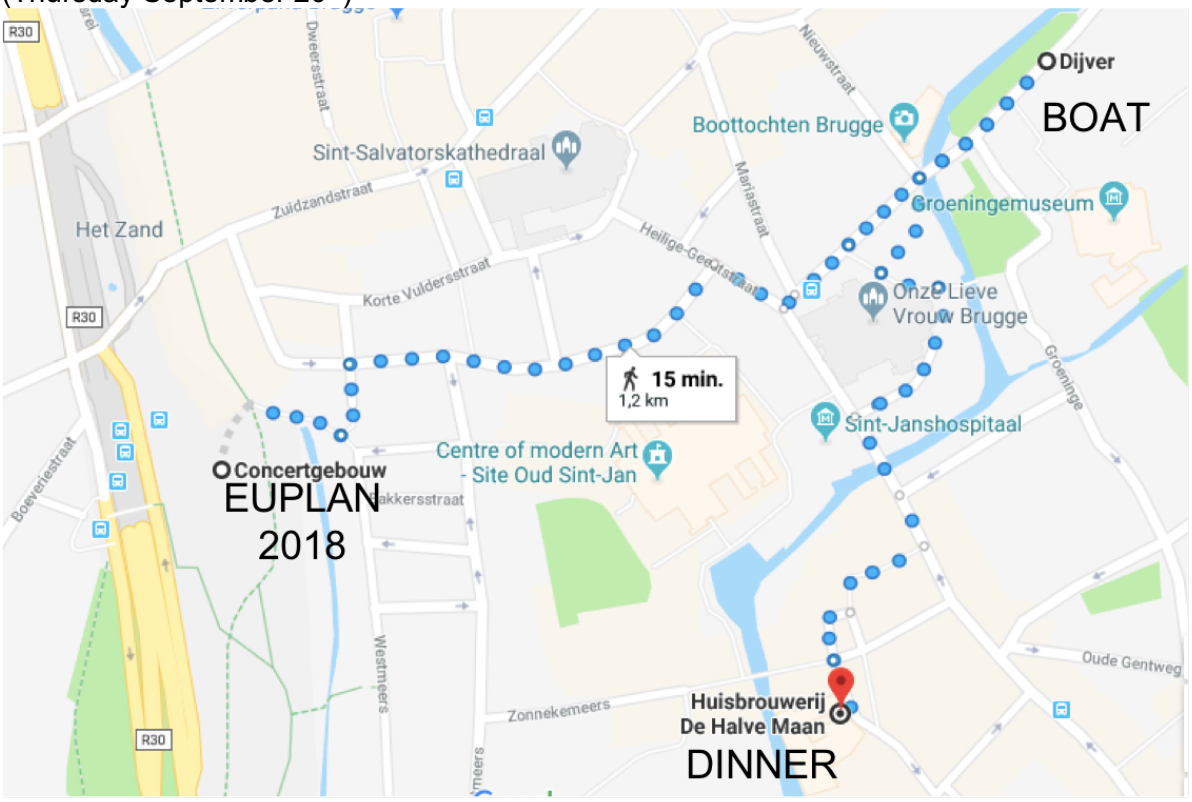
Contact on site (in case of emergency):

- Jasper Van Steenberghe (ELAND) +32 470 83 81 00
- Joni Vanpeteghem (KULAK) +32 494 81 90 85

Directions from EUPLAN venue to city hall (Wednesday September 19th)

Directions from EUPLAN venue to boat departure and Brouwerij De Halve Maan (Thursday September 20th)



Program

Wednesday September 19th

15.30 registration

16.45 Welcome address by Hans Deckmyn

17.00 Session 1: keynote lectures

Chair: Hans Deckmyn and Peter Presek

KL1 The lung is a platelet factory and a reservoir for blood progenitors: new evidence for an old hypothesis
Emma Lefrançais

KL2 Advances in *ex vivo* platelet production
Dominique Baruch

18.30 Opening ceremony in the city hall of Bruges

Thursday September 20th

8.30 Session 2: keynote lectures

Chair: Cécile Oury and Johan Heemskerk

KL3 Duality of platelets in inflammation: how platelets repair neutrophil dependent vascular injury
Benoît Ho-Tin-Noé

KL4 Platelet ITAM receptors: new regulators of inflammation
Julie Rayes

9.30 Session 2: short oral communications

OC1 AMPK-ACC signaling modulates platelet phospholipids content and potentiates platelet function and thrombus formation
Sophie Lepropre

OC2 Oxidized high density lipoprotein promotes pro-thrombotic phenotype by platelet adhesion and coagulation factor modulation
Felipe Simon

OC3 Novel role for adipose triglyceride lipase in thrombogenesis
Madeleine Göritzer

OC4 Platelet lammelipodia formation is not required for thrombus formation and stability
Markus Bender

10.30 coffee break

11.00 Session 3: short oral communications

Chair: Kathleen Freson and Christian Gachet

OC5 Ikaros: a new downstream player of AhR-dependent responses favoring platelet biogenesis by an enrichment of a megakaryocytic precursor
Valentin Do Sacramento

OC6 Mutations in cell-cycle MASTL kinase promotes thrombocytopenia by deregulating actin cytoskeleton dynamics in platelets
Begoña Hurtado

OC7 MGL-1/2 play a key role in the clearance of desialylated platelets through Kupffer cells
Carsten Deppermann

OC8 Deoxyribose-1-phosphate: a platelet derived molecule that stimulates angiogenesis and tissue repair via redox dependent activation of the NFkB transcription factor in endothelial cells
Giordano Pula

OC9 A combination of proteomic approaches identifies a panel of circulating extracellular vesicle proteins related to the risk of suffering cardiovascular disease in obese patients
Maria Barrachina

OC10 A synthesis approach of mouse studies to identify genes and proteins in arterial thrombosis and bleeding
Constance Baaten

13.00 lunch break

14.00 Session 4: keynote lectures

Chair: Simon De Meyer and Ilaria Canobbio

KL5 Understanding the pathways of platelet-mediated immunoregulation
John Semple

KL6 Platelets in inflammation and infection
Waltraud Schrottmaier

15.00 Session 4: short oral communications

OC11 Platelet induced intracellular Ca^{2+} rises in neutrophils
Magdolna Nagy

OC12 A novel P2X1-G-CSF-CCL5 axis controls hemostasis via platelets and neutrophils in intestinal inflammation
Cécile Oury

OC13 Selective inflammatory activation of megakaryocytes and platelets by transgenic expression of IKK2 results in a hyporeactive platelet phenotype
Manuel Salzmann

OC14 Histone release in trauma patients induces the formation of procoagulant, immunomodulatory platelet balloons and microparticles
Paul Vulliamy

16.00 coffee break

16.30 poster session I

18.00 Session 5: keynote lecture

Chair: Tomas Lindahl and Pierre Fontana

KL7 Platelets at the crossroad between cardiovascular and Alzheimer's disease
Ilaria Canobbio

18.30 evening program and dinner (optional)

Friday September 21st

8.30 Session 6: keynote lectures

Chair: Karen Vanhoorelbeke and Steve Watson

KL8 New involvement of bioactive lipids in the dialogue between platelets and metastatic cancer cells
Olivier Peyruchaud

KL9 Platelets and colorectal cancer: how do tumors escape the immune system by educating platelets?
Cécile Oury

9:30 Session 6: short oral communications

OC15 Platelet-stored antibodies potently diminish viral infection in vitro and in vivo
Waltraud Schrottmaier

OC16 Prolonged histone exposure induces a subset of unresponsive platelets
Scarlett Gillespie

OC17 Biophysical characterization of clot retraction in platelet rich plasma of patients with primary anti-phospholipid syndrome
Jolan Harsfalvi

OC18 Structural features of acute ischemic stroke thrombi
Senna Staessens

10.30 coffee break

11.00 poster session II

12.30 lunch break

14.00 Session 7: short oral communications

Chair: Hans Deckmyn and Mauro Torti

OC19 Targeting CXCR7 inhibits thrombotic response through cGMP-cAMP mediated mechanism: implications in coronary artery disease
Madhumita Chatterjee

OC20 Differential effects of PDE3 and-5 on platelet function under hemostatic and inflammatory conditions
Judith Cosemans

OC21 Platelet-specific factor XIII-A: A novel role in the regulation of platelet activation
Jo Mitchell

OC22 Anti-CUB1 or anti-spacer antibodies that increase ADAMTS13 activity act by allosterically enhancing metalloprotease domain function
An-Sofie Schelpe

OC23 Open ADAMTS13 conformation in immune-mediated thrombotic thrombocytopenic purpura is induced by anti-ADAMTS13 autoantibodies and corresponds with an ongoing ADAMTS13 pathology
Elien Roose

OC24 Circulating biomarkers with the ability to predict and monitor therapeutic response in CRPC
Jonas Nilsson

15.30 Session 7: keynote lecture

KL10 Tumor-educated platelet RNA for blood-based liquid biopsies
Myron Best

16.00 closing and future outlook

Keynote lectures

KL1 **Emma Lefrançais**
Institut de Pharmacologie et de Biologie Structurale – CNRS Université de Toulouse, Toulouse, France

The lung is a platelet factory and a reservoir for blood progenitors: new evidence for an old hypothesis

Platelets are critical for haemostasis, thrombosis, and inflammatory responses, but the events that lead to mature platelet production remain incompletely understood. The bone marrow is a major site of platelet production, although there is indirect evidence that the lungs might also contribute to platelet biogenesis. Here, by directly imaging the lung microcirculation in mice, we show that a large number of megakaryocytes circulate through the lungs, where they dynamically release platelets. Megakaryocytes that release platelets in the lungs originate from extrapulmonary sites such as the bone marrow where we observed large megakaryocytes migrating out of the bone marrow space. The contribution of the lungs to platelet biogenesis is substantial, accounting for approximately 50% of total platelet production or 10 million platelets per hour. Furthermore, we identified populations of mature and immature megakaryocytes along with haematopoietic progenitors in the extravascular spaces of the lungs. Under conditions of thrombocytopenia and relative stem cell deficiency in the bone marrow, these progenitors can migrate out of the lungs, repopulate the bone marrow, completely reconstitute blood platelet counts, and contribute to multiple haematopoietic lineages. These results identify the lungs as a primary site of terminal platelet production and an organ with considerable haematopoietic potential.

Bio
Following an engineering degree in cell and molecular biology, Emma Lefrançais received her PhD in 2012 from the University of Toulouse. From 2013 to 2017, Emma moved to the University of California, San Francisco for her postdoctoral fellowship in Mark Looney laboratory where she used advanced imaging approaches to understand lung physiology and innate immunity during pneumonia and hemostasis. She has recently discovered that the lung circulation is an important participant in the production of platelets and that the lung is a site of megakaryocyte residence. She has just obtained a permanent CNRS research position in Jean-Philippe Girard laboratory at the Institute of Pharmacology and Structural Biology in Toulouse where she is studing spatio-temporal characteristics of the immune response by lung real-time 3D microscopy.

KL2 **Dominique Baruch**
INSERM UMR-S 1140, Univ Paris Descartes, Sorbonne Paris Cité, Paris, France

Advances in ex vivo platelet production

Megakaryopoiesis is a complex mechanism leading hematopoietic stem cells (HSC) to produce mature megakaryocytes (MK) characterized by their large size, polyploidy and extended cytoskeleton, the demarcation membrane system which is the source of the future platelet membranes. Thrombopoiesis is the final stage of MK development and results in platelet formation from mature MK. Contrary to the proliferation and maturation of MK precursors, thrombopoiesis does not take place in the bone marrow but rather requires passage of large MK fragments into sinusoid vessels present in bone marrow. These MK fragments are carried by the bloodstream into the venous circulation, where their fragmentation continues to yield proplatelets, indicating that shear forces exerted on the vessel wall are involved in platelet production outside the bone marrow environment.
We will present recent literature to review the different models allowing *ex vivo* platelet formation in dynamic conditions. We will describe our dedicated microfluidic platform, which is based on the strong biochemical interaction between VWF and GPIb at high shear rates and accelerates proplatelet formation and platelet release. We will show its potential in different models, allowing studies at the single cell level as well as at large-scale for collecting platelets and characterizing their *in vitro* and *in vivo* properties. Finally, in keeping with the role of the lung arteries and capillaries as a site of intense platelet production, suggesting a role of oxygen tension, we will present data on the role of oxidative stress and mitochondrial dynamics in platelet formation under normal and pathological conditions.

Bio
Dominique Baruch (MD, PhD) is a senior research director at INSERM (French national institute for medical research) and University Paris Descartes, expert in research in the field of thrombosis and hemostasis. D. Baruch has a long-standing know-how of platelet adhesion and aggregation in real-time under various flow conditions and shear stress. The group has also developed the model of platelet production from megakaryocytes in real-time under flow conditions allowing exposure to high shear rates.

KL3 **Benoit Ho-Tin-Noé**
Inserm Unit 1148 – Laboratory for Vascular Translational Science
Paris Diderot University, Paris, France

Duality of platelets in inflammation: how platelets repair neutrophil dependent vascular injury

Platelets have long been considered primarily through the lens of hemostatic platelet plug formation and thrombosis, who both rely on integrin-mediated platelet aggregation. They are, however, increasingly recognized as major actors of inflammatory reactions and immune responses. Platelets intervene at various stages of inflammatory reactions. They promote edema formation and leukocyte infiltration, and also regulate several effector functions of innate immune cells including degranulation, oxidative activity, phagocytosis, or the formation of neutrophil extracellular traps. Besides these proinflammatory effects, studies based on the combination of mouse models of inflammation and severe thrombocytopenia have shown that platelets continuously secure inflamed vessels and limit collateral damage to the host by preventing bleeding in inflamed organs from the very onset of inflammation. This protective action of platelets was reported in many different models of inflammation including, immune-complex mediated dermatitis and glomerulonephritis, endotoxin- and bacteria-induced acute lung injury, myocardial and cerebral ischemia-reperfusion injury, models of viral infection and solid tumors. Strikingly, in several of these models, prevention of inflammatory bleeding was maintained in mice compromised for platelet aggregation. Although the underlying mechanisms of this recently appreciated hemostatic function of platelets remain to be fully elucidated, there is evidence that it involves mobilization of individual platelets to the vessel wall where they interact with leukocytes and appear to seal gaps that arise between endothelial cells at sites of neutrophil infiltration.

Bio
B. Ho-Tin-Noé received his PhD from Paris Diderot University in 2005 for his work on the mechanisms and consequences of plasminogen activation by vascular cells and neurons. He then joined the Wagner Lab (HMS, Boston) where he worked for 3 years as a postdoctoral fellow. There, he started studying the role of platelets in the maintenance of vascular integrity in inflamed organs and tumors. He was recruited in 2009 as a tenured researcher by INSERM to work in the Laboratory for Vascular Translational Science in Paris. His research projects focus on 1) the prevention of bleeding by platelets in inflamed organs, 2) the contribution of platelet/leukocyte interactions to the pathogenesis of acute ischemic stroke, and 3) the regulation of the tumor vascular and inflammatory microenvironment by platelets.

KL4 **Julie Rayes**
Institute of Cardiovascular Sciences, University of Birmingham, Birmingham, UK

Platelet ITAM receptors: new regulators of inflammation

Platelets are best known for their role in hemostasis and thrombosis. They also play crucial roles in host defense, inflammation and tissue repair. Many of these roles are regulated by the immune-like receptors GPVI and CLEC-2, which signal through an immunoreceptor-tyrosine-based-activation-motif (ITAM). GPVI is activated by collagen in the sub-endothelial matrix and by fibrin and fibrinogen in the thrombus. CLEC-2 is activated by the transmembrane protein podoplanin which is found outside of the vasculature and is up-regulated in inflammation and cancer. We have recently shown that CLEC-2 and GPVI regulate immune cell recruitment and activation in septic mice. CLEC-2 deletion inhibits macrophage recruitment to the site of infection and exacerbates the cytokine storm during sepsis. Despite sharing a common signaling pathway leading to platelet activation, CLEC-2 and GPVI play opposite roles in inflammation. CLEC-2-podoplanin axis is anti-inflammatory and GPVI is largely pro-inflammatory. My talk will address our recent findings on the role of CLEC-2 and GPVI in inflammation, infection and tissue repair. I will also present and discuss recent evidences on the beneficial role of impaired vascular integrity-mediated bleeding in tissue repair.

Bio
Julie Rayes completed her PhD in 2007 at the University of Denis Diderot-Paris VII in France working on the role of von Willebrand factor and ADAMTS13 mutations in thrombotic and bleeding disorders. During her post-doctoral fellowships, she was interested in the crosstalk between hemostatic and coagulation proteins and the regulation of innate and adaptive immunity. Since 2014, she joined the Birmingham platelet group at the University of Birmingham focusing on the non-hemostatic functions of platelets, in particular in inflammation, infection and tissue repair.

KL5 **John Semple**
Division of Haematology and Transfusion Medicine, Lund University, Lund, Sweden

Understanding the pathways of platelet-mediated immunoregulation

Platelets are the primary cellular mediators of hemostasis and this function intimately acquaints them with a variety of inflammatory processes. For example, when aggregated on damaged vessel walls, activated platelets can secrete and express many pro- and anti-inflammatory molecules that attract and capture circulating leukocytes and focus them on inflamed tissue. Platelets secrete several chemokines and cytokines that affect local innate immune responses by, for example, attracting neutrophils to sites of inflammation. In addition, platelets may directly regulate adaptive humoral immune responses via their expression and secretion of CD40/CD40L molecules. These studies have also suggested that platelets represent an important linkage between inflammation and thrombosis. Platelets also avidly bind to microorganisms and do so by expression of Toll-like receptors (TLR). It appears that platelets may act as circulating sentinel cells that first encounter blood borne infectious products for presentation and activation of innate immune responses. More recently, it has been shown that platelets and their parent cells, the megakaryocytes, can process and present both foreign- and self-antigens to CD8+ T cells giving them an ability to directly alter adaptive immune responses. This lecture will highlight several attributes of platelets that draw them into the realm of immunity and will discuss how platelets may be responsible for directly and indirectly controlling immune functions.

Bio
John W. Semple obtained his PhD in Immunology from Queen's University at Kingston and was a Diabetes Canada Fellow at the Banting and Best Department of Medical Research in the C.H. Best Institute, University of Toronto. He joined St. Michael's Hospital in Toronto in 1990 and became Head of the Toronto Platelet Immunobiology Group at St. Mike's and a Professor of Pharmacology and Medicine at the University of Toronto. In 2016, he moved to Lund University where he is currently a Professor of Transfusion Medicine and Group Leader in Platelet Immunology. In addition to being the recipient of many awards, he is a member of several organizations and has delivered over 160 presentations at international meetings. He is currently the Scientific Secretary of the International Society of Blood Transfusion, a member of the Scientific Committee on Megakaryocytes and Platelets of the American Society of Hematology and serves on the editorial boards of the Blood and Transfusion journals. His primary research interests include the pathogenesis of immune thrombocytopenia (ITP) where he studies animal models of platelet immunity. His lab also focuses on the pathogenic mechanisms that lead to Transfusion Related Acute Lung Injury (TRALI). His lab also fist described Toll-like receptor expression on platelets in 2004 and since then, he has had an interest in platelet-immune interactions. He has authored more than 175 publications in the area of platelet immunology and TRALI.

KL6 **Waltraud Schrottmaier**
Department of Vascular Biology and Thrombosis Research, Medical University Vienna, Vienna, Austria

Platelets in inflammation and infection

Platelets do not only get activated during inflammation and infection but are sensitive fine-tuners of immune responses. In both acute and chronic inflammation such as pulmonary injury and atherosclerosis platelets exacerbate cytokine release, thereby boosting recruitment of innate leukocytes and their host-damaging activities including neutrophil phagocytosis and monocyte/macrophage differentiation into foam cells. However, platelets walk a fine line between thrombosis and immunoregulation, while also maintaining vascular integrity and promoting tissue repair.
Platelets can get directly activated by bacterial and viral stimuli, launching rapid and direct responses and thus representing an important first line of defence against invading pathogens. These leukocyte-activating functions of platelets are beneficial for pathogen clearance, as platelet inhibition accelerates bacterial and viral burden. However, upon excessive immune stimulation, e.g. in severe sepsis, the immunomodulatory functions of platelets appear to be quickly overshadowed by their haemostatic functions, and while capture of pathogens in microthrombi can locally curtail pathogen dissemination, systemic thrombosis leads to vessel occlusion and tissue hypoxia.
Chronic or re-occurring inflammatory conditions, however, seem to affect platelet function in a different way. Platelets seem to be less responsive and potentially exhausted in patients with chronic inflammatory disease. While persistent inflammation directly affects megakaryocytic development and platelet production, also intrinsic factors generated during inflammatory diseases such as adipositas or re-occurring endotoxaemia render platelets to a hypo-reactive phenotype. This mechanism could provide an evolutionary advantage by diminishing the risk of thrombotic complications in inflammation and add one more layer of complexity to the multifaceted role of platelets in immune responses and immunothrombosis. Hence, even though platelets are only fine-tuners of the immune system, they represent double-edged swords and depending on the underlying condition they may tip the balance in inflammation and infection to either counteract or exacerbate disease severity.

Bio
Waltraud Schrottmaier received her master's degrees in 2012 from the University of Vienna where she studied Genetics/Microbiology and Anthropology. For her PhD she joined the lab of Alice Assinger at the Medical University of Vienna from which she graduated in 2016. During her PhD she investigated interactions of platelets with innate leukocytes, in particular the role of platelet PI3K signalling in the context of inflammation and infection. For her postdoctoral training she joined the lab of Mattias Forsell at Umeå University, Sweden, where she studied the physiologic function of platelet-derived IgG for virus neutralization. Since September 2018 she is back at A. Assinger´s lab and her research focuses on platelets in viral infections.

KL7 **Ilaria Canobbio**
Department of Biology and Biotechnology, University of Pavia, Pavia, Italy

Platelets at the crossroad between cardiovascular and Alzheimer's diseases

Abnormal accumulation of amyloid peptides (Aβ) in brain parenchyma is correlated with the progression of Alzheimer's disease (AD). Amyloid peptides also accumulate in cerebral vessel walls causing cerebral amyloid angiopathy (CAA) which frequently co-occurred in AD patients. The precise origin of circulating amyloid peptides is still uncertain, but some evidence demonstrates that amyloid peptides have pathological roles in the vascular system. Aβ40 and Aβ42 induce platelet adhesion and activate platelets through distinct receptors, increase platelet intracellular calcium concentration, activate tyrosine kinases and small GTPases, promote cytoskeleton reorganization and granule release, stimulate ROS formation, and potentiate thrombus formation (Canobbio et al., 2015). Aβ peptides stabilize fibrin clot and promote coagulation via the FXII-driven contact system (Zamolodchikov et al, 2016). Also, amyloid peptides are toxic to endothelial cells, leading to alteration of the blood-brain barrier and to cerebrovascular lesions (Yamazaki and Kanekiyo, 2017). All these effects trigger a prothrombotic phenotype in vivo and result in an increased risk of cardiovascular disorders, as observed in AD patients as well as in AD/CAA murine models (Sevush, 1998; Jarre et al, 2104; Canobbio et al., 2016). Conversely, cardiovascular complications (ischemic stroke or myocardial infarct) are often associated with the progression of AD (de Bruijn and Ikram, 2014). Deciphering the molecular basis of Aβ toxicity in the circulation is crucial to develop new and powerful strategies to minimize cardiovascular risks associated with AD or to delay the onset of dementia and to improve the quality of life of AD patients.

Canobbio et al., Front Cell Neurosci. 2015; 9:65
Zamolodchikov et al, J Thromb Haemost. 2016; 14:1489
Yamazaki and Kanekiyo, Int J Mol Sci. 2017;18
Sevush et al., Arch Neurol. 1998; 55: 530
Jarre et al, Cell Signal. 2014; 26: 2040.
Canobbio et al., Cell Signal. 2016; 28:1863
de Bruijn and Ikram, BMC Med. 2014; 12: 130

Bio
Ilaria Canobbio graduated in Biology at the University of Pavia in 1998. She obtained a PhD in Biochemistry and Molecular Physiology of Membrane Systems in 2002 at the University of Pavia and subsequently a Master in Communication of Science and Sustainable Innovation at the University of Milan in 2012. Her principal research interests are related to the biochemical mechanisms of platelet activation and signal transduction in platelets with attention to von Willebrand factor-induced platelet activation, to the role of ITAM receptors, Src family kinases, PI-3kinase, focal adhesion kinases and small GTPases. Recently, she focused her attention on the possible roles of Alzheimer's disease-related amyloid peptides and amyloid precursor protein APP in platelet functionality and cardiovascular disorders.

KL8 **Olivier Peyruchaud**
INSERM U1033, Université Claude Bernard Lyon 1, Lyon, France

New involvement of bioactive lipids in the dialogue between platelets and metastatic cancer cells

Metastases are the main cause of death in cancer patients. Platelets play a crucial role in the survival of metastatic cells in the blood circulation. Therefore, targeting the dialogue between platelets and cancer cells may represent a new alternative therapy against metastasis. Interaction of tumor cells with platelets leads to the production of plethoric platelet factors with protumoral and prometastatic properties. Among which we have shown in the past that the bioactive lipid lysophosphatidic acid (LPA) produced by activated platelets can act on the type 1 LPA receptor (LPA_1) promoting cancer cell proliferation, motility, and secretion of pro-osteoclastic cytokines (IL-6, IL-8, MCP-1, GMC-SF) explaining why silencing LPA_1 in osteotropic cancer cells or pharmacological blocking of LPA_1 inhibit the formation and the progression of ostolytic bone metastases in animal models. In addition, Autotaxin (ATX) that is a lysophospholipase D responsible for basal concentrations of LPA in blood is uptake from the blood circulation by platelets and stored in □-granules. Functionally active ATX is eventually released following tumor cell-induced platelet aggregation, thereby promoting metastasis. In order to define new anti-metastasis therapies exhibiting a high specificity for the pathological interaction between tumor cells and blood platelets without abrogating normal platelet functions we developed a high throughput assay and found hits that targeted the cysteinyl leukotriene receptor type 1 (CysLT1). Cysteinyl leukotrienes (Cys-LKT) are pro-inflammatory bioactive lipids highly involved in inflammatory diseases such as asthma. Intriguingly our breast cancer cell lines do not express CystLT1 and platelets are well known to be deprived in 5-lipoxygenase activity. Therefore, platelets cannot produce Cys-LKT and our tumor cell lines cannot respond to Cys-LKT stimulation. We found that our breast cancer cells secrete Cys-LKT and that conditioned media collected from these cells sensitized platelets to low concentrations of ADP in platelet aggregation assays that were blunted in presence of a CysLT1 antagonist. In vivo, using two different independent animal models we found that the treatment with a CysLT1 antagonist remarkably inhibited by 90% the capacity of both human and murine breast cancer cells to induce bone and lung metastases.
In conclusion, bioactive lipids are novel important factors involved in the reciprocal dialogue between platelets and cancer cells throughout the metastasis process that offer novel anti-metastasis therapeutic options.

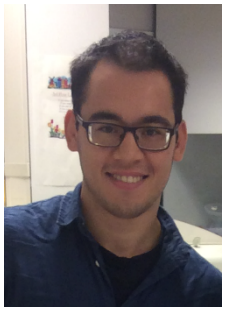
Bio
Olivier Peyruchaud obtained his Ph.D in 1995 at the University of Bordeaux II (France) with work on GPIIb-IIIa alloantigens and mutations in Glanzmann's thrombasthenia. After postdocs with AT Nurden (Pessac, Fr, 1996), DF Mosher (U Wisconsin, USA 1997-8), D Delmas (Lyon, Fr, 1999-2001), he promoted within INSERM to become consecutively Group leader in 2001 and Research Director in 2015 and Leader of Team #3 INSERM U1033 (Lyon, Fr) with Lysophospholipids and Bone Pathophysiology as subject.

KL9 **Cécile Oury**
GIGA Cardiovascular Sciences, University Hospital Sart Tilman, Liège, Belgium

Platelets and colorectal cancer: how do tumors escape the immune system by educating platelets

Chronic inflammation drives a lot of cancers. A prime example of this is the strong association between inflammatory bowel disease and colon cancer. Studies indicate that components of the hemostatic system, including thrombin and fibrinogen, promote colitis-associated cancer (CAC) progression. Interestingly, we recently showed that platelets also play an instrumental role in CAC initiation, which can be prevented by use of P2Y12 receptor inhibitors. We found that local thrombin generation in inflamed colonic mucosa supports platelet-mediated events that help create the immunosuppressive microenvironment necessary for tumor growth. Importantly, these findings highlight the fundamental importance of the hemostatic system in early events that drive tumorigenesis. Platelets have the potential to affect inflammatory pathways in colitis through a myriad of potential mechanisms. Activated platelets express cell surface proteins, such as CD40 ligand and P-selectin, which can directly affect inflammatory cell function. The platelet secretome also contains a significant number of proinflammatory mediators which act on inflammatory cell activation and/or recruitment. Our study adds to the repertoire of platelet functions in inflammatory settings by showing that platelets mediate a distinctly immune suppressive role by supporting the recruitment/expansion of myeloid derived suppressor cells (MDSCs). We uncovered platelet-derived serum amyloid A (SAA) proteins as one important mechanism by which platelets may modulate MDSC biology. In other cancer settings, platelets regulate the tumor immunological microenvironment through the GARP-TGF-□ axis, but whether this pathway contributes to CAC remains unknown. While the mechanistic details will require further study, our work point to platelets as a crucial bridge between the hemostatic and inflammatory systems, providing important pro-inflammatory and immunosuppressive signals that shape the early tumor microenvironment to drive tumorigenesis. Given the link between inflammation and the development of colorectal cancer (CRC) of distinct etiologies and of other cancers (pancreas, lung, liver, stomach,…), the implication of platelets in cancer initiation might go far beyond CAC. Our data may provide a mechanistic explanation for the finding that long-term aspirin use appears to reduce the incidence and mortality of CRC. They also suggest that antiplatelet regimen targeting P2Y12 may represent a new paradigm for rational prevention of cancer in chronic inflammatory settings. P2Y12 inhibitors, through their ability to inhibit immunosuppressive myeloid cell expansion at pre-tumoral inflammatory stage, may allow more efficient immune eradication of tumors. Furthermore, we postulate that platelet SAA levels may represent a novel accurate biomarker for early detection of patients at risk of developing cancer.

Bio
After Cécile Oury obtained her PhD in molecular and cellular biology from the University of Liège, Belgium, she was trained in thrombosis and hemostasis during a 6-year postdoctoral stay at KU Leuven (1997-2003). Currently she is co-director of the GIGA-Cardiovascular Sciences research unit at the University of Liège and head of the Laboratory of Thrombosis and Hemostasis and Valvular Heart Diseases. She has published more than 50 research articles related to platelets in high impact journals leading to international recognition in the platelet community. Her present research interest focuses on the elucidation of the cellular and molecular mechanisms underlying the fine balance between thrombosis and bleeding, particularly in VHD patients. Our VHD program includes both basic science and clinical studies with the goal of translating the developed knowledge into patient benefits. An important line of research deals with prosthetic heart valve complications.



KL10 **Myron Best**

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Tumor-educated platelet RNA for blood-based liquid biopsies

Blood-based liquid biopsies have emerged as promising biomarker sources for non-invasive detection of cancer. Confrontation of blood platelets with tumor cells via transfer of tumor-associated biomolecules (tumor-educated platelets; TEPs) is an emerging concept. TEPs are implicated as central players in the systemic and local responses to tumor growth, thereby altering their RNA profile. We performed RNA-sequencing of >1000 platelet samples covering multiple tumor types and individuals without cancer. We demonstrated that our self-learning particle swarm optimization (PSO)-enhanced algorithms enable efficient selection of RNA biomarker panels from platelet RNA-seq libraries, and allow for accurate blood-based identification of early- and late-stage non-small-cell lung cancer, and breast and brain tumors. In addition, across six different tumor types, the location of the primary tumor was correctly identified with 71% accuracy. We demonstrate that platelet subpopulations and intra-platelet RNA-binding protein activity may be altered in patients with cancer, potentially resulting in the TEP RNA profiles. In all, our results indicate that platelets provide a valuable platform for cancer diagnostics. The unprecedented ability of TEPs to pinpoint the location of the primary tumor advances the use of liquid biopsies for cancer diagnostics.

Bio
Myron Best is an MD/PhD-student, who conducts his research at the Amsterdam UMC, VU University medical center Amsterdam and the Cancer Center Amsterdam. After he obtained his bachelor's degree in Medicine, he applied for the MD/PhD-trajectory in which he aims to both obtain his master's degree in Medicine as his PhD thesis. His research is focused on the use of blood platelets to diagnose cancer and non-cancerous diseases in blood, using novel molecular techniques combined with sophisticated bioinformatic algorithms. In addition, he and his colleagues perform studies that aim to develop algorithms that capture changes in blood during tumor treatment, and unravel the biological role of blood platelets in human diseases. His work is published in leading scientific journals including *Cancer Cell* and covered by national and international media.

Oral communications

OC1 **Sophie Lepropre**

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AMPK-ACC signaling modulates platelet phospholipids content and potentiates platelet function and thrombus formation

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Co-author(s) Marie Octave, Marie-Blanche Onselaer, Gregory R. Steinberg, Bruce E. Kemp, Alexandre Hego, Odile Wéra, Sanne Brouns, Frauke Swieringa, Martin Giera, Victor M. Darley-Usmar, Jérôme Ambroise, Bruno Guigas, Johan Heemskerk, Luc Bertrand, Cécile Oury, Christophe Beauloye, and Sandrine Horman

Introduction: AMP-activated protein kinase (AMPK) α1 is activated in thrombin- or collagen-stimulated platelets, and as consequence phosphorylates and inhibits the acetyl-CoA carboxylase (ACC).

Objective: Since ACC is crucial for the synthesis of fatty acids, which are essential for platelet activation, we hypothesized that this enzyme plays a central regulatory role in platelet function and thrombus formation.

Methods: To investigate this, we used a double knock-in (DKI) mouse model in which the AMPK phosphorylation sites Ser79 on ACC1 and Ser212 on ACC2 were mutated to prevent AMPK-signaling to ACC. Thrombus formation was measured *ex vivo* using a flow chamber assay. Hemostasis was assessed via the measurement of bleeding time. *In vivo*, thrombosis was studied upon ferric chloride-induced carotid artery injury.

Results: We show that, of the two isoforms, ACC1 is the major isoform expressed in platelets. As expected, thrombin or collagen stimulation led to a rapid increase in ACC phosphorylation in WT platelets. However, baseline and thrombin- or collagen-induced ACC phosphorylation remained undetectable in ACC DKI platelets. Suppression of ACC phosphorylation promoted *ex vivo* thrombus formation on collagen-coated surfaces under flow. *In vivo*, DKI mice had a shorter bleeding time and exhibited an increased thrombus growth, reflected by a higher increment in thrombus surface area over a 10-minute time interval, compared to WT mice (7242 μm^2±1265 for DKI mice; 2520 μm^2±1219 for WT mice, $P<0.05$). Mechanistically, the lack of AMPK-ACC signaling amplified thromboxane generation and subsequent dense granules release, specifically in response to collagen. Lipidomic analysis revealed that ACC DKI platelets had increased levels of arachidonic acid containing phosphatidylethanolamine plasmalogen, a major contributor of arachidonic acid and thromboxane generation following platelet stimulation.

Conclusions: In conclusion, AMPK-ACC signaling is coupled to the control of thrombosis by specifically modulating thromboxane and granule release in response to collagen. It appears to achieve this by increasing platelet phospholipid content required for the generation of arachidonic acid, a key mediator of platelet activation.

OC2 **Simon Felipe**

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Oxidized high density lipoprotein promotes pro-thrombotic phenotype by platelet adhesion and coagulation factors modulation.

Co-author(s): Lorena Perez

Systemic inflammatory diseases are characterized by the activation of the coagulation system determined by two events: platelet adhesion dependent on expression of adhesion molecules such as P-selectin[1-4], and clot formation dependent on the balance between pro-coagulant and anti-coagulant/fibrinolytic factors[5,6,7]. During systemic inflammatory diseases, oxidative stress is increased in the bloodstream promoting oxidative modifications to several macromolecules including lipoproteins, generating the oxidized forms of low-density lipoprotein (LDL) and high-density lipoprotein (HDL): oxLDL and oxHDL[8-10].
Despite that native HDL promotes antithrombotic and fibrinolytic activity[11], effects of oxHDL on blood coagulation are not known. Here, we investigated whether oxHDL induces platelet adhesion and promotes a pro-thrombotic phenotype.
We used primary rat mesenteric endothelial cells (RMEC) and platelets isolated from rats. RMEC were stimulated with vehicle, HDL or oxHDL for 12 h, and TF and t-PA expression and platelet adhesion was determined. Cultured RMEC exposed to vehicle, HDL or oxHDL were co-cultured with platelets to measure platelet adhesion to endothelial cells (EC). Also, rats were administrated with vehicle, HDL or oxHDL for 24 h and D-dimer levels and coagulation time was determined.
Our results indicated that EC exposed to oxHDL showed increased P-selectin expression, compared to cells exposed to vehicle and native HDL. OxHDL-induced P-selectin increase was dependent on the activity of the oxLDL receptor, LOX-1, suggesting a potential interaction between oxHDL and LOX-1. In according to this, EC co-cultured with platelets showed a significant increase in platelet adhesion to EC in response to oxHDL treatment, compared to HDL- or vehicle-treated cells. Increased platelet adhesion was severely decreased when LOX-1 activity was inhibited. Additionally, oxHDL-treated EC showed both, increased the pro-coagulant protein tissue factor (TF) expression and decreased the fibrinolytic protein plasminogen activator (t-PA) expression, compared to HDL- or vehicle-treated cells, suggesting the generation of a pro-thrombotic condition. Furthermore, changes in TF and t-PA expression were abolished using LOX-1 activity inhibitor.
Noteworthy, rats injected with oxHDL showed increased plasma D-dimer levels compared to HDL- or vehicle-treated rats. Furthermore, the coagulation time was reduced in oxHDL-treated rats. These results suggest that oxHDL administration induces a pro-thrombotic condition in rats.
All together, we conclude that the oxidized form of HDL, oxHDL, contributes to generate a pro-thrombotic state mediated by platelet adhesion and coagulation factors modulation.

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OC3 **Madeleine Göritzer**

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Novel role for adipose triglyceride lipase in thrombogenesis

Co-author(s): Stefanie Schlager, Nemanja Vujic, Juliane Dorow, Sanja Curcic, Dagmar Kolb, Rufina Schuligoi, Akos Heinemann, Uta Ceglarek, Branislav Radovic, and Dagmar Kratky

Background: In platelets, the presence of lipid droplets and the role of their lipolytic machinery to degrade triglycerides have not been investigated yet. According to genome-wide RNA-sequencing data from human and mouse platelets, the main neutral lipase adipose triglyceride lipase (ATGL) is expressed in platelets. However, its role in platelet function has never been addressed.

Methods: Atgl-deficient (-/-) mice were used to study the role of ATGL in platelets. Expression of lipases in murine platelets was determined by PCR and western blotting. Platelet spreading and morphology were visualized by electron microscopy. Agonist-induced platelet activation was analyzed by flow cytometry. In vitro thrombus formation assays were performed, in which platelets in whole blood were stained with a fluorescent dye and perfused over collagen-coated channels.

Results: We found that Atgl-/- platelets do not accumulate lipid droplets as observed in other cells and tissues. While stimulation with agonists such as ADP, thrombin, and thromboxane A2 resulted in comparable platelet activation, collagen and collagen-related peptide were not able to stimulate Atgl-/- platelets to the same extent as wild-type (wt) platelets. Although surface expression levels of GPVI were comparable between wt and Atgl-/- platelets, our results indicate a modulation of the intracellular signaling pathway downstream of GPVI. The absence of ATGL in mice was associated with an anti-thrombogenic phenotype, as evident by reduced in vitro thrombus formation and collagen-induced platelet activation.

Conclusions: Our data suggest that genetic deletion of ATGL reduces platelet reactivity in mice, revealing a novel role for ATGL in thrombogenesis.

OC4 **Bender Markus**

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Platelet lamellipodia formation is not required for thrombus formation and stability

Co-author(s): Yvonne Schurr, Andreas Sperr, Sarah Beck, Bernhard Nieswandt, Laura Machesky

Background: During platelet spreading, the actin cytoskeleton undergoes rapid rearrangement, forming filopodia and lamellipodia. The latter structure displays a circumferential zone with orthogonally arrayed short actin filaments. Controversial data have been published on the role of lamellipodia in thrombus formation and stability, mainly based on inhibitor studies or analyzing platelets with additional functional defects. One study showed that Rac1 deficiency leads to defective lamellipodia formation and a specific ITAM signaling defect, resulting in impaired platelet adhesion and defective thrombus formation on collagen under flow. This defect could be fully rescued by supplementation with ADP and a thromboxane A2 analogue, thus providing indirect evidence that lamellipodia formation might be dispensable for thrombus formation and stability. The WAVE regulatory complex, which has been shown in other cell types to drive lamellipodia formation by enhancing actin nucleation via the Arp2/3 complex, is activated by Rac1 interaction with the WAVE complex subunit Cyfip1 (Cytoplasmic FMR1-interacting protein, also known as Sra-1). Based on this, we suggest that Cyfip1 might function as an important regulator of platelet lamellipodia formation. However, the role of Cyfip1 in platelet function is unknown.

Aims. The aim of this study was to investigate the impact of Cyfip1 deficiency on platelet physiology, and the role of platelet lamellipodia in thrombus formation and stability.

Methods: We generated megakaryocyte- and platelet-specific Cyfip1-deficient mice (*Cyfip1*$^{fl/fl}$ *Pf4-Cre*), and performed flow cytometry, immunoblotting, confocal and electron microscopy as well as *in vitro* and *in vivo* thrombus formation studies.

Results: *Cyfip1*$^{-/-}$ mice displayed normal platelet counts and a slight reduction in platelet volume, but unaltered glycoprotein surface expression. Activation of mutant platelets was only moderately reduced to all tested agonists as measured by αIIbβ3 integrin activation and P-Selectin surface exposure. Cyfip1-deficient platelets adhered to multiple adhesive surfaces including fibrinogen under static conditions but, strikingly, lamellipodia formation was completely abolished and the platelets rather exhibited an increased length and number of filopodia. Despite defective lamellipodia formation under static conditions, *Cyfip1*$^{-/-}$ platelets formed stable thrombi, when perfused *ex vivo* over collagen fibers at intermediate shear rates and *in vivo* in two models of occlusive arterial thrombosis. Similarly, the hemostatic function was unaltered in the mutant mice. Investigation of control and *Cyfip1*$^{-/-}$ platelet morphology in an induced thrombus under flow by scanning electron microscopy revealed that platelets rather form filopodia in the thrombus shell, and are flattened with filopodia-like structures when in direct contact to collagen fibers at the bottom of the thrombus.

Conclusions: Here, we provide for the first time direct evidence by analyzing Cyfip1-deficient mice that surprisingly platelet lamellipodia formation is not required for stable thrombus formation, and that morphological changes of platelets differ between a static spreading assay and thrombus formation under flow. We are currently investigating, whether platelet lamellipodia formation plays a role in other platelet-dependent processes *in vivo*.

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OC5 **Valentin Do Sacramento**

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Ikaros: a new downstream player of AhR-dependent responses favoring platelet biogenesis by an enrichment of a megakaryocytic precursor

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Background: The aryl hydrocarbon receptor (AhR), a transcription factor better known for its detoxifying activity via the regulation of the expression of P450 cytochromes such as CYP1B1(canonical pathway), also participates in several physiological processes via other pathways. Recently, a new role of AhR has been revealed in the regulation of megakaryocyte differentiation and platelet biogenesis. An antagonist of AhR, stemregenin-1 (SR1), was found to promote the generation of a thrombocytogenic $CD34^+CD41^{low}$ megakaryocytic precursor, i.e. capable to mature into MK displaying a high potential to generate (pro)platelets (Strassel et al., Blood, 2016).

Aim: This study aimed to decipher the mechanisms associated to AhR inhibition during *in vitro* magakaryocytopoiesis and more specifically to delineate the respective involvement of the AhR-canonical and -non-canonical signaling pathways and their molecular determinants.

Methods: $CD34^+$ progenitors were differentiated into MKs using a 2-step procedure (expansion of MK precursors for 7 days, followed by a maturation step). The role of AhR was assessed in the second step. The role of the canonical pathway was evaluated by a loss of function approach using CYP1B1 inhibitors (tetramethoxystilbene (TMS) and alpha-naphthoflavone (ANF)) and CYP1B1 shRNA knock-down. Alternatively, an unbiased approach was used; the expression program associated to the emergence of $CD34^+CD41^{low}$ cells when AhR is inhibited was analyzed. For this purpose, $CD41^+$ cells a day 7 and different cell populations obtained at day 10 in the presence or the absence of SR1 were isolated, their transcriptomes determined by RNA-seq and compared.

Results: CYP1B1 inhibitors did not impact the emergence of the thrombocytogenic $CD34^+CD41^{low}$ megakaryocytic precursor and platelets production. The down-regulation of CYP1B1 by shRNA did not affect the capacity of MKs obtained in the presence of SR1 to generate proplatelets. These results downplay a role of the AhR-canonical pathway in $CD34^+CD41^{low}$ cells emergence. Transcriptomic analysis of the $CD34^+CD41^{low}$ cells generated in the presence or the absence of SR1 revealed a two-fold increase in *IKZF1*, coding for Ikaros, in the SR1-treated cells (p=1,48E-07). Moreover, the genetic profile of these cells was intermediate between those of $CD41^+$ cells at day 7 and those of more mature CD41+ CD34- megakaryocytic cells obtained at day 10, in particular genes such as *PF4, GP1BA* were less expressed, in agreement with their already reported downregulation induced by Ikaros overexpression. Interestingly, a treatment with lenalidomide, an inducer of Ikaros degradation, reduced by two-fold the emergence of SR1-induced $CD34^+CD41^{low}$ cells.

Conclusions: This study indicates that the canonical pathway of AhR has a weak impact on MK differentiation. In contrast, the non-canonical AhR-dependent control of Ikaros expression and/or activity could be a major driver of this differentiation. Of note, rather than inducing a dramatic change in gene expression, this pathway appears to prompt the emergence of a $CD34^+CD41^{low}$ subpopulation by delaying MK differentiation. This AhR-Ikaros dependent checkpoint controlling the stages of MK differentiation revealed by the current study opens new perspectives to improve platelet production in culture.

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OC6 **Hurtado Begoña***

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Mutations in cell-cylce MASTL kinase promotes thrombocytopenia by deregulating actin cytoskeleton dynamics in platelets

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Introduction: MASTL is a mitotic kinase involved in the maintenance of the mitotic state by inhibiting PP2A-B55 complexes[1–4]. A specific mutation (E167D) in the human MASTL (located in 10p11-12) was found to perfectly segregate with nonsyndromic autosomal dominant thrombocytopenia or thrombocytopenia-2 (THC2)[5,6]. These patients were characterized by incomplete differentiation of megakaryocytes and moderate thrombocytopenia with certain propensity toward easy bruising and minor bleeding. However, the possible connections between the cell cycle machinery and this human disease remained unexplored. MASTL is a mitotic kinase involved in the maintenance of the mitotic state by inhibiting PP2A-B55 complexes[1–4]. A specific mutation (E167D) in the human MASTL (located in 10p11-12) was found to perfectly segregate with nonsyndromic autosomal dominant thrombocytopenia or thrombocytopenia-2 (THC2)[5,6]. These patients were characterized by incomplete differentiation of megakaryocytes and moderate thrombocytopenia with certain propensity toward easy bruising and minor bleeding. However, the possible connections between the cell cycle machinery and this human disease remained unexplored.

Methods: We generated genetically-engineered mice with specific ablation of Mastl in megakaryocytes and platelets and a new Mastl E166D knock-in model carrying the thrombocytopenia-associated mutation. We assay the megakaryocytopoiesis in both mice and *in vivo* models were used to assess the effect of Mastl depletion/mutation in platelets, including tail bleeding, carotid artery thrombosis and pulmonary thromboembolism. Further, isolated platelets were obtained and compared using activation, adhesion assays and biochemical techniques. Finally, we perform phosphoproteome analysis of resting and activated null/mutant platelets and validated the results by western blot.

Results: We observed that Mastl ablation/mutation results in reduced platelet counts in both mice models. We first show here that this mutation in Mastl does not result in reduced activity as originally thought[5,7], but is accompanied by increased phosphorylation of Cdk/PP2A substrates, suggesting a gain-of-function alteration that results in decreased PP2A activity. Whereas genetic ablation of Mastl results in deficient maturation of megakaryocytes *in vivo*, Mastl E166D mutant mice display defective dynamics in the actin cytoskeleton during platelet activation accompanied by an altered pattern of protein phosphorylation, mimicking the effect of PP2A inhibition. These defects can be partially rescued both in vitro and in vivo by downregulating critical upstream kinases involving in outside-in signaling, such as protein kinase C (PKC), a molecule that regulates the actin cytoskeleton during platelet activation.

Conclusion: These results unveil an unexpected function for Mastl in sustaining actin cytoskeleton in platelets, and suggest that the thrombocytopenia-associated mutation in MASTL is a pathogenic dominant mutation that alters platelet cytoskeletal dynamics.

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OC7 **Carsten Deppermann**

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MGL-1/2 play a key role in the clearance of desialylated platelets through Kupffer cells

Co-author(s): Paul Kubes

Background: 100 Billion platelets are produced every day from megakaryocytes in the human bone marrow. Human platelets patrol the vasculature for about 10 days and while circulating lose the sialic acid content from their surface. At the end of their lifespan, desialylated platelets get cleared in the liver [1]. Recent studies report that desialylation causes platelet clearance via the Ashwell-Morell receptor on hepatocytes [2], however, this process has never been observed *in vivo.*

Aim: To study the process of platelet clearance *in vivo* using intravital microscopy of the liver.

Methods: Spinning-disk intravital confocal microscopy was performed on the liver of anesthetized mice. Mice were either treated with sialidase to induce *in vivo* desialylation of platelets or *ex vivo* desialylated platelets were labeled with CellTracker dye and transfused into mice. Kupffer cells (KCs) and endogenous platelets were labeled using fluorescent anti-F4/80 and anti-CD49b antibodies, respectively.

Results: Both *in vivo* as well as *ex vivo* desialylated platelets rapidly accumulated on KCs and not hepatocytes or endothelium. Platelet adherence was specific for sialidase treatment of platelets, since untreated transfused platelets continued circulating in the liver vasculature. Pre-treatment of wildtype animals with asialofetuin, which has previously been used to block the Ashwell-Morell receptor, significantly reduced the amount of desialylated platelets binding to KCs. Surprisingly, in Ashwell-Morell receptor-deficient mice, desialylated platelets adhered to KCs to the same degree as in wildtype animals. We therefore hypothesized that there is another receptor besides Ashwell-Morell receptor that is blocked by asialofetuin. Analysis of Kupffer cells revealed another receptor known to be blocked by asialofetuin, namely MGL-1. Indeed, blocking MGL-1/2 with antibodies dramatically reduced desialylated platelets binding to Kupffer cells.

Conclusion: Our data reveals for the first time that Kupffer cells play an important role in the clearance of desialylated platelets and that this interaction is mediated by MGL-1/2. Our findings might lead to new strategies to improve half-life of transfused platelets. Future studies will tell if MGL-1/2 is also part of the mechanisms underlying immune thrombocytopenia.

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OC8 **Pula Giordano**

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Deoxyribose-1-phosphate: a platelet-derived molecule that stimulates angiogenesis and tissue repair via redox-dependent activation of the NFkB transcription factor in endothelial cells.

Co-author(s): Joanna M Swarbrick, Harry Mellor, Matthew Burgess, Kate Wicks, Kimberly Mace, Shaun Reeksting, Anneke Lubben, Caroline PD Wheeler-Jones, and Dina Vara

Deoxyribose-1-phosphate (dRP) is a pro-angiogenic paracrine signal released by human platelets and other cell types, including macrophages and cancerous cells [1]. We have demonstrated previously the paracrine activity of this molecule as platelet primer modulating the response to physiological platelet agonists [2] and as chemokinetic stimulus promoting endothelial cell migration [3-4]. Here, we investigated the mechanism of action of dRP and its ability to promote angiogenesis and tissue repair *in vivo*.
Liquid chromatography-mass spectrometry (LC-MS) was utilized to quantify dRP in human plasma, which revealed the release of micromolar concentrations of this molecule by platelets in human blood. Live endothelial cell imaging, electron paramagnetic resonance (EPR) for the detection of superoxide anions generation, pull-down of dRP-interacting proteins from endothelial cell extracts followed by immunoblotting, gene silencing of different NOXs and their regulatory co-subunits by siRNA transfection, and experiments with pharmacological inhibition and siRNA-dependent silencing of the sugar transporter GLUT1 were utilized to demonstrate that dRP released by platelets stimulates angiogenesis following GLUT1-dependent internalization and NADPH oxidase 2 (NOX2) activation in endothelial cells. In turns, the increased superoxide anion generation in response to endothelial NOX2 activation leads to redox-dependent activation of the transcription factor nuclear factor kappa B (NFkB), which induces VEGF receptor 2 (VEGFR2) upregulation. Using endothelial tube formation assays, gene silencing by siRNA and pharmacological inhibitors, we demonstrated that the activation of NFkB and the upregulation of VEGFR2 are necessary for the angiogenic responses elicited by dRP. The dependence of the angiogenic response induced by dRP on NOX2 activation and VEGFR2 upregulation has been confirmed *in vivo* in excisional wound repair and matrigel plug vascularisation assays using $NOX2^{-/-}$ mice [5]. Interestingly, dRP resulted able to markedly accelerate tissue repair in a murine model of diabetes- and age-dependent impaired wound healing.
Taken together, this study describes a novel molecular mechanism underlying the pro-angiogenic and tissue regenerative activity of dRP, which involves the sequential activation of NOX2 and NFkB and the upregulation of VEGFR2 in endothelial cells. Therefore, the release of dRP by human platelet represents an important example of how platelets in addition to their recognized role in haemostasis also perform a critical physiological function in tissue repair by promoting angiogenesis and vascular regeneration.

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OC9 **Maria N. Barrachina**

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A combination of proteomic approaches identifies a panel of circulating extracellular vesicle proteins related to the risk of suffering cardiovascular disease in obese patients

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Plasma-derived extracellular vesicles (EVs) have been extensively described as putative biomarkers in different diseases. More precisely, increased levels of EVs subpopulations are well-known to associate with obesity. The goal of this study was to identify EVs-derived biomarkers in plasma from obese patients in order to predict the development of pathological events associated with obesity and cardiovascular disease.

The study was performed combining two different proteomic approaches (2D-DIGE and label-free LC-MS/MS). Samples were obtained from 22 obese patients, with no associated comorbidities, and their lean-matched controls, and divided into two cohorts: one for a 2D-DIGE-based study, and the other one for a label-free LC-MS/MS-based approach. EVs were isolated following a serial ultracentrifugation protocol.

We detected 22 and 23 different regulated protein features, the former is from 2D-DIGE approach and the latter from the label free LC-MS/MS analyses. Most of the differentially regulated proteins identified are involved in the coagulation and complement cascades. Interestingly, there was a clear up-regulation of complement C3, complement C4 and fibrinogen in obese patients following both approaches, which correlates with a pro-inflammatory and prothrombotic state of those individuals. Indeed, fibrinogen was also found to be upregulated in circulating EVs from ST-elevation myocardial infarction patients (Vélez et al. 2014). On the other hand, we showed a down-regulation of adiponectin. Adiponectin is an important adipocytokine which plays a crucial role in glucose and lipid metabolism, inflammation and oxidative stress. Interestingly, high concentrations of adiponectin have been associated with a reduction in the risk of coronary artery disease.

As a conclusion, our results suggest the relevance of considering plasma-derived-EVs proteins as a source of potential biomarkers for the development of atherothrombotic events in obesity. Our study led to the identification of a panel of EVs-related proteins altered in obesity.

OC10 **Constance Baaten**

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A Synthesis Approach of Mouse Studies to Identify Genes and Proteins in Arterial Thrombosis and Bleeding

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Current antithrombotic therapies effectively, albeit partly, reduce cardiovascular diseases by preventing arterial thrombosis and thromboembolism, but are mostly accompanied by an increased risk of bleeding. Arterial thrombosis studies using genetically modified mice have been invaluable for identification of new molecular targets. However, because of low sample size and heterogeneity in approaches or methodologies, a formal meta-analysis to compare studies of mice with a single gene defect encountered major limitations.
To overcome these, we developed a novel synthesis approach to quantitatively scale 1431 published studies of arterial thrombus formation (*in vivo* and *in vitro*), thromboembolism and tail bleeding using genetically modified mice. Using a newly defined consistency parameter (CP), indicating the strength of published data, a comparison was made of 409 mouse genes, of which 17 consistently contributed to thrombus formation without affecting hemostasis. Ranking analysis indicated high correlations between collagen-dependent thrombosis models *in vivo* ($FeCl_3$ injury or ligation/compression) and *in vitro*. Integration of scores and CP values resulted in a network of protein interactions in thrombosis and hemostasis (PITH), which was validated with databases of genetically linked human bleeding disorders. The network revealed 2,946 nodes connected to modifying nodes for thrombus formation or bleeding, mostly for genes with expression along the megakaryocyte lineage.
Studies with novel knockout mice revealed that 4/8 (*Apoe*, *Fpr2*, *Ifnar1*, *Vps13a*) new genes were modifying in thrombus formation. Thereby, the PITH network: *(i)* revealed a homology of murine and human thrombotic processes, and *(ii)* identified multiple new proteins with a possible role in thrombosis and hemostasis.

OC11 **Magdolna Nagy**

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Platelet induced intracellular Ca^{2+} rises in neutrophils

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Introduction: Neutrophils are involved in pathological thrombus formation at an early stage, by interacting with platelets and the vessel wall. Although it is known that neutrophils communicate with platelets by multiple receptor-ligand interactions and chemokines, it has remained unclear which platelet-derived factors can cause stimulation of these leukocytes. Our aim was to unravel the underlying mechanisms behind this communication in the context of thrombus formation.

Methods: Human thrombi with highly activated platelets were formed *in vitro* by whole blood perfusion over collagen microspots at arterial shear rate. Neutrophil responses were recorded by real-time fluorescence microscopy: *i.e.* adhesion, chemotaxis, Ca^{2+} fluxes (Fluo-4 loading), secretion (CD66b, MPO), production of reactive oxygen species (ROS) and endocytosis. Confirming measurements were performed by flow cytometry.

Results: Platelets in human thrombi with high activation markers, CD62P, CD63 and phosphatidylserine, were highly attractive for neutrophils (CD15+ leukocytes). Neutrophils adhered via multiple receptor-ligand interactions, degraded and endocytosed collagen, produced ROS, and gradually expressed myeloperoxidase (MPO) and CD66b. A fraction of about 60% of the neutrophils displayed continuous oscillatory Ca^{2+} rises of low frequency, which were in part related to their crawling and preceded other neutrophil activation markers. Platelet post-silencing by iloprost (prostacyclin analogue) strikingly suppressed the Ca^{2+} oscillations in neutrophils, however, it did not affect neutrophil responses in absence of platelets, thus pointing to a continuous production by platelets of neutrophil-activating factors. Inhibition of specific platelet derived substances pointed to a major role of the platelet produced chemokine CXCL7 (NAP-2), and lesser roles of CXCL4 (PF4) and CCL5 (RANTES). Furthermore, platelet silencing caused neutrophil dissociation by preventing the cells to stay at the same thrombus. In contrast, post-silencing of platelet cyclooxygenase with indomethacin was without effect on neutrophil Ca^{2+} signaling or crawling.

Conclusions: Persistent neutrophil attraction to and activation by platelets in a thrombus relies on the continuous release of platelet biomolecules, in particular CXCL7. This multi-staged process of neutrophil adhesion, crawling and activation can be suppressed by platelet silencing with iloprost.

OC12 **Cécile Oury**

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A novel P2X1-G-CSF-CCL5 axis controls hemostasis via platelets and neutrophils in intestinal inflammation

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Introduction: Platelets play a key role in inflammation notably by secreting chemokines that regulate neutrophil extravasation. Among them, CCL5 is one of the main chemokines secreted by platelets that has been involved in leukocyte arrest on inflamed endothelia. It may also participate in the control of G-CSF-dependent neutrophil expansion and function. Platelets maintain vascular integrity at the site of inflammation by sealing breaches caused by neutrophils, which relies on release of platelet granule content. However, whether these mechanisms underlie inflammatory bleeding in the intestinal vascular bed remains unknown. The ATP-gated P2X1 ion channel expressed on both platelets and neutrophils might play a major role.

Aims: We sought to investigate the mechanisms of platelet-neutrophil interactions in acute intestinal inflammation and their respective contribution to the associated hemostatic imbalance.

Methods: We used a mouse model of acute colitis induced by oral administration of dextran sodium sulfate (DSS) for 7 days. The effects of P2X1 deficiency, neutrophil-depleting antibody and the role of G-CSF were compared on the following parameters: disease activity index (diarrhea, bleeding, weight loss), differential blood cell counts, and hematocrit. Plasma and colon levels of cytokines were analysed by multiplex assays. Neutrophil phenotype was analysed by flow cytometry and neutrophil extracellular trap (NET) formation by blood neutrophils was evaluated ex vivo.

Results: Upon DSS administration, P2X1-deficient mice showed more intestinal bleeding and had more severe colitis than wild-type (WT) mice. Unexpectedly, DSS-treated P2X1-deficient mice displayed a significant increase of mean platelet volume (MPV), a possible indicator of in vivo platelet activation. G-CSF plasma levels were more elevated in P2X1-deficient mice than in WT mice, leading to elevated blood neutrophil count. Neutrophil infiltration in the colon of these mice was increased and correlated with the hematocrit drop. Although neutrophil depletion limited inflammatory bleeding, treatment with an anti-G-CSF blocking antibody decreased neutrophil count and MPV but had no effect on intestinal bleeding. DSS-induced increase of plasma CCL5 was abrogated in P2X1-deficient mice compared to WT mice, suggesting that a defect of platelet secretion might contribute to bleeding. In agreement with a contribution of both platelet defect and neutrophils to bleeding in P2X1-deficient mice, injection of G-CSF (filgrastim) in DSS-treated WT mice stimulated neutrophil production without causing bleeding. Strikingly, neutrophils from P2X1-deficient mice showed an activated phenotype with increased CD11b and decreased CD62L expression, whereas neutrophils from mice that received G-CSF displayed decreased CD11b, CD62L and increased ICAM-1 and CXCR2 expression. Neutrophils from the two mice were more prone to form NET as compared to those from DSS treated WT mice, suggesting that NET formation did not contribute to bleeding in P2X1-deficient mice. Accordingly, mice with neutrophil-specific PAD4 deficiency were not protected from bleeding upon DSS treatment.

Conclusion/Perspectives: In acute colitis, P2X1 is instrumental for the regulation of G-CSF-dependent neutrophil expansion and activity and for maintaining intestinal vascular integrity, two processes that may involve platelet release and CCL5. We are currently studying further the role of CCL5 in the prevention of inflammatory bleeding.

OC13 **Manuel Salzmann**

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Selective inflammatory activation of megakaryocytes and platelets by transgenic expression of IKK2 results in a hypo-reactive platelet phenotype

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Background and Objective: Megakaryocytes can sense inflammatory signals, but little is known how this might change platelet function. Most inflammatory signalling pathways converge at IκB kinase 2 (IKK2) activating the transcription factor NF-κB. Although platelets lack a nucleus, non-genomic effects of IKK2 have been described for platelets, which were in some reports postulated to be inhibitory[1,2] while others claimed that IKK2 is important for platelet activation[3,4]. Our aim was to clarify the non-genomic effect of persistent inflammation and NF-κB activity on platelet function.

Methods: Mice with a PF4-promoter driven expression of constitutively active IKK2 (caIKK2) were used as a model of persistent inflammation in megakaryocytes and platelets. Platelet count and lifespan were determined and function was tested *in vitro* by agonist-induced degranulation and aggregation and *in vivo* by tail bleeding, *intra vital* microscopy of ferric chloride induced thrombus formation and mechanical aorta injury. Additionally, RNA-sequencing, proteome and phospho-proteome analyses of megakaryocytes as well as resting and activated platelets was performed.

Results: Platelet count and lifespan were unaltered in Pf4/caIKK2 mice, however aggregation, degranulation as well as GPIIb/IIIa activation were decreased in caIKK2 expressing platelets upon stimulation with ADP and PAR4 receptor agonist. Consistently, *in vivo* thrombus formation was impaired both in the ferric chloride- and the mechanical injury-induced model and furthermore tail bleeding times were significantly increased in these mice.

Conclusion: In contrast to the common hypothesis that inflammation causes a hyperreactivity of platelets, we observed increased bleeding times and deficiencies to form a stable platelet thrombus in mice with an inflammatory activation of megakaryocytes and platelets. It is known that inflammation induces the expression of several plasmatic coagulation factors, thereby increasing thrombotic risks. Our findings indicate that platelets are hyporeactive under these conditions, thereby counteracting thrombotic processes through yet unknown IKK2-mediated mechanisms.

OC14 **Paul Vulliamy**

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Histone Release in Trauma Patients Induces the Formation of Procoagulant, Immuno-modulatory Platelet Balloons and Microparticles

Co-author(s): Scarlett Gillespie, Paul C Armstrong, Harriet E Allan, Timothy D Warner, Karim Brohi

Introduction: Injuries account for 10% of the global burden of disease and of the 5 million trauma deaths every year, around half will die as a consequence of hemorrhage. Platelets rapidly become dysfunctional within minutes of major injury and contribute to an overall trauma-induced coagulopathy which is associated with poor outcomes. The mechanisms responsible for this are unknown. We hypothesized that platelets become rapidly 'exhausted' during traumatic haemorrhage due to exposure to damage molecules released from injured tissues.

Methods: We examined platelets and platelet responses in blood samples taken from trauma patients (TP) immediately on arrival to the trauma center. Two cohorts of patients were included. In the first cohort (n=280), we performed impedance aggregometry, thromboelastometry and prothrombin fragment quantification, to identify signature alterations in platelet behavior after injury. We then performed detailed investigation of platelet activity in the second cohort (total n=63) using flow cytometry, transmission electron microscopy (TEM) and imaging flow cytometry (IFC). Healthy volunteers (HV, n=10) acted as a control group for the second cohort. Results are expressed as proportion of all platelets and reported as the mean ± SEM unless stated.

Results: Using hierarchical clustering analysis, we identified two clusters of patients who displayed reduced platelet aggregation in response to multiple agonists, but massively elevated thrombin generation. This signature was associated with the greatest severity of injury and the highest mortality. Using TEM (n=3) and IFC (n=48), we identified the presence of procoagulant ballooning platelets in the peripheral circulation. Balloons were significantly elevated in critically injured patients compared to those with minor injuries (8±2% vs 3±1%, $p<0.001$) and were not identified in healthy volunteers. Because histones are released into the bloodstream after injury and are known to influence platelet function, we next investigated histone-platelet interaction as a cause of this response. We found that platelets from trauma patients (n=12) expressed histone H4 on their surface (MFI: TP 89±12 vs HV 20±4, $p=0.005$) and that histones were detectable on the surface of platelet balloons. Exposure of platelets from healthy volunteers to recombinant histone induced platelet ballooning (H4 12.4±0.6% vs vehicle (V) 0.8±0.4%, $p<0.001$) and procoagulant transformation (Annexin V-positive platelets: H4 31±5% vs V 0.8±0.3%, $p<0.001$). Simultaneously, histone H4 triggered release of platelet-derived microvesicles (H4 1.9±0.4 $x10^7$/ml vs V 0.3±0.05 $x10^7$/ml, $p=0.001$) which were procoagulant by annexin V expression (H4 84±2% vs V 31±7%, $p<0.001$) and retained histone H4 on the surface (81±5% vs 6±5%, $p<0.001$).

Conclusion: The results presented in this study identify a fundamental switch in platelet behaviour during traumatic hemorrhage which develops as a direct response to tissue damage. We propose a model in which histones released into the circulation by mechanically damaged or ischemic tissues induce platelet ballooning, a phenomenon described here for the first time in human disease, leading to exuberant thrombin generation at the expense of platelet aggregation.

OC15 **Waltraud Schrottmaier**

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Platelet-stored antibodies potently diminish viral infection in vitro and in vivo

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Background: Besides their primary role in haemostasis, platelets are actively involved in immune responses as they respond to an array of inflammatory stimuli, including microbial infection. Further, platelets contain intracellular IgG, but their physiologic function remains unknown.

Aim: We aimed to elucidate the function of platelet-derived IgGs and their effect on viral infections.

Methods: Human and murine wildtype or α-granule -deficient platelets were analysed for IgG levels and α-degranulation by ELISA (IgG, CXCL4) and flow cytometry (IgG, P-Selectin). Plasma IgG uptake of platelets was assessed *in vitro* and *in vivo* via flow cytometry of naïve murine platelets that had been incubated with anti-PE IgG positive plasma or labelled and transfused into PE-immunized mice, respectively. For *in vitro* neutralization assays virus-infected Madin-Darby canine kidney cells (MDCK) or human umbilical vein endothelial cells (HUVEC) were incubated or perfused with platelets or plasma of seropositive or –negative donors before quantification of infection by IF or qPCR. As *in vivo* model, thrombocytopenic PF4cre iDTR mice were transfused with seropositive or -negative platelets, infected with IAV and pulmonary viral load was quantified after 7 days.

Results: Human and murine platelets contained IgG which were released upon shear stress. However, IgG loss did not correlate with P-Selectin exposure or CXCL4 release and α-granule deficient (Nbeal2-/-) platelets failed to show reduced IgG content and release, indicating an extragranular IgG storage site within platelets. While platelet IgG could derive from megakaryocytes that have taken up IgG from the bone marrow microenvironment, naïve platelets were also able to rapidly take up IgG directly from plasma *in vitro* and *in vivo*. Murine platelets from anti-IAV IgG seropositive mice reduced IAV infection *in vitro* and *in vivo* more efficiently than plasma containing comparable levels of anti-IAV IgG. *In vitro* virus neutralization by seropositive platelets was abrogated by inhibiting COX-1, P2Y12 or PI3Kβ signalling. Further, human platelets from anti-CMV IgG seropositive but not seronegative donors also potently neutralized *in vitro* CMV-infection of HUVEC under microvascular shear stress.

Conclusion: Our data indicate that IgG storage in platelets may not be restricted to α-granules. Further, our results show that platelets have the potential to mediate potent IgG-mediated antiviral effects both *in vitro* and *in vivo* directly at foci of infection. This indicates that platelet-derived IgG may represent a yet unexplored mechanism for focused serological immunity.

OC16 **Scarlett Gillespie**

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Prolonged histone exposure induces a subset of unresponsive platelets

Co-author(s): Paul Vulliamy, Karim Brohi

Introduction: Following critical injury platelet function is rapidly altered. We have previously reported marked decreases in platelet aggregation post trauma. What causes this alteration in platelet function is not known. Circulating histones are readily detected in the vascular space after trauma. Histones bind to cell membranes and alter cell functions. Whether release of histones from damaged tissues may contribute to the altered platelet function is unclear.

Aim: Our aim was investigate whether histones could be detected on the platelets of trauma patients and investigate the effect that this had on membrane permeability and calcium signaling.

Method: Whole blood taken from healthy volunteers was incubated with histones or vehicle, and stained with anti-CD61 and anti- histone H4 (H4) antibody. Additionally, adult trauma patients at a major trauma centre were recruited and underwent a baseline blood draw. Whole blood from patients was also incubated with anti-CD61 and anti-H4 antibody. Samples were fixed and processed using stand and imaging flow cytometry. Lactate dehydrogenase (LDH) post H4 (25µg/ml, 60mins) incubation was measured in washed platelets using a commercially available colorimetric assay. Calcium signaling following exposure to TRAP-6 (T6, 10µM) or H4(25µg/ml) was monitored in washed platelets loaded with Fluo-3AM from healthy volunteers. Fluorescence dynamics were recorded for 5mins post challenge, and following prolonged exposure (60mins). A subset of samples were exposed to H4 for 60mins, followed by T6(10µM) challenge with fluorescence dynamics were recorded for a further 5mins. Data presented as median ± SD.

Results: Exposure of platelets to H4 in vitro leads to binding of histones to the platelet cell surface (MFI: H4 154±39vs vehicle 2±1, p=>0.05). Blood samples taken from critically injured trauma patients demonstrated the presence of platelets bound by H4 in comparison to those circulating in healthy controls (14±4% vs 4±3%, p=>0.05). Exposure of platelets to histones at concentrations comparable to reported in injured patients induced a population of platelets with sustained intracytosolic calcium which persisted over a 60min period which was in stark contrast to rapid, transient response seen in T6 challenged platelets (25 secs, H4: 5±4% vs T6: 97±1%, p=>0.05, 60 mins H4: 66±6% vs T6 11.4±5%, p=>0.05). Furthermore, prolonged exposure to H4 led to the development of a discrete platelet population (26±6%) that were unable to respond to subsequent agonist stimulation. Platelets incubated with H4 released LDH in a concentration dependent manner (% of max lysis, H4: 3±1% of maximum vs T6: 1±0.5, p=>0.05).

Conclusion: Histones are readily detected on platelets following critically injury. Prolonged H4 exposure induced sustained cytosolic calcium, and induced a subset of unresponsive platelets. Release of LDH indicates the ability to H4 to destabilise the platelet membrane. These findings highlight the ability of histones to alter platelet responses, through the production of a population of hypofunctional platelets and directly inducing cell membrane damage. Delineating whether anti-histone therapies could reverse these effects and restore platelet function merits further investigation.

OC17 **Jolan Harsfalvi**

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Biophysical Characterization of Clot Retraction in Platelet Rich Plasma of Patients with Primary Anti-phospholipid Syndrome

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Background: anti-phospholipid syndrome (APS) is an autoimmune process that leads to thrombotic disorders. The most significant autoantibody is the one against beta-2-glycoprotein I (or Apolipoprotein H, β2GPI). The complex of β2GPI and its antibody is targeted in the platelet thrombus [1]. Anti-cardiolipin also binds to β2GPI and associates to thrombosis.

Our question: is, what are the biophysical characteristics -viscoelasticity (hysteresis area and force difference), contractility (baseline force)- of the platelet-rich fibrin network during clot formation and degradation in platelet rich plasma (PRP) of APS patients versus controls.
Patients with APS or venous thromboembolism (VTE) as controls were selected in the study. Both groups (age and therapy matched) were under vitamin K antagonists (VKA) or direct oral anticoagulants (DOAC; II- or Xa-Inhibitors) therapy. None of them had inter-current disease in the previous one year. Ethical approval: SE TUKEB116/2017

Method: is atomic force microscopy (AFM)-based nano-thrombelastography (nTEG) [2]. Citrated blood was centrifuged for PRP at room temperature (150 g, 10 min). An AFM cantilever was submerged in a 300 µL sample and cyclically moved up and down with 1 µm amplitude and 1 µm/s speed. The sample contained PRP and Ca^{2+} 10 mM, clotting was initiated with thrombin, 1 IU/ml final activity. As the sample clotted, the cantilever increasingly deflected during its vertical travel.

Results showed that when platelet count was set to 50G/L, the medians of the delay ([sec]) until the first force signals for the APS group was 2-3x that of the controls; slope of the force generation ([nN/sec]) was about ½; maximal force difference (ΔF, [nN]) of each cycle -calculated from the maximum deflection signal- was 1/3.
The median of fibrinogen level for the APS patients was 3.17 g/L and 2.95 for the controls.

Conclusion: Changes in the viscoelastic properties during platelet contractility and fibrin network formation of human PRP-derived clot (in Platelet Rich Plasma) can be characterized quantitatively.
The AFM based measurement is a standardisable biophysical method, provides nano-scale, numerical parameters. The group of Litvinov recently published a relative macro scale measurements on contractile stress and clot contraction [3], and they showed a decreased contraction of blood clot of SLE patients [4].
Our statistical analysis implicate that both the anti-B2GPI and anti-cardiolipin antibodies influence the clot retraction, but for this analysis more antibody positive patient is to be selected.

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OC18 **Senna Staessens**

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Structural features of acute ischemic stroke thrombi

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Background
Acute ischemic stroke is one of the leading causes of death and disability worldwide. Despite the huge clinical, economical and social burden associated with ischemic stroke, only two strategies are currently available to remove the occluding thrombus and restore blood flow to the brain: (i) pharmacological thrombolysis using tissue plasminogen activator (t-PA) and (ii) mechanical removal of the thrombus via endovascular thrombectomy. However, more than half of the patients receiving t-PA do not respond to the therapy due to so called 't-PA resistance'. In addition, the thrombectomy procedure is not successful in 10-20 % of patients. The factors contributing to such therapy resistance are not known but are most likely linked to specific characteristics of the thrombus that is occluding the blood vessel in stroke patients.

Aims: The aim of this study was to analyze ischemic stroke thrombi retrieved from patients treated with thrombectomy to better understand thrombus characteristics and structure.

Methods: A total of 176 thrombi were collected from endovascular treated ischemic stroke patients at Groeninge Hospital (Kortrijk, Belgium). Fresh thrombi were fixed in 4 % paraformaldehyde, embedded in paraffin and cut into 5 µm sections. Histological analysis was performed using hematoxylin and eosin, martius scarlet blue staining, Feulgen staining, and both immunohistochemical and immunofluorescence analysis of von Willebrand factor (VWF), platelets (GPIb), fibrin, DNA and white blood cells (CD45). Multicolor immunofluorescent analysis was performed on a randomly selected subset of thrombi (n = 8).

Results: Stroke thrombi are heterogenous. Nevertheless, histological analysis reveals common structural features. In general, stroke thrombi are composed of red blood cell (RBC)-rich areas, interspersed with platelet-rich areas. On a microstructural level, RBC-rich areas consist of red blood cells that are trapped within a relatively loose fibrin network in a honeycomb structure in which platelets and VWF are absent. In contrast to RBC-rich areas, platelet-rich zones consist of much denser fibrin structures that in addition are lined with VWF. These fibrin-VWF organizations are filled with platelets. White blood cells tend to accumulate on the boundaries between RBC-rich and platelet-rich areas. DNA networks are seen throughout the thrombus in some but not all thrombi and are particularly present in the platelet-rich areas and in the boundary zones.

OC19 **Madhumita Chatterjee**

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Targeting CXCR7 inhibits thrombotic response through cGMP-cAMP mediated mechanism: implications in coronary artery disease

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Background and objectives*:* Elevated platelet surface expression of the chemokine CXCL12 and its receptors CXCR4-CXCR7 in acute coronary syndrome (ACS) influences functional recovery following myocardial infarction, and prognosis. CXCR7 ligation by physiological agonists exerts a prosurvival and anti-thrombotic effect. This study explores the anti-thrombotic and anti-thromboinflammatory effects of a pharmacological CXCR7 agonist and its potential therapeutic implications.

Methods and Results: Coronary artery disease (CAD) patients with >median CXCR7 expression on platelets have significantly lower aggregation response to ADP. CXCR7-agonist binds to CXCR7 on platelets causing receptor internalization without affecting CXCR4. CXCR7 ligation counteracted activation-induced platelet degranulation (CD62P expression, ATP release), integrin activation, aggregation, thrombus formation, (*ex vivo* flow chamber assay, T-TAS), in both healthy subjects and ACS patients *ex vivo*. CXCR7-agonist regulated spreading and morphology dynamics of platelets on collagen and fibrinogen coated surface visualized by scanning ion conductance microscopy. CXCR7 ligation counteracted CRP-induced phosphorylation of PLC-γ, therefore generation of diacylglycerol and subsequent activation of PKC; it also counteracted activation induced phosphorylation of Src, PI3K, Akt, p38MAPK, and checked CRP triggered intracellular calcium mobilization. However, CXCR7 agonist induced cGMP levels and stabilized cAMP (LC-MS) levels and subsequent PKG-PKA dependent VASP phosphorylation to regulate platelet activation. Therefore CXCR7 imposed inhibitory effects were counteracted in presence of a GC inhibitor (ODQ), AC inhibitor SQ22536, by both PKG (KT5823)–PKA (KT5720) inhibitors. The platelet inhibitory effect of CXCR7 agonist was partially reduced in *sGC*$^{-/-}$ mice. CXCR7-agonist decreased activation-induced externalization of phosphatidylserine and relative percentage of procoagulant platelets, checked platelet dependent (PRP) but did not affect plasma dependent (PPP) thrombin generation (thrombinoscopy) or clot formation (thromboelastography). *In vivo*, CXCR7-agonist administration counteracted platelet activation, thrombus formation in the injured carotid artery, without affecting bleeding time or coagulation profile (APTT, PT). CXCR7 agonist not only checked thrombotic response but also controlled thromboinflammatory potential of platelets by counteracting the release of pro-inflammatory lipid mediators like arachidonic acid, thromboxane, HHT, HETE, ceramides (UHPLC-QTOF-ESI-MS/MS). Moreover, formation of platelet leukocyte aggregates following thrombus formation in *ex vivo* flow chamber assay and following *in vivo* carotid injury ($FeCl_3$-induced) was also checked by CXCR7 agonist supplementation to the experimental system.

Conclusion: CXCR7-ligation as an anti-thrombotic strategy bears the potential to counteract platelet hyper-reactivity and subsequent thromboinflammatory consequences without compromising physiological haemostasis.

Reference: *Chatterjee M, et al Thromb Hemost. 2016*

OC20 **Judith Cosemans**

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Differential effects of PDE3 and -5 on platelet function under hemostatic and inflammatory conditions

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Co-author(s): Hugo J. Albers, Alexandra C.A. Heinzmann, Rory R. Koenen, Andries D. van der Meer, Judith M.E.M Cosemans

Background: Platelet-rich thrombi, formed after atherosclerotic plaque rupture, may cause myocardial infarcts and strokes. Current treatment is focused on suppressing thrombus growth by inhibiting platelet activation. However, recurrent events are still occurring in about one third of the patients. Recently, we found that platelets in thrombi remain active for a prolonged time. We hypothesize that platelet granule secretion is pivotal in mediating not only acute, but also prolonged thrombus activities. The aim of this study is to examine the effect of suppressing platelet granule secretion, by (in)directly elevating cAMP and/or cGMP, on key functional thrombus activities.

Methods: The effect of PDE3/5 inhibitors on platelet $\alpha_{IIb}\beta_3$ activation and α- and δ-granule secretion was measured with flow cytometry. Microfluidics were used to study whole blood thrombus formation at 1000 s^{-1} over a collagen type I + tissue factor surface or over a confluent monolayer of human umbilical vein endothelial cells treated overnight with tumor necrosis factor-α (10 ng/ml). Platelet extracellular vesicle (EV) release was measured with a prothrombinase assay.

Results: Cilostazol (PDE3i, 5 μM) significantly inhibited platelet $\alpha_{IIb}\beta_3$ activation and platelet α- and δ-granule secretion of collagen-related peptide-treated washed platelets (p=0.0006, p=0.0001 and p=0.01). Tadalafil (PDE5i, 10 nM) tended to affect these processes (p>0.05). Both cilostazol and tadalafil significantly decreased platelet EV release upon stimulation with convulxin or thrombin. In whole blood perfusion, PDE3 (50 μM) or -5 (100 nM) inhibition did not affect platelet adhesion but showed a tendency to decrease thrombus size. PDE3 inhibition did significantly increased time to fibrin (p=0.03 vs. p=0.17 with PDE5 inhibition). Strikingly, a ten-fold lower dose of cilostazol (5 μM) or tadalafil (10 nM) gave a strong and significant decrease in platelet adhesion on inflamed endothelial cells.

Conclusion: These data indicate that PDE5, and especially, PDE3 are key controlling enzymes in the regulation of platelet adhesion, activation and granule secretion. Herein, platelet adhesion and thrombus formation appear to be differentially regulated on collagen versus inflamed endothelium. Interestingly, the effect of PDE inhibition is more pronounced on EV release than on α- and δ-granule secretion. Combination of PDE3/5 inhibition with current antiplatelet medication might be the key in the regulation of acute and prolonged thrombus activities.

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OC21 **Jo Mitchell**

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Platelet-specific factor XIII-A: A novel role in the regulation of platelet activation

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Background: Platelets contain an abundance of the cross-linking enzyme factor XIII-A (FXIII-A) in their cytoplasm. FXIII-A is exposed on the stimulated surface and is functional in extracellular cross-linking reactions where it stabilises thrombi against fibrinolysis. FXIII-A has many intracellular substrates, including actin, amongst other cytoskeletal proteins. The abundance of platelet FXIII-A in the cytoplasm and its functionality in external cross-linking reactions suggests it may also have intracellular roles and is potentially involved in platelet function and clot formation and retraction in addition to fibrinolysis.

Aims: To determine whether platelet FXIII-A has roles in platelet function and clot retraction.

Methods: Platelets were isolated from a FXIII-deficient patient and normal healthy donors. Platelet spreading and immunocytochemistry were performed on normal and FXIII-deficient platelets. A FITC-labelled anti-FXIII-A antibody was used to detect FXIII-A, FXIII activity was detected using a fluorescent probe and actin detected using fluorescently labelled phalloidin. Fibrinogen binding to platelets stimulated with CRP-XL, TRAP-6, ADP and the TxA2 mimetic U46619 was detected using flow cytometry. FXIII-depleted plasma with washed normal or FXIII-deficient platelets were used to form clots or Chandler model thrombi under flow to measure clot retraction and thrombus lysis, respectively. In some experiments a transglutaminase inhibitor (TGI) was included to inhibit the action of FXIII.

Results: FXIII-A antigen and activity co-localized with the actin cytoskeleton in normal platelets, suggesting FXIII-A may be cross-linking intracellular actin. Immunocytochemistry confirmed FXIII-deficient platelets contained no detectable FXIII-A. Inclusion of FXIII-deficient platelets into thrombi had no effect on the lysis of FXIII-depleted thrombi compared to the rate of lysis seen with normal platelets. In line with this, a TGI did not affect the fibrinolysis rate in FXIII-depleted plasma thrombi with incorporated FXIII-deficient platelets, but did increase the lysis rate of thrombi with normal platelets. This data confirms previous observations that platelet-mediated protection of FXIII-depleted thrombi against fibrinolysis is attributable to platelet FXIII-A.Incorporation of TGI into clots formed from FXIII-depleted plasma with added normal platelets significantly reduced clot retraction (clot weight 0.046g vs 0.099g $P<0.05$).Retraction of clots with FXIII-deficient platelets in FXIII-depleted plasma was reduced compared to normal platelets (clot weight 0.090 vs 0.046g), whilst the TGI had no effect on clot retraction with FXIII-deficient patient platelets (clot weight 0.096) confirming the effect is due to platelet FXIII-A. TGI caused a significant reduction in fibrinogen binding in response to TRAP-6 (2506 MFI vs. 687, $P<0.0001$) and U46619 (8227 MFI vs. 1822, $P<0.0001$), but not ADP or CRP-XL. The TGI had no effect on fibrinogen binding in FXIII-deficient platelets and FXIII-deficient platelets had reduced fibrinogen binding compared to normal donors.

Conclusions: Fibrinogen binding and clot retraction are attenuated in platelets deficient in FXIII-A and in normal platelets treated with a transglutaminase inhibitor. These data suggest that platelet FXIII-A is actively involved in these processes, possibly by mediating intracellular clot retraction.

OC22 **An-Sofie Schelpe**

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Anti-CUB1 or anti-spacer antibodies that increase ADAMTS13 activity act by allosterically enhancing metalloprotease domain function

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Background: ADAMTS13 circulates in a folded conformation, which is mediated by interactions between the C-terminal CUB domains and its central Spacer domain. Binding of ADAMTS13 to the VWF D4-CK domains disrupts the CUB-Spacer interaction, inducing a structural change that extends ADAMTS13 into an open conformation that enhances catalytic efficiency ~2-fold. This mechanism supports a modelin which ADAMTS13 unfolding induces exposure of an exosite in the Spacer domain that interacts withthe VWF A2 domain, increasing the affinity between the two molecules, and, therefore, the rate of proteolysis. The D4-CK-mediated conformational activation of ADAMTS13 can be mimicked in vitro with the use of antibodies (Abs) that disrupt the CUB-Spacer interaction, such as the previously published anti-CUB antibody, Ab17G2. We recently generated a novel, activating antibody against the Spacer domain (Ab3E4).

Aim: To characterize the mechanism by which the Ab17G2 and Ab3E4 enhance the catalytic efficiency of ADAMTS13.

Methods: The effects of the Ab17G2 and Ab3E4 on the activity of ADAMTS13 were studied using FRETS-VWF73. The effects of the Ab17G2 and Ab3E4 on the kinetics of VWF96 (VWF G1573-R1668) proteolysis were characterized using an in-house assay. ELISA was used to investigate conformational changes in ADAMTS13 induced by the Ab17G2 and Ab3E4.

Results: Both Ab17G2 and Ab3E4 enhanced FRETS-VWF73 proteolysis by ~1.7-fold. This result was reproduced using the VWF96 substrate; the Ab17G2 and Ab3E4 enhanced the catalytic efficiency (kcat/Km) of ADAMTS13 by ~1.8- and ~2.0-fold, respectively. The activation was dependent on the conformational extension of ADAMTS13, since the Abs could not enhance the activity of an ADAMTS13 variant that lacks the TSP2-CUB2 domains (MDTCS). Surprisingly, ADAMTS13 activation was not mediated through exposure of the Spacer or Cys-rich domain exosites as previously proposed, as the Ab17G2 and Ab3E4 efficiently enhanced proteolysis of VWF96 variants in which the Spacer/Cys-rich exosite binding sites were disrupted. Kinetic analysis of VWF96 proteolysis showed that the Ab17G2- and Ab3E4-induced activation of ADAMTS13 is primarily manifest through a ~1.5- to ~2-fold increase in enzyme turnover (kcat). Thus, contrary to the current model, this suggests that the conformational extension of ADAMTS13 influences the functionality of the active site, and not substrate binding affinity (Km). Incubating ADAMTS13 with either Ab17G2 or Ab3E4 exposed a cryptic epitope in the metalloprotease domain that was specifically detected by an Ab binding to this cryptic site in ELISA, further corroborating that the Abs induce a conformational change in ADAMTS13 affecting the M domain.

Conclusion: Abs can be used as tools for understanding the structure/function of enzymes. Using activating Abs against the Spacer and CUB1 domains of ADAMTS13, we show for the first time that the activation of ADAMTS13 following its unfolding is not a result of exposure of a functional exosite in Spacer/Cys-rich domain that increases affinity to VWF. Rather, our data are consistent with an allosteric activation mechanism upon the metalloprotease domain. We propose that ADAMTS13 unfolding causes a conformational change in the active site that further activates the enzyme. We are currently investigating whether the D4-CK-induced enhancement of ADAMTS13 proteolytic activity is also mediated by conformational changes in the active site.

OC23 **Elien Roose**

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Open ADAMTS13 conformation in immune-mediated thrombotic thrombocytopenic purpura is induced by anti-ADAMTS13 autoantibodies and corresponds with an ongoing ADAMTS13 pathology

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Background: Deficient ADAMTS13 activity (TS13:act <10%) induced by anti-ADAMTS13 autoantibodies (autoAbs) causes immune-mediated thrombotic thrombocytopenic purpura (iTTP). Recently we showed that an open ADAMTS13 conformation is characteristic for acute iTTP patients, while folded ADAMTS13 was found in 78% of iTTP patients in remission with an TS13:act >50%. However, also iTTP patients in remission with a persistent (<10%) or moderately restored (10-50%) TS13:act have been described, but their ADAMTS13 conformation is unknown. Intriguingly, the factor responsible for inducing open ADAMTS13 in iTTP patients remains elusive. Identifying the cause of open ADAMTS13 in iTTP will help better understand the pathophysiology of iTTP and could help appreciate the prognosis and better manage the prevention of subsequent relapses.

Aim: Determine ADAMTS13 conformation in plasma of iTTP patients during acute TTP and remission when TS13:act is <10%, moderately restored (10-50%) or >50% and investigate if anti-ADAMTS13 autoAbs induce conformational changes in ADAMTS13.

Methods: TS13:act was determined in 120 iTTP plasma samples from 4 different centers (Marseille, Milan, Budapest, Utrecht). Samples were categorized according to the presence of clinical symptoms (acute *versus* remission) and their TS13:act in remission (>50%, 10-50%,<10%). Next, ADAMTS13 conformation was determined in all samples using our ADAMTS13 conformation ELISA. Additionally, presence of anti-ADAMTS13 autoAbs was also determined via ELISA. Finally, IgG's from 18 acute iTTP plasma samples were purified and added to folded ADAMTS13 from healthy donor (HD) plasma to test whether iTTP IgG's are able to induce the open HD ADAMTS13 conformation.

Results: Of the 120 iTTP plasma samples, 46 were obtained during the acute (clinical signs present) and 74 during the remission phase (clinical signs absent). Further subdividing remission samples showed that TS13:act was >50% in 41, 10-50% in 14 and <10% in 19 samples. ADAMTS13 was open in 98% (45/46) of the acute samples and folded in 71% (29/41) of the remission samples with TS13:act >50%, confirming our previous results. Interestingly, ADAMTS13 was open in 93% and 89% of remission samples with TS13:act 10-50% and <10%, respectively (chi square, $P<0.0001$). Since anti-ADAMTS13 autoAbs influence TS13:act in iTTP patients, we next could demonstrate that open ADAMTS13 conformation was linked with presence of anti-ADAMTS13 autoAbs (chi square, $P<0.0001$) suggesting that anti-ADAMTS13 autoAbs could be a factor able to induce an open ADAMTS13 conformation in iTTP patients. To further test this hypothesis, we purified IgG's from 18 acute iTTP plasma's with open ADAMTS13 and added them to HD plasma containing closed ADAMTS13, where 14 of the 18 patient IgG pools (78%) did induce the open conformation in HD ADAMTS13, indicating that patient anti-ADAMTS13 autoAbs indeed can induce conformational changes in ADAMTS13.

Conclusion: We show that ADAMTS13 is not only in the open conformation in iTTP patient plasma during the acute phase but also in remission when TS13:act is <10% or 10-50%. Hence, the presence of open ADAMTS13 in those remission patients indicates that the underlying pathophysiology is still ongoing, emphasizing the need for a close monitoring of those patients. In addition, anti-ADAMTS13 autoAbs were identified as a factor responsible for the change in conformation in ADAMTS13 in iTTP.

OC24 **Jonas Nilsson**

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Circulating biomarkers with the ability to predict and monitoring therapeutic response in CRPC

Co-author(s): Lee-Ann Tjon-Kon-Fat, Linda Köhn, Pernilla Wikström, Tom Wurdinger

Background: Novel therapies for castration resistant prostate cancer (CRPC) have been introduced in the clinic with possibilities for individualized treatment plans. Best practice of those expensive drugs requires predictive biomarker monitoring. In this study, different platforms of circulating biomarker have been used to follow cancer-derived information that has been implicated in therapy resistance.

Method: Longitudinal blood collections from patients before and during therapy have been used for both isolation of the platelet population as well as circulation-free DNA (cfDNA) from plasma. From cfDNA changes in genomic copy numbers and mutations were analyzed and in platelets tumor-derived transcripts were analyzed. ddPCR and targeted sequencing were used to identify the selected biomarkers in CRPC patient's before and during androgen synthesis (AS)-directed therapies. The association between biomarker status and therapy response, progression-free survival (PFS) and overall survival (OS) was examined.

Results: In a cohort receiving AS-directed therapy cancer-associated biomarkers were present both within the platelet fraction as well as in the cfDNA. The analyzed platelet derived biomarkers in the AS-directed group were associated with shorter PFS, and with an increased risk of therapy failure, as well as short OS. Similarly, cfDNA alterations showed a shorter PFS as well as OS. Both cfDNA and tumor-derived information in platelets could be used to longitudinal monitor tumor progression.

Conclusions: Analyzing circulating biomarkers in the platelet population enables prediction on who will benefit from AS-directed therapy with high accuracy, and platelet based analysis of cancer derived biomarkers may be used in treatment stratification of patients scheduled for AS-directed therapies. The addition of cfDNA improved accuracy of therapy stratification in this AS-therapy naïve cohort indicating benefit of biomarker combinations.

Poster abstracts

P01 **Mohammad Abo-Hassan**

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ILK role on kindlin regulation and integrin β_3 clustering in platelet function

Co-author(s): Chris Jones

Integrin $\alpha IIb\beta_3$ activation and clustering is a vital part of platelet activation and is mediated in part through the association of kindlin with integrin β_3. ILK is a known effector molecule which recruits kindlin to bind cytoplasmic tail of β_3. However, the temporal interaction between kindlin-β_3 and the mechanisms of $\alpha IIb\beta_3$ clustering are not fully understood. The objective of this study was to investigate the time course of integrin β_3 clustering, its association with kindlin and the role of ILK in this process.

Method: Platelet-rich-plasma (PRP) was treated with ILK inhibitor Cpd-22 (3μM) and activated with thrombin (1U/mL) in the presence of GPRP for 0-300s, fixed with 2% (v/v) formal saline and permeablised with BD Phos Flow Perm Buffer III. Kindlin and integrin β_3 were identified with primary and alexa fluor 647 and 555 labelled secondary antibodies, respectively. Labelled platelets were washed then adhered overnight to poly-D-lysine coated slide. Clustering of β_3 and kindlin-β_3 association were assessed using super resolution STORM microscopy. To examine the impact of ILK inhibition of signal downstream of β_3, human washed platelets were pretreated with Cpd-22 or vehicle control, and stimulated with thrombin (1U/mL) for up to 300s, then lysed in SDS-PAGE Laemmli sample buffer. Blots were immunoblotted for tyrosine phosphorylation of β_3(Y773), Src(575) and Syk(Y525/526).

Results: Upon stimulation with thrombin the percentage of β_3 molecules within β_3-β_3 clusters increased, peaking at approximately 60s before falling at 90s and then increasing 150s and falling again 300s. Following stimulation with thrombin the percentage of kindlin molecules within kindlin-β_3 clusters initially showed the opposite trend (decreasing from a high in resting platelets) before following the same pattern as the β_3-β_3 clusters. Pretreatment of platelets with Cpd-22 caused a significant reduction in the % of β_3 clustering in response to thrombin at late (90, 150, 300s) but not early time points, and Kindlin-β_3 association at early time points (0, 30 and 90s). β_3, Src, and Syk phosphorylation occurred at late time points, corresponding with 2nd peak of β_3 clustering. Treatment of platelets with Cpd-22, caused a reduction in the phosphorylation of β_3 at (60, 90 and 150s), Src at (150 and 300s) and Syk at (150 and 300s).

Conclusion: We have developed a STORM microscopy method and used it to quantify the temporal changes in the co-clustering of kindlin with integrin β_3, and the biphasic clustering of integrin β_3 in human platelets following thrombin stimulation. ILK is essential for kindlin- β_3 association, the later clustering of β_3 molecules and for signalling downstream of integrin β_3.

P02 **Marta Zarà**

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Molecular mechanisms of breast cancer cell-induced platelet aggregation and release of pro-metastatic microparticles.

Co-author(s): Guidetti Gianni, Canobbio Ilaria, Visconte Caterina, Canino Jessica, Vismara Mauro, Calvaruso Cristina, Torti Mauro

Platelet-cancer interplay influences both the tumor metastatic spread and the development of thrombotic complications in cancer patients (1). In this frame, tumor cell-induced platelet aggregation (TCIPA) is considered critical to increase cancer cell survival in the circulation, mostly because platelets are assumed to create a protective shield against mechanical stresses and immune responses. Nevertheless, several studies suggested that the cross-talk between platelets and cancer cells is far more complex and involves an intricate set of intercellular signals and cellular responses (2).

In this study, we have investigated the molecular mechanisms and the functional consequences of platelet aggregation induced by MDA-MB-231, an aggressive breast cancer cell line .
We found that TCIPA occurs exclusively in the presence of autologous plasma indicating that, differently from what proposed previously, the direct interaction of purified platelets with cancer cells is not sufficient to stimulate platelet aggregation. In the presence of plasma, platelet-cancer cell interaction triggers the generation of low amounts of thrombin which is strictly required for TCIPA, as indicated by the ability of the serine protease inhibitor PPACK to completely suppress aggregation.

TCIPA completely depends on platelet activation and is accompanied by the stimulation of phospholipase C, the increase of intracellular Ca2+ concentration, and the activation of the small GTPase Rap1b. Pharmacological inhibition of phospholipase C, but not of phosphatidylinositol 3-kinase or Src family kinases prevents tumor cell-induced platelet aggregation. Tumor cells also induce platelet dense granule secretion, and the stimulation of the P2Y12 receptor by released ADP is necessary for complete platelet aggregation. By contrast, prevention of thromboxane A2 synthesis by aspirin does not alter the ability of cancer cells to induce platelet aggregation.

Importantly, breast cancer cells also promote a massive release of platelet-derived microparticles (PMPs) that is strongly compromised when TCIPA was prevented by pharmacological inhibitors. By confocal microscopy and TEM analyses, we found that MDA-MB-231 cells can bind and internalize PMPs. Moreover, cancer cell-induced PMPs are able to signal back to MDA-MB-231 cells, triggering remarkable responses, including the phosphorylation of p38MAPK and myosin light chain and resulting in the stimulation of migration and extracellular matrix invasion.
In conclusion, this study provides a molecular characterization of platelet activation induced by breast cancer cells and indicates a novel link between TCIPA and the release of PMPs able to potentiate the aggressiveness of cancer cells.

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P03 **Harriet E. Allan**

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Platelet activation causes mitochondria loss and extracellular vesicle production

Co-author(s): Plinio M. F. Ferreira, Marilena Crescente, Timothy D. Warner

Background: Platelets are fundamental for the physiological process of haemostasis but also drive pathological thrombosis. The processes of activation, aggregation and secretion are extremely energy dependent for which reason platelets contain a small number of mitochondria. Beyond the traditional role of mitochondria in ATP synthesis, recent reports have detailed a critical role for platelet mitochondria in calcium buffering as well as in apoptosis. However, their role during activation remains poorly defined. Here we have investigated the consequences of activation on platelet mitochondria.

Methods: Blood was obtained by venepuncture from healthy volunteers into 3.2% sodium citrate and centrifuged for 15 minutes (175 x g) to obtain platelet rich plasma. Further platelet purification was achieved by centrifugation (1000 x g, 10 min) and resuspension in modified Tyrodes HEPES buffer. Immunostaining and confocal microscopy were used to visualise platelet mitochondria (MitoTracker Orange CMTMRos) in unstimulated and TRAP-6 stimulated platelets. Fixed platelets were permeabilized and stained using mouse monoclonal anti-α Tubulin and detected with a secondary Alexa Fluor 488-conjugated antibody. Samples were imaged using a Zeiss LSM880 airyscan microscope and analysed using Image J and GraphPad Prism. Traditional flow cytometry and Image Stream X MKII analysis were used to characterize extracellular vesicles produced from unstimulated and TRAP-6 stimulated platelets, staining for MitoTracker Orange CMTMRos, CD61-APC and Annexin V fluorescence. Results were analysed using FlowJo v.8, IDEAS 6.2 and GraphPad Prism. Results are expressed as mean±sem, with significance established at $p<0.05$.

Results: Using confocal microscopy, we observed that platelet activation by TRAP-6 caused a significant decrease in average mitochondria number per platelet from 8.1±0.5 in resting platelets to 3.5±0.1 in activated samples ($n=6$, $p<0.001$). This was accompanied by a decrease in platelet cross-sectional area from 5.8±0.5μm^2 to 4.3±0.2μm^2. Flow cytometry revealed that the extracellular vesicles produced following TRAP-6 stimulation exhibited a higher number of MitoSpy Green positive events than unstimulated samples (18411±6446 AU vs. 2575±678 AU). Using Image Stream X flow cytometry we were able to visualise the presence of mitochondria within extracellular vesicles produced by TRAP-6 stimulation, accounting for 21±4% of the total population of vesicles.

Conclusions: Using confocal microscopy, we have shown platelet stimulation causes mitochondrial loss as well as a reduction in platelet size. Consistent with this data we have shown that mitochondria are present within extracellular vesicles produced by platelets following stimulation. Further work is required to establish why platelets release their mitochondria in vesicles and the functional significance of this process to other cells.

P04 **Christina Yip**

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Quantitative Platelet Proteomics analysis between individuals with Essential Thrombocythemia, Reactive Thrombocytosis and Healthy donors.

Co-author(s): Yew Mun LEE, Clarice Choong, Chun Tsu LEE, Eng Soo YAP, Qingsong LIN, Te Chih LIU

Background: Essential thrombocythemia (ET) is a member of chronic myeloproliferative neoplasm (MPN) characterized by persistently elevated platelet count of ≥450x10^9/L for more than 6 months and with an increased thrombohemorrhagic risk. Thrombotic complications are one of the major causes of morbidity and mortality in ET with the rate of thrombotic events, predominantly arterial and microvascular thrombosis, ranging from 2% to 4% patient-years. Studies showed that ET patients with microvascular disturbances have shortened platelet survival, increased beta-thromboglobulin, platelet factor 4, and thrombomodulin levels, and increased urinary thromboxane B2 excretion, indicating platelet-mediated thrombotic processes. The use of aspirin to inhibit platelet cyclooxygenase-1 is followed by relief of microvascular disturbances and normalisation of platelet activation indices. Thrombotic risk remained as a major factor in stratifying patients with MPN. While thrombotic risk factors such as age and previous thrombotic events have been defined, specific biomarkers leading to thrombosis requires further investigation. This is a pilot study to explore the differential platelet proteomic expressions in ET patients in comparison to patients with transient raised platelet count due to reactive causes or reactive thrombocytosis (RT) and healthy individuals. We aimed to characterise the pathogenic role of aberrant platelet protein expression in ET in comparison to patients with RT and healthy individuals.

Methods: Total proteins were extracted from peripheral blood platelets of four ET patients, three RT patients and four healthy individuals as normal control. We performed quantitative mass spectrometry proteomics protocol using tandem LC-MS/MS (SCIEX Triple TOF 5600) coupled to 8-plex isobaric tags for relative and absolute quantitation (iTRAQ) labelling to elucidate the differences in the proteome of platelets derived from pooled peptides samples from ET, RT and healthy individuals. Protein lists generated were analysed using Ingenuity Pathway Analysis (IPA) and STRING for relevant pathways and protein functional networks.

Results: Preliminary findings showed that the top 20 differentially expressed proteins in ET compared to RT identified proteins are involved in coagulation system, cell death and survival, cellular growth and proliferation and cell signaling. MIF, BAG6, SHMT2, SH3BGRL3and HADHB were up-regulated. As predicted, reduced protein expression of PF4, PPBP and TPM4 were found in ET patients who were on aspirin therapy.

Conclusions: Quantitative proteomics has become an important method contributed to the discovery of novel diagnostic or therapeutic targets for the management of cancer. Interestingly, a number of dysregulated proteins seen in ET such as MIF and SH3BGRL3 were associated with other cancers but not previously reported in ET. Further study is required to validate and elucidate the role of aberrant protein expression in ET.

P05 **Paul Armstrong**

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Potentiation of $P2Y_{12}$ inhibitor efficacy through combination with cyclic nucleotide modulators

Co-author(s): Plinio Ferreira, Melissa V.Chan, Chih-C Shih, Martina H. Lundberg-Slingsby, Timothy D. Warner

Background: Prostacyclin and nitric oxide, derived from the endothelium, act upon platelets to stimulate adenylyl cyclase and guanylyl cyclase, respectively, to elevate intra-platelet levels of the cyclic nucleotides cAMP and cGMP. In this way platelet quiescence is maintained. A number of approved treatments for pulmonary hypertension exist which target cyclic nucleotide generation or degradation by inhibiting phosphodiesterase (PDE), or more recently modulating soluble guanylyl cyclase. We, and others, have previously demonstrated a synergistic relationship exists between cyclic nucleotides and $P2Y_{12}$ inhibition (1-3). The aim of this study was to determine *in vivo* and *ex vivo* the anti-thrombotic efficacy of these approved drugs in combination with a $P2Y_{12}$ inhibitor.

Methods: Mice (C57Bl6) were administered sub-maximal doses of prasugrel (0.3mg/kg i.v; vehicle 0.2%DMSO) or cinaciguat (0.3mg/kg i.v; vehicle 2% DMSO) plus dipyridamole (2.0mg/kg i.p; vehicle 0.01M HCL), or combination of all three (PCD). Mice were anaesthetized (ketamine 100mg/kg & xylazine 20mg/kg, i.p.) and ferric chloride (10%)-induced thrombosis of carotid artery performed with blood flow being monitored to determine the time to occlusion, defined as cessation of blood flow for 60 seconds. In other experiments, blood was taken from the inferior vena cava of mice into lepirudin (0.25mg/ml final) and whole blood aggregometery (4) performed using as agonists arachidonic acid (1mM, AA), collagen (10µg/ml), a PAR-4 receptor agonist GYPGKF (30µM) or thromboxane A_2 mimetic U46619 (3µM). Data are presented as mean s.e.m. and compared using t-test or one-way ANOVA with Tukey post-hoc test, as appropriate.

Results: Combination of prasugrel with cinaciguat and dipyridamole (PCD) caused a significant increase in the time to occlusion (1472±210seconds) compared to vehicle (432±50seconds; $p<0.05$, n=6). Treatment with individual components, i.e. prasugrel alone or cinaciguat plus dipyridamole, did not significantly alter thrombotic response compared to vehicle (438±87seconds and 482±110seconds, respectively). This alteration in *in vivo* response corresponded with *ex vivo* aggregation studies in which samples from mice treated with PCD demonstrated significantly lower aggregatory responses than samples from vehicle-treated animals (AA, 62±10% vs 22±16%; collagen, 53±9% vs 22±9%; PAR-4 amide, 37±9% vs 7±4%; U46619, 48±11% vs 13±6%; $p<0.05$ for all, n=5-8).

Conclusion: Our studies demonstrate that the efficacy of prasugrel can be significantly potentiated through combination with cinaciguat and dipyridamole. In this way it is possible to achieve strong anti-platelet effects at lower doses than required when the drugs are used individually. This lower dose combination represents a novel anti-thrombotic therapeutic regimen by which it may be possible to achieve focused anti-platelet effects with reduced side effects.

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P06 **Stuart Wallis**

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Extracellular fibrinogen binding protein: deriving a novel anti-inflammatory agent

Co-author(s): Ian Eggleston, Giordano Pula, Stefan Bagby

Platelet-leukocyte complexation via P-selectin-PSGL-1 interaction is essential for the initiation of inflammation. Neutrophils trigger inflammation by recruiting platelets to vascular surfaces. The recruited platelets play a major role in facilitating the enrolment of aberrant leukocytes to these vascular surfaces; this can contribute to a range of cardiovascular pathologies including atherosclerosis. Controlling platelet-leukocyte complexation by disrupting P-selectin-PSGL-1 interaction could restrict the growth of atherosclerotic plaque, alleviating inflammatory damage of blood vessels. Efb (Extracellular fibrinogen binding protein), a Staphylococcus aureus protein, has been shown to bind to P-selectin, disrupting P-selectin-PSGL-1 and thus platelet-leukocyte interaction.

Determining the smallest Efb fragment that can still disrupt the interaction between P-selectin and PSGL-1 is one possible route to designing an Efb-based platelet-leukocyte interaction inhibitor.
Four peptides were used from the N-terminal of Efb, the region involved in the binding interaction with P-selectin. Each Efb peptide was FITC-labelled and incubated with platelets. Flow cytometry was used to determine which peptide binds most strongly to platelets, narrowing down the active sites of the protein. The results suggest two binding sites and support previous literature suggesting two binding mechanisms.

Those peptides with the highest affinity to platelets will be tested for their capacity to inhibit the P-selectin/PSGL-1 interaction. The next step will be to elucidate structural, biochemical and biophysical details of this interaction. The subsequent phase will use the interaction analyses to guide mutations of the peptides whilst retaining their inhibitory capacity of the P-selectin-PSGL-1 interaction and to test their efficacy.

Finally, the peptides with the highest inhibitory efficacy will be tested to see if they inhibit platelet-leukocyte complex formation in human whole blood, in resting and vascular flow conditions, and how effectively they do so. We aim to develop an agent that slows down or even prevents development and progression of major cardiovascular pathologies associated with vascular inflammation and atherosclerosis.

P07 **Lloyd Atkinson**

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Cyclic Nucleotide induced platelet reversibility is matrix type and concentration dependent

Co-author(s): Yusra Ahmed, Mohammed Yusuf, Steve Thomas, Khalid Naseem and Simon Calaminus

Vascular extracellular matrix components and their biophysical properties are critical in haemostasis. Alteration in matrix components in arteries is linked with arterial stiffening and excessive thrombus formation. Importantly, we have shown that part of the mechanism to control thrombus growth is cyclic nucleotide-mediated inhibition of the actin cytoskeleton in activated platelets via stress fibre dissolution.

This causes structural weakness in the thrombus in the high shear arterial environment, leading to thrombus embolisation and reduction in thrombus size *in vitro*. Interestingly, we now show that alterations in matrix concentration alter the reversibility of platelets to both prostacyclin (PGI_2) and nitric oxide (NO). On high density (100□g/ml) immobilised fibrinogen, PGI_2 or NO reversed platelet stress fibre formation and induced a reduction in platelet surface area. However, platelets spread on low density (3□g/ml) fibrinogen, were larger in size in agreement with previously published data, but also did not induce actin nodules nor reverse their stress fibres or reduce their surface area in response to PGI_2 or NO. The differences in the reversibility of platelet spreading by matrix concentration changes is not dependent on the secondary mediators ADP and TxA_2. Importantly if cAMP increases were induced by forskolin, reversal of stress fibre formation was achieved on both high and low density fibrinogen.

Critically not only was reversal of platelet matrix concentration dependent, but also matrix type dependent, as platelets spread on collagen, both in the absence and presence of ADP and TxA_2, did not reverse their stress fibres, nor reduce their surface area. This indicates that platelet reversibility to cyclic nucleotides is matrix type and concentration specific, indicating that alteration of the matrix components of the arteries could dramatically affect platelet responses to key platelet inhibitory signalling.

P08 **Ahmed Muhammad Usman**

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Targeting GPVI in a growing thrombus promotes disaggregation

Co-author(s): Nicolas Receveur, Martine Jandrot-Perrus, Christian Gachet, Pierre Mangin

Background: The platelet specific collagen and fibrin(ogen) receptor GPVI, is considered as a potentially safe anti-thrombotic target. So far, it has been reported that the absence or blockade of GPVI prevents thrombus formation in several experimental models of arterial thrombosis. However, the effect of anti-GPVI agents on thrombi already formed has never been assessed. Evaluating the impact of anti-GPVI agents on preformed thrombi appears of importance to delineate their potential mechanism of action in therapeutic use.

Method: This study was based on an *in vitro* flow-based model of arterial thrombosis in which aggregates were pre-formed by perfusing human whole blood through collagen coated microfluidic devices.

Results: Real-time video-microscopy showed that the GPVI-blocking fragment (Fab) of the antibody ACT017, promotes efficient disaggregation of thrombi preformed on collagen. ACT017-induced disaggregation was sensitive to rheological conditions as it occurs under arterial (>300 s^{-1}) but not venous (150 s^{-1}) blood flows and gradually increases with wall shear rate to reach a maximum at 3,000 s^{-1}. ACT017-induced disaggregation was not the result of steric hindrance due to antibody binding, since another anti-platelet antibody, ALMA.16, which binds GPIX had no effect. Moreover, a non-blocking anti-GPVI Fab fragment, 3J24, did not promote disaggregation, suggesting that the effect of ACT017 resulted from its ability to block ligand binding to GPVI. Surprisingly, soluble GPVI-Fc did not significantly promote thrombus disaggregation when compared to ACT017, suggesting that directly blocking the target is more efficient than using a competitive approach. Both real-time video-microscopy and nanoscale imaging by scanning electron microscopy indicated that ACT017 promotes loosening of the aggregates with individual platelets losing their contracted shape. This result suggests that the desaggregating effect of ACT017 results from impaired platelet activation during thrombus growth. In agreement, we observed that blockade of several key actors of GPVI signaling, namely src-kinases, Syk, PI3K or Phospholipase C, resulted in a similar kinetic of disaggregation.

Conclusion: This study shows that blockade of GPVI with the blocking antibody ACT017 promotes thrombus disaggregation in whole blood under flow.

P09 **Silvia Barbieri**

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Subchronic stress unveils prothrombotic phenotype in BDNFVal66Met heterozygous mice

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Traditional risk factors are not able to fully explain the occurrence and progression of cardiovascular disease (CVD), and environmental stressors, physical and psychosocial, are now identified as new potential risk factors for this pathology (1).

Under stressful conditions, the hyperactivation of the hypothalamic–pituitary–adrenal (HPA) axis and stimulation of the sympathetic adrenomedullary system lead to enhanced release of cortisol and catecholamine in the circulation (2). These hormones usually play a key function in acute stress adaptation (3), but when stress exposure persists they stimulate megakaryo- and thrombopoiesis, induce platelet activation (4,5) and endothelial dysfunction, enhance coagulation pathway activation and reduce fibrinolysis (6), predisposing to CVD and thrombosis. In addition, stressors can also reveal the phenotypic expression of such susceptibility genes, summarizing the central concept of gene-environment interaction (GxE) in multifactorial diseases (7).

In this scenario, a single nucleotide polymorphism of BDNF (BDNFVal66Met) associated with mood disorders and with acute coronary syndrome has also been related to increased susceptibility to stress (8-11). In particular, BDNF heterozygous Met (BDNF+/Met) mice display anxiety/depressive-like behavior phenotype only under stressful conditions (12). However, the interplay between stress and BDNF+/Met in relation to thrombosis is completely unknown.

Here, we showed that adult BDNF+/Met mice display a greater propensity to arterial thrombosis than WT mice after seven days of subchronic restraint stress. Subchronic stress markedly increased the number of leukocytes and platelets, and it induced hyper-responsive platelets, increasing the percentage of circulating platelet/leukocyte aggregates and enhancing the expression of P-selectin and GPIIbIIIa in heterozygous mutant mice. In addition, stressed BDNF+/Met mice have a greater number of large and reticulated platelets but comparable number and maturation profile of bone marrow megakaryocytes compared to stressed WT mice. Interestingly, subchronic stress led to a significant reduction of BDNF expression accompanied by an increase of tissue factor activity in the aorta of both BDNF+/Met and WT mice.

In conclusion, we provide evidence that stress unveils prothrombotic phenotype in heterozygous Met mice. Human studies will be crucial to understand whether this GxE interaction need to be taken into account in risk stratification of CAD patients.

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P10 **Anne Dominique Terrisse**

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Profiling of phosphoinositide molecular species in resting and activated human and mouse platelets by mass spectrometry: unprecedented insights into lipid signaling

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Phosphoinositides are bioactive lipids playing critical roles in the regulation of cell signaling, cytoskeleton reorganization and membrane dynamics. Their metabolism is highly active during blood platelets activation. Two well identified pathways involving phospholipase C and phosphoinositide 3-kinases (PI3K) have been shown to be strongly involved in platelet activation. Using a sensitive high-performance liquid chromatography-mass spectrometry method recently developed, we monitored for the first time the profiling of phosphatidylinositol (PI), PIP, PIP2 and PIP3 molecular species (fatty-acyl profiles) in human and mouse platelets during the course of stimulation by thrombin and collagen-related peptide. Moreover, using class IA PI3K p110a or p110b deficient mouse platelets and a pharmacological inhibitor, we show the crucial role of p110b and the more subtle role of p110a in the production of PIP3 molecular species following stimulation. Our study reveals new information on phosphoinositides biology, similarities and differences in mouse and human platelets and unexpected dramatic increase in low-abundance molecular species of PIP2 during stimulation. In conclusion, this comprehensive platelet phosphoinositides profiling provides important resources for future studies and open new perspectives in phosphoinositide signaling in platelets.

P11 **Nagy Béla**

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Everolimus suppresses endothelial cell activation via transcriptional and post-transcriptional regulatory mechanisms after drug-eluting coronary stenting

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Background: We have previously detected higher level of platelet and endothelial cell (EC) activation in patients who suffered from in-stent restenosis after bare-metal stenting compared to subjects who underwent drug-eluting stenting (DES) showing no complications (Szük T et al., Platelets, 2016; 27:410-9.).

Aims: In this study, we investigated the potential transcriptional and post-transcriptional regulatory mechanisms by which everolimus suppresses EC activation after DES intervention.

Methods: We studied the effect of everolimus on E-selectin (SELE) and VCAM1 mRNA and protein levels when human coronary artery (HCAECs) and human umbilical vein ECs were challenged with recombinant TNF-α (100 ng/mL) for 1-24 hours in the presence or absence of everolimus using 0.5 μM concentration locally maintained by DES. EC activation was evaluated via the levels of IL-1β and IL-6 mRNAs with miR-155 expression by RT-qPCR as well as the nuclear translocation of nuclear factor kappa beta (NF-κB) p65 subunit detected by fluorescence microscopy. To investigate the transcriptional regulation of E-selectin and VCAM-1, TNF-α-induced enhancer RNA (eRNA) expression at p65-bound enhancers in the neighboring genomic regions of SELE and VCAM1 genes, including SELE_-11Kb and VCAM1_-10Kb, were measured in HCAECs. Mature and precursor levels of E-selectin and VCAM-1 repressor miR-181b were quantified to analyze the post-transcriptional regulation of these genes in HCAECs. Circulating miR-181b was analyzed in plasma samples of stented subjects by stem-loop RT-qPCR, and its levels were statistically correlated with soluble E-selectin and VCAM-1 concentrations by Pearson test.

Results: We found that TNF-α considerably elevated E-selectin and VCAM-1 mRNAs at transcriptional level in ECs compared to baseline samples. In contrast, everolimus in the presence of TNF-α lowered these mRNAs and the relevant protein expressions measured by ELISA in HCAECs ($P<0.001$). Furthermore, this drug decreased EC activation via attenuated IL-1β and IL-6 expression. The levels of mature, pre- and pri-miR-181b were repressed in ECs by TNF-α, while everolimus acted as a negative regulator of EC activation via inhibited translocation of NF-κB p65 subunit into cell nuclei, lowered eRNA expression at SELE and VCAM1 genes-associated enhancers ($P<0.05$) and modulated expression of their post-transcriptional repressor miR-181b ($P<0.05$). Significant negative correlation was observed between plasma miR-181b and soluble E-selectin ($r=-0.375$, $P=0.049$) and VCAM-1 ($r=-0.441$, $P=0.019$) in stented patients.

Conclusions: Everolimus directly attenuates EC activation via reduced NF-κB p65 translocation causing decreased E-selectin and VCAM-1 expression at transcriptional and post-transcriptional level after DES implantation.

This study is supported by the GINOP-2.3.2-15-2016-00043-IRONHEART project.

P12 **Bernadett Szilagyi**

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Modulated level of microRNAs in platelets and megakaryocytes via altered Dicer1 level among septic conditions

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Background: We have recently described lower expression of activation-related microRNAs (miRNAs) in platelets that might contribute to their elevated activation status in sepsis. However, the regulatory mechanisms of abnormal platelet miRNA levels as well as the relevant clinical conditions are largely unknown.

Aims: In this study, we examined whether Dicer1 level is altered in platelets of septic patients that modulate mature miRNA expression. In parallel, Dicer1 was analyzed in stimulated platelet samples *in vitro* as well as in megakaryocyte (MK) cell cultures after different inflammatory treatment.

Methods: Dicer1 mRNA levels were first investigated in leukocyte-depleted platelet samples obtained from 10 individuals suffering from sepsis and in 12 age- and gender-matched clinical controls. We quantified miR-223 and miR-26b levels with their target P2RY12 and SELP (P-selectin) mRNAs by RT-qPCR, respectively, in patient samples. These measurements were also performed in stimulated platelets by lipopolysaccharide (LPS) (O55:B5, 100 ng/mL) in the presence of lipoprotein binding protein (100 ng/mL) and soluble CD14 (150 ng/mL), or tumor necrosis factor-α (TNF-α, 100 ng/mL) for 90 min, and as non-inflammatory control, PAR1 agonist TRAP (30 μM) was used for 30 min. The effect of sepsis in MKs was then analyzed via mature and precursor miRNAs (pre-miRNAs) as well as Dicer1 levels in MEG-01 and matured K562 MK cells after those stimuli above for 1-24 hours. Finally, to model the role of Dicer1 function in sepsis, its level was downregulated by transfection of Dicer1-specific siRNA (40 pmol, Ambion) for 48 hours in comparison to negative control samples with NEG-01 siRNA. Inflammatory conditions of cells were evaluated by miR-155 expression and IL-1β/IL-6 mRNA level by RT-qPCR and via nuclear translocation of p65 subunit of NF-κB pathway by fluorescence microscope. To prove the effect of LPS, TLR4 expression was also observed on platelets and MKs by flow cytometry and fluorescence microscope.

Results: Septic conditions resulted in elevated miR-155 and IL-1β or IL-6 mRNA levels in platelets and MKs in the *ex vivo* and *in vitro* experiments ($P<0.05$) via TLR4 receptors that could be detected on the surface of both cell types (platelets: 3-7%; MKs: 8-12% positivity). Platelets obtained from septic patients and *in vitro* stimulated samples by LPS or TNF-α showed lower Dicer1 mRNA levels resulting in decreased miR-223 and miR-26b with upregulated P2RY12 and SELP mRNA levels ($P<0.05$) similarly to TRAP activation. Furthermore, LPS-treated MK cultures showing substantial p65 translocation into nuclei demonstrated less Dicer1 level causing attenuated mature miR-223 and miR-26b, while Dicer1-independent miR-451 expression was not altered. Interestingly, pre-miRNAs were also decreased in these septic samples.

Conclusions: Abnormal Dicer1 level is produced by septic milieu that modulate the levels of certain platelet and MK miRNAs in sepsis. Further investigation is being performed to analyze this enzyme alteration at protein level.

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P13 **Sanne Brouns**

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Glycoprotein VI-dependent interactions of (anti)coagulation pathways in platelet-fibrin thrombus formation under flow: alterations in patients with factor V deficiency

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Background: Platelet activation and coagulation are linked by mechanisms that are not completely understood. Here, we studied the interactions of platelet responses and (anti)coagulation mechanisms during platelet-fibrin thrombus formation under defined flow conditions.

Methods: Recalcified human blood was perfused at defined shear rates over nine microspotted surfaces, five of which allowing platelet activation via glycoprotein VI (GPVI). Kinetics of platelet adhesion and activation as well as thrombus and fibrin formation were assessed in real-time by multicolor fluorescence microscopy.

Results: Blood perfusion over microspots with(out) tissue factor (TF) per run resulted in 82 parameters over time of platelet adhesion, ensuing platelet activation, and thrombus and fibrin clot formation. Co-coating with TF ensured maximal enhancement, whereas thrombomodulin or activated protein C (APC) delayed the processes of platelet-dependent clotting. Both at high and low shear rates (1000/s or 150/s), GPVI-activating surfaces were most active in enhancing the parameters of platelet activation and coagulation. Suppression of either the extrinsic (factor VII/ tissue factor) or intrinsic (factor XII) coagulation pathway delayed the thrombus-formation kinetics, again most strongly for GPVI-activating surfaces. Modelling elucidated that the GPVI-activating surfaces performed the best to predict the presence of extrinsic and/or intrinsic coagulation triggering. On these surfaces we were able to characterize blood from patients with factor V deficiency. This revealed a clear loss of function in (the kinetic) parameters of thrombus buildup and fibrin formation, linked to both intrinsic and extrinsic triggering. Markedly, these changes were mostly compatible with but exceeded those in thrombin generation profiles measured in the patient plasmas.

Conclusions: This work provides the basis for a novel approach of multi-parameter phenotyping of platelet activation and coagulation sub-processes using flowing whole blood. The assay is optimized for assessment of loss-of-functions in platelet-dependent coagulation.

P14 **Katrijn Six**

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Cold storage increases the procoagulant capacity of Intercept treated platelets

Co-author(s): Rosalie Devloo and Hendrik B. Feys

Background: Cold storage at 4°C slows down bacterial growth and may thus be used to elongate the shelf-life of platelet (PLT) concentrates for transfusion as alternative to standard room temperature (RT) storage. Cold storage however has an impact on PLT quality because it induces shape change leading to slightly activated spherical PLTs. Although still functional, these PLTs are cleared from the patient's circulation faster than RT stored PLTs. While pathogen inactivation methods are being implemented globally, interest in the combined impact of cold storage and pathogen inactivation on PLT function is growing. This study investigated this combined effect using the Intercept pathogen inactivation method (Cerus Corporation, Concord, CA) in combination with cold storage.

Methods: Control untreated PLTs were compared with paired Intercept treated PLTs during either RT or cold storage (n=4). Surface receptor expression of GPIbα, P-selectin, phosphatidylserine and activated integrin αIIbβ3 (PAC-1) was investigated by flow cytometry. Platelet aggregation, thrombin generation and platelet adhesion with concurrent coagulation in a collagen-coated microfluidic flow chamber were also studied. Data were collected from both study arms before storage and after 3 days of storage either at RT with constant agitation or at 4°C without agitation.

Results : Intercept pathogen inactivation of RT stored PLTs caused the expected decrease in integrin αIIbβ3 activation and PLT aggregation. On the same line, cold storage of control untreated PLTs caused the typical changes including increased mean platelet volume, P-selectin and phosphatidylserine expression compared to standard RT storage. Cold storage only marginally increased the procoagulant properties of control untreated PLTs as shown by higher peak thrombin concentrations (264 nM at baseline *vs* 354 nM after 3 days cold storage, P=0.08) in a thrombin generation assay. However, when cold storage and Intercept pathogen inactivation were combined, the PLT procoagulant properties increased significantly with a peak thrombin concentration of 519 nM compared to 220 nM for Intercept treated PLT before storage ($P < 0.001$) and compared to 354 nM for RT stored untreated control platelets ($P = 0.01$). These data were confirmed in collagen-coated microfluidic flow chambers where the onset of coagulation by Intercept treated cold stored PLTs was faster (802 s) than for untreated cold stored PLT (952 s). This observation was unexpected because prior to storage Intercept treated PLTs were actually slower (1097 s) compared to untreated control PLTs (865 s) to promote coagulation. This cold-induced procoagulant effect may be a consequence of increased phosphatidylserine expression after cold storage of Intercept
treated *vs* untreated PLTs ($P < 0.05$) but cannot be a consequence of increased microparticle formation because the fraction of microparticles after cold storage was significantly lower for Intercept treated PLTs (11%) compared to untreated control PLTs (23%) ($P = 0.002$).

Intercept pathogen inactivation treatment generally affects PLT function, as was demonstrated here by a reduction in procoagulation using standard blood banking conditions. However, this reduction disappeared when stored at non-standard cold temperatures. The data thus suggest that cold storage can restore the coagulation potential of PLTs treated with Intercept pathogen inactivation.

P15 **Katrijn Six**

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GPIbα shedding is a consequence of damage to cryopreserved platelets, not a cause

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Background: Cryopreservation of platelet concentrates is under consideration as an alternative storage method to assure patient care in circumstances where liquid preservation is impossible. However, cryopreservation significantly damages platelets, including significant GPIbα ectodomain shedding. It is not clear if inhibition of GPIbα shedding is a cause or a consequence of the cryopreservation-induced damage.

Methods: Non-cryopreserved control platelets were compared to paired cryopreserved platelets (n=6) either with or without 10 µM of marimastat, a broad spectrum sheddase inhibitor. GPIbα shedding was monitored by flow cytometry and by western blotting of the GPIbα ectodomain (glycocalicin) in cell-free supernatant. Platelet function was investigated by ristocetin agglutination (1.25 mg/mL) and by platelet adhesion to immobilized collagen in microfluidic flow chambers at a shear rate of 1000 s^{-1}.

Results: Cryopreservation without marimastat caused a significant decrease of GPIbα expression in flow cytometry (1.0 x 10^5 MFI (median fluorescence intensity)) compared to 1.8 x 10^5 for non-cryopreserved controls (P = 0.02). As expected, marimastat efficiently conserved GPIbα expression after cryopreservation (MFI 1.8 x 10^5). In comparison to controls, the glycocalicin signal was increased 2.3-fold in cryopreserved platelets without marimastat, while remained stable at 1.1-fold in the presence of marimastat. These data indicate that marimastat is an efficient inhibitor of GPIbα shedding during cryopreservation. However, marimastat could not improve platelet agglutination after cryopreservation since no significant difference in agglutination amplitude is found with (29%) or without (32%) including marimastat (P = 0.40). In addition, adhesion of cryopreserved platelets to collagen under flow was significantly lower compared to non-cryopreserved controls (P = 0.05) irrespective of marimastat addition (P = 0.84).

Conclusion: We conclude that GPIbα shedding is a consequence of platelet cryopreservation and that inhibition of GPIbα shedding cannot improve cryoplatelet function in agglutination and adhesion experiments.

P16 **Jessica Cedervall**

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Platelets and Neutrophil extracellular traps (NETs) as mediators of cancer-associated organ failure

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Cancer is associated with systemic pathological effects that contribute largely to mortality, such as thrombosis and general organ failure. Insufficient kidney function, a frequent condition in cancer-patients, prevents efficient anti-cancer therapy and may contribute directly to mortality due to renal failure. The reason for cancer-associated renal insufficiency is not well explored, and the only treatment option available is dialysis.

We have found that tumor-induced formation of neutrophil extracellular traps (NETs) is a previously unknown cause of kidney injury in mice with cancer. Our previous data show that NETs, associated with activated platelets, accumulate in peripheral vessels of tumor-bearing mice and cause vascular hypoperfusion and neutrophil infiltration, indicative of renal dysfunction. Clinical parameters, such as impaired creatinine clearance, reveal that these mice indeed display signs of kidney injury. Removal of NETs by short-term treatment with DNase I or pharmacological inhibition of the enzyme peptidylarginine deiminase 4 (PAD4), required for NET formation, was sufficient to restore kidney function. Tumor-induced systemic inflammation and impaired perfusion of the peripheral vasculature was also reverted by treatment with the PAD4 inhibitor. In line with our previous observations, we have recently found that mice with cancer show signs of heart dysfunction, with upregulation of factors such as growth differentiation factor 15 (GDF-15) and B-type natriuretic peptide (BNP) in heart tissue. Removal of NETs by DNase I treatment or PAD4 inhibition could suppress the levels of these factors, indicating an improved heart function. It has previously been described that platelets promote NET induction during infectious conditions, but the role of platelets in cancer-associated NET formation remains to be explored. Preliminary data from our group suggest that platelets promote NET formation in tumor-bearing mice, since platelet depletion significantly suppress levels of NETs-associated markers in blood samples.

Our data indicate that NETs, or platelets due to their NET promoting role, could represent a therapeutic target to prevent organ failure in cancer patients.

P17 **Melissa Chan**

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Platelet reactivity in individuals over 65 years of age

Co-author(s): Ming-Huei Chen, Temo Barwari, Jennifer E. Huffman, Paul C. Armstrong, Peter Santer, Stefan Kiechl, Johann Willeit, Manuel Mayr, Andrew D. Johnson & Timothy D. Warner

Introduction: Platelets are central to the processes of myocardial infarction (MI) and thrombotic stroke. The incidences of these events increase with age. However, there are few studies of platelet function in older people. Therefore, using the Bruneck Study cohort, we have phenotyped platelet responses in an elderly population and determined whether there are associations between age and platelet reactivity.

Methods: Blood was taken into citrate (0.105M) vacutainers from fasted participants of The Bruneck Study. Platelet-rich (PRP) and -poor plasma were obtained by centrifuging whole blood at 175*xg* for 15 min and 12000*xg* for 2 min, respectively. Light transmission aggregometry (LTA) was performed in PRP stimulated by arachidonic acid (AA; 1mM), ADP (5 and 20µM), collagen (0.4, 4 and 10µg/ml), TRAP-6 amide (25µM) and U46619 (10µM), as well as modified Optimul aggregometry[1,2] (AA: 0.3-1.5mM; ADP: 1-30µM; collagen: 0.4-30µg/ml; epinephrine: 0.6-10µM; ristocetin: 0.1-1.5mg/ml; TRAP-6: 0.1-25µM; U46619: 0.01-10µM). Data were analysed after calculating % aggregation using multivariate analysis using R, where aspirin use was classified as final aggregation responses to 1mM AA in LTA as <30%. Data is reported as mean±s.e.m.

Results: Platelet reactivity was studied in 338 people (51% male) with a mean age of 76.1±7.1 years. The population had a low incidence of MI (4.7%), stroke (6.5%) and diabetes (6.2%). Aspirin was strongly correlated with lower platelet reactivity outcomes. When data was adjusted for aspirin use, there was no influence of age seen against any response except for that to the highest concentration of TRAP-6 using Optimul (25µM; beta=-0.087, se=0.030, p=0.004).

Conclusions: This is the first study showing platelet reactivity in a large, elderly and healthy population using traditional LTA and Optimul aggregometry with a broad range of platelet agonists. Our study shows that although aspirin affects platelet reactivity age does not. With further analysis, we will determine whether disease in aging contributes to changes in platelet reactivity.

P18 **Ingmar Schoen**

Royal College of Surgeons in Ireland

Morphometry of spread platelets reveals mechanical structure-function relationships

Co-author(s): Martin Kenny, Sebastian Lickert, Viola Vogel

Contractile forces generated in the platelet's actomyosin cytoskeleton are necessary for all steps during primary haemostasis, but the requirements for adhesion, aggregation, and compaction of the thrombus differ. We hypothesize that adhesion receptor identity and mechanotransduction are essential to differentiate between these needs and to shape ultrastructural rearrangements. To address this question, we developed a morphometric image analysis that extracts characteristic features of f-actin and vinculin which are representative of the geometry by which spread platelets generate and transmit tractions [1]. The majority of platelets adhering to fibrinogen or fibronectin aligned their actin cytoskeleton into parallel bundles, very different than on collagen or laminin. Blocking integrin αIIbβ3 binding by RUC-4 or eptifibatide led to more circular arrangements in a dose-dependent manner, as did a titration of the fibrinogen surface density, as well as decreasing substrate stiffness below ~10 kPa. The same characteristic circular arrangement was observed in platelets from a patient with Glanzmann Thrombasthenia that had 5-7% residual αIIbβ3 surface expression. Reducing myosin IIa activity by blebbistatin led to less bundling and a more irregular arrangement. Our results show that mechanotransduction by a high number of functional αIIbβ3 integrins is necessary to achieve a highly contractile phenotype that might allow efficient force generation and transmission during aggregation. The developed image analysis scripts are available and could help to standardize the analysis of platelet morphology between different labs.

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P19 **Marilena Crescente**

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Selective deletion of platelet COX-1 reveals pro-thrombotic effects of aspirin in mice

Co-author(s): Paul C. Armstrong, Melissa V. Chan, Matthew L. Edin, Jing Jiao, Tania Maffucci, Harriet E. Allan, Charles A Mein, Carles Gaston-Massuet, Graeme S. Cottrell, Nicholas S. Kirkby, Jane A. Mitchell, Darryl C. Zeldin, Harvey R. Herschman, Timothy D. Warner

Introduction: Aspirin is the cornerstone of anti-thrombotic protection, being regularly taken by millions of individuals. While it is well known that aspirin inhibits the enzyme cyclooxygenase (COX)-1 in platelets, it also has less understood actions at off-platelet COX-1 potentially limiting its effect. Distinguishing the platelet and off-platelet effects of aspirin has been so far hampered by the lack of proper mouse models. For this reason, we have developed for the first time a platelet COX-1 knockout mouse to replicate the effect of low-dose aspirin.

Results: COX-$1^{fl/fl}$ mice, generated by flanking the exons 3 and 5 of the COX-1 gene with lox P sites, were crossed with Pf4-Cre mice to obtain COX-$1^{fl/fl}$Pf4-Cre, carrying the platelet COX-1 deletion. COX-$1^{fl/fl}$Pf4-Cre mice had no COX-1 in platelets, while it was still expressed in the leukocytes, aortic endothelial cells and other tissues. Loss of COX-1 in platelets reduced platelet aggregation and TxA_2 synthesis but did not affect the production of endothelial prostacyclin. Mass spectrometry analysis identified eicosanoids with opposing effects on platelet and endothelial function which are either unique of platelet COX-1 activity or derive from other sources. Finally, by using a $FeCl_3$-induced carotid artery thrombosis model, we showed that loss of platelet COX-1 products prolongs the time for occlusive thrombosis. However, subsequent administration of aspirin, blocking the synthesis of eicosanoids from non-platelet sources, has a net pro-thrombotic effect.

Conclusion: Characterisation of our unique mouse model has revealed that platelet and non-platelet COX-1 activity affect eicosanoid pathways with contrasting effects on platelet and endothelial function. As a consequence, we report that aspirin has pro-thrombotic effects *in vivo* when acting at non-platelet sites.

P20 **Parvathy Sasikumar**

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SUMOylation: A post translational modification that regulates platelet function

Co-author(s): Gasper,R; Little,G; Ayiad, A .M; Kriek, N; Bye,A

Background: SUMOylation results in the covalent attachment of the small ubiquitin like modifier (SUMO) proteins to specific lysine residues via an iso-peptide bond. Reversible SUMOylation of proteins is controlled by an enzymatic cycle. SUMOylation has been shown to play roles in the range of cellular processes, including DNA repair and cell cycle control, and is implicated in diseases such as cancer and Alzheimer's disease.

Aim: To investigate whether protein SUMOylation regulates platelet function.

Method: Immunofluorescence microscopy, immunoprecipitation and western blotting was performed on human platelets using specific SUMO 2/3 antibodies. The role of protein SUMOylation in regulating platelet function *in vitro* was established using platelet aggregation, secretion and thrombus formation assays using SUMOylation inhibitor, Ginkgolic acid (25μM) on human platelets. Immunofluorescence, immunoprecipitation and Western blotting was performed on human platelets using specific SUMO 2/3 antibodies.

Results: In our study, the presence of components of the multi-step post translational modification SUMOylation were identified in megakaryocytic cell line, Meg01 and human platelets. Using immunoblot analysis we reveal that platelets and Meg01cells express the key enzymes for the SUMOylation cycle, SUMO-activated enzyme SAE2 (E1), SUMO conjugating enzyme (Ubc9, E2) and the de-conjugating enzyme SENP-7. We identified a number of SUMOylated proteins in platelets and found that SUMOylation increases upon activation with PAR-1 agonist TRAP6. SUMOylation inhibitor Ginkgolic acid (E1 enzyme inhibitor) inhibited the activation of platelets in response to stimulation with GPVI agonist (CRP-XL), and TRAP6, resulting in diminished secretion and aggregation. Exposure to SUMOylation inhibitor also reduced integrin $\alpha_{IIb}\beta3$ outside-in signalling and thereby reduced the ability of platelets to stimulate clot retraction. Thrombus formation on collagen, under arterial flow conditions was also decreased following inhibition of global SUMOylation. Using immunofluorescence microscopy and immunoprecipitation analysis of platelet SUMO proteins we have identified tubulin as substrate for SUMOylation. In the presence of ginkgolic acid, the rate tubulin depolymerisation during platelet activation is reduced indicating that SUMOylation may regulate tubulin protein function and thus platelet secretion and activation.

Conclusions: Our study demonstrates the SUMOylation may represent a new paradigm for the control of platelet function.

P21 **Katharina Cullman**

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Megakaryocyte and Platelet Production from iPS Cells by Inducible Overexpression of Supporting Factors

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Induced pluripotent stem cells (iPSC) are interesting sources for patient-specific cellular therapies. iPSC-derived platelets can be transfused and due to their ability to store proteins in their granules and release them after activation, they may represent a novel system for delivery of therapeutics.

Although differentiation of platelets from iPSC is possible, the recovered numbers are low. Megakaryopoiesis and platelet biogenesis are induced by expression of transcription factors (TF) and small GTPases. Therefore, to optimize MK and platelet output from murine iPSC, we overexpressed the TF Gata-1, Nfe2 and Pbx1, as well as the small GTPases RhoA and Cdc42 by gammaretroviral vectors. To avoid off-target effects during pluripotency and differentiation and to induce transgene expression at optimal time points during lineage specification, we generated iPS cells from mice that contain the transactivator in the Rosa26-locus and utilized Tet-inducible vectors. Differentiation of iPSC was initiated by embryoid body (EB) formation and hematopoietic commitment supported by addition of SCF and IL-3. Induced overexpression of Gata-1 during or after EB dissociation generated expandable CD41-positive cells by co-cultivation on OP-9 feeder cells and supplementation of Thpo. 5-10% of the cells co-expressed CD42d 2-3 weeks after starting the co-culture, confirming the megakaryocytic surface phenotype. Cytomorphological analysis identified the typical MK morphology with enlarged cells and multilobulated nuclei. Platelet numbers from Gata1-overexpressing cultures were 8-fold increased compared to GFP-expressing control cultures. Also the platelet phenotype with absence of a nucleus, different types of granules and the open canalicular system was shown by electron microscopy. Similarly, overexpression of Cdc42 generated MK with elevated surface expression of CD41 and CD42d while Nfe2, RhoA and Pbx1 expression did no influence MK and platelet output. The functionality of platelets is investigated in an in vitro flow chamber assay.

P22 **David Riley**

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Investigation of the role of coronin proteins in platelet function

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Background: Remodelling of the actin cytoskeleton is essential for platelet function including shape change and spreading. The actin-binding protein coronin modulates a variety of actin-mediated cellular processes including phagocytosis, macropinocytosis and cell locomotion. There are 7 mammalian coronins (1-7), of which 1-3 and 7 are present in platelets. Coronin 1 is expressed in cell types including glial cells and Langerhans cells and is abundantly expressed in all cells of the hematopoietic lineage except red blood cells. Coronin is a protein with a variety of functions in different cell types: ablation of coronin 1 leads to defects in macropinocytosis and phagocytosis in macrophages; a lack of cell survival via the calcium/calcineurin pathway in T cells; and increased apotosis in neurophils. However, ablation of coronin 1 in natural killer cells and mast cells revealed that this protein is dispensable for their function, although the later presented higher level of F-actin. Humans and mice with a loss of function mutation in the coronin 1 gene (coro1A) experience T lymphocytopenia and behaviour abnormalities. This is presented in humans clinically by diagnosis of attention deficit hyperactivity disorder and a rare type of severe combined immuodeficiency. Coronin binds actin and can modulate the functions of actin regulatory proteins important for platelet function including the Arp2/3 complex, cofilin and Rac1. Coronin 1, 2 and 3 are abundant in platelets but their roles have not been investigated.

Results: Western blot analysis showed that coronin 1, 2 and 3 are abundantly expressed in human and murine platelets. Coronin 1 and 3 associate with the Triton X-100 insoluble F-actin pellet and this association increases upon stimulation with thrombin or collagen. Subcellular fractionation revealed that coronin 1 is abundant in the cytosol and a fraction is membrane associated. Latrunculin B mediated actin depolymerisation causes most actin, but only some coronin to leave the membrane fraction, demonstrating that the association of coronin 1 to membranes is only partly mediated by actin. Immunoprecipitation revealed that coronin 1 and 2 bind to components of the Arp2/3 complex. Immunostaining of platelets revealed that coronin 1, 2 and 3 colocalise with F-actin at actin nodules and the cell cortex of spread platelets.

Conclusion: Coronins are abundant in platelets, associate with the actin cytoskeleton and bind to the actin modulating Arp2/3 complex, suggesting involvement in cytoskeleton remodelling upon agonist stimulation. Coronin is only part actin associated and a fraction remains in the membrane upon F-actin depolymerisation suggesting additional roles that are not actin-related. To gain insight into the functional role of coronin 1 in platelets we are characterising the effect of coronin 1 ablation on key processes like aggregation, spreading, platelet activation and secretion in a mouse model.

P23 **Shannen Deconinck**

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Inhibiting ADAMTS13 in a preclinical ovine left ventricular assist device model does not rescue the loss of high molecular weight von Willebrand factor multimers

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Background: Acquired von Willebrand syndrome has been linked to the bleeding diathesis observed in patients treated with an LVAD as these patients have a loss of high molecular weight (HMW) von Willebrand factor (VWF) multimers. At this meeting we show for the first time that by specifically blocking ADAMTS13, using the inhibitory anti-ADAMTS13 monoclonal antibody (mAb) 3H9, the loss of HMW VWF multimers was prevented in an in vitro Heartmate II™ and Impella CP® LVAD circuit with human blood. However, it remains to be determined if blocking ADAMTS13 is an effective novel therapy to rescue the loss of HMW VWF multimers in a preclinical animal model.

Aim: To investigate if an inhibitory anti-ADAMTS13 mAb can (1) prevent the loss of HMW VWF multimers in an in vitro Impella CP® system with ovine blood and (2) can rescue the loss of HMW VWF multimers in an in vivo preclinical ovine Impella CP® model.

Methods: Since our mAb 3H9 does not cross-react with sheep ADAMTS13, we used a novel inhibitory anti-ADAMTS13 mAb 17C7 (30 µg/mL), which potently inhibits both human and sheep ADAMTS13 and a non-inhibitory anti-ADAMTS13 mAb 5C11. Ovine blood was circulated through an in vitro Impella CP® system (n=4) and blood was sampled 5 minutes (min) before and 5, 30 and 60 min after start of the pump. Plasma samples were analysed for VWF multimers.
For the in vivo study, Impella CP® pumps were implanted in sheep (n=8). One dose of 600 µg/kg of the inhibitory mAb 17C7 (n=4) or phosphate buffered saline (PBS) (n=4) was injected 1 day after Impella implantation. Blood was sampled before implantation, 30 minutes and 1 day after pump implantation and 1 and 2 days after injection of mAb 17C7 or PBS. ADAMTS13 inhibition and the loss of HMW VWF multimers were determined.

Results: In vitro, control experiments (using mAb 5C11) led to a 54% reduction of HMW VWF multimers in the Impella circuit, 60 min after pump initiation ($p=0.003$). In contrast to our previous in vitro data with human blood and the mAb 3H9, blocking ovine ADAMTS13 using the mAb 17C7 did not prevent the loss of HMW VWF multimers in the in vitro Impella CP® circuit as HMW VWF multimers were still 42% decreased, 60 min after start of perfusion ($p=0.003$). The efficacy of mAb 17C7 was also tested in a preclinical Impella ovine model. As expected, HMW VWF multimers significantly decreased one day after Impella implantation (n=8, 55% decrease, $p<0.0001$) and HMW multimers remained decreased when PBS was injected (n=4, 53% decrease). However, in agreement with the in vitro experiments, injection of mAb 17C7 did not rescue the HMW VWF multimers (n=4, 57% decrease) although ADAMTS13 activity was blocked in all sheep (n=4, 8.2 ± 4.7% ADAMTS13 activity, 1 day after injection of 17C7 compared to 81.0 ± 53.6% before injection). To prove that the mechanism of shear induced VWF proteolysis is different between sheep and humans, we performed an in vitro Impella experiment with human blood and showed that addition of mAb 17C7 (as observed with mAb 3H9) did prevent the loss of HMW VWF multimers (13% decrease versus 70% with mAb 5C11).

Conclusions: In contrast to human blood, blocking ADAMTS13 activity in an in vitro Impella CP® circuit with ovine blood, could not prevent the loss of HMW VWF multimers. Although ADAMT13 activity could be inhibited in a preclinical ovine Impella model, the shear-induced proteolysis of VWF could not be rescued. Taken together, these data indicate that in sheep other mechanisms than ADAMTS13 are responsible for the loss of HMW VWF multimers.

P24 **Tomasz Przygodzki**

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Decreased CD61 expression on platelet membranes is a common characteristics of type 2 diabetic patients, streptozotocin-diabetic rats and *in vitro* model of hyperglycemia. Consequences for platelet adhesion

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Background: It is believed that diabetes is associated with an increased activation of blood platelets and endothelium. Adhesion of blood platelets to activated endothelium is considered an important step of development of atherosclerosis. It could be assumed that a prevalence of atherosclerosis in diabetic patients is due to the fact that diabetic hyperactive platelets are more prone to interact with vascular endothelium. Such a tendency of platelets has been described by our group in animal model of diabetes. The question remains to what extent hyperreactivity of platelets and endothelium dysfunction contribute to this phenomenon. To verify this we assessed ability of blood platelets from diabetic patients, diabetic animals and platelets exposed *in vitro* to high concentration of glucose to adhere in flow conditions to vWf and fibrinogen.

Methods: Blood platelets were collected from diabetic patients and from rats with steptozotocin (STZ)-induced diabetes. In another sets of experiments platelet-rich plasma from healthy human donors were incubated with either glucose or mannitol. Adhesion of platelets to fibrinogen and vWF was assessed with the use of VenaFlux (Cellix) system. Expression of CD61 on platelets was analyzed with flow cytometry. Concentrations of soluble CD61 were analyzed in plasma of diabetic patients and in plasma subjected to glucose *in vitro*. Extent of protein glycation was analyzed in platelets in the above experimental setups.

Results: No differences were found in adhesion of platelets from diabetic and non-diabetic individuals to fibrinogen and vWF. Adhesion of normal platelets exposed *in vitro* to glucose or mannitol was significantly lower than that of platelets incubated in normoglycemic conditions. Adhesion of platelets from diabetic rats measured after 1 and 5 months of diabetes was not different from non-diabetic animals, interestingly it was significantly lower after 2.5 months of diabetes.
Expression of CD61 on platelets of diabetic humans was significantly lower than in the case of non-diabetic ones. Complementary to these results was the finding that the concentrations of soluble CD61 in plasma of these patients were increased. Expression of CD61 on platelets incubated in hyperglycemic conditions and with mannitol became decreased and the plasma concentration of soluble CD61 was higher in the incubates. Proteins of platelets in diabetic patients and in platelets incubated in hyperglycemic conditions were glycated to higher extent.

Conclusions: Contrary to our expectations, adhesion of blood platelets to fibrinogen and vWf in flow conditions was not increased in diabetic humans, in STZ rats and in the *in vitro* model of hyperglycemia. Lower expression of CD61 on platelets with simultaneously increased soluble CD61 suggest enhanced shedding of this integrin in hyperglycemic conditions. This shedding may explain the lack of increased adhesion of diabetic platelets or even decreased adhesion in some experimental setups. Since the *in vitro* exposure of platelets to mannitol exerted similar effects on CD61 expression and on platelet adhesion as glucose, we suggest that the effect is partially due to osmotic alterations. We conclude that enhanced adhesion of platelets to endothelium in diabetic animals observed *in vivo* is driven by endothelial dysfunction rather than by altered function of platelets.

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P25 **Charlotte Dekimpe**

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A novel ADAMTS13 conformation ELISA shows conformational activation of rat ADAMTS13 with exposure of cryptic epitopes in vitro

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Introduction: Thrombotic thrombocytopenic purpura (TTP) is characterized by a severe deficiency in the multidomain metalloprotease ADAMTS13, consisting of a metalloprotease (M), disintegrin-like (D), cysteine-rich (C), spacer (S), 8 thrombospondin type 1 repeats (T1-8) and 2 CUB domains. Human ADAMTS13 can adopt different conformations and several monoclonal anti-T2-CUB2 antibodies (mAbs) are able to induce an increased proteolytic activity (FRETS-VWF73 assay) by opening ADAMTS13, and subsequently revealing cryptic epitopes in human ADAMTS13. Recently, our group has shown that ADAMTS13 adopts an open conformation in acute immune-mediated TTP. Therefore, animal models mimicking conformational changes in ADAMTS13 would be of great importance to further elucidate the pathophysiology of TTP.

Aim: We aimed at inducing conformational changes in rat ADAMTS13 using anti-ADAMTS13 mAbs and to develop an ELISA to discriminate between open and folded rat plasma ADAMTS13.

Methods: First, cross-reactivity of the activating anti-human ADAMTS13 mAbs 17G2 (anti-CUB1) and 19H4 (anti-T8) with rat ADAMTS13 was tested in ELISA. Next**,** rat plasma ADAMTS13 activity was determined in the absence/presence of the activiating mAb 19H4, using the FRETS-VWF73 assay. Thereafter, a panel of anti-ADAMTS13 mAbs recognizing cryptic epitopes in human ADAMTS13 were screened for cross-reactivity with cryptic epitopes in rat ADAMTS13 (pre-incubated with 19H4) using ELISA. Finally, an ELISA was developed to discriminate between rat plasma ADAMTS13 in the absence (**folded**) and presence (**open**) of the activating mAb 19H4.

Results: Of the activating anti-human ADAMTS13 mAbs, only 19H4 bound to rat ADAMTS13 in ELISA. In addition, mAb 19H4 induced a 2.3 fold increase in rat plasma ADAMTS13 activity in the FRETS-VWF73 assay. The cloned patient anti-S Ab I-9, which recognizes a cryptic epitope in human ADAMTS13, cross-reacted with rat ADAMTS13 but only after pre-incubation of rat ADAMTS13 with mAb 19H4. Finally, a novel rat ADAMTS13 conformation ELISA was developed, in which only open rat ADAMTS13 is captured by Ab I-9 e.g. upon pre-incubation of rat ADAMTS13 with mAb19H4, and detected by biotinylated crossreacting polyclonal rabbit-anti-mouse ADAMTS13 Abs.

Conclusion: Activating conformational changes can be induced in rat ADAMTS13 upon addition of the anti-T8 mAb 19H4. The folded vs open rat ADAMTS13 conformation can be discriminated in our novel in-house ELISA by using the anti-S Ab I-9, which recognizes a cryptic epitope in ADAMTS13. This unique tool can now be used to further investigate conformational changes in ADAMTS13 *in vivo* and to elucidate whether exposure of cryptic epitopes in ADAMTS13 can evoke an anti-ADAMTS13 immune response in the rat.

P26 **Viola Maria Popov**

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Evaluation of platelet membrane potential in assessing the risk of thrombosis in patients with chronic myeloproliferative neoplasms

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Background: Patients with chronic myeloproliferative neoplasms (MPNs) had qualitative and quantitative modifications of platelet membrane receptors that are involved in alteration of platelet function. These modifications of platelet functions determined thrombotic complications. The aim of our study was to determine if changes of platelet membrane potential could be correlated with alterations of platelet function and increased risk of thrombosis

Methods: This retrospective study included 170 patients with MPNs (97 female and 73 male), median age 61.1 (female-59.3, male 64.6) and 10 healthy volunteers. Mutational analyses identified in 109 patients presence of JAK2 mutation, 25 patients have CALR mutation presence and 36 patients have no mutation. The group of patients was splitted in group with thrombotic complications (heart infarct, pulmonary emboli, stroke, splanchnic thrombosis) represented by 51 patients and without thrombosis – 119 patients. The determination of platelet membrane potential was done by fluorescence method using 3,3'-dipropyl-2,2'-thiadicarbocyanine iodide (DiSC3-5)
Results and discussion Patients with chronic myeloproliferative neoplasms (MPN) have a higher membrane potential than controls (- 65 mV vs -57 mV), p=0.05. Patients with MPN and thrombotic complication present higher resting membrane potential compared with MPN patients without thrombosis (median value - 76 mV vs - 62mV). Thrombosis was present in 36 MPNs patients JAK2 positive, 1 MPN patient CALR positive and 14 MPN patients without any mutations. We do not obtain any difference in resting membrane potential in MPN group splitted by presence of JAK or CALR mutation presence.

Conclusions: The resting of platelet membrane potential is higher in chronic myeloproliferative neoplasms compared with controls, the difference being higher in patients with thrombotic complication. This hyperpolarization could be a result of Cl(-) entry through Cl(-) channels. The Cl(-) entry was identified as important factor in maintaining the driving force for Ca(2+) entry and phosphatidylserine exposure, an important step in starting of thrombotic process.

P27 **Timea Feller**

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Mechanical changes during clotting in platelet rich plasma

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Blood clotting involves spatially and temporally controlled mechanical changes associated with fibrin and platelets, where the underlying molecular mechanisms are still not fully understood. We developed a nano-thrombelastography method (nTEG), based on atomic force microscopy (AFM) that gives information about the viscoelastic properties and contractility of fibrin network during clot formation and degradation in human plasma.

Our aim is to investigate the effect of the presence of platelets on the evolution of the mechanical properties of the clot. Therefore we evaluated the nTEG method for nanomechanical characterization of platelet driven clot formed in platelet rich plasma (PRP).

An AFM cantilever was submerged into a 300-µL sample and moved cyclically up and down with an amplitude of 1 µm and a speed of 1 µm/s. The sample contained PRP and 10 mM Ca^{2+}. Clotting was initiated with thrombin or collagen at 1 IU or 5 µg/ml final concentration, respectively. As the sample clotted, a local microenvironment formed around the cantilever caused its deflection. PRP was prepared by centrifugation of the citrated blood at room temperature and 150g for 10 minutes. Blood was collected into Vacuette tube (sodium citrate 3.2%) from five untreated and eight coumarine treated individuals.

In each measurement we followed the changes in three parameters. The force difference of each vertical cycle characterizes the elastic properties of the formed network. The hysteresis loop informs us about the dissipated energy, hence about the viscous properties of the formed network. The contracting platelets retract the network that causes a constant deflection in the position of the cantilever. This third parameter gives us information about the function of the platelets.

The force difference of each cycle, calculated from the deflection signal (max signal 297 nN), increased with the formation of the elastic platelet-rich fibrin network. The delay until the first force-difference signal was 100-200 sec, and the delay increased three-fold in lieu of thrombin. The area of the hysteresis loop began to increase in advance of the force-difference signal, suggesting that the viscous properties were more prominent than the elastic properties in the early stages of clot formation. After activation, platelets retracted the clot that caused a constant deflection in the position of the cantilever in a platelet-number-dependent manner. By following the temporal evolution of this deflection, we gain information about the clot retraction.

We evaluated nTEG for platelet rich plasma samples and by the method the force generation during clot formation can be characterized. Coumarine doesn’t seem to have any effect on these parameters.

P28 **Gina Perrella**

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Monomeric GPVI and D-dimer: the variables to consider for the solution of a dilemma

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GPVI is a platelet glycoprotein receptor with a crucial role in thrombus formation. On resting platelets, GPVI is mostly present as a monomer and undergoes dimerization upon activation. The best known GPVI ligand is collagen which stimulates powerful platelet aggregation. Recently it has been shown that fibrin and fibrinogen can also bind to GPVI. While it is known that collagen binds to dimeric GPVI, there is a debate whether fibrin(ogen) binds to monomeric or dimeric GPVI, or not at all[1,2,3,4,5]. We aim to narrow down the variables that could cause these discrepancies.
In order to solve the conundrum about whether monomeric or dimeric GPVI binds to fibrin, we started with the most commonly performed assay: the ELISA binding assay. This allows us to detect binding between a protein coated on the surface and another one in solution. The structure of fibrin varies with the experimental conditions and this therefore provides a potential explanation for the variation in results. Fibrin has been reported to bind to GPVI through the D-dimer region[3] which has a more clearly defined composition. Further, D-dimer can also be used to estimate non-specific binding.
Commercial, plasmin-generated D-dimer competed with coated D-dimer lacking the stalk for binding to recombinant monomeric GPVI composed of the D1 and D2 immunoglobulin domains but lacking the stalk region[6]. The IC50 for displacement was approximately 350 nM. In contrast, there was no binding of dimeric GPVI to D-dimer. Revacept is a recombinant, dimeric form of GPVI containing the two Ig domains and the stalk region that is in Phase II clinical trial in patients with acute coronary syndromes. In agreement with the above, we did not observe specific binding of Revacept to D-dimer. In contrast, and in confirmation with previous reports, we observed binding of Revacept to immobilised collagen. D-dimer can be generated by digestion of fibrin with plasmin or trypsin. We therefore investigated the binding of monomeric GPVI lacking the stalk region to the trypsin-generated D-dimer. In contrast to the results with plasmin-generated D-dimer, binding of trypsin-digested immobilised D-dimer to monomeric GPVI was not displaced by soluble D-dimer suggesting that the binding is non-specific.
These results demonstrate that plasmin-derived but not trypsin-derived derived D-dimer binds to monomeric but not to dimeric GPVI and this is not influenced by the stalk. A complete understanding of the basis of the published differences will require sharing of reagents and mimicking the experimental conditions used by each laboratory.

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P29 **Renato Simoes Gaspar**

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Contribution of thiol isomerases and NADPH oxidases to overall reactive oxygen species production in platelets

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Background: Reactive oxygen species (ROS) are key components of signaling pathways in virtually all eukaryotic cells, acting either as second messengers or oxidants to target proteins. It has been reported that NADPH oxidases (Nox) and protein disulphide isomerase (PDI) may interact physically and functionally to fine tune ROS production. In platelets, both Nox and PDI contribute to platelet activation, even though the mechanisms by which these proteins interact on platelet membrane and whether they contribute to ROS production is poorly understood. Understanding how platelets produce ROS and ultimately how Nox and PDI interact might provide better tools to treat and prevent thrombotic events.

Aim: To determine the contributions of PDI and Nox to overall platelet ROS production and how these proteins interact on platelet membrane.

Methods: Human washed platelets (WP) were used in all experiments. ROS were quantified by two methods: fluorescence intensity after 30 min incubation with 20 μM DCFDA (excitation 495 nm and emission 525 nm) measured with spectrofluorimeter and median fluorescence intensity acquired using a BD Accuri C6 flow cytometer. Reductase activity was measured in whole platelets using a fluorescent oxidised glutathione coupled with eosin (Di-E-GSSG, excitation 510 and emission 545 nm) quantified using a spectrofuoremeter. All inhibitors were incubated for 10 minutes.

Results: Both specific (Bepristat 2a and CxxC peptide) and broad-spectrum inhibitors of PDIA1 reduced overall ROS production induced by collagen and collagen-related peptide (CRP), with broad-spectrum inhibitor causing a 50% greater inhibition than Bepristat 2a and CxxC peptide. Out of all Nox inhibitors tested, only ML 171, a Nox-1 specific inhibitor, decreased platelet ROS production, by a degree comparable to Bepristat 2a and CxxC peptide. Interestingly, Nox inhibitors ML 171 and Nox2ds-tat increased reductase activity on platelet membrane.

Conclusion: Overall, both PDI and Nox proteins contribute to ROS production, though the first seems to be of greater importance than the latter. Since the broad-spectrum thiol isomerase inhibitor had an effect that was greater than the selective inhibitors, it is possible that other proteins from the PDI family are important for ROS production. Finally, the increase in reductase activity seen with Nox and specific PDI inhibitors was counter-intuitive, providing novel insight into how Nox activity affects thiol isomerases and how different PDI inhibitors modulate overall reductase activity.

P30 **Simla Olgaç**

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Virtual Screening for Small Molecule Inhibitors of Glycoprotein VI (GPVI)

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Antiplatelet drugs in the market cannot be totally effective against occlusive blood vessels because of their side effects. Current limitations of antiplatelet therapy continuously trigger the search to develop safer, novel and potent antiplatelet agents without bleeding risk and side effects. One of new attractive targets in platelet receptors is Glycoprotein VI (GPVI) receptor interacting with collagen at an early stage of platelet activation [1]. To find GPVI inhibitors by testing millions of compounds *in vitro* takes too much time. Instead of this, it can be chosen to apply virtual screening against GPVI as a quick and easy way to screen whole compound library. In my project, a compound database, which contains seven millions of in-stock compounds [2], was virtually screened by using high performance computing (HPC) technologies and applied an e-pharmacophore model based on the GPVI antagonist published in the article after the filter such as Lipinski's rule of five (Figure 1). The filtered compounds were docked to GPVI's binding site (PDB Code: 2GI7) [3]. After these steps, the compounds which show similar structural interactions to newly published indole derivative, a new GPVI antagonist, were chosen to evaluate their *in vitro* antiplatelet activity. The studies about finding compounds that show significant inhibition to the GPVI agonist collagen, but don't show much inhibition to strong platelet inducer thrombin are under investigation. More studies are needed to develop specific antiplatelet drug candidates as GPVI inhibitors.

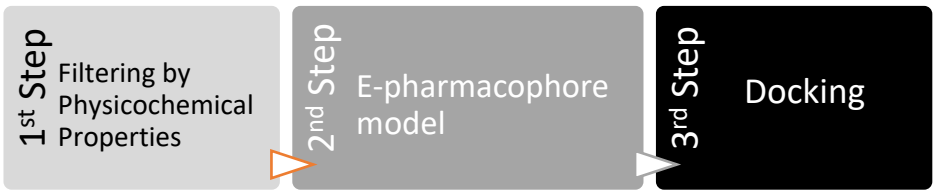

Figure 1. Multi-step protocol of virtual screening.

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P31 **Hamel Hadja**

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Predictive value of the 4Ts scoring system for heparin-induced thrombocytopenia

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Introduction: Heparin-induced thrombocytopenia type II (TIH II) is a rare complication related to heparin therapy, defined as a drop in platelet levels during heparin therapy. Thrombocytopenia is the consequence of the elimination of platelets on which the ternary immune complex has been fixed: IgG-heparin-chemokine (usually PF4). This is a descriptive retrospective study aimed at studying the interest of the 4Ts score in the suspicion of TIH typeII.

Patients: 240 patients treated with LMWH hospitalized in services: intensive care, cardiology

Methods: 1) Confirmation of thrombocytopenia: 2) Calculation of the score: is based on the careful study of the clinico-biological anamnesis that meets the 4Ts criteria. This score classified the patients into three levels of clinical-biological probability: low probability, intermediate probability and high probability.

Results: Of the 240 patients treated with LMWH, 07 patients (2.9%) had developed thrombocytopenia during their treatment, one with a high score, 05 patients with an intermediate score and one patient with a low score. Therefore, an intermediate or high score necessarily requires further investigation with the search for anti-PF4 antibodies and the performance of functional tests to confirm the accountability of heparin .

Our conclusion is consistent with a meta-analysis of Cuker A et al.

P32 **Marie Octave**

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The ACC inhibitor TOFA decreases platelet mitochondrial function and thrombin-induced activation

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Introduction: Acetyl-CoA carboxylase (ACC) is phosphorylated/inhibited by AMPK upon platelet stimulation but its role in platelets has never been investigated. ACC is a key regulator of lipid synthesis and oxidation.

Objective: Given the primary roles of lipids in platelet energy storage and signaling, we hypothesized that sustained ACC inhibition might have consequences on platelet bioenergetics and functions.

Methods: Platelets were treated with 30 µM TOFA, an ACC inhibitor, for 2 hours before thrombin stimulation. Mitochondrial oxygen consumption rate (OCR) was measured using the Seahorse Flux Analyzer. Platelet functions were assessed by aggregometry and flow cytometric studies.

Results: Indices of mitochondrial function assessed by OCR (pmoles/min/µg prot) before and after sequential injection of mitochondrial respiratory chain inhibitors (oligomycin, FCCP, rotenone/antimycin A) were decreased in TOFA-treated platelets relative to control (basal: control 9.01±0.53, TOFA 6.81±0.30; ATP-linked: control 7.79±0.65, TOFA 2.70±0.30; reserve capacity: control 8.19±1.37, TOFA 1.77±0.89; $p<0.05$). In addition, TOFA also suppresses mitochondrial function in response to thrombin, notably through a decrease in reserve capacity and ATP-linked respiration. These bioenergetics changes were accompanied by a significant defect in dense granules secretion and aggregation in response to low thrombin concentrations, whereas alpha-granules secretion was not affected, suggesting that the default in aggregation likely resulted from a lower autocrine effect of ADP. Underlying mechanisms involve reduced PKC activity, and in particular two PKC substrates activity, cytohesin-2 and PKD, known to control dense granule secretion and aggregation.

Conclusion: It is concluded that the inhibitory effect of TOFA on platelet secretion and on aggregation may be mediated by the reduction of mitochondrial energy production and PKC pathway activation.

P33 **Handtke Stefan**

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Ca^{2+} determines the functional capability of large and small platelets.

Co-author(s): Raghavendra Palankar, Andreas Greinacher, Thomas Thiele

Background: It is widely anticipated that large platelets are more reactive than small platelets because several studies found a stronger response of large platelets to stimulation by various agonists. However, many of these studies were performed in Ca^{2+}-free media only. As Ca^{2+} is important for platelet activation, we sought to determine the influence of the extracellular Ca^{2+} concentration and intracellular Ca^{2+} storage on the response of large and small platelets to TRAP.

Methods: Large and small platelet fractions were separated from healthy blood donors by differential centrifugation. Quantification of Ca^{2+} in intracellular stores was performed by flow cytometry using Fluo-4 AM single staining and cell size independent Fluo-4 AM/Fura Red double staining (1) after inhibition of SERCA 2b and SERCA 3 Ca^{2+}-pumps. Mobilization of Ca^{2+} from intracellular stores was measured using a Ca^{2+} release assay based on labelling of free intracellular Ca^{2+} with a Fluo-4 AM fluorescent dye. Platelet function was determined by CD62P expression in tyrode buffer without Ca^{2+} or in Tyrode buffer supplemented with 1 mM Ca^{2+} using flow cytometry as well as by light transmission aggregometry in citrated or hirudinated autologous plasma after TRAP stimulation. Each experiment was performed with platelets from at least 6 different subjects.

Results: The prepared large and small platelet fractions significantly differed in their MPV: 12.03 fl ± 0.88 vs. 7.76 fl ± 0.53, $p<0.0001$. Ca^{2+} content in the intracellular Ca^{2+} stores of large platelets is 40-45% higher in the dense tubular system ($p=0.0156$) and in the lysosome-like acidic organelles ($p=0.0469$) and large platelets mobilized more Ca^{2+} from intracellular stores after stimulation with TRAP (0.276 MFI ± 0.11) compared to small platelets (0.223 MFI ± 0.096; $p=0.0156$). In Ca^{2+}-free buffer, large platelets showed higher CD62P-expression after stimulation with TRAP compared to small platelets: 45.1 MFI ± 4.06 vs. 40.3 MFI ± 3.6; $p=0.0313$. In a Ca^{2+} supplemented buffer, no difference in CD62P-expression was observed between large platelets and small platelets (53.7 MFI ± 7.07 vs. small platelets 53.1 MFI ± 6.81, $p=0.9193$). Aggregation of large platelets was stronger (70% ± 9.97 vs. 52% ± 15.91 $p=0.0313$) and faster (5s ± 2 vs. 10s ± 2.3, $p=0.0305$) after TRAP-stimulation compared to small platelets in Ca^{2+} free medium. In Ca^{2+} supplemented buffer, both platelet fractions showed a similar response (80% ± 8.5 maximum aggregation, $p=0.1563$ and 5s ± 3.4 lag time, $p=0.5$).

Conclusion: We show for the first time, that large and small platelets have similar functional capabilities after activation of the thrombin receptor, when extracellular Ca^{2+} is available. The higher reactivity of large platelets upon TRAP stimulation is only present in Ca^{2+} free media and explained by increased Ca^{2+} mobilization in large platelets from intracellular stores.

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P34 **Marion Mussbacher**

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Pf4-promoter-mediated expression of constitutive active IKK2 decreases atherosclerosis in ApoE-deficient mice

Co-author(s): Manuel Salzmann, Waltraud C. Schrottmaier, Julia B. Kral-Pointner, Alice Assinger and Johannes Schmid

Atherosclerosis is a chronic inflammatory disease, strongly associated with hypercholesteremia and accumulation of immune cells within the intima of the vessel wall. Over the last decades, platelets have been identified as important modulators of this disease as they do not only facilitate infiltration of leukocytes via formation of platelet-leukocyte aggregates but also contribute to the generation of an inflammatory environment by the secretion of cytokines and chemokines from their respective granules. We have shown that mice expressing constitutively activate IκB kinase 2 (caIKK2) in platelets via a Pf4-promoter exert a hypo-reactive platelet phenotype that is associated with prolonged bleeding and impaired vessel occlusion after ferric chloride-mediated or mechanical injury.

To characterize this unique role of IKK2 in platelets in the context of atherosclerosis, we crossed these mice on ApoE KO background and fed them a hypercholesteremic diet. Analysis of aortas after 12 weeks of hypercholesteremic diet revealed significantly decreased plaque area in caIKK2^{PF4} mice that was associated with reduced plasma cholesterol levels. Moreover, caIKK2^{PF4} platelets formed less platelet-monocyte aggregates after activation, and showed less localization to the liver.
In summary, we were able to show that PF4 promoter-mediated expression of constitutively activated IKK can modulate the development of atherosclerosis presumably by reduced platelet/leukocyte aggregates; effects on liver inflammation and subsequently hepatic cholesterol metabolism.

P35 **Marta Hergueta-Redondo**

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Platelets from obese microenvironments modulate breast cancer tumor cell behavior

Co-author(s): Sara Sanchez-Redondo, Marina S. Mazariegos, Diego Megias and Héctor Peinado

Obesity has drastically increased to become one of the most serious health problems worldwide and is now recognized as a risk factor for breast cancer incidence, progression, and prognosis (1,2). We are investigating the local crosstalk between the adipose tissue and tumor cells, analyzing the role of platelets as systemic players in the metastatic process. The interaction of cancer cells with platelets is believed to confer a number of advantages that promote not only successful metastasis, also protection from immunological assault and evasion of immune supervision (3,4). Thrombosis and thrombocytosis are frequent complications of breast cancer patients and are associated with poor prognosis highlighting the importance of platelets in the pathology of cancer (5). Our laboratory is interested in understanding the mechanisms underlying the establishment of pre-metastatic niches and the contribution of platelets both in metastasis and in other pathologies.

In this project we aimed to understand if platelets derived from obese microenvironments could modulate metastatic behavior. Tumor cell-induced platelet aggregation has been previously reported to facilitate hematogenous metastasis by increasing the arrest of tumor cell emboli in the microcirculation (6). Confocal microscopy on co-cultured tumor cells and platelets showed that HFD-derived platelets increase their aggregation and interaction with AT3 and Eo771 mouse breast cancer cells. We next investigated whether obesity affects experimental metastasis via intravenous co-injection of tumor cells with lean or HFD-derived platelets. Our preliminary analysis using the Eo771 cell line (n=2), shows that metastasis is increase when Eo771 cells are co-injected with HFD-derived platelets. Importantly, platelets derived from ob/ob models also showed metastasis reinforcement (n=3).

Finally, we analyzed circulating platelets in Obese patients without cancer, and our preliminary data shows a strong correlation between IMC and platelet number, and we are currently recruiting breast cancer patients to analyze these interactions in human.

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P36 **Isuru Induruwa**

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Platelet receptor Glycoprotein VI-dimer is overexpressed in ischaemic but not haemorrhagic stroke. Results of the GlYcoProtein VI in StrokE (GYPSIE) study.

Co-author(s): Masaaki Moroi, Harriet McKinney, Carly Kempster, Joana Batista, Patrick Thomas, Willem Ouwehand, Kate Downes, Richard Farndale, Elizabeth Warburton and Stephanie Jung

Introduction: Currently, large artery atherosclerotic (LAS) and cardioembolic (CES) stroke aetiologies are treated using either antiplatelets or anticoagulation, creating a dichotomy in stroke treatment leaving many patients still at risk of stroke. Platelets, through the active form of platelet receptor glycoprotein (GP) VI; *the GPVI-dimer*, play a key role in thrombus formation in ischaemic stroke[1]. The unique ability of GPVI to bind both collagen and fibrin, the two substrates underpinning thrombus formation in LAS and CES respectively[2–4], suggests that its inhibition could represent a viable anti-thrombotic target in multiple stroke aetiologies.

Methods: We recruited 125 patients presenting to a large tertiary hospital in Cambridge, UK within 8h of acute ischaemic or haemorrhagic stroke. Each ischaemic stroke was then subclassified into a subcatagory according to its aetiology as per the TOAST [5] criteria [LAS, CES, small vessel occlusion (SVO), other, or undetermined]. Using flow-cytometry, patient blood was analysed for surface expression of total platelet GPVI (using HY101) and GPVI-dimer (using 204-11 Fab), as well as fibrinogen binding and P-selectin expression on resting platelets at day 0 and 90 post-stroke. Results were compared against that of a control population of 91 individuals with no pre-existing medical conditions.

Results: The mean age of the stroke population was 73.5±15.5 compared to the control population; 61.9±10.6 ($P<0.01$). Total GPVI surface expression was not significantly different between the two groups ($P=0.55$). However, the stroke population demonstrated overexpression specifically of GPVI-dimer at day 0 ($P<0.0001$) persisting to day 90 ($P<0.0001$). Resting, non-activated platelets from stroke patients exhibited higher P-selectin expression ($P<0.0001$) and fibrinogen binding ability ($P<0.0001$) compared to controls. Strokes subclassified into CES ($P<0.0001$), LAS ($P=0.002$) and small vessel disease ($P=0.009$) showed significantly raised GPVI-dimer expression compared to controls, whereas haemorrhagic stroke did not ($P=0.25$).

Conclusion: Stroke patients appear to exhibit more active resting platelets compared to a healthy population. Furthermore, they significantly overexpress GPVI-dimer specifically in all ischaemic stroke aetiologies, persisting up to 3-months post-stroke. Inhibition of GPVI-dimer, and subsequent platelet activation and thrombus formation, could represent a specific pharmacological target in ischaemic stroke.

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Trem-Like Transcript-1: a more sensitive marker of platelet activation than P-selectin in humans and mice

Co-author(s): Christopher W. Smith, Zaher Raslan, Lola Parfitt, Abdullah O. Khan, Pushpa Patel, Yotis A. Senis, Alexandra Mazharian

Background: Recent findings suggest thrombi can be divided into a core of tightly packed, highly activated P-selectin positive platelets, and a shell of loosely packed, P-selectin negative platelets. TREM-like transcript-1 (TLT-1) is an immunoreceptor tyrosine-based inhibition motif (ITIM)-containing receptor expressed in □-granules of platelets and megakaryocytes, which is rapidly upregulated to the surface upon activation. TLT-1 has been proposed to bind fibrinogen and facilitate platelet activation. However, TLT-1 function in thrombus formation and stability has yet to be defined.

Aims: To determine the expression of TLT-1 relative to P-selectin during platelet activation *in vitro* and thrombus formation *in vivo*, and to investigate the role of TLT-1 in haemostasis and arterial thrombosis.

Methods: TLT-1 and P-selectin surface expression were analyzed in activated mouse platelets and megakaryocytes *in vitro* by flow cytometry and confocal microscopy, and during thrombus formation following laser injury of cremaster arterioles and ferric chloride in carotids in mice. Tail-bleeding times and ferric chloride-induced thrombus formation in the carotid artery were assessed in TLT-1 deficient mice.

Results: TLT-1 is more rapidly and robustly upregulated to the surface of mouse and human platelets compared to P-selectin upon activation with thrombin and the GPVI-specific agonist collagen-related peptide. Collagen stimulation of megakaryocytes also resulted in a greater upregulation of TLT-1 compared to P-selectin. Similarly, TLT-1 was more rapidly translocated to the surface of activated platelets than P-selectin during laser-induced thrombus formation, with TLT-1 detected throughout thrombi, whereas P-selectin was restricted to a highly localized region directly adjacent to the site of injury. Interestingly, in $FeCl_3$-induced thrombosis model, the platelet core and shell are not discernible. However, consistent with our previous results, TLT-1 was detected much faster than P-selectin and is present throughout the entire thrombus. In platelets and megakaryocytes, TLT-1 and P-selectin are both present in platelet □-granules but some did not colocalize, suggesting differential distribution within □-granules or localization to other as yet unidentified compartments. Mice lacking TLT-1 exhibited a tendency for extended tail-bleeding times, however ferric chloride-induced thrombus formation was normal.

Conclusions: In conclusion, TLT-1 is a more sensitive marker of platelet and megakaryocyte activation than P-selectin, which can be detected in both the core and shell of thrombi *in vivo*. This opens the possibility of TLT-1 being utilized as a biomarker for early detection of platelet activation in various pathologies, including coronary artery disease and deep vein thrombosis, as well as megakaryocyte activation in the bone marrow in myeloproliferative disorders and myelofibrosis. Preliminary findings also suggest that TLT-1 facilitates haemostasis, but does not play a role in thrombosis. Further investigation is needed to elucidate the physiological function of TLT-1 in platelet aggregation and clot formation.

This work was supported by the British Heart Foundation.

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APAC, a dual antiplatelet and anticoagulant, attenuates platelet procoagulant activity and targets vascular injury sites

Co-author(s): Karina Barreiro, Riikka Tulamo, Mikko Parry, Marja Lemponen, Anders Albäck, Brian Cooley and Riitta Lassila

Introduction: Activation of platelets and coagulation is the key pathogenesis for occlusive thrombosis, including in association with vascular interventions. While managing local problems, systemic antithrombotics and their combinations predispose to bleeding complications. We have developed APACs, mimics of mast cell-derived heparin proteoglycans[1], which possess both antiplatelet and anticoagulant activities against collagen and thrombin in both *in vitro* and *in vivo* models.[2,3]

Aims: We further characterized APAC efficacy on 1) platelet aggregation in human blood and platelet-rich plasma (PRP) with co-administered acetylsalicylic acid (ASA) and global coagulation; and 2) *in vivo* targeting of injured porcine vessels and collagen-triggered thrombosis in mouse arteries.

Methods: 1) APAC-spiked citrated blood of healthy volunteers was studied in platelet aggregation via collagen (3.2 µg/ml), ristocetin (0.77 mg/ml) and ADP (6.4 µM) in Multiplate, and via collagen (0.5 or 2 µg/ml) in PRP, with and without ASA, and in rotational thromboelastometry (ROTEM) for intrinsic, extrinsic and fibrin activity in blood. 2) Porcine arterial balloon denudation and arterio-venous fistula (AVF) on femoral artery and vein were studied by exposing APAC-biotin (0.5 mg/ml) for 2 min to the injury sites before releasing blood flow. In collagen-induced thrombosis mice model (epigastric artery inserted in carotid artery) fluorescent-APAC (0.5 mg/kg IV) was studied for vessel targeting and inhibition of rhodamine 6G-labeled platelet accumulation.

Results: APAC dose-dependently prolonged lag time and decreased the slope of aggregation both in blood and PRP. In blood APAC (150 µg/ml) inhibited collagen- and ristocetin-, but not ADP-induced aggregation by 58±15% (mean±SD) and by 25±2%, respectively, unlike unfractionated heparin (UFH). In PRP APAC inhibited collagen (0.5 µg/ml)-induced aggregation dose-dependently (n=9): 1 µg/ml by 55±31%, 3 µg/ml by 75±15%, 10 µg/ml by 79±21%, and 30 µg/ml by 85±11%. APAC and ASA showed a synergistic effect on inhibiting platelet aggregation, requiring 2 µg/ml of collagen to induce aggregation. In ROTEM APAC (3 µg/ml) had a broad effect, attenuating fibrin elasticity and prolonging clotting 4.5-8-fold, in extrinsic and intrinsic pathways, while UFH (3 µg/ml) only prolonged the latter. APAC bound to both porcine balloon-injured arteries and AVF anastomosis, adjacent artery and vein, and co-localized with von Willebrand factor and laminin (>70%). Binding was limited when PECAM or podocalyxin was present (<30%). APAC targeted the collagen-exposing vessel in the mice model and reduced *in situ* platelet accumulation ($p<0.05$, n=6 vs. saline).

Conclusions: APAC inhibits collagen-, but not ADP-induced, platelet aggregation in citrated blood and PRP. APAC worked synergistically with ASA to inhibit collagen-induced platelet aggregation, suggesting a low-dose and safe administration to control local thrombosis at vascular injury sites. In ROTEM APAC globally attenuates platelet procoagulant activity and fibrin elasticity. APAC co-localized with multiple vascular matrix components, excluding PECAM and podocalyxin. APAC inhibits collagen-driven thrombosis in a mice model. Vascular interventions may benefit from the ability of APAC to provide simultaneous antithrombotic potential and local targeting and integration to injury sites.

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The Impact of Antiretroviral Therapy on Platelet Activation

Co-author(s): Kirk Taylor, Alan Winston, Michael Emerson

Abacavir (ABC) is a nucleoside reverse transcriptase inhibitor (NRTI) often administered with another NRTI, lamivudine (3TC), plus a 3rd antiretroviral (ARV) agent to treat HIV. ABC use has been linked with an increased risk of myocardial infarction, potentially mediated by altered platelet function.

A recent randomised controlled trial demonstrated that a switch away from ABC to a tenofovir alafenamide based regimen resulted in a decrease in platelet reactivity in response to collagen. In light of these findings, we aimed to investigate the pharmacological impact of antiretrovirals on platelets in the absence of confounding issues such as HIV infection.

Platelet rich plasma (PRP) and washed platelets from HIV negative donors were incubated with ARV agents at a range of concentrations above and below C_{max}, and platelet aggregation was assessed in response to collagen, TRAP-6, thrombin or ADP using a 96 well plate-based assay. A mouse model of thromboembolism was used to examine the effect of ARV agents on platelet responses *in vivo*.

There was no significant difference in platelet aggregation *in vitro* following incubation of platelets with ABC or 3TC and 3TC had no effect on platelet activation in response to collagen *in vivo*. Flow cytometric analysis is ongoing to establish the effect of ARV agents on markers of platelet activation.

These results suggest that ABC and 3TC do not have a direct pharmacological effect on platelet activation. Given that these agents are pro-drugs, further research is required to ensure concentrations of the active metabolites are clinically relevant. Additionnally as these drugs are administered as combination therapy, it is important to explore the effect of drug combinations on platelet activation.

P40 **Caroline Kardeby**

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Synthetic glycopolymers and natural fucoidans cause human platelet aggregation via PEAR1 and GPIb

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Fucoidans are sulfated fucose-based polysaccharides which activate platelets and have pro- and anti-coagulant effects and thus may have therapeutic value. In the present study we show that two synthetic sulfated α-L-fucoside-pendant glycopolymers (with average monomeric units of 13 and 329) and natural fucoidans activate human platelets through a Src- and phosphatidylinositol 3-kinase (PI3K)-dependent and Syk-independent signaling cascade downstream of the Platelet Endothelial Aggregation Receptor 1 (PEAR1). Synthetic glycopolymers and natural fucoidans stimulate marked phosphorylation of PEAR1 and Akt but not Syk.

Platelet aggregation and Akt phosphorylation induced by synthetic glycopolymers and natural fucoidan are blocked by a monoclonal antibody (mAb) to PEAR1. Direct binding of sulfated glycopolymers to epidermal like growth factor (EGF)-like repeat 13 of PEAR1 was established by AVEXIS technology. In contrast, synthetic glycopolymers and natural fucoidans activate mouse platelets through a Src- and Syk-dependent pathway regulated by C-type Lectin-like Receptor 2 (CLEC-2). Mouse platelets lacking the extracellular domain of GPIbα and human platelets treated with GPIbα-blocking antibodies have a reduced aggregation response to synthetic glycopolymers showing that binding to GPIbα facilitates the interaction with CLEC-2 or PEAR1, respectively. Our results establish PEAR1 as the major receptor for synthetic glycopolymers and natural fucose-based polysaccharides in human but not in mouse platelets. We believe that sulfated α-L-fucoside-pendant glycopolymers are unique tools for further investigation of the physiological role of PEAR1.

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Identification of Hsp47 collagen binding sequences that support platelet function.

Co-author(s): Parvathy Sasikumar, Dominque Bihan, Richard Farndale, Jon Gibbins

Background: HSP47 is a collagen binding chaperone protein found on the surface and within platelets, and the levels of surface exposure are elevated upon platelet activation. We have reported that inhibition of HSP47 using a small molecule inhibitor (SMIH) abolished collagen stimulated aggregation and reduced dense granule secretion in response to collagen and the GPVI specific agonist CRP-XL. Treatment with SMIH also reduced GPVI mediated signaling. Additionally, thrombus formation in mouse cremaster muscle arterioles was found to be reduced and bleeding times increased in platelet/megakaryocyte specific HSP47 deficient mice, all suggesting HSP47 plays a role in the regulation of platelet function in response to collagen.

Aim: To identify amino acid sequences within collagen to which HSP47 binds and to determine whether peptides containing these sequences are able to modulate normal functional responses.

Methods: His-tagged recombinant HSP47 was screened against a library of collagen toolkit peptides to identify potential binding sequences. Collagen toolkits are a series of overlapping peptides containing the primary sequence of Collagens II and III flanked by GPP sequences which maintain the triple helical structure of full length collagen. Anti-His antibodies were used to identify sequences that HSP47 bound to and these sequences were used to explore whether they were able to modulate functional responses. Selected sequences were used in a range of functional assays using washed platelets including adhesion, spreading and aggregation (using both standard light transmission and a plate-based end point assay). SMIH (10μM) was used to confirm whether these effects were mediated through HSP47.

Results: Toolkit peptides Coll II 13, 14, 20, 26 and Coll III 5a bound to His-tagged recombinant HSP47. Experiments to explore the ability of platelets to adhere and spread on these peptides were conducted and identified that platelets are able to spread on the peptides selected from the screen, a response which is inhibited using SMIH. Adhesion to Coll III 5a was partially inhibited using 6F1 antibody, which binds the α2-subunit of integrin α2β1 and blocks interaction with collagen. Platelets aggregate in response to Coll II 13, but none of the other peptides, an effect that was inhibited by SMIH.

Conclusion: HSP47 is able to bind peptides from the Collagen toolkit library, and these sequences are able to modulate platelet function selectively through HSP47, identified by the reduction in response after SMIH treatment. There was, however, significant variation in response to different peptides suggesting that the sequences responsible for the different effects are found along the length of the collagen fibers. Partial inhibition of platelet adhesion to Coll III 5a in the presence of 6F1 may suggest a role for α2β1 in the interaction between HSP47 and collagen. It is not known whether HSP47 interacts with other proteins on the cell surface in order to function. In future work the shortest sequence within collagen that supports platelet HSP47 binding function will be determined and will enable the mode of action of this protein on the cell surface to be established.

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TB-secreted antigens drive platelet-controlled tissue destruction in pulmonary tuberculosis.

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Introduction: Approximately 10.4 million people develop tuberculosis (TB) annually, resulting in 1.7 million deaths. Each year there is increasing drug resistance to first line therapies and there are few new drugs available. As a consequence, there is increased interest in host directed therapy and a need to understand disease mechanisms. Pulmonary TB is characterised by inflammation and immune-mediated tissue damage mediated by enzymes, particularly matrix metalloproteinases (MMPs). MMP-1, which degrades Type 1 collagen, the main structural component of the lung, and MMP-10, which activates MMP-1, are important in driving pulmonary disease. Although increasingly recognised as immune regulators, very little is known about the role of platelets in immunopathology in TB. We hypothesized that platelets have a key role in inflammatory tissue destruction in pulmonary TB.

Methods: Plasma samples were obtained from 50 TB patients before and during treatment, and from 50 age- and sex-matched healthy controls, in Lima, Peru. Platelet-derived markers were measured using Luminex multiplex assay. Ethical approval was obtained from the Universidad Peruana Cayetano Heredia and from participating clinical centres.
Next, fresh platelets from healthy donors were incubated with live, virulent *Mycobacterium tuberculosis* (M.tb) laboratory strain H37Rv, TB-derived secreted antigens, or control medium for 30 minutes. Platelet function was assessed using light transmission aggregometry. Monocytes were cultured with autologous platelets ±M.tb for 24h. Supernatants were analysed by ELISA and gene expression by qPCR. To assess functional MMP enzyme activity, M.tb-infected monocytes ±platelets were cultured for 24h on Type 1 collagen that fluoresces upon degradation, then evaluated using confocal microscopy.

Results: Baseline plasma platelet factor 4 (PF4) concentrations were significantly higher in TB patients than healthy controls (median 1,129 [IQR 1,769] vs 462.5 [IQR 693] ng/ml, $p<0.0001$). PF4 concentrations transiently increased at treatment day 14 and normalised by day 60 (612.7 [IQR 1,806] ng/ml, $p=0.073$ vs controls). CD40L, PDGF-BB, and PTX-3 concentrations followed similar trends.

Incubation with M.tb did not affect platelet secretion *in vitro* but soluble TB antigens increased PF4 secretion from 141.8 ±6.6 (SEM) to 255.8 ±13.8 ng/ml ($p=0.0017$). Functional platelet responses measured using light transmission aggregometry were altered in response to both bacterial lipopolysaccharide and TB antigens. Platelets significantly increased MMP-1 and -10 secretion from M.tb-infected monocytes from 231.9 ±29.1 to 1,820 ±59.2 pg/ml ($p=0.0017$) and from 530.8 ±45.3 to 1,341 ± 84.2 pg/ml ($p=0.014$) respectively. MMP-1 and -10 gene expression were similarly upregulated. Type 1 collagen degradation was minimal in M.tb-infected monocytes alone and greatly enhanced when platelets were present.

Conclusion: Platelet activity is increased in TB patients and normalizes with treatment. This is driven by TB-secreted antigens. Platelets drive tissue-degrading enzyme secretion by M.tb-infected monocytes, and these enzymes are functionally active. Platelets may contribute to TB immunopathology, and may be a potential therapeutic target to limit harmful inflammation in TB.

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Variation in platelet function in the healthy population

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Platelet activation and aggregation underpin both haemostasis and thrombotic disease. Platelet function in individuals is determined by the sum of multiple factors that inhibit platelets or increase their reactivity: external signals present in the plasma, e.g. PGI_2, NO, collagen or ADP, and intrinsic characteristics of the platelets such as the copy number and phosphorylation status of receptors and components of signalling pathways.

We are carrying out a cohort study to investigate the variation in platelet function in the healthy population and the contribution of different signalling pathways and biomarkers to this. In order to measure platelet activation in detail, we have developed and validated a standardised flow cytometry test for large scale platelet function studies measuring binding of fibrinogen to activated integrin $\alpha_{IIb}\beta_3$ and P-selectin exposure. This utilises pre-prepared 96-well plates containing serial dilutions of a panel of freeze-dried agonists to reduce variability and increase throughput and data density. To facilitate the analysis of the large data sets that are generated, we have developed scripts coded in R that output the data as dose-response curves to provide two distinct estimates of platelet reactivity for each agonist: sensitivity to the agonist (EC_{50}) and magnitude of the response (maximum gain) that are summarised as dot-plots or within spreadsheets. The code has been developed in a modular fashion with the advantage that the plate layout can be changed without the need to change the code.

We have recruited healthy, fasted volunteers aged between 30 and 65 years of age and measured a set of physical parameters such as BMI, body fat composition and blood pressure. In addition to detailed platelet function, we have measured metabolic markers in the serum such as fasting glucose, insulin, cholesterol, triglycerides, HDL and LDL. We have also analysed by flow cytometry expression levels of a panel of important receptors and signalling molecules on the platelet surface.

To validate our methodology, we have tested our data for correlations between GPVI levels on the platelet surface and activation by Collagen-Related Peptide (CRP) as Jones *et al* (2007) have previously reported a positive correlation for these factors. We were able to confirm this correlation between GPVI levels and EC_{50} for CRP. We also found that surface expression of CD148, a receptor-like tyrosine phosphatase that has been identified as a global regulator of platelet activation (Senis *et al,* 2009), positively correlated with maximum gain for CRP, whereas levels of tetraspanin (CD9), a scaffold protein that interacts with GPVI in the membrane (Protty *et al,* 2009), positively correlated with both EC_{50} and maximum gain for CRP.

In summary, we have developed and validated a flow cytometry based method that uses pre-prepared assay plates to allow high-throughput, reproducible and detailed characterisation of platelet function in individuals. This standardised test has the potential to be utilised to aid diverse clinical and basic research studies.

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Oxidized low-density lipoprotein modulates the activity of RhoGTPases altering the reversal of platelet activation to PGI_2

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Atherosclerosis is a major element in cardiovascular disease and a significant health concern in developed countries. Part of the mechanism by which platelets are linked to this process is via their activation by oxidised Low Density Lipoproteins (oxLDL), which drives both atherogenesis and thrombosis. However, the mechanism by which oxLDL drives platelet activation and thrombosis is unclear.

The combinations of matrix protein within the vascular wall and within the thrombus, especially if oxLDL is present can affect the size and strength of the thrombus formed. We have previously shown that platelets bind effectively to fibrinogen, but that this can be reverse by prostacyclin (PGI_2). We now demonstrate that the combination of fibrinogen and oxLDL as a matrix protein induced a significant increase in platelet adhesion and spreading in a dose-dependent manner. Furthermore, we identified that oxLDL partially blocked the reversal of spread platelets by PGI_2, as the surface area of the platelet was unaffected in contrast to those platelets spread only on fibrinogen. This suggested that oxLDL modulated the reversal of platelet spreading by PGI_2. However, oxLDL mediated inhibition of surface area reduction by PGI_2 was independent of the secondary mediators ADP and TxA_2.

The inhibitory effect of oxLDL on platelet reversal (surface area unaffected, whilst stress fibres were reversed) indicated that the RhoGTPases were differentially controlled within the platelets, with Rac activity maintained whilst Rho was inhibited. Therefore, platelets were spread in the presence of the Rho kinase inhibitor Y27632 and the Rac inhibitor, NSC23766. We found that surface area of platelets is reduced in the presence of NSC23766 compared to Y27632. These data identify addition of oxLDL to the vasculature induces a reduction in sensitivity to PGI_2, through differential control of RhoGTPases, potentially increasing the chance of excessive thrombus formation.

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MicroRNA-126 is a regulator of platelet-mediated thrombin generation

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I**ntroduction:** Platelets are an important source of microRNAs (miRNAs). Platelet miRNA expression profiles correlate with platelet reactivity, which raises the possibility of finding novel miRNA-based therapeutic targets and disease biomarkers for the recurrence of cardiovascular events in patients. MiR-126, the second most abundant miRNA in human platelets, is among the most promising candidates in this regard. However, the impact of miR-126 on thrombus formation is unknown.

Methods: We generated transgenic zebrafish with thrombocyte-specific overexpression of miR-126 as well as the green fluorescent protein (Tg(*cd41*:EGFP)). Laser injury of the caudal vein of 5 day-old larvae was performed with or without anticoagulant pre-treatment, in the transgenic line and controls. Thrombocyte-associated fluorescence in thrombi forming at the site of vessel injury was quantified, as well as vessel occlusion at 2 minutes post-laser. We used plasma samples of a cohort of stable aspirin-treated cardiovascular patients to validate our results (n=185).

Results: Control larvae developed a thrombocyte-rich thrombus at the site of vessel injury that never occluded the vessel (n=22). In contrast, the transgenic line developed a thrombocyte-poor thrombus that occluded the vessel in 75% of the larvae (n=20). Pre-treatment with the direct thrombin inhibitor argatroban, but not aspirin, completely prevented laser-induced vessel occlusion in the transgenic line (0% occlusion, n=18). The plasma level of miR-126, but not a control platelet-derived miRNA, correlated with thrombin generation markers such as prothrombin fragment F1+2 in cardiovascular patients ($r=0.39$, $p<0.01$).

Conclusions: We show that thrombocyte-specific overexpression of miR-126 is associated with larger laser-induced thrombi than in controls, an effect which is prevented by a direct thrombin inhibitor. The positive correlation between levels of miR-126 and thrombin generation markers in human plasma support a key role for miR-126 in platelet-mediated thrombin generation.

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mRNA sequencing of lung educated platelets as a specific biomarker for pulmonary hypertension

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Background: Pulmonary hypertension (PH) encompasses a group of rare conditions for which there is presently no cure. PH is characterized by narrowing and obliteration of small arteries in the lung, almost invariably leading to right heart failure and premature death. Idiopathic pulmonary arterial hypertension (IPAH) and chronic thromboembolic pulmonary arterial hypertension (CTEPH) are two distinct clinical entities characterized by remodeling of the lung vasculature, increased pulmonary vascular resistance and right ventricular failure [(1)]. A diagnostic challenge is to separate IPAH and CTEPH from PH due to Left Ventricular Diastolic Dysfunction(LVDF), where PH is induced by passive transmission of an elevated left ventricular end diastolic pressure, with or without secondary remodeling of the pulmonary vascular bed [(2)]. Circulating blood platelets contain ~5000 different mRNAs. Previously we have shown that tumor-educated platelets-derived RNA signatures may have predictive value for tumor type-specific diagnostics [(3)].

Hypothesis: Here, we hypothesize that platelet RNA derived from "lung vasculature-educated" platelets can be exploited to select unique biomarker RNA-panels for the detection of PH.

Methods: We collected platelet pellets, isolated from whole blood by differential centrifugation from 109 pulmonary arterial hypertension (PAH) patients at moment of diagnosis, as well as 109 age and gender-matched healthy controls. Platelet pellets were subjected to total RNA isolation, SMARTer cDNA amplification, and libraries were sequenced on the Illumina HiSeq platform. Raw data (~20 M reads per sample) was mapped to the human reference genome and only intron-spanning spliced RNA reads were used for downstream analysis. RNA panels were calculated by ANOVA statistics, and classifications performed using a support vector machine (SVM)-algorithm.

Results: We successfully performed RNA-sequencing from 218 platelet pellets for all subjects. By differential expression analysis, we identified 222 RNAs to have increased spliced RNA levels in platelets of PAH patients. Hierarchical clustering (Figure 1A) of RNAs with a false discovery rate <0.03 among these groups identified significant separation between PAH patients and healthy controls ($p<0.001$, Fisher's exact test). Evaluation of an SVM-based classification model, which utilizes a leave-one-out cross-validation approach, allowed for the separation of PAH patients from healthy controls with high accuracy (91% accuracy, Area Under the Curve: 0.95, Figure 1B).

Conclusion: This proof-of-concept study demonstrates that massive parallel sequencing of platelet RNA, paired with a support vector machine (SVM)-based diagnostic algorithm, is a highly efficient tool to discriminate patients with lung vascular disease from age-matched healthy subjects. Profiling "lung-vasculature-educated platelets" potentially enables liquid biopsy-based diagnostics for PAH. Large-scale validation is ongoing and the most up to date results of the PAH cohort will be presented.

P47 **Robert Wolf**

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Platelet adhesion and thrombus formation is significantly reduced by specific inhibition of MRP4/ABCC4.

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The multidrug resistance protein 4 (MRP4/ABCC4) is a versatile efflux transporter in platelets, known to transport second messenger molecules such as cAMP, cGMP and several lipid mediators, i.e. certain eicosanoids like thromboxane B2 and prostaglandin E2. Recently, the platelet-derived bioactive signaling lipid sphingosine-1-phosphate (S1P) has been identified as an additional substrate of MRP4.

The aim of the present study was to characterize the effects of the novel selective MRP4 inhibitor Ceefourin-1 on different signaling pathways in platelets and on thrombus formation *in vitro*.

Human platelet activation was determined by light transmission aggregometry as well as by flow cytometry (CD62P surface expression; binding of FITC-labeled fibrinogen) after stimulation with different agonists. As a surrogate marker for intracellular cAMP- and cGMP-levels, the magnitude of vasodilator-stimulated phosphoprotein (VASP) phosphorylation was quantified by a flow cytometric approach, while intracellular calcium concentrations in Fura-2 loaded platelets were measured by ratiometric fluorescence spectroscopy. Whole blood with FITC-Anti-CD42a-labeled platelets was perfused through collagen-coated microchannels under high arterial shear conditions to analyze platelet adhesion and thrombus formation. In addition, transport studies were performed with ^{3}H-labeled cGMP, FITC-labeled S1P and TxB_2 using membrane vesicles from MRP4-overexpressing SF9 cells and human platelets.

In *ex vivo* aggregometry studies, Ceefourin-1 (10 µM) significantly inhibited platelet aggregation by about 30 to 50% when ADP or collagen were used as activating agents, respectively. Furthermore, Ceefourin-1 significantly lowered the ADP- and CRP-XL-induced activation of integrin $\alpha_{IIb}\beta_3$, determined by binding of FITC-fibrinogen (e.g. 25% reduction at 50 µM Ceefourin-1 and 125 ng/ml CRP-XL). In comparison, P-selectin (CD62P) surface exposure as indicator of platelet degranulation was affected to a minor extend. VASP-phosphorylation induced by PGE_1 (1 µM) and Cinaciguat (1 µM) was elevated in platelets preincubated with Ceefourin-1 (50 µM) by 1.5 fold and 1.7 fold, respectively, while platelet calcium-influx was attenuated by about 15%. Moreover, selective MRP4 inhibition significantly reduced both the total area covered by thrombi and the average size of thrombi by about 40% in the flow chamber model. Studies of ATP-dependent transport in isolated MRP4-SF9 membrane vesicles revealed a potent inhibition of cGMP- and TxB_2-transport by Ceefourin-1 (IC_{50} of 6 µM and 4 µM, respectively). FITC-S1P-transport was also significantly affected (52 ± 33% and 85 ± 22% inhibition at 50 µM Ceefourin-1 in MRP4-SF9 and human platelet vesicles, respectively; Mean ± SEM).

In conclusion, the selective MRP4 inhibitor Ceefourin-1 causes both reduced platelet adhesion and thrombus formation under flow conditions. This finding is mechanistically reflected by the inhibition of integrin $\alpha_{IIb}\beta_3$ activation, elevated VASP-phosphorylation and reduced calcium influx, which might be the result of an impaired cyclic nucleotide extrusion or sequestration due to MRP4 inhibition. Beside the potent inhibition of cyclic nucleotide transport, Ceefourin-1 also affects the transport of pro-inflammatory mediators such as S1P.

P48 **Paul Armstrong**

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Platelets more readily interact with pro-inflammatory monocytes than neutrophils in response to stimulation

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Background: Platelet-leukocyte interactions have been linked to a range of cardiovascular and inflammatory disease states. These interactions continue to be the subject of study, however their fundamental purposes remain unclear. To date, the mechanisms of platelet-neutrophil and platelet-monocyte interactions have been studied independently with no indication of which cells platelets prefer to bind with. We therefore set out to address this question by coupling low volume whole blood stimulation, using a low volume 96-well plate approach, with flow cytometric analysis.

Methods: In brief, blood was collected by venepuncture from healthy volunteers. 45µl aliquots of blood were then placed into the individual wells of half-area 96-well plates with TRAP-6 amide (3µM), U46619 (3µM), CRP (0.1µg/ml) or vehicle (PBS) and gently mixed (200rpm for 10mins, 37°C). Following this, samples were immediately fixed and flow cytometric analysis was performed to elucidate platelet P-selectin (CD62P) expression and also identify platelet binding to the following leukocyte subsets: neutrophils ($CD66b^+$), classical monocytes ($CD14^+CD16^-$), intermediate monocytes ($CD14^+CD16^+$) and non-classical monocytes ($CD14^-CD16^+$). Interactions between platelets and cells were calculated as a percentage (%) of cell subset number and then normalised for paired vehicle control. Data presented as mean ±s.e.m (n=4 for all)

Results: All agonists induced significantly higher platelet P-selectin expression than vehicle. TRAP-6 stimulated blood exhibited the following order of platelet interactions: intermediate monocytes (55±11%); classical monocytes (46±12%); non-classical monocytes (12±2%); neutrophils (3±3%). CRP stimulated blood exhibited the same order of preferential binding: intermediate (43±9%); classical (40±9%); non–classical (11±6%); neutrophils (0±2%). Finally, a similar pattern was observed following U46619 stimulation, intermediate (27±4%); classical (20±5%); neutrophils (2±3%); non-classical (0±3%).

Conclusion: Our data indicates that following *in vitro* stimulation across a range of agonists, platelets preferentially bind to intermediate and classical monocytes which are commonly characterised as pro-inflammatory monocyte subsets. In contrast, despite activation and induced P-selectin expression, platelet did not favour binding to neutrophils. This preliminary data indicates that platelets may influence monocyte behavior more readily than neutrophil behavior during response to stimulation and inflammation.

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A novel combinatorial technique for the quantification of oxygen radicals in platelets reveals the NADPH oxidase-dependent regulation of haemostasis

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Introduction: The regulation of platelets by oxidant molecules formed during the process of cell activation is critical for vascular health and remains poorly understood [1]. Superoxide anions ($O2^{\bullet-}$) have a pre-eminent role in biology and pathophysiology, as it serves as a progenitor for formation of hydrogen peroxide (H2O2), ONOO•− and hydroxyl radical (HO•) and plays a key role in the post-translational oxidative modification of proteins [2].
In this study, we utilised the EPR/turbidimetry [3,4,5] technique to clarify the dynamics of oxygen radical generation and activation in human platelets in response to different physiological and pathological stimuli. The use of NOX1- and NOX2-selective peptide inhibitors allowed the identification of some key differences in the involvement of these two enzymes in the response to different platelet agonists and modulators. The application of this technique will further our understanding in redox-dependent platelet regulation and may have important consequence in cardiovascular drug discovery.

Method & Results: Here, electron paramagnetic resonance spectroscopy and turbidimetry were combined to monitor contemporaneously platelet activation and oxygen radical generation. This analysis revealed that intracellular superoxide anions generated by NADPH oxidases are necessary for the responses to collagen and thrombin, but their dismutation to hydrogen peroxide is required for thrombin-dependent activation. NADPH oxidase 1 is the main source of oxygen radicals in response to collagen, while NADPH oxidase 2 is critical for the response to thrombin. Finally, weak agonists/positive modulators oxidised low density lipoproteins and β amyloid peptides also modulate platelet response via NADPH oxidase activation.

Conclusion: In summary, the scavenging of superoxide anions or the selective inhibition of NADPH oxidase isoenzymes are viable approaches to regulate haemostasis and combat the pro-thrombotic states associated with vascular diseases.

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RASA3 (GAP1-IP4BP) interacts with integrin and cytoskeletal proteins in human platelets

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Introduction: RASA3 (also known as GAP1-IP4BP) is a dual activity Ras and Rap GAP that is present on the inner surface of the platelet plasma membrane. The protein binds the phosphoinositides PI(3,4,5)P3 (PIP3), PI(4,5)P2 and (1,3,4,5)IP4 (IP4) with high affinity. Recent studies on transgenic mice have shown that RASA3 is a major regulator of Rap1 and any defect in its expression or function leads to unregulated Rap1 activation, integrin activation and thrombocytopenia. It has also been suggested to be the major mechanism for ADP-P2Y12-PI3K mediated irreversible activation of platelets. However the molecular mechanisms involved with how PIP3 and/or IP4 affects RASA3 and Rap1 activity are not known. Further the initial in vitro liposomal studies suggested that IP4-RASA3 stimulated GAP1 activity towards Ras and Ras function in platelets is still not understood. In this study we sought to understand better which proteins RASA3 interacts with in platelets.

Methods: Freshly prepared platelets were stimulated with thrombin, collagen or with PGE1 and after lysis in Triton based buffers, cytoskeleton and lysates were prepared for immunoprecipitation using RASA3 antibodies. In some experiments the immunoprecipitates were subjected to in gel digestion followed by LC-MS/MS. The MS/MS data was screened against the human protein database. Assignments were accepted with >99.9% protein probability and proteins listed with a minimum 3 identified peptides with manual exclusion of keratin and IgG contaminants.

Results: Platelet activation by thrombin and collagen resulted in RASA3 increasingly associated with the cytoskeleton. In lysates activation was associated with an increase of tyrosine phosphorylation of RASA3 with a slightly slower mobility in SDS-PAGE gels. In immunoprecipitation experiments we could not observe co-immunoprecipitation of Rap1 proteins with RASA3 suggesting that the direct interaction was not stable during the extraction procedures. In proteomic analysis of RASA3 imunoprecipitations we found RASA3 to bind to specific integrin subunits, integrin associated proteins and important components of the cytoskeleton. Immunoprecipitation of these RASA3 binding proteins confirmed the analysis.

Conclusions: RASA3 has been shown to be an important regulator of Rap1 and integrin activation. Our results suggest that upon activation a portion of RASA3 is tyrosine phosphorylated and there is increased association with the cytoskeleton. An analysis of RASA3 immunoprecipitates suggest that RASA3 undergoes partial tyrosine phosphorylation and cytoskeletal association. Any direct binding of RASA3 to Ras or Rap1 appears not to survive the extraction procedure, thus Rap or Ras may interact with RASA3 via RASA3 binding proteins.

P51 **Bibian Tullemans**

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Anti-platelet effects of tyrosine kinase inhibitors used for cancer treatment: pazopanib as a proof of principle

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Background: Multiple tyrosine kinase inhibitors (TKIs) are in clinical use for cancer treatment aiming to prolong progression free survival. Most of these compounds are directed against the conserved catalytic ATP-binding site of a spectrum of tyrosine kinases (TKs). The targeted TKs are mostly receptors of growth factors present on tumour and endothelial cells. Importantly, in megakaryocytes and platelets, TKs are crucial for platelet production and platelet signalling, especially via (hem)ITAM-linked receptors, such as glycoprotein VI (GPVI). Whereas patient treatment with TKIs can increase the bleeding risk, it is often unclear if this is related to an effect on platelets. Therefore we aimed to *(i)* review the reported effects of TKIs on platelet function and haemostatic activity; and *(ii)* investigate these links in patients treated with the TKI pazopanib.

Methods and Results: We first compared the reported inhibitory profiles of 26 different TKIs, all in clinical use or in clinical trials, regarding their potential to inhibit TKs present in platelets or megakaryocytes. Most of these TKIs showed relatively low affinities for TKs that are relevant for platelet signalling (i.e., Src family kinases, Btk, Tec and Syk). For 13 of the TKIs, bleeding symptoms have been described, and for 1 thrombosis. For 7 TKIs, decreased platelet counts are reported, with as possible targets the above-mentioned TKs, or B-Raf or Kit. For a similar panel of TKIs, alterations in platelet functional responses are reported *ex vivo* or *in vitro*, usually upon GPVI stimulation. In almost all cases, the antiplatelet effects of TKIs appeared to be off-target.
Next, treatment effects of the TKI pazopanib on quantitative and qualitative platelet traits were examined in patients with renal cell carcinoma (RCC). Three out of ten studied patients reported minor bleeding complications (epistaxis); an increased bleeding risk with this drug is confirmed in the literature. Pazopanib significantly reduced the platelet count by 19% (median, interquartile range: 6-32%; P = 0.0049). After 14 days of treatment, whole blood thrombus formation under flow was essentially unchanged, except for a significant reduction in platelet phosphatidylserine exposure. *In vitro* treatment of washed platelets with pazopanib resulted in a potent, dose-dependent inhibition of GPVI-induced platelet responses, including tyrosine phosphorylation, secretion, aggregation and phosphatidylserine exposure; effects that were antagonized by the presence of plasma. *In vitro* treatment of platelets also caused a moderate reduction of whole blood thrombus formation. This pointed to a 'protective' effect of plasma.

Conclusions: For the majority of TKIs examined, an increased risk of bleeding on-treatment can be linked to lower platelet counts and/or reduced platelet functional properties. As a proof-of-principle, treatment of RCC patients with pazopanib reduced the platelet count, and moderately suppressed platelet activation *ex vivo*, in contrast to its potent inhibitory action *in vitro*. Together, this indicates that – even with low bioavailability – TKIs like pazopanib can affect haemostasis by combined quantitative and qualitative effects on platelets. This should raise awareness for off-target effects of TKIs, which may be enhanced in the presence of antithrombotic drugs.

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GPVI surface expression and signaling pathway activation are increased in platelets from obese patients: elucidating potential anti-atherothrombotic targets in obesity

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Platelets play a fundamental role in the increased atherothrombotic risk related to central obesity since they show hyperactivation and lower sensitivity to antiplatelet therapy on obese patients.

The main goal of this study was to identify platelet biomarkers related to the risk of suffering atherothrombosis in obese patients, confirm platelet activation levels in these patients, and identify altered activation pathways.

Platelets were obtained from cohorts of obese patients and age- and sex-matched lean controls. Biochemical and proteome analyses were by two-dimensional differential in-gel electrophoresis (2D-DIGE), mass spectrometry, and immunoblotting. Functional and mechanistic studies were by aggregation assays and flow cytometry.

Among others, we confirmed an up-regulation of αIIb and fibrinogen isoforms in platelets from obese patients. Besides, a complementary platelet aggregation approach showed platelets from obese patients are hyper-reactive in response to collagen and collagen-related peptide (CRP), revealing the collagen receptor Glycoprotein VI (GPVI) signalling as one of the altered pathways. We also found the active form of Src (pTyr418), fundamental for αIIb and GPVI signalling, is up-regulated in platelets from obese individuals, which links proteomics and aggregation data. Moreover, we show that CRP-activated platelets present higher levels of tyrosine phosphorylated PLCγ2 in obese patients, confirming alterations in GPVI signalling. In line with the above, flow cytometry studies show higher surface expression levels of total GPVI and GPVI-dimer in obese platelets, both correlating with BMI.

In conclusion, our results suggest a higher activation state of SFKs-mediated signalling pathways in platelets from obese patients, with a primary involvement of GPVI signalling.

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Differential Impact of HIV Antiretrovirals on Platelet Function

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Despite the success of antiretroviral therapy, cardiovascular risk is elevated for people living with HIV and cardiovascular disease is the leading cause of mortality in this population. Multiple, but not all, prospective and retrospective observational studies provide clinical evidence of an increased risk of myocardial infarction (MI) in patients receiving the antiretroviral abacavir (ABC) sulphate, however, the mechanism and role of platelets is not fully elucidated. ABC and tenofovir are nucleos(t)ide reverse transcriptase inhibitors and their active metabolites are guanosine and adenosine analogues, respectively.

We investigated the hypothesis that ABC and tenofovir differentially impact platelet activation. Platelet function was assessed using *in vitro* and *ex vivo* analysis of blood samples collected from HIV-negative volunteers and subjects from a Phase I clinical trial. Furthermore, we evaluated murine platelet activation using an *in vivo* aggregation model and flow cytometric approaches.

None of the antiretrovirals tested directly affected platelet aggregation *in vitro* at clinically-relevant concentrations. ABC interrupted nitric oxide (NO)-mediated inhibition of platelet aggregation and increased *in vivo* aggregation in mice. Granule release in response to collagen, but not ADP or thrombin, was enhanced in both human and mouse platelets. In contrast, tenofovir prodrugs (tenofovir disoproxil fumarate [TDF] or tenofovir alafenamide fumarate [TAF]) did not affect platelet function *in vitro* or *in vivo*. Furthermore, aggregation and activation of platelets isolated from twenty subjects taking clinically-relevant doses of TDF or TAF was comparable to baseline values.

ABC can therefore enhance platelet activation, independently of the confounding variables (e.g. HIV status), found in earlier clinical studies, suggesting a potential pharmacological effect that is absent with tenofovir in mouse and human studies. Mechanistically, interruption of NO-mediated platelet inhibition is proposed to underpin enhanced activation in the presence of ABC but not TDF or TAF. This hypothesis is supported by our data indicating that ABC treatment leads to: 1) enhanced aggregation *in vivo* and 2) *in vitro* responses that are resistant to inhibition by NO. Although an association between ABC and CVD has not been confirmed in large prospective randomized trials, these findings provide evidence of a mechanistic link between platelet activation and antiretroviral therapy. Future work should evaluate the impact of antiretrovirals on platelet function in people living with HIV, and those taking antiretrovirals as post-exposure prophylaxis.

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Adenosine receptor agonists enhance the inhibition of platelet aggregation by $P2Y_{12}$ antagonists

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Several adenosine receptor (AR) agonists were shown in the past to possess anti-platelet potential, however, the adjunctive role of AR agonists in anti-platelet therapy with the use of $P2Y_{12}$ receptor inhibitors has not been elucidated so far.

In this *in vitro* aggregation-based study we investigated whether the inhibition of platelet function mediated by cangrelor or prasugrel metabolite can be potentiated by selected AR agonists. The effect of non-selective (AR1), A_{2A}-selective (AR2, AR3, AR4) and A_{2B}-selective AR agonists (AR5) on platelet function has been evaluated in relation to their toxicity, specificity towards adenosine receptor subtypes, structure and solubility.

AR1, AR2, AR3, AR4 were found to be more or less potent inhibitors of ADP-induced platelet aggregation when acting alone and at the same time they remained non-cytotoxic to the cells. These AR agonists were also effective in the potentiation of the effects exerted by $P2Y_{12}$ antagonists. Considering the estimated IC_{50} value, AR2, showing a relatively high binding affinity to A_{2A} adenosine receptor, has been identified as the most potent anti-aggregatory agent. This compound diminished platelet aggregation at nanomolar concentrations and further augmented platelet inhibition by $P2Y_{12}$ antagonists by approx. 60% ($P<0.01$).

Our results indicate the importance of adenosine receptors as therapeutic targets and point out challenges and potential benefits of therapeutic use of a combined therapy of $P2Y_{12}$ antagonist and AR agonist in cardioprotection. The comparative analysis of the effects of AR agonists on platelet response in plasma and whole blood may indirectly suggest rather little contribution of other blood morphology elements to the inhibition of platelet function by AR agonists.

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Quercetin and its methylated metabolites possess anti-platelet and anti-thrombotic properties, and interact with aspirin to enhance anti-platelet efficacy

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Background: Flavonoids are a class of plant secondary metabolites, and are found in diets globally. One such flavonoid, quercetin, has demonstrated anti-CVD effects, with cohort studies linking quercetin intake to a reduced risk of ischemic heart disease. These effects are thought, at least in part, to be due to anti-platelet activity. Upon consumption, however, quercetin is extensively metabolised in the small intestine and liver, and there is a paucity of information regarding the anti-platelet actions of these metabolites. As such, the precise mechanisms of action of Quercetin *in vivo* have yet to be fully elucidated.

Aims: To establish the mechanisms through which quercetin and its methylated metabolites tamarixetin and isorhamnetin exert anti-platelet and anti-thrombotic actions, and investigate the interactions of these flavonoids with the anti-platelet effects of aspirin.

Methods: The effects of flavonoids on platelet aggregation were investigated using light transmission aggregometry, and granule secretion and integrin activation were measured using flow cytometry. Calcium mobilisation was measured using a fluorescent calcium-binding dye, FURA-2 AM. *In vitro* thrombus formation under physiological shear conditions was investigated using microfluidic blood perfusion channels. A laser-injury model in C57/BL6 mice was used to assess thrombosis in mice. Combined effects of flavonoid:aspirin dual treatment on platelet aggregation was investigated using a 96-well, plate-based aggregation assay.

Results: Quercetin, tamarixetin and isorhamnetin inhibited platelet aggregation, granule secretion, integrin activation, and calcium mobilization at physiologically achievable plasma flavonoid concentrations. *In vitro* thrombus formation was inhibited significantly by all three flavonoids at 10μM. Tamarixetin was most potent, indicating a 4'-methyl group in increased potency. Isoquercetin treatment over a 48 and 72-hour period resulted in a significantly reduced thrombus size in a murine laser injury model of thrombosis. Quercetin, tamarixetin and isorhamnetin all significantly enhanced the anti-platelet effects of aspirin, in a more-than-additive manner; indeed, the IC_{50} values for the inhibition of aggregation by aspirin were reduced by an order of magnitude upon co-administration of 10μM flavonoid.

Conclusion: Quercetin and its methylated metabolites inhibit platelet function at physiologically achievable concentrations, and these effects are maintained in platelet rich plasma, as well as in whole blood through an inhibition of thrombus formation, demonstrating the ability of these flavonoids to inhibit platelet function despite high plasma-binding properties. This effect was maintained in a murine model of thrombosis. This may offer insight into the ability of a diet high in flavonoids, such as quercetin, to reduce CVD risk, and with their interaction with aspirin, offer the potential to reduce therapeutic aspirin doses and alleviate associated symptoms such as gastric bleeding.

Conflict of interest: None declared

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Roles of endothelial thrombomodulin and heparan sulphates in the control of platelet activation and coagulation under flow conditions

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Background: In the intact vessel wall, the endothelium forms a barrier between the blood and the remaining vascular structures, serving to maintain blood fluidity and preventing platelet activation and clotting. Endothelial cells (ECs) dispose of several protective mechanisms, including production of platelet inhibitors, and expression of NTDPases, thrombomodulin (activating the anticoagulant protein C via the binding of thrombin), tissue factor pathway inhibitor (TFPI) and the thrombin-inactivating glycocalyx. We developed a vascular cell-on-a-chip model with a non-confluent EC layer on an active matrix to investigate the key endothelial regulatory mechanisms of platelet and coagulation inhibition under flow conditions.

Results: On a collagen/tissue factor coated surface in microfluidics chambers, seeded ECs (40-60% coverage) potently delayed and suppressed fibrin formation during perfusion of recalcified blood, even at low shear rates of 50 s^{-1}. Platelets deposited and fibrin fibres formed distantly from the ECs, and remained restricted to the inter-endothelial gaps, pointing to a potent antiplatelet and anticoagulant property of the non-confluent ECs. The kinetics of fibrin formation were additionally determined and revealed that fibrin coverage in the initial phase of flow perfusion was decreased in the presence of ECs. Fibrin fibres ultimately formed from activated platelets adhering in between ECs. The deposition of platelets remained unaltered in time and was not different in conditions with(out) ECs. The suppression of fibrin formation was antagonized by heparinase III treatment (removing the glycocalyx heparan sulphate chains) and by blocking thrombomodulin on the EC surface. The latter effect, however, was restricted to low tissue factor coating. In agreement with this, co-coating of purified thrombomodulin delayed the collagen- and platelet-dependent fibrin formation.

Conclusions: In the 'vascular cell on-a-chip' system: (i) activated platelets are the primary site of fibrin clot formation, (ii) ECs suppress fibrin formation, and (iii) the EC glycocalyx has a larger anticoagulant capacity than endothelial thrombomodulin.

P57 **Katrijn Six**

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Platelet production in a microfluidic device using hematopoietic stem cells from umbilical cord blood and peripheral blood

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Background: *In vitro* production of human platelets offers a promising alternative for donor-derived platelet concentrates in the field of transfusion. Multiple sources of hematopoietic stem cells (HSC) are currently used for culture and differentiation of megakaryocytes as precursors for platelets *in vitro*.

Methods: In this study, $CD34^+$ HSC isolated either from umbilical cord blood (UCB), regular non-mobilized peripheral blood (PB) or mobilized peripheral blood (mPB) were evaluated for expansion and differentiation towards megakaryocytes during a 12-day culture. Platelet production was obtained by perfusion of the cultured cells over von Willebrand factor-coated microfluidic flow chambers. Platelet production yield during perfusion in the flow chamber was compared. Surface receptor expression on the produced platelets was also investigated.

Results: The percentage of isolated HSC per total nucleated cells from PB was significantly lower (0.06%) compared to UCB (1.33%) ($P<0.0001$) and to mPB (1.36%). Proliferation of HSC from PB was significantly lower than from UCB shown by a 24-fold increase in cell number on day 12 for PB *versus* 76-fold for UCB ($P<0.0001$). Proliferation of HSC from mPB was 18-fold on day 12. Differentiation of HSC to megakaryocytes was less efficient when starting from PB where only 44±9% of the cultured cells were positive for platelet markers CD42b and CD41, compared to 76±11% for UCB ($P<0.0001$). Despite this, platelet production *in vitro* under flow was more efficient for megakaryocytes cultured from PB with 7.4 platelet-like particles per input cell, compared to 4.2 for UCB ($P=0.02$). The yield of platelet-like particles per input cell was 6.6 starting from mPB. Platelet surface receptor expression was comparable on platelets produced from UCB and PB.

Conclusion: *In vitro* production of platelet-like particles is possible starting from HSC isolated from UCB, PB and mPB. In absolute numbers, more of these platelets are obtained from UCB because of more efficient isolation and proliferation. In relative numbers, the efficiency of platelet production per cultured megakaryocyte is higher from (m)PB.

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Migration and clearance of bacteria by platelets

Co-author(s): David Riley, Jean Pieters, Francisco Rivero

Background: Platelets are commonly known for their role in maintaining haemostasis, where they alter their morphology and function through the reorganisation of the actin cytoskeleton, as to promote the formation of a platelet plug. However, recent breakthrough research supports the idea that platelets can also function as immune cells to prevent the progression of systemic infections and/or sepsis. Emerging evidence also supports the idea that platelets encompass the capacity to migrate towards certain stimuli i.e. invading microbes. Although various approaches towards quantifying platelet motility have been reported, in general this aspect has been poorly investigated and methods have not been standardised. In this study we utilise human platelets isolated from blood collected from healthy volunteers to optimise assay conditions required to investigate the migrating capacity of platelets.

Results: Platelets were either isolated and maintained in plasma or washed and resuspended in Tyrode's buffer, allowed to sit on coverslips coated with fluorescently labelled fibrinogen, fixed and stained with fluorescently labeled phalloidin to visualise F-actin distribution. Platelets diluted in plasma showed the potential to migrate after 60 min but not earlier. However, washed platelets lacked the capacity to displace from their original position. Interestingly though, washed platelets resuspended back into plasma had the capacity to migrate. Migration only occurs when the plasma is not diluted below 50%. Only a small proportion (about 20%) of platelets actually migrate. Other conditions like the effect of plasma pH, the concentration of fibrinogen or the time to application to the matrix coated surface after platelet isolation were also investigated.

Conclusion: Platelets have the capacity to migrate on fibrinogen surfaces, supporting the idea that they can actively migrate and scavenge their proximity to clear any potential invading microorganisms. An ideal set of conditions to investigate migration of human platelets was obtained from the optimization experiments. Our results will aid the designing of experimental conditions needed to investigate the role of the cytoskeleton and signaling components in platelet migration in a variety of conditions.

P59 **David Scully**

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Platelet-based applications for skeletal myogenesis and muscle stem cells with implications for regenerative medicine

Co-author(s): Peggy Sfyri, Sandrine Verpoorten, Petros Papadopoulos, Ahmed Aburima, Laura Gutierez, Khalid Naseem, Antonios Matsakas

Introduction: Due to the ease of access, cost-effectiveness and safety-orientated nature of platelet-based applications, they have recently become a popular method of delivering growth factors to injured tissue, in an attempt to promote skeletal muscle healing. However, there is currently contrasting evidence on the role of platelets as biomaterials in injuries. Given that the molecular mechanisms involved in platelet-mediated regeneration are poorly understood, the aim of this study was to establish the effect of platelet releasate on skeletal myogenesis *in vitro* and muscle stem cells *ex vivo*.

Methods: We analysed the effect of platelet releasate on proliferation and differentiation of C2C12 myoblasts and single muscle fibres and isolated satellite cells by means of cell proliferation assays, immunohistochemistry and gene expression. Most importantly, we expanded *in vitro* cell culture findings on by establishing the effect of platelet releasate on murine skeletal muscle stem cells using protein expression profiles for key myogenic regulatory factors.

Results: Here we show that that platelet releasate increases myoblast proliferation in a dose-dependent manner, which is mitigated by vascular endothelial growth factor- (VEGF) and platelet-derived growth factor- (PDGF) receptor pharmacological inhibition. Key cell culture findings were validated on skeletal muscle stem cells, providing similar outcomes. We show that platelet releasate upregulates MyoD expression on skeletal muscle stem cells and promote their myogenic potential to regenerate injured muscle fibres through the VEGFR and PDGFR pathways. Furthermore, application of platelet releasate can either attenuate fusion of myoblasts into myotubes or promote terminal myogenic differentiation in a temporal manner, indicating that the timing of releasate application is crucial. Key components of the platelet releasate were identified by immuno-based assays.

Conclusion: Taken together, *in vitro* and *ex vivo* data provide supporting evidence on a positive effect of platelet releasate on myoblast proliferation, with temporal effects on cell differentiation. The effects are mediated through the VEGF and PDGF signalling pathways. Platelet-based methodologies aiming to provide autologous biomaterials have implications for regenerative medicine and muscle healing.

P60 **Joanne Clark**

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Elevation of cyclic AMP inhibits platelet aggregation stimulated by collagen but phosphorylation is unaffected

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Platelets are exposed to prostacyclin and nitric oxide at the endothelium, which elevate cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate GMP (cGMP) respectively. cAMP and cGMP are cyclic nucleotides that act as second messengers through activation of protein kinase pathways. Elevation of these nucleotides in platelets causes powerful platelet inhibition and is shown to block several stages of platelet activation such as aggregation, granule release and adhesion. cAMP levels are also raised by activation of the G protein-coupled receptor, adenosine A_{2A} which signals through its stimulatory G protein (G_s).

Using collagen which signals through the tyrosine kinase-linked GPVI and integrin $\alpha_2\beta_1$ platelet receptors, the aim of this study was to investigate the cross-talk between adenosine A_{2A} and GPVI receptors in platelet regulation via cAMP formation. Using NECA (5'-N-ethylcarboxamidoadenosine), a high affinity bioactive adenosine analogue and forskolin, an adenylyl cyclase activator, we show that cAMP significantly inhibits low concentration collagen and collagen related peptide (CRP)-induced aggregation and ATP secretion and to a lesser extent high concentration collagen-induced aggregation. cAMP does not significantly affect high or low concentration collagen-induced tyrosine phosphorylation of the downstream signalling proteins: PLCγ2, Syk and LAT.

Investigations of human platelet spreading on collagen-coated coverslips show that cAMP has no significant effect on platelet spreading or adhesion. Using single molecule super resolution microscopy, direct stochastic optical reconstruction microscopy (dSTORM), we show that neither NECA nor forskolin had a significant effect on GPVI receptor dimerisation and clustering along collagen fibres. We therefore proposed that cAMP was affecting platelet activation by collagen downstream of PLCγ2 signalling, possibly by inhibiting IP_3-induced Ca^{2+} release via PKG-IRAG-IP_3 receptor complex. Results confirm this to not be the case and showed that cAMP had no significant effect on the calcium response. The partial inhibition of collagen-induced aggregation and secretion by adenosine or forskolin is therefore mediated downstream of Ca^{2+} signalling.

P61 **An-Sofie Schelpe**

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Generation of anti-idiotypic antibodies to detect anti-spacer antibody idiotopes in acute thrombotic thrombocytopenic purpura patients

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Introduction: In an autoantibody (autoAb)-mediated autoimmune disease, patients develop autoAbs against self-antigens. AutoAb profiling allows to stratify patients and to link autoAb with disease severity and outcome. In immune-mediated (i)TTP patients, the anti-ADAMTS13 autoimmune response is polyclonal but almost all iTTP patients have anti-ADAMTS13 autoAbs against the spacer domain. However, patient stratification according to Ab profiles and their link with disease outcome and severity has not been fully explored.

Aim: We aimed at developing a new autoAb profiling assay for iTTP using anti-idiotypic Abs that recognize specific idiotopes on anti-ADAMTS13 autoAbs. We next aimed at determining anti-spacer idiotope profiles in iTTP patients and at investigating if these idiotope profiles could be linked with disease outcome or treatment.

Methods: Mice were immunized with either cloned human anti-spacer autoAb II-1, TTP73 or I-9, to generate anti-idiotypic Abs. Next, the developed anti-idiotypic Abs (17H9, 9G12 and 7D10) were coated to capture their respective anti-spacer idiotope from 148 acute iTTP plasma samples. Finally, we investigated if the anti-spacer idiotope profiles of 95 iTTP patients could be linked with disease outcome or treatment.

Results: We developed 3 anti-idiotypic Abs (17H9, 9G12 and 7D10) that recognized particular idiotopes in the anti-spacer autoAbs II-1, TTP73 and I-9 that are involved in ADAMTS13 binding as they inhibited the binding of the respective anti-spacer autoAbs II-1, TTP73 and I-9 to rhADAMTS13.
Of the 148 iTTP patients, respectively 35% (52/148), 24% (36/14) and 42% (62/148) of the patients were positive for Abs with the anti-spacer II-1 idiotope (positive on anti-idiotypic Ab 17H9; 57.0 ± 41.6 ng/mL), anti-spacer TTP73 idiotope (positive on 9G12; 261.8 ± 208.8 ng/mL) and anti-spacer I-9 idiotope (positive on 7D10; 89.6 ± 104.9 ng/mL). We next stratified the patients according to 1 out of the 8 anti-spacer idiotope profiles (presence of either 1, 2, 3 or none of the 3 anti-spacer idiotopes). Finally, we showed that none of the 8 anti-spacer idiotope profiles could be linked with a Benhamou score of ≥3 (chi square, ns), which was shown previously to be predictive for early death. Also, none of the 8 anti-spacer idiotope profiles could be linked with treatment (plasma exchange (PEX) with/without rituximab versus PEX with/without rituximab together with additional therapies).

Conclusion: Our data shows that anti-idiotypic Abs are well-suited to determine Ab profiles in iTTP patients. By using anti-idiotypic antibodies, the antigen itself is no longer needed for the profiling assay. Even though the resulting idiotope profiles could not be linked with disease outcome or treatment, future development of anti-idiotypic Abs against anti-ADAMTS13 autoAbs that are linked with disease severity, outcome and relapse will be a promising approach to personalize treatment of iTTP patients.

P62 **Maria Csilla Csanyi**

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Axial structure of von Willebrand multimers

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Von Willebrand factor (VWF) mediates interactions between injured vessel wall and platelets at high shear and elongation forces. It is a multidomain glycoprotein secreted into blood from endothelial cells and platelets as large multimers that comprise various numbers of linearly connected dimers (1 to >40). Reduction in the level and activity of the VWF or the degree of its multimerization lead to bleeding (VW disease). By contrast, increasing these parameters cause thrombotic events.

As force and the multimer size of VWF determine its activity, we aimed at analysing the relationship between them with atomic force microscopy (AFM).

VWF was isolated from HaemateP, by using HiTrap Heparin HP column and NaCl gradient elution (0.0-0.3M in HEPES buffer) which also resulted in a size fractionation of VW multimers. Aliquots were kept at -20°C for 3 months. Prior to AFM analysis, the samples were diluted to $4x10^{-8}$g/L with phosphate-buffered saline containing 50% glycerol. 0.01mL of the solution was dropped onto freshly cleaved mica (d=12mm) and incubated for 1 min to allow equilibration to the surface. Alternatively, the molecules were extended by molecular combing[1]. Unbound molecules were washed away with ultrapure water. The sample was then dried with N_2. VWF length was measured on AFM images by tracing the contour of individual multimers.

VW mulitmers adsorbed on mica were 80 to 2410 nm long and displayed a beads-on-string structure. A smaller and a lager (medians 15 and 21 nm) could be identified, corresponding to the C- and D-domain knots[2]. In surface-equilibrated molecules, the large globules were close to each other, and the rod-like parts were randomly visible. Elongated VW molecules (n=16) were seen as an effect of molecular combing with a median distance between large globules of 93 nm, IQR= 63-121 nm, which corresponds to dimers. Small globules were located in the centre of the axial repeats. Next, we selected three group of samples containing short (S), medium (M) and long (L) multimers. Based on electrophoresis (ELFO), the number of dimers in the groups: S =1-3, in M=1-10 and in L >10. We could demonstrate that the median contour length (and IQR) of the molecular combed multimers were S=105 (88-131) nm, M=293 (163-389) nm and L=371(262-545) nm. In the latter fractions, 800-2410 nm long chains were present with low frequency. The length in the three categories were shorter than it was expected based on ELFO and EM, probably because our samples were not denatured. Thus, effect of intramolecular forces in the skeleton of the linearly connected dimers has to be considered.[3]

We found good correlations between maximum contour length and activity (n=21, r=0.85, p<0.0001) and multimer size by molecular mass (n=40, r=0.90, p<0.0001).

In summary, the length of VW multimers, measured here by AFM, is a sensitive parameter of the degree of VWF multimerization. Understanding the role of intermolecular forces in the structure of non-denatured VW multimers requires further investigations.

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P63 **Manuel Salzmann**

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A Novel Method for Automated Assessment of Megakaryocyte Differentiation and Proplatelet Formation

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Background and Objective: Transfusion of platelet concentrates represents an important treatment for various bleeding complications. However, the short half-life and frequent contaminations with bacteria restrict the availability of platelet concentrates and raise a clear demand for platelets generated *ex vivo*. Therefore, *in vitro* platelet generation from megakaryocytes represents an important research topic. A vital step for this process is accurate analysis of thrombopoiesis and proplatelet formation, which is usually conducted manually. We aimed to develop a novel method for automated classification and analysis of proplatelet-forming megakaryocytes *in vitro*.

Methods: Murine bone marrow cells were differentiated *in vitro* into mature megakaryocytes. To induce proplatelet formation, megakaryocytes were cultured on fibrinogen- or fibronectin-coated slides for 5 hours. Fixed cells were fluorescently labelled and megakaryocytes were automatically categorized and analysed with a novel pipeline of the open source software *CellProfiler*. The applicability and sensitivity of our novel method was verified in comparison to manual assessment and furthermore by treatment of megakaryocytes with dasatinib, a reported inhibitor of proplatelet formation[1].

Results: Our new workflow accurately recognises and classifies large numbers of megakaryocytes in an automated manner and is able to detect changes in megakaryocyte differentiation. In contrast to earlier reports, which discriminate only between megakaryocytes that are positive or negative for proplatelet formation, our method classifies four distinct subtypes of megakaryocytes during thrombopoiesis: proplatelet-forming, spreading, pseudopodia-forming and terminally differentiated, anucleated megakaryocytes.

Conclusion: This novel method enables rapid, unbiased and reliable in-depth analysis of proplatelet formation based on morphological characteristics, leading to the quantification of four distinct subsets of differentiating megakaryocytes. Similar attempts of subclassification are emerging in recent reports[2,3] and underline the necessity to redefine and standardise stages of megakaryocytes undergoing thrombopoiesis.

P64 **Shannen Deconinck**

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Inhibiting ADAMTS13 activity prevents the loss of high molecular weight von Willebrand factor multimers in in vitro left ventricular assist devices

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Background: Gastrointestinal (GI) bleedings are one of the leading adverse events of left ventricular assist device (LVAD) therapy and have been associated with acquired von Willebrand syndrome (aVWS). All LVAD patients have a selective loss of high molecular weight (HMW) von Willebrand factor (VWF) multimers and hence loss of their haemostatic function. The loss of HMW VWF multimers might be explained by an increased shear-induced proteolysis of VWF by ADAMTS13 (A Disintegrin And Metalloprotease with Thrombospondin type 1 repeats, number 13) due to the high shear stress occurring in the LVAD device. Consequently, inhibiting ADAMTS13 would be an efficient way to rescue the loss of HMW VWF multimers in LVAD patients.

Aim: To investigate whether ADAMTS13 inhibition prevents the loss of both HMW VWF multimers and VWF activity, in two different in vitro LVAD circuits.

Methods: Two different in vitro LVAD circuits were used: one with a HeartMate II™ (HM II, long-term support, n=3) pump and one with a Impella CP® (short-term support, n=5). Citrate-anticoagulated human blood was circulated to study ADAMTS13 mediated VWF proteolysis in the presence of the inhibitory anti-ADAMTS13 monoclonal antibody (mAb) 3H9 (20 µg/mL). A non-inhibitory anti-ADAMTS13 mAb 5C11 (20 µg/mL) or phosphate buffered saline (PBS) was used as a control. Blood samples were taken 5 minutes (min) before and 5, 30, 60 and 180 min after the onset of perfusion in the LVAD system. Plasma samples were analysed for VWF multimers, VWF antigen (VWF:Ag), VWF collagen binding activity (VWF:CB) and VWF ristocetin cofactor activity (VWF:RCo).

Results: A time-dependent decrease in HMW VWF multimers was observed in both LVAD systems in the presence of the non-inhibitory mAb 5C11 or PBS, leading to a 70% reduction of HMW VWF multimers, 180 min after the start of perfusion (p=0.01 for HM II and p=0.0003 for Impella). This was also reflected by a severely decreased VWF:CB/VWF:Ag ratio (0.59 ± 0.11 and 0.52 ± 0.10 at 180 min versus 1.00 ± 0.06 and 1.07 ± 0.09 before perfusion, for the HM II (p=0.03) and Impella (p=0.001) respectively) and moderately decreased VWF:RCo/VWF:Ag ratio (0.94 ± 0.07 and 0.81 ± 0.04 at 180 min versus 1.00 ± 0.11 and 1.03 ± 0.05, for the HM II (p=0.34) and Impella (p=0.007) respectively). Interestingly, blocking ADAMTS13 activity using the anti-ADAMTS13 mAb 3H9 at 20 µg/mL prevented the loss of HMW VWF multimers in both circuits: HMW VWF multimers were not decreased in function of time (p=0.50 for the HM II and p=0.06 for the Impella, 180 min after the start of perfusion). The preservation of HMW VWF multimers was also reflected by normal VWF:CB/VWF Ag (0.92 ± 0.16 and 0.97 ± 0.11 versus 0.93 ± 0.09 and 1.19 ± 0.12 for the HM II (p=0.75) and Impella (p=0.06) respectively) and VWF:RCo/VWF:Ag ratios (0.98 ± 0.21 and 0.98 ± 0.07 at 180 min versus 1.03 ± 0.20 and 0.90 ± 0.05 for the HM II (p=0.25) and Impella (p=0.25) respectively).

Conclusion: Using an inhibitory anti-ADAMTS13 antibody, we were able to prevent the loss of HMW VWF multimers in both the in vitro HeartMate II™ and Impella CP® device. Our data unequivocally show that the loss of HMW VWF multimers is due to shear-induced ADAMTS13 proteolysis. Hence, inhibiting ADAMTS13 function might be an effective way to treat aVWS in LVAD patients.

P65 **Sofia Ramström**

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Lysis of blood cells by arachidonic acid causes ADP-dependent platelet activation responses in platelet function tests using whole blood.

Background: The use of arachidonic acid (AA) to stimulate platelets is considered the most specific approach to study aspirin treatment efficacy. However, very high concentrations of AA are used, and it has been previously reported that AA can induce cell lysis. Several clinical studies have reported decreased responses to AA in whole blood tests in the presence of clopidogrel.

Aim: To investigate whether cell lysis could lead to unspecific effects contributing to platelet activation in situations where AA is used as platelet agonist.

Methods: Blood from healthy donors was collected after informed consent in a procedure approved by the local ethics committee. Sub-samples were collected during AA-induced platelet activation in light transmission aggregometry (LTA) in citrated platelet-rich plasma (PRP), and in two assays using hirudinized whole blood, multiple electrode aggregometry (MEA, Multiplate®) and flow cytometry. The appearance of platelet and red blood cell fragments as well as activated platelets was detected using flow cytometry.

Results: We found that cell lysis, especially of red blood cells, does occur at the concentrations of AA used in the clinically used tests (0.5-1 mM) and that this induces ADP-dependent aggregation responses. In flow cytometry, very limited platelet activation was detected before reaching AA concentrations where cell lysis also occurred, making it problematic to develop a reliable flow cytometry test using AA as platelet agonist.

Conclusion: We conclude that cell lysis and ADP release contribute to AA-induced platelet responses, most markedly in whole blood assays. This finding could potentially explain some differences between studies comparing methods using whole blood and platelet rich plasma and also how clopidogrel treatment could influence AA-induced aggregation results in previously published studies. Our findings highlight some issues with AA as reagent for platelet activation, which also have an impact on how platelet activation assays using AA should be interpreted.

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The presence of anti-ADAMTS13 autoantibodies does not change ADAMTS13 antigen levels measured by an in house developed ADAMTS13 antigen ELISA

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Background: Immune-mediated thrombotic thrombocytopenic purpura (iTTP) is characterized by the presence of autoantibodies (autoAbs) against ADAMTS13. ADAMTS13 is a metalloprotease which consists of multiple domains: metalloprotease (M), disintegrin-like (D), cysteine-rich (C), spacer (S), 8 thrombospondin type 1 repeats (T1-8) and 2 CUB domains. ADAMTS13 antigen (Ag) levels are moderately to severely reduced in most iTTP patients, indicating that antibody-mediated clearance is the major pathogenic mechanism. In addition, a severe reduction in ADAMTS13 Ag has recently been shown to be associated with a higher mortality rate.

Aims: We aimed at validating our in-house ADAMTS13 Ag ELISA in terms of sensitivity, reproducibility and background and investigated whether ADAMTS13 Ag determination is influenced by the presence of anti-ADAMTS13 autoAbs.

Methods: In our in-house ADAMTS13 Ag ELISA, ADAMTS13 is captured by the anti-ADAMTS13 monoclonal antibody (mAb) 3H9 (anti-M) and detected using a mixture of biotinylated anti-ADAMTS13 mAbs 17G2 (anti-CUB1) and 19H4 (anti-T8) and HRP-labeled streptavidin. Detection limit (DL, 3xSD above mean of blank) and quantification limit (QL, minimum measurable concentration with coefficient of variation (CV) < 20%) were determined by performing six replicates of serial dilutions of normal human plasma (NHP), in six assays. The influence of iTTP autoAbs on ADAMTS13 Ag determination was tested using 20 purified iTTP IgG samples. First, NHP was pre-incubated with the purified iTTP IgGs and ADAMTS13 Ag levels in this mixture were determined. Next, the presence of overlapping epitopes between the assay mAbs (3H9, 17G2 or 19H4) and iTTP IgGs were studied in a competition ELISA on coated recombinant human (rh)ADAMTS13.

Results: The DL of our in-house ADAMTS13 Ag ELISA is 2.52 ng/mL (1/396.9 NHP dilution) and the QL is 7.00 ng/mL (1/142.9 NHP dilution). The CV for each NHP dilution (1/6.7; 1/11.1; 1/18.5; 1/31.0; 1/51.4; 1/85.7; 1/142.9) was 3.0%, 3.5%, 3.1%, 3.0%, 2.6%, 2.3% and 3.6%, respectively. Additionally, the ADAMTS13 Ag measurements in NHP were similar with or without pre-incubation of purified iTTP IgGs, indicating that iTTP autoAbs do not interfere with the ADAMTS13 Ag determination. Moreover, a competition ELISA in which the binding of the assay mAbs (3H9, 17G2 and 19H4) to rhADAMTS13 in the presence of purified iTTP IgGs was tested, showed that none of the purified iTTP IgGs competed with 3H9 or 17G2 for rhADAMTS13 binding, and only one iTTP IgG sample competed with 19H4 for rhADAMTS13 binding.

Conclusion: Our in-house ADAMTS13 Ag ELISA has been validated in terms of sensitivity, reproducibility and background. Additionally, we showed that the presence of anti-ADAMTS13 autoantibodies does not change ADAMTS13 antigen levels. The high affinity of our murine anti-ADAMTS13 mAbs for plasma ADAMTS13 possibly explains why the presence of low(er) affinity patient anti-ADAMTS13 autoAbs do not interfere in the ELISA.

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Von Willebrand factor deficiency does not influence angiotensin II-induced abdominal aortic aneurysm formation in mice

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Background: Abdominal aortic aneurysm (AAA) refers to a permanent, localized dilation of the abdominal aorta that exceeds its normal diameter by 50%. When left untreated, AAAs have a high risk of rupture, which is associated with a high mortality rate. AAA pathophysiology is characterized by progressive inflammation, proteolytic degradation, vessel wall destabilization and thrombus formation. Von Willebrand factor (VWF) is a multimeric plasma glycoprotein, known for its role in hemostasis, thrombosis, vascular stability and inflammation. Clinical studies have reported elevated VWF levels in AAA patients and suggested VWF as a potential biomarker for AAA growth. However, the exact role of VWF in AAA pathophysiology is currently not clear.

Aim: To investigate the potential thromboinflammatory involvement of VWF in AAA pathophysiology using an angiotensin II continuous infusion-induced AAA mouse model.
Methods: AAA formation was induced in both wild type and VWF-deficient mice by subcutaneous implantation of an osmotic pump, which continuously released a dose of 1 µg/kg/min angiotensin II for 28 days. Survival in both groups was monitored for the duration of the experiment. After the infusion period, the surviving mice were sacrificed to harvest the abdominal aortas in order to determine AAA incidence and severity. Cryosections of the suprarenal segments were analyzed with different histological procedures to visualize the aneurysm and the associated intramural thrombus.

Results: After implantation of the angiotensin II-infusing osmotic pumps, 19% (3/16) of VWF-deficient mice did not reach the end of the 28 day-infusion period. In comparison, all wild type mice (n=15) survived; however, this difference was not statistically significant (p=0.08). After 28 days, both AAA incidence and severity were assessed from the isolated suprarenal segments. Overall AAA incidence was not statistically different between VWF-deficient mice (7/13; 54%) and wild type animals (5/15; 33%) (p=0.4). Also, overall severity was not significantly different between the two groups after classification of the mice based on the appearance of the abdominal aorta. Accordingly, the maximal abdominal aortic diameter in wild type animals (1.6 ± 0.7 mm) was comparable to the maximal diameter in VWF-deficient mice (1.9 ± 0.6 mm, p = 0.3). Despite increased VWF plasma levels (169 ± 45%) in the wild type mice after the angiotensin II infusion period, these levels were not correlated with AAA severity. Additionally, detailed histological analyses of important hallmarks of AAA, including elastic degradation, intramural thrombus formation and leukocyte infiltration were assessed. Four wild type mice (27%) developed an aneurysm with an intramural thrombus containing VWF. Interestingly, 4 VWF-deficient mice (31%) also formed an intramural thrombus in the absence of VWF. Measurement of both fibrin and red blood cell content in these thrombi did not reveal differences in thrombus composition between both groups. In addition, staining of leukocytes in the suprarenal aortic tissue revealed a similar extent of inflammation in both wild type and VWF-deficient mice.

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The VWF-GPIb interaction mediates thrombo-inflammation in experimental stroke via recruitment of monocytes, neutrophils and T-cells to the brain

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Introduction: Von Willebrand factor (VWF) is crucial for hemostasis by capturing platelets at sites of vascular damage and by serving as a carrier molecule for FVIII. Recently, also an inflammatory role for VWF has emerged. Previously, we and others have shown that mice deficient in VWF are protected from ischemic stroke. Intriguingly, mainly VWF-mediated platelet adhesion and not VWF-mediated platelet aggregation was found to be detrimental in the acute phase of ischemic stroke. Hence, VWF contributes to stroke progression in a way that is not strictly related to thrombus formation, but most likely also involves an acute inflammatory component. However, how VWF mediates thrombo-inflammatory ischemic stroke brain damage is currently unclear.

Aim: To investigate the potential inflammatory role of the VWF A1- platelet GPIb interaction in ischemic stroke.

Methods: VWF knockout (KO) and wildtype (WT) mice were subjected to 60min of cerebral ischemia, followed by 23h of reperfusion. To study the role of the VWF - platelet GPIb interaction in VWF-mediated inflammation, mice were treated with a nanobody specifically blocking the GPIb-binding site in the VWF A1 domain (KB-VWF-006bi) or a control nanobody (KB-VWF-004bv), 1h after stroke onset. Twenty-four hours after stroke, mice were neurologically scored, cerebral infarct sizes were measured, and flow cytometric and immunohistological analysis of immune cell recruitment to the brain was performed.

Results: In a first set of experiments, the acute cerebral immune response after stroke was compared between VWF WT and KO mice by flow cytometric analysis. Twenty-four hours after stroke, the amount of recruited white blood cells in the ipsilesional hemisphere of VWF KO mice was 2-times less as compared to VWF WT mice ($p=0.02$). Upon further analysis, we found two-fold less proinflammatory monocytes ($p=0.03$), five-fold less neutrophils ($p=0.01$) and four-fold less T-cells ($p=0.002$) in the ischemic brain of VWF KO mice compared to VWF WT mice. Interestingly, immunohistological analysis revealed that most of the recruited neutrophils and T-cells were located within the microcirculation of the ipsilesional hemisphere.

Next, we investigated whether inhibition of VWF-mediated platelet adhesion could reduce immune cell recruitment to the brain and thereby protect mice from ischemic stroke brain damage. To test this, we used a nanobody specifically inhibiting the interaction between the VWF A1 domain and the platelet receptor GPIb. Interestingly, inhibition of the VWF-A1–platelet GPIb interaction led to a reduced recruitment of white blood cells to the ischemic brain compared to control mice. More specifically, recruitment of proinflammatory monocytes was two-fold less ($p=0.02$), neutrophil recruitment was five-fold less ($p=0.0003$) and T-cell recruitment was two-fold less ($p=0.02$) in VWF A1 nanobody treated mice compared to mice receiving a control nanobody. Importantly, this reduced recruitment of white blood cells to the ipsilesional hemisphere was accompanied by an improved neurological outcome ($p=0.02$) and reduced cerebral infarct sizes ($p=0.001$).

Conclusions: Inhibition of the interaction between VWF and platelet GPIb reduces the pro-inflammatory effects of VWF in experimental stroke, revealing an inflammatory component of the VWF-GPIb interaction in the ischemic brain. This puts forward inhibition of the VWF-GPIb interaction as a promising strategy to reduce thrombo-inflammation in ischemic stroke brain damage.

P69 **Bernard Payrastre**

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Platelet activation and prothrombotic properties in a mouse model of peritonitis sepsis

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Thrombocytopenia and microvascular thrombosis are often associated with sepsis. Several studies have described an implication of platelets in this pathology but their kinetics of activation and behavior remain poorly characterized. Using a mouse model of peritonitis we show the appearance of platelet-rich thrombi in organ microvessels correlated with organ damage and thrombocytopenia. Our data indicate that complementary methods are necessary to characterize platelet activation during sepsis as circulating soluble markers and platelet-monocyte aggregates revealed early platelet activation, while surface activation markers were detected at later stage.

A microfluidic based *ex-vivo* thrombosis assay demonstrated that platelets from septic mice have a prothrombotic behavior at shear rate encountered in microvessels. Interestingly, we found that even though phosphoinositide-3-kinase beta-rdeficient platelet mice formed less thrombi in liver microcirculation, peritonitis sepsis activates a platelet alternative pathway to compensate the otherwise mandatory role of this lipid-kinase to form stable thrombi at high shear rate. In conclusion, platelets are rapidly activated during sepsis. Thrombocytopenia can be attributed in part to platelet-rich thrombi formation in capillaries and platelet-leukocytes interactions. Platelets from septic mice have a prothrombotic phenotype at a shear rate encountered in arterioles. Further studies are now necessary to unravel the molecular mechanisms leading to this prothrombotic state of platelets in order to guide the development of future treatments of polymicrobian sepsis.

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Functional validation of microRNA-126-3p as a platelet reactivity regulator using human hematopoietic stem cells

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Background: Platelets are involved in important pathological processes including inflammation, cell proliferation, immune modulation and thrombus formation. The identification of determinants of platelet reactivity may have a major impact in diseases where platelets play a role. Platelets are an abundant source of miRNAs. MiRNAs expression profile in platelets correlates with platelet reactivity. The vast majority of studies designed to address the role of miRNAs in platelet reactivity regulation are association-type studies investigating correlations between miRNAs levels and platelet reactivity or recurrence of ischemic events (1-3). Functional approach is needed to understand the mechanisms involved and to validate the direct role of miRNAs on platelet function. However, the biological relevance of these associations remains uncertain and functional validation of these candidate miRNAs on human-derived cells is lacking.

Aims: To functionally validate the candidate miR-126-3p as a regulator of platelet reactivity in platelet-like structures (PLS) derived from human hematopoietic stem cells using a flow-based assay, and to identify gene targets of miR-126-3p regulating PLS reactivity.

Methods: CD34+-derived megakaryocytes were transfected with miR-126-3p and differentiated in PLS (4). PLS reactivity was assessed by perfusion in a fibrinogen-coated flow chamber. Selected gene target of miR-126-3p was validated by quantitative PCR, protein quantification and reporter gene assay.

Results: MiR-126-3p transfection procedure leads to a 2-fold increase in miR-126-3p level and does not affect megakaryocytes differentiation or PLS production. Overexpression of miR-126-3p is associated with an increase of 156% of PLS adhesion compared to control. Silencing one of the *in silico* predicted gene targets of miR-126-3p, plexin B2 (an actin dynamic regulator), led to similar functional results than with miR-126-3p transfection. Moreover, miR-126-3p transfection was associated with downregulation of mRNA as well as protein level of plexin B2. Finally, we validated by reporter gene assay that miR-126-3p directly downregulates plexin B2 expression by targeting its 3'UTR sequence.

Conclusions: Taken together, using a flow chamber assay, we functionally validate miR-126-3p as a regulator of platelet reactivity on PLS derived from human hematopoietic stem cells. Moreover, plexin B2 was validated as a platelet reactivity regulator, supporting the hypotheses that miR-126-3p modulates platelet reactivity, at least in part, through downregulation of plexin B2.
This work represents the first validation of the functional impact of one candidate miRNA on platelet function in human-derived cells. Validation of putative regulators of platelet function could lead to identification of new biomarkers of platelet reactivity in diseases where platelets play a major role.

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Flow cytometry for comprehensive platelet function assessment

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Introduction: Platelet functional activity can be impaired due to various conditions: trauma, surgery, pre-term birth, inflammation, sepsis, tumor, chemotherapy and many others. Evaluation of such impairments is of vital importance for research and clinical purposes, especially in the newborns and children, where blood volume and blood collection methods could be limited. We performed a simple flow cytometry based screening method of comprehensive whole blood platelet function testing on a range of pediatric and adult samples in the hematology hospital setting, including classic inherited platelet function disorders, healthy term newborns and pre-term newborns.

Methods: Venous blood was collected into vacuum plastic tubes with sodium citrate, final concentration 3.8%. 20 µL of blood was diluted 1:20 in HEPES-buffered Tyrode buffer. For platelet activation, a mixture of collagen-related peptide (10 µg/mL), PAR-1 activating peptide SFLLRN (12.5 µM) and 2.5 mM $CaCl_2$ was used. We determined forward scatter (FSC), side scatter (SSC), CD42bPE, CD61-PE, CD62P-Alexa Fluor 647, PAC1-FITC, annexin V-Alexa Fluor 647 binding and mepacrine release levels.

Results: Platelet functional activity of healthy adults (n=72), healthy children aged 1 to 16 years (n=17), pre-term neonates (33-34 gestation weeks, n=10), term neonates (>37 gestation weeks, n=10) and pediatric patients (n=12) with various inherited bleeding disorders, including type I Glanzmann's thrombasthenia (GT), Bernard-Soulier syndrome (BSS), MYH-9 type thrombocytopenia and Wiscott-Aldrich syndrome (WAS) was analyzed. Platelet response to stimulation in pre-term neonates was slightly lower than normal, but they had significant pre-activation of procoagulant platelets. Healthy newborns had normal parameters in resting platelets. However after activation PAC1, CD62P and dense granule release level were clearly below normal range. The same tendency, though a bit milder, was observed in the older children. As for pediatric platelets, FSC, as well as SSC, was normal in type I GT, greatly decreased in microthrombocytopenic WAS, increased in BSS and even more so in MYH-9, reflecting the macrothrombocytopenic nature of these disorders. CD62P and dense granule release level followed that distribution as well. CD42b was normal in GT, decreased in WAS, profoundly increased in MYH-9 and naturally absent in BSS. CD61 and PAC1 in resting and activated platelets were within normal range in BSS and absent in type I GT. Both markers were high in MYH-9 and low in WAS.

Conclusion: Flow cytometry analysis revealed differences in platelet functional activity between pre-term neonates, term neonates, pediatric patients with various inherited bleeding disorders and healthy adults.

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Integrin αIIbβ3 outside-in signaling stimulation of mTORC2 activity and Akt phosphorylation is dispensable for platelet spreading

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Integrin αIIbβ3 -mediated adhesion of murine platelets to fibrinogen stimulates PI3Kβ activity by a Src- and Pyk2-dependent mechanism, and leads to the subsequent activation of Akt and the small GTPase Rap1 (1). Inhibition of PI3Kβ prevents platelet spreading on fibrinogen (1), and independent studies on transgenic mice have suggested also a role for both Rap1 and Akt (2,3). However, the contribution of Akt to PI3K-dependent Rap1 activation downstream integrin αIIbβ3 has not been addressed. Full activation of Akt is supposed to require phosphorylation on Ser473 by the kinase mTORC2. Regulation of platelet Akt activity by mTORC2-mediated phosphorylation was demonstrated in thrombin- and IGF1-stimulated platelets, but was found to be isoform specific (4).

In this work we investigated the role of mTORC2 in Akt phosphorylation and activation downstream of integrin αIIbβ3 and we addressed its role in Rap1 activation as well as in platelet spreading on fibrinogen. Adhesion of human platelets to immobilized fibrinogen triggered the time-dependent phosphorylation of Akt and GSK3β Both events were inhibited by wortmannin as well as by the PI3Kβ specific inhibitor TGX221, and GSK3β phosphorylation was prevented by the Akt inhibitor MK2206. Phosphorylation of Akt on Ser473 was not affected upon selective inhibition of mTORC1 activity by rapamycin, but was completely prevented by the dual mTORC1 and mTORC2 inhibitor AZD8055, suggesting that mTORC2 mediates Akt phosphorylation on Ser473 downstream of integrin αIIbβ3. Similar results were also obtained using a different mTOR inhibitor, PP242. Moreover, phosphorylation of Akt on Thr308 was reduced by AZD8055 and PP242, as well. Platelet adhesion to fibrinogen caused the rapid phosphorylation of mTOR on Ser2481, a recognized autophosphorylation site. Accordingly, this event was inhibited by AZD8055, but was not affected by TGX221, indicating that mTOR activation by integrin αIIbβ3 is PI3Kβ-independent. Platelet spreading on fibrinogen was significantly reduced by TGX221, but occurred normally upon mTORC inhibition with AZD8055. Blockade of Akt activity by MK2206 prevented GSK3β phosphorylation but did not affect platelet spreading. Accordingly, pharmacological inhibition of GSK3β by CHIR99021 failed to restore spreading of TGX221-treated platelets. Integrin αIIbβ3 also promoted the stimulation of the small GTPase Rap1 in human platelets, which was dependent on PI3Kβ activity but occurred normally upon inhibition of mTORC or Akt.

These results identify mTORC2 as the kinase responsible for Akt phosphorylation downstream of PI3Kβ activation during integrin αIIbβ3 outside-in signaling, but indicate that the mTORC2/Akt/GSK3β axis is not required for platelet spreading on fibrinogen. Therefore, different effectors regulated by the lipid products generated by PI3Kβ are likely to support spreading of human platelets. Our evidence argues for a possible role of the small GTPase Rap1.

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Evaluation of a new range of platelet agonists for the diagnosis of inherited or acquired platelet dysfunctions

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Introduction: Diagnosis of platelet dysfunctions (inherited or acquired) are performed by light transmission aggregometry (LTA) using specific agonists targeting the major receptors (thrombin: PAR-1; ADP: $P2Y_1$ & $P2Y_{12}$; epinephrine: □2A; collagen: GPVI) and arachidonic acid signaling pathways (thromboxane: TxA_2). The aim of this study was to evaluate the performance of a new range of agonists (Arachidonic Acid, ADP, Collagen, Epinephrine and TRAP-6) by determining reference intervals in healthy donors and diagnosing of platelet dysfunction in comparison with reference agonists (CE Marked).

Methods: LTA (aggregometer TAV8, SD-Medical) was performed using citrated platelet-rich plasma (cPRP). Platelet aggregation was induced by different agonists (Agro-Bio a Stago brand and reference CE marked agonists) at different concentrations (low and high): ADP (2 and 10 µM), Arachidonic acid (1 mM), Collagen (2 and 10 µg/mL), Epinephrine (5 and 25 µM) and TRAP-6 (10 and 50 µM). Statistical analyzes (outliers research, reference intervals, Bland & Altman, Correlation tests) were carried out using the MedCalc® software.

Results: Reference intervals (min, max) were defined on 71 to 81 healthy donors with platelet count between 152 - 402 G/L using CLSI C28-A3 robust method and ranged from 53% to 100% for all agonists regardless of concentration, except for ADP at 2 µM where we found 7 low responder of 75 included healthy donors. However, a good correlation can be observed between Agro-Bio reagents as compared to reference. The performance of these agonists was evaluated in the diagnosis of inherited thrombopathies (□-storage pool disease; n=2, CalDAG-GEFI and Kindlin-3 deficiency; n=3), Glanzmann's diseases were mimicated with anti-GPIIb-IIIa (Abciximab; n=5) and in the diagnosis of acquired thrombopathies (Clopidogrel; n=6, Aspirin; n=14 or dual therapies; n=18) and showed good matching in LTA, Bland & Altman or Correlation tests (data not shown) for all agonists. Nevertheless, we observed a slight decrease in aggregation induced by collagen (2 µmL) and in a lesser extent by TRAP-6 (10 µM) for CalDAG-GEFI and Kindlin-3 deficiency, Aspirin , dual therapy treated patient as compared to the reference reagent, suggesting a better sensitivity for the diagnostic of thrombopathies involving collagen and thrombin receptors.

Conclusion : This new range of agonists have shown the same effectiveness as the existing references in the diagnosis of patient with acquired or inherited platelet disorders.

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Targeting Von Willebrand Factor A -domains with single chain antibodies

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When the vascular wall has been compromised, Von Willebrand Factor (VWF) acts as an adhesive molecule to capture platelets at sites of exposed sub-endothelial collagen. Investigation into the collagen binding behaviour of the VWF can improve molecular understanding of Von Willebrand disease, a disorder characterised by a disrupted haemostatic response leading to excessive bleeding or thrombus formation. VWF has three A-domains (A1, A2 and A3) that play a central role in this process. A1 interacts with heparin, platelets (via glycoprotein 1b-GPIb) and collagen; A2, a calcium binding I domain, contains a metalloproteinase cleavage site that allows the activity of VWF to be regulated; and A3 interacts with collagen exclusively. The collagen binding motif for A3 domain has been identified recently.
Due to the lack of crystal structure of the complete A-domain fragment, not much is known about how the A-domains interact with each other. This study attempts to shed light on their conformation by isolating single chain variable fragments (scFv) that can bind to each isolated A-domain using phage display technology. Numerous scFv were isolated using this approach and a subset of these were purified. Several scFv were unable to bind to their respective VWF A-domains in the context of the complete A1-A2-A3 protein fragment, suggesting that all three A-domains are hidden to some extent due to intramolecular interactions. The scFv isolated will provide a useful experimental tool for future work on the conformation of the VWF A-domains, including two scFv that are able to inhibit the A3 domain from binding to collagen.

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An assessment of platelet activation status and platelet reactivity in Multiple Myeloma, Smouldering Myeloma and MGUS Patients

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Thrombotic events are a major cause of death and morbidity in haematological cancer patients, being reported in up to 10% of patients with Multiple Myeloma (MM), Smouldering Myeloma (SM) and the premalignant disorder, monoclonal gammopathy of undetermined significance (MGUS). Platelet dysfunction, arising from the disease state or associated treatment regimes, is a key modulation of thrombotic risk reported in these patients which is incompletely characterised. In addition, recent evidence has pointed towards a relationship between platelets and tumour cells in MM patients via P-Selectin Glycoprotein Ligand and IL-1β mechanisms, highlighting the need to characterise platelet dysregulation in these patients.1,2 Platelet hyperactivity and the influence of circulating paraprotein levels on platelet activity is investigated in MM, SM and MGUS patients and healthy controls in this study.

Fibrinogen receptor activation (PAC-1) and platelet activation (CD62P, CD63) were assessed by flow cytometry at baseline and following treatment. Phosphatidylserine exposure (Annexin V) was also assessed. In addition, changes in platelet activation markers in response to ADP and TRAP-6 were assessed by flow cytometry. Platelet-Leucocyte Aggregates (CD61/CD45) were also assessed for the first time in MM patients. An *in-vitro* flow cell assay was used to assess thrombus formation and influence of paraprotein presence on this process. Platelet production was examined by quantification of reticulated platelets. The influence of paraprotein on platelet activity was also investigated by assessing paraprotein coating of platelets in vivo.

Results of this study indicate that platelets are hyperactivated in MM patients and in pre-malignant SM and MGUS patients. Platelet-Monocyte aggregates are found also to be increased for all patients versus healthy controls. Platelet reactivity to ADP and TRAP-6 show dysregulation in patient groups. Identifying activated pathways in these patients will also allow further study of the role of key regulators in these pathways, including cytoskeletal proteins, and may highlight alternative therapeutic targets in the prevention of myeloma associated thrombosis.

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FcγRIIa/CD32A - expressing platelets determine the severity of experimental anaphylaxis

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Platelets are key regulators of vascular integrity; however, their role in anaphylaxis, a life-threatening systemic allergic reaction characterized by the loss of vascular integrity and vascular leakage, remains unknown. Anaphylaxis is a consequence of inappropriate cellular responses triggered by antibodies to generally inoffensive antigens, resulting in a massive mediator release and rapidly occurring organ dysfunction. Human platelets express receptors for IgG antibodies and can release potent mediators, yet their contribution to anaphylaxis has not been previously addressed in mouse models, probably because mice do not express IgG receptors on platelets.

Here, we investigated whether platelets contribute to IgG anaphylaxis in human IgG receptor expressing mouse models and a cohort of patients suffering from drug-induced anaphylaxis. We found that platelet counts dropped immediately and dramatically at anaphylaxis induction, only when they expressed the human IgG receptor FcγRIIA/CD32A. Platelet depletion attenuated anaphylaxis whereas thrombocythemia drastically worsened its severity. FcγRIIA-expressing platelets were directly activated by IgG immune complexes in vivo and were sufficient to restore susceptibility to anaphylaxis in resistant mice. Serotonin released by activated platelets contributed to anaphylaxis severity. Data from a cohort of patients suffering from drug-induced anaphylaxis indicated that platelet activation was associated with anaphylaxis severity and that anaphylaxis occurrence was accompanied by a reduction in circulating platelet numbers.

Our findings identify platelets as critical novel players in IgG anaphylaxis and provide a rationale for the design of platelet-targeting strategies to attenuate the severity of anaphylactic reactions.

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Dihomo-γ-linolenic acid potentiates aspirin-mediated inhibition of platelet aggregation and alters the proteomic releasate profile

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Introduction: Aspirin (ASA) is routinely used as prophylactic protection against thrombotic events and, as is well known, inhibits the production of platelet eicosanoids. Dihomo-γ-linolenic acid (DGLA), an omega-6 fatty acid, has anti-aggregatory effects on platelets both directly (1) and following dietary intake. Previous studies have focused on prolonged exposure to DGLA, however, DGLA may also acutely influence platelet function by modulating eicosanoid production similar to ASA (2). Here we have examined the direct effects of DGLA on platelet aggregation and the proteomic releasate profile, both in the presence and absence of ASA, to gain further insight into its influences on platelet function.

Methods: Blood from healthy volunteers was collected and platelet-rich plasma (PRP) isolated by centrifugation (200*g*, 10min). Platelets were incubated with 30μM ASA or vehicle (0.05% ethanol/PBS) for 30min and light transmission aggregometry (LTA) was used to measure aggregation in response to 3μM TRAP-6 in the presence of 1mM DGLA or vehicle (0.1% ascorbic acid/PBS). To determine the platelet-released proteome we used label-free liquid chromatography– _tandem mass spectrometry (LC-MS/MS). Washed platelets were isolated from PRP by further centrifugation (2000*g*, 10min) in the presence of prostaglandin E1 and resuspended in HBSS buffer. Platelets were treated with 30μM ASA for 30min and incubated with 50μM DGLA for 3min. Following stimulation with 1U/ml thrombin, the platelet releasate was collected, precipitated, and digested with trypsin. Proteins were detected via LC-MS/MS and analysed using the *Perseus* and *Ingenuity Pathway Analysis* softwares.

Results: DGLA significantly inhibited maximal aggregation of platelets stimulated with TRAP-6 from 72±2% to 28±13% (n=6). In the presence of ASA, DGLA had an even greater inhibitory effect reducing aggregation from 64±4% to 6±2% (n=5). Using LC-MS/MS, 580 proteins were detected in 3 biological replicates of platelet releasate stimulated with thrombin following exposure to ASA and/or DGLA. In absence of ASA, DGLA altered the activation profile of 17 proteins involved in cell activation and blood coagulation. Significant changes in the levels of 180 proteins were observed when platelets were co-incubated with ASA and DGLA compared to DGLA and ASA alone. Pathway enrichment analysis revealed that DGLA reduced the levels of proteins involved in platelet aggregation, leukocyte activation and cell death while increasing levels of proteins involved in angiogenesis, vasculogenesis and endothelial cell activation.

Conclusion: DGLA significantly inhibited platelet aggregation in response to TRAP-6 to a greater extent than ASA, and in synergy with ASA when used together. This suggests that ASA and DGLA inhibit platelet aggregation via alternative pathways possibly involving the production of different eicosanoids (3). To further investigate the functional changes in platelets, the profiles of protein releasates were studied. DGLA showed particularly marked effects upon the protein profile when platelets were pre-treated with ASA, notably reducing proteins involved in inflammatory responses while increasing proteins that play roles in tissue regeneration. Together, this offers promising insights into the possibility of a dual treatment approach of DGLA and ASA to protect against thrombosis. Further research is required to elucidate the mechanisms underlying these effects particularly into the roles of eicosanoids.

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Platelet function and its association with bleeding risk in adult patients with chronic immune thrombocytopenia before and on romiplostim treatment

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Introduction: A quantitative platelet defect may cause bleeding in immune thrombocytopenic purpura (ITP), however, platelet count poorly correlates with bleeding severity in ITP. There is increasing evidence that not only platelet quantity, but also platelet function may be impaired in this disease. Still, there is insuffucient information with regard to the actual evidence of platelet function abnormality in ITP and its importance for clinical phenotype. The effects of thrombopoietin mimetics romiplostim and eltrombopag approved for ITP treatment on platelet function are also poorly understood.

Methods: We studied evolution of platelet function and clinical bleeding symptoms in 58 adult chronic ITP patients with and without romiplostim treatment using flow cytometry, thrombelastography (TEG), transmission electron microscopy, whole blood thrombus formation in the flow perfusion chamber. Blood samples were collected before romiplostim treatment, and then for 6 months with a 1-month interval for first cohort study (n=32, age median 62 years, 25 f and 7 m). Romiplostim was given weekly as a monotherapy following a standard treatment protocol (1-4 μg/kg starting dose). For additional analyses, a second cohort of the patients either before treatment (n=17 age median 66 years, 15 f and 2 m) or on romiplostim (n=9 age median 57.5 years, 8 f and 1 m) was recruited. Comparison of the parameters between patients and healthy donor groups was performed using Mann-Whitney non-parametric test. The significance level was set as 95%. To evaluate specificity/sensitivity of the parameters or their combinations, ROC-analysis was used.

Results: The resting platelets of the untreated patients were pre-activated (by PAC1, CD62P, annexin V, electron microscopy) and increased in size (forward scatter, CD42b, CD61, mepacrine uptake) compared with healthy controls. However, they responded normally to potent stimulation (collagen-related peptide 10μg/mL + SFLLRN 12.5 μM) and responded better than normal to weak stimulation (ADP 5μM), and thrombus formation was not affected by platelet pre-activation. The TEG lag time parameters R and K were increased in the ITP patients, while α angle and MA were decreased. The size, pre-activation and TEG changes were significantly greater in the patients with severe bleeding phenotype compared with those who had no hemorrhage. Evaluation of their predictive capabilities with regard to bleeding risks produced AUC values between 0.78 and 0.91, suggestung possibility of a risk calculator development. Romiplostim treatment led to a rapid (<1 month) disapperance of procoagulant annexin V-positive platelets, followed by a more gradual normalization of platelet size and decrease of PAC1-positive platelets over several months. Platelet-associated antibodies were affected, and soluble glycocalicin levels were increased by romiplostim. In some patients, disappearance of clinical bleeding syndromes on romiplostim was associated with normalization of platelet function parameters in the absence of platelet count increase.

Conclusion: To summarize, our data indicate that: a) platelets in chronic ITP are pre-activated and large, and clotting onset is slow; b) these changes are associated with bleeding independently of platelet count, and can be used for risk stratification, although they do not necessarily reflect platelet function defects; c) romiplostim treatment decreases platelet size and pre-activation.

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cAMP- and cGMP-elevating inhibitors of human platelets maintain and even enhance GPIbα- and GPVI-specific activation of Syk

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Background: The cAMP-dependent protein kinase A (PKA) and the cGMP-dependent protein kinase G (PKG) inhibit different checkpoints of platelet signaling and activation when under physiological conditions endothelial cells release prostacyclin and nitric oxide, respectively. However, regulation of GPIbα- or GPVI-induced platelet upstream signaling by these major platelet inhibitory pathways is not known so far. In this study the selective GPIbα-agonist, echicetin-coated polystyrene beads (Navdaev A., PLoS One, 2014) and the specific GPVI-agonist, convulxin were used to investigate the effects of cAMP-/cGMP-elevating inhibitors on GPIbα- and GPVI-mediated activation and signaling of human platelets.

Methods: Echicetin was purified from *Echis carinatus* venom, validated by mass spectrometry, and used to prepare echicetin beads (EB) as described (Navdaev A., PLoS One, 2014). The stable prostacyclin analog iloprost and the soluble guanylate cyclase stimulator riociguat were used to induce cAMP/PKA and cGMP/PKG pathways, respectively. Aggregation of human platelets was analyzed by light transmission aggregometry and platelet signaling by immunoblot analysis of phosphoproteins.

Results: EB and convulxin induced full platelet aggregation associated with fast but transient phosphorylation of Syk, LAT, PLC□2, p38 and delayed phosphorylation of Akt. Stimulation of PKA or PKG pathways by iloprost or riociguat strongly inhibited platelet aggregation and AktS473, p38Y180/T182 phosphorylation, but maintained and even enhanced phosphorylation of SykY352, SykY525/526, LATY191 and PLC□2Y759.
Conclusion: Both GPIbα- and GPVI-specific signaling include phosphorylation of SykY352, SykY525/526, LATY191, PLC□2Y759, AktS473 and p38Y180/T182. In contrast to Akt and p38 phosphorylation, tyrosine phosphorylation of Syk and PLC□2 was not impaired by cAMP- or cGMP-elevating platelet inhibitors. These data indicate that activation of Syk and PLC□2 as upstream responses of GPIbα- and GPVI-mediated signaling is not blocked by PKA/PKG pathways in human platelets.

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The molecule KI3112 as a potential antiplatelet agent able to inhibit GPVI activation.

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Introduction: Ischaemic heart disease and stroke have been the two major causes of death worldwide over the last 15 years (1). As platelets play an essential role in the development and progression of these cardiovascular diseases, antiplatelet agents represent the treatment of choice in these patients. Nevertheless, the increasing emergence of non-responding individuals and the high rates of bleeding-related side effects highlight the need of developing new antiplatelet agents with an improved benefit-risk balance.
Glycoprotein VI (GPVI) is the major collagen receptor in platelets and has been proposed as a promising target since it is exclusively expressed in platelets and megakaryocytes, and due to its essential role in thrombosis while no required for hemostasis (2). In this context, we recently showed an up-regulation of GPVI signaling in ST-elevation myocardial infarction patients (3).

Methods: The aim of this study was to identify novel antiplatelet molecules by focusing on GPVI. We performed a cell- based screen of intracellular calcium mobilization upon platelet activation with the GPVI-specific agonist Collagen- related peptide (CRP). We tested a chemical library composed by biologically annotated kinase inhibitors. Molecules that showed inhibitory activity were then tested by LTA (Light transmission aggregometry) upon platelet activation with CRP, collagen and thrombin. The potential platelet toxicity of the selected molecules was evaluated by calcein- based platelet viability assays.

Results: We identified a number of kinase inhibitors as potential hits. We then tested commercially available molecules and found that KI3112 inhibited the intracellular calcium release upon GPVI activation, with an EC50 of 1.3μM. Furthermore, KI3112 efficiently blocks CRP- induced aggregation whereas collagen-induced aggregation is moderately inhibited. Besides, thrombin-induced aggregation did not significantly changed, suggesting selectivity of this compound for the GPVI-mediated signaling pathway. No significant changes in platelet viability were found upon the addition of this drug.
We are currently evaluating the effectiveness of this drug at inhibiting platelet adhesion to collagen and testing its efficacy in in-vivo assays.

Conclusions: The kinase inhibitor KI3112 seems to inhibit effectively and selectively GPVI-induced platelet activation. Further confirmation of the preclinical platelet aggregation efficacy might support the repurposing of drugs targeting kinases as antiplatelet agents.

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Clinical Utility of Immature platelet fraction [IPF] parameter in Sysmex XN Series as a predictor in all cases of Thrombocytopenia to determine etiology immune nonimmune and to determine indication for platelets transfusion.

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Introduction: Platelets plays an essential role in preserving vessel wall integrity, so it is associated with a defect of primary haemostasis. Megakaryocytes are precursor of blood platelets. Proliferation and maturation of megakaryocytes is controlled by thrombopoietin [TPO] or c-mpl ligand, IL3, IL6, IL11 Thrombocytopenia appears in the background of a multisystem disorder. It is classified as:
1.The failure of the bone marrow to produce adequate numbers of platelets.
2.Increased peripheral destruction or sequestration of platelets.
Thrombocytopenia can be: Due to decreased platelet production [Acquired], immune mediated thrombocytopenia or non-immune mediated thrombocytopenia. In the evaluation of thrombocytopenia, quantification of immature platelets can be used as index of bone marrow thrombopoietic activity. Immature platelet fraction [IPF] reflect the rate of thrombopoiesis. This reticulated platelets contains mRNA & are the youngest forms and functionally & metabolically active than the resting ones. They express more glycoprotein Ib&IIb/IIIa receptors.
Aim : As care pathways are different in each case of thrombocytopenia, our aim was to evaluate IPF parameter in all cases of thrombocytopenia as a predication of platelets recovery transfusion regulator and a marker of thrombocytopenia [bone marrow activity]

Inclusion criteria: Patients who had platelets <50,000/cumm by impedence were screened

Methods and Materials : The study was done at tertiary hospital in Mumbai ,India. 200 Patients were screened with different history of thrombocytopenia. Blood collected in K2EDTA (B.D.vacutainers). Lab.temperature maintained was ambient (20-26°C). Samples were tested within 4 hrs of draw time.

Rejection criteria was followed: Gross hemolysed, clotted, frozen or delayed transport samples were not included in this study. Immature platelet fraction [IPF] was identified by flow cytometry technique & enumerated the use of a nucleic acid specific dye Oxazine [fluorescent dyes] in PLT-F Channel of sysmex XN1000 analyser.

A case of ITP undergoing treatment
It was observed that IPF increased in ITP ,SEPSIS ,DIC,MDS [Leukemic stage], liver disease malaria parasites, where increased IPF suggests increased destruction / consumption. Decreased IPF was seen in Cancer ,AA/PNH ,Bleeding cases which suggests decreased bone marrow production. In dengue cases IPF has strong correlation with the recovery of platelets counts.
A case of DENGUE serially followed during treatment
It was observed that in Dengue patients 21% Patients showed recovery within 24 hrs of the rise of IPF above 6.6 %. 8% patients showed recovery within 24-48 hrs when the IPF was > 10%.

Conclusion : IPF can be useful test in the initial evaluation of patients with thrombocytopenia.
Turn around time is rapid and available with the CBC
Patients with ITP ,DIC , Sepsis and liver disease with increased IPF supports that thrombocytopenia is due to peripheral destruction.
Patients with decreased / low normal IPF in the presence of thrombocytopenia suggests that thrombocytopenia is due to bone marrow failure & bleeding is more likely to occur.
Monitoring IPF can guide and limit the use of prophylactic platelet transfusions in view of anticipated imminent recovery of the platelet count..Transfusion can be considered when the % IPF value are not rising which would indicate poor intrinsic thrombopoietic activity

KEY MESSAGE: IPF differentiates increased platelets destruction or consumption from bone marrow failure / suppression. High IPF suggests increased thrombopoiesis where as low IPF suggests decreased thrombopoiesis.

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Analysis of glycoprotein VI (GPVI) binding to fibrin clots

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Background: GPVI has recently been proposed to act as a receptor for fibrin, a role separate from its function as a collagen receptor (1,2). However, whether monomeric (3) or dimeric GPVI (4) serves in this capacity remains controversial. Furthermore, the level of GPVI binding to fibrin measured by different laboratories, to immobilized fibrin(ogen) substrates, have produced contradictory results: Either significant levels of binding were reported (1–3) or only low levels (4,5). These discrepancies may arise from the difficulties in measuring binding to fibrin with ELISA or other methods using immobilised fibrin, where physiological clot formation is not assured and fibrin fiber characteristics may be different from fibers formed physiologically. We report here our analyses of GPVI-binding to fibrin clots formed in solution with defined components, without fibrin immobilisation, which is a model closer to physiological conditions

Methods: Recombinant GPVI-dimer (GPVI-Fc_2) (6), fibrinogen, and control protein (albumin or human Fc) in the presence or absence of factor XIII (FXIII) + Ca2+ were mixed and converted to a fibrin clot by adding thrombin. The amount of GPVI-dimer bound to fibrin clots was quantitated. Effects of GPVI-dimer specific antibody (mFab-F) and Factor XIII (FXIII) were analysed. Confocal images were obtained for washed platelets bound to fibrin clots (GPVI and integrin αIIbβ3 staining).

Results: GPVI-Fc_2 shows dose-dependent, saturable binding to fibrin clots. The affinity constant (K_d) was calculated to be 1.19 μM (n=3). GPVI-Fc_2 also bound to cross-linked fibrin; its affinity (K_d = 0.508 μM, n = 4) was increased about fourfold compared to that of non-crosslinked fibrin. The GPVI-dimer antibody mFab-F dose-dependently inhibited GPVI-dimer binding to fibrin. Control proteins, albumin or Fc fragment, did not bind significantly to the fibrin clot. Confocal images (GPVI and integrin αIIbβ3 staining) showed that platelets bind to the fibrin fibres (formed from fluorescently labeled fibrinogen) and are spread and aggregated. In contrast, platelets treated with Eptifibatide (αIIbβ3 inhibitor) are not spread but still bound to the clot.

Conclusions: GPVI-Fc_2 binds to fibrin clots, with a typical receptor–ligand binding curve. The calculated K_d values are similar to those for the binding of GPVI-Fc_2 to D-fragment and D-dimer (4), consistent with the involvement of this region of the fibrinogen molecule in the binding to GPVI. Because non-crosslinked fibrin fibers can bind to GPVI-Fc_2, this indicates that crosslinking is not required. However, crosslinking fibrin does increase the affinity of GPVI-Fc_2 for fibrin. Our findings suggest that the GPVI-binding site in fibrinogen becomes exposed after fibrin fiber formation or cleavage to fragment D. Our data raises the possibility that the low amount of binding to immobilised fibrinogen may be attributable to partial exposure of the binding site due to immobilisation of fibrinogen, which is the condition used in ELISA assays and flow adhesion studies. *Acknowledgments*: These studies are supported by British Heart Foundation grants (SP/13/7/30575 and PG/18/36/33811).

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Plasma lipid environment in hyperlipidemic and hypercholesterolemic mice enhances platelet and coagulation activation independent of age

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Rationale: Dyslipidemia as well as aging are well-established risk factors for cardiovascular diseases (CVD). Familial hyperlipidemia (FH) is the most common genetic predisposition for CVD. However, in the heterogeneous group of hyperlipidemic and hypercholesterolemic patients it is unclear how differences in plasma lipids affect a prothrombotic phenotype.

Objective: To investigate how elevation of plasma lipids, modified by genetic modification and age, influence platelet and coagulation tendency. Mice deficient in apolipoprotein E (ApoE) or low-density lipoprotein receptor (LDLR) were used as models for human FH, with *Apoe*-/- mice modelling hyperlipidemia and *Ldlr*-/- mice modelling hypercholesterolemia.

Methods and Results: Plasma cholesterol levels were higher in (aged) *Apoe*-/- mice than in *Ldlr-/-* mice. Thrombus formation *ex vivo* was investigated in blood from young and aged *Apoe*-/- and *Ldlr*-/- versus wild-type (WT) mice by perfusion over microspots of thrombogenic surfaces. Parameters of coagulant thrombus formation over time were more increased for *Apoe-/-* mice than for *Ldlr-/-* mice, Independent of age. This difference was reflected in the plasma coagulant activity. Parameters of noncoagulant thrombus formation under flow showed a more pronounced gain-of- platelet-function in *Ldlr*-/- mice than in *Apoe*-/- mice when compared to WT, again independent of age. Surprisingly, proteomes of *Ldlr*-/- and *Apoe*-/- platelets were highly comparable to that of WT platelets. Platelet lipidomic profiles indicated increases in both cholesterol levels and glycosphingolipids in platelets from both knock-out mice. Transplantation of WT or *Ldlr-/-* bone marrow into *Ldlr-/-* recipient mice resulted in similarly, high gain-of-platelet-function. Hence, the prothrombotic phenotype of *Ldlr*-/- platelets appeared to be adapted from the hyperlipidemic environment.

Conclusions: The prothrombotic propensity of *Apoe-/-* and *Ldlr-/-* mice appears to be age-independent, and driven by a differential gain-of-function of platelet activity (*Ldlr-/-* > *Apoe-/-*) and of coagulant activity (*Apoe-/-* > *Ldlr-/-*). These changes are driven by the altered plasma lipid environment, rather than by differences in platelet protein profiles. These results plead for a more tailored anti-thrombotic therapy in patients with hyperlipidemia or hypercholesterolemia.

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New type of nanocapsules as potential carriers of adenosine receptor agonists in antiplatelet strategy.

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Background: Agonists of adenosine receptors (AR) are promising candidates in antiplatelet therapy. For numerous compounds their poor solubility in water is a significant restraint when using AR agonists in scientific or clinical approaches.

Aim: The aim of this *in vitro* study was to evaluate the antiplatelet properties of two encapsulated AR agonists, HE-NECA and UK-432,097, in DMSO-free media.

Methods: HE-NECA and UK-432,097 were separately encapsulated in the polysaccharide-based nanocapsules templated on oil cores, according to the procedure described by Szafraniec and colleagues [1]. The homogeneity and diameters of obtained nanocapsules were verified by Scanning Electron Microscopy (SEM) and Dynamic Light Scattering (DLS), respectively. Thus formed nanocapsules (10^{10} objects/ml) were incubated at 37º with aliquots of human whole blood for the increasing time intervals (3, 15 and 45 minutes). Flow cytometric assay for calcein staining was used to determine platelets' viability. The antiplatelet activity of non-capsulated (dissolved in DMSO) and capsulated HE-NECA and UK-432,097 was evaluated on the basis of the surface membrane expression of P-selectin and activated integrin complex $\alpha_{IIb}\beta_3$ in platelets stimulated with ADP (20 μM).

Results: SEM analysis confirmed the spherical structure of nanocapsules. According to the DLS measurements, the diameter of nanocapsules containing corn oil was over twofold greater compared to nanocapsules made of oleic acid, regardless of whether HE-NECA or UK-432,097 were present. We did not observe any significant effect of pure nanocapsules (containing only OA or CO) on the viability of blood platelets. The surface expression of P-selectin and activated integrin $\alpha_{IIb}\beta_3$ on platelets stimulated with ADP was significantly reduced following their incubation with encapsulated HE-NECA and UK-432,097, compared to blood treated with nanocapsules not containing AR agonists. When comparing the antiplatelet activity of encapsulated and non-encapsulated AR agonists, we found higher inhibition of platelet response to ADP after 3 minutes of incubation with encapsulated HE-NECA and UK-432,097, in relation to non-capsulated AR agonists, dissolved in DMSO. For longer incubation times the inhibitory effects of enclosed and free AR agonists on platelet *in vitro* reactivity was at similar level.

Conclusions: In the presented study we proved that the oil-core nanocapsules with hyaluronate-based shell can be applied to monitor the antiplatelet properties of AR agonists, HE-NECA and UK-432,097, under DMSO-free *in vitro* conditions. Further *in vivo* studies are essential to confirm the usefulness and effectiveness of the used nanocapsules as a new type of biocompatible carriers of water-insoluble AR agonists in a living organism.

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P85 **Sirima Kraisin**

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The role of von Willebrand factor in experimental malaria-associated acute respiratory distress syndrome

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Background: Malaria remains a global health problem, with 216 million documented cases resulting in 445,000 deaths in 2016. One of the most lethal symptoms is malaria-associated acute respiratory distress syndrome (MA-ARDS). Recent patient studies have demonstrated that severe malaria is associated with acute endothelial cell activation, accumulation of highly active von Willebrand factor (VWF) multimers, and decreased ADAMTS13 activity.

Aim: To investigate the role of VWF in malaria pathogenesis by using a murine model of MA-ARDS.

Methods: 10^4 *Plasmodium berghei* (*Pb*) NK65 parasites were injected intraperitoneally in wild-type (WT) C57BL/6 and VWF knockout (*Vwf*$^{-/-}$) mice. Blood samples were taken to assess plasma VWF and ADAMTS13 levels as well as platelet counts. Parasitemia levels were monitored by examination of Giemsa-stained blood smears. Total protein levels in bronchoalveolar lavage fluid were assessed as a measure of pulmonary edema. Quantitative RT-PCR technique was used to determine parasite accumulations in the lungs. Reticulocyte counts were evaluated using flow cytometry.

Results: In accordance with patient studies, infected mice had increased VWF levels and reduced ADAMTS13 levels ($p<0.0001$ and $p<0.01$, respectively). VWF multimer patterns were normal until the end-stage of disease (8/9 days-post infection (p.i.)), at which high molecular weight VWF multimers were significantly decreased ($p<0.0001$). Malaria-associated thrombocytopenia was VWF-independent, as thrombocytopenia was observed in both WT and *Vwf*$^{-/-}$ mice. Depletion of macrophages, however, significantly restored platelet counts in *Pb*NK65-infected WT mice. Alveolar permeability, as measured by protein leakage, was 2-fold lower in *Vwf*$^{-/-}$ mice compared to WT animals ($p=0.02$). This was accompanied by a significant reduction of leukocyte infiltration in the lungs ($p=0.03$). Despite reduced lung edema and inflammation, *Vwf*$^{-/-}$ mice died more rapidly compared with WT (9 days p.i. vs 10 days p.i., $P=0.003$), which possibly could be attributed to the significantly higher parasitemia levels and parasite accumulations in the lungs ($P<0.0001$ and $P=0.0003$, respectively). Interestingly, *Vwf*$^{-/-}$ mice showed increased numbers of reticulocytes compared to WT mice, which can account for the faster development of parasitemia in *Vwf*$^{-/-}$ mice.

Conclusion: Similar to patient studies, *Pb*NK65-mediated malaria infection in mice is associated with early elevated levels of plasma VWF, and decreased ADAMTS13 activity. Macrophages, but not VWF, contribute to malaria-associated thrombocytopenia. VWF contributes to alveolar leakage, potentially by recruiting leukocytes in the lungs. VWF deficiency is associated with increased numbers of reticulocytes, faster development of parasitemia, and shortened survival.

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