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NURSES

NUR 01.1 | Physical Activity during First Year after Pulmonary Embolism and its Association with Respiratory Symptoms, Lung Function and Functional Capacity. A Longitudinal Observation Study

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Background: Acute pulmonary embolism (PE) is a major cause of hospitalization and morbidity. Common symptoms are dyspnea and respiratory associated chest pain (RACP). The association between physical activity (PA) and respiratory symptoms is not previously studied.

Aims: To prospectively describe how physical activity associate with respiratory symptoms, lung function and functional capacity the first year after PE.

Methods: Sixty-four consecutive patients with first time PE were investigated during hospitalization and at 3, 6 and 12 months after discharge. The investigations included spirometry, six-minute walk test as well as ratings of PA, dyspnea and RACP. All participants gave their written consent to participate in the study. The study was approved by the Ethical Review Board in Gothenburg (Ref number: 063-11) and supported by The Local Research and Development Board for Gothenburg and Södra Bohuslän (grant no: VGFOUGSB-29591) and the Reneé Eander Foundation.

Results: Median PA per week increased from 4 (0-27) hours to 7 (0-29) hours, while ratings of dyspnea and RACP decreased during the year. Lung function, measured as forced expiratory volume in one second, increased between discharge and 3 months, 88.8 % predicted (SD 2.7) to 98.5 (SD 2.3). Functional capacity, measured as six-minute walk distance, increased during the whole year, 80.5 % predicted (SD 2.8) to 98.9 (SD 2.1). Patients with higher PA ratings (> 4 h/week) had greater functional capacity after 6 months. Fear of respiratory symptoms was associated with a decrease in PA.

Conclusions: Respiratory symptoms, lung function and functional capacity improved during the first year after a PE. High PA (>4 h/week) 6 months after discharge was associated to greater functional capacity. Fear of respiratory symptoms was associated with a decrease in PA. By identifying patients with fear of respiratory symptoms to encourage physical activity, sedentary behaviour after PE may be prevented.

NUR 01.2 | Psycho-social Status and Coping Strategies among Women Carrier for Hemophiliac - A Descriptive Study from India

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Background: Women carriers for hemophilia experience psychological issues and which has a major effect on children and family. The effect of carrier status may impact on individual and . family level where it is an long term psychological impact.

Aims: To assess the stress & anxiety, and coping strategies among women with carrier of hemophilia.

Methods: A prospective survey design was adopted, and the data were collected from women carrier for hemophilia. A total of 47 women were interviewed by doing a household visit and with a informed consent. Using an interview technique, the questionnaire on stress and anxiety, coping was administered and data were gathered. data were analysed with descriptive and inferential statistics.

Results: The mean age of the women carriers was 41.9 ± 6.4 (SD) years, and most (25.1 %) were 36 to 50 years of age. The majority 12(50.2%) of the women carrier had higher secondary education. All the 47 (100%) women with carrier were living with spouse with children. Majority 38 (74%) of the women carrier had reported moderate level of stress and anxiety and 7 (14.8%) had major stress and anxiety. The mean stress & anxiety scores are 38.7 ± 0.8 (SD). The common coping strategies used were turning to work or other activities to take my mind off things, finding comfort in religious or spiritual beliefs, getting advice or help from other people about what to do, learning to live with it.

Conclusions: The women carrier are experience moderate to severe anxiety as they are carrier for disease.

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NUR 01.3 | The Management of Isolated Distal Deep Vein Thrombosis in Pediatrics: A Survey of CPTHN and ISTH SSC Members

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Background: There are no published studies on the management of isolated distal deep vein thrombosis (IDDVT) in pediatrics. In adults, there is a lack of consensus on the optimal management of IDDVT. Systematic review and meta-analysis by Lim et al. (2017) reviewed 14 studies of 2918 adult patients with IDDVT. The authors concluded that rates of proximal extension and/or pulmonary embolism were lower in patients who were anticoagulated, however there is limited data to support optimal length of treatment.

Aims: To investigate the current clinical practices and medical management of IDDVT in a pediatric population.

Methods: Members of the Canadian Pediatric Thrombosis and Hemostasis Network (CPTHN) and International Society of Thrombosis and Hemostasis (ISTH) Pediatric and Neonatal Scientific and Standardized Committee (SSC) were surveyed using a multiple choice questionnaire. Survey questions consisted of 2-5 responses with a section for qualitative comments. Questions gathered information on the estimated number of patients with IDDVT, method of diagnosis, management and reoccurrence. The survey was distributed as hard copies at the CPTHN 2018 and ISTH SSC 2018 meetings and electronically to all members of the ISTH.

Results: 119 people completed the survey. The majority were physicians (88%). 38% managed 1-2 patients, 21% managed 3-5 patients, 27% managed greater than 5 patients, with IDDVT in the last 24 months. 93% were diagnosed with Doppler ultrasound. 91% of symptomatic patients received anticoagulation. 44% received anticoagulation for 6-12 weeks. 3% of patients were clinically observed with serial ultrasounds. Patients were followed for 1-6 months (31%), 6-12 months (26%) and >12 months (27%). 71% reported no reoccurrence and 68% reported no proximal extension/pulmonary embolism.

Conclusions: The survey identified current pediatric clinical practices and medical management of IDDVT. The majority of respondents confirmed diagnosis with Doppler ultrasound, symptomatic patients were anticoagulated 6-12 weeks, with a low rate of reoccurrence, proximal extension, and/or pulmonary embolism.

NUR 01.4 | Scapular Site Injection of Low Molecular Weight Heparin in Neonates: Study Update

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Background: Clinically, low molecular weight heparin (LMWH) is given subcutaneously in the arms or legs of neonates who require anticoagulation for venous thromboembolism. There is no evidence-based guideline to support these locations for injection. There is concern regarding the lack of subcutaneous fat on the extremities of a neonate. Subcutaneous fat is initially laid down at the nape of the neck, inter-scapular area in a developing fetus. This study investigates scapular site for injection (SSI) as a potential area for the administration of LMWH.

Aims: To investigate the absorption of LMWH as measured by anti-Xa levels, to assess bleeding and/or bruising, and to evaluate parental and nursing satisfaction, using SSI.

Methods: Prospective non-randomized pilot study of neonates at McMaster Children's Hospital. Following consent, SSI is demonstrated to parents and nursing staff. Absorption of LMWH is evaluated using anti-Xa levels with a therapeutic range of 0.5-1.0U/ml. Patient/nursing satisfaction and bleeding/bruising are evaluated using a 5 item Likert scale.

Results: 7 neonates have been enrolled in the study from August 2017 to December 2018, with an average gestational age of 34 weeks. Neonates received an average of dose of 2.1 (1.45-2.78) mg/kg of LMWH. 76% of anti-Xa levels taken during the study were within the therapeutic range, 19% measured below 0.5 U/ml and 5% measured above 1.0 U/ml. No bleeding events and minimal bruising have been reported. Both parents and nurses expressed satisfaction with ease of administration of the SSI in the post-study Likert scale.

Conclusions: Despite a small sample size, the interim analysis reveals that SSI seems to allow adequate absorption of LMWH to achieve therapeutic anti-Xa levels in neonates. Parents and nurses report satisfaction with no bleeding, limited bruising and a preference for this route of administration. Enrollment is ongoing.

NUR 01.5 | Trends of Anticoagulation Usage and Patient Characteristics in a Tertiary Hospital Anticoagulation Service in Western Australia 2008 - 2018 Gurugram

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Background: In 2007 an anticoagulation program was developed to provide best practice to anticoagulated patients and respond to the geographic challenges within Western Australia (WA). The service incorporated point of care technology and integrated with existing community based services to administer a safe, cost effective approach to the management of anticoagulation.

Aims: To report the anticoagulation usage and patients characteristics referred to the Anticoagulation service at Sir Charles Gairdner Hospital.

Methods: single centre retrospective review of data collected by the anticoagulation service.

Results: Between 2008 and 2018 10,417 patients (mean age 66.23 years; 95%CI 65.93,66.53); have been referred to the anticoagulation service. 59.8% of these patients were male. Cardiology and Cardiothoracic surgery have consistently been the largest source of referrals at 15.3 and 9.1% respectively. Other specialties for example gastroenterology have a noticeable increase from 0.7% in 2017 to 2.5% in 2018.

International guideline changes in the management of atrial fibrillation and venous thromboembolism have led to the increase of patients being prescribed Direct Oral Anticoagulants (DOAC) and the reduction of the numbers of patients requiring warfarin, resulting in a reduction in the length of stay within the service from a mean 8.91 days (95%CI 8.52, 9.30) in 2008 to 4.15days (95%CI 4.03, 4.26). Despite a reduction in the home visits associated with INR monitoring the service has continued to grow. It continues to incorporate effective team collaboration and focuses on correct assessment of anticoagulation choices, high quality patient engagement and education, as well as identifying concerns during the early phases of anticoagulation and therapy changes.

Conclusions: The trends in international anticoagulation therapy are reflected within our service and have resulted in practice changes. This includes an increase in the use of DOACs and a reduction in the length of stay of patients in our service. This presentation will present other characteristic of these patients.

NUR 01.7 | A Nurse Led Thromboprophylaxis Re-assessment Tool to Improve the Safety of Patients at Risk of Developing a Hospital Acquired Thrombosis

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Background: English Hospital Trusts attach CQUIN payment to Venous Thromboembolism Risk Assessment (RA) to improve uptake of RA on admission. Welsh Health Boards' have no financial incentives. 2012-Nurse led Thromboprophylaxis (TP) Re-Assessment (Re-A) tool

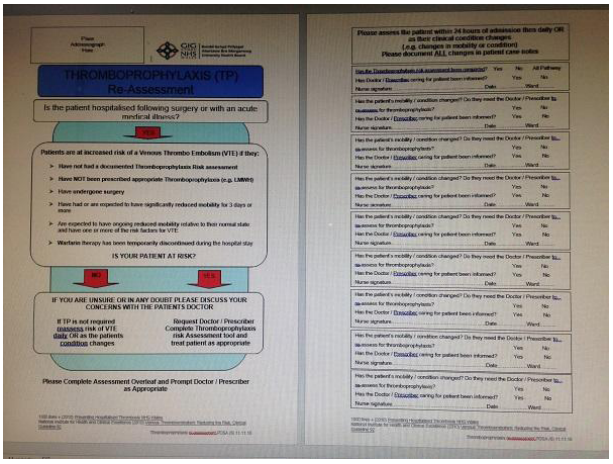

FIGURE 1 Nurse Led Thromboprophylaxis Re-Assessment Tool

was implemented on wards in Princess of Wales Hospital (POWH). Before March 2012, focus had been on completing the TP RA during admission, predominantly by Doctors. Doctors continued to complete initial TP RA but now nurses complete TP Re-A for the duration of the patient's admission.

Aims: The Aim of the tool is to increase the number of patients RA on admission to reduce the incidence of Hospital Acquired Thrombosis (HAT). The TP Re-A tool has 2-fold benefit:

1. Nurses prompt clinicians to complete TP RA improving number of patients risk assessed and treated appropriately on admission.
2. Nurses Re-A patients daily or as condition alters.

Methods: The Thromboprophylaxis Re-Assessment innovation was piloted POWH from March to July 2012. The Improving Quality Together Model for Improvement was utilised and following the completion of Plan, Do, Study, Act (PDSA) Cycles, the Re-A tool was added to The Welsh Care Metrics Audit tool in 2013 to measure quality of care at ward level.

Results: Graph A demonstrates situation in POWH prior to implementation of TP Re-A tool, the low inconsistent uptake was common throughout the hospital.

Graph B and C demonstrate continuous improvement and sustainment of TP RA & Re-Assessment rate. Graph data taken from Welsh Care Metrics monthly audit results.

Graph D demonstrates reduction in HAT's in POWH 2013-2018.

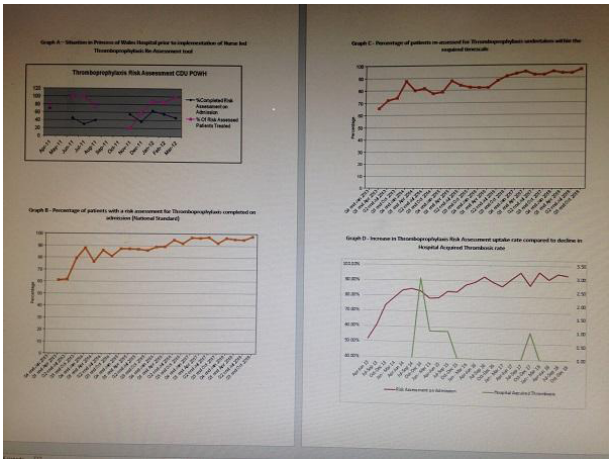

FIGURE 2 Graphs, A, B, C and D demonstrating pre and post implamentation of the nurse led TP Re-A Tool

Conclusions: The power of collaborative working between all clinicians is essential to the success of the innovation. The introduction of the TP Re-Assessment tool has resulted in an increase in the Thromboprophylaxis Risk Assessment uptake and a decrease in the number of HAT's resulting in the provision of a safer service within POWH.

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ARTERIAL THROMBOEMBOLISM

OC 02.1 | Thymidine Phosphorylase Is a Novel Anti-atherothrombotic Target

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Background: Atherothrombotic event is the major cause of myocardial infarction and ischemic stroke, yet the mechanism is under investigated. We recently found that thymidine phosphorylase (TYMP) enhances platelet activation and thrombosis; however, whether it increases atherothrombotic risk is not studied.

Aims: We tested the hypothesis that TYMP enhances inflammation and development of atherosclerosis, and thus increases risk of atherothrombosis.

Methods: Wild type (WT) and *Tymp*$^{-/-}$ mice were fed with a western diet (WD) or a high fat diet (HFD) for 8 to 16 weeks. Weight gain and organ weight were monitored. Leukocyte adhesion and rolling on the artery wall, and $FeCl_3$ injury-induced arterial thrombosis were assessed using intravital microscopy. Organ inflammation was assessed by Western blot assay of MAPKs and NF-κB activation. The inhibitory effects of tipiracial hydrochloride (TPI), the TYMP inhibitor, on atherogenesis and hemostasis were studied on *Apoe*$^{-/-}$ mice fed with WD and WT mice on chow.

Results: TYMP deficiency dramatically inhibited *in vivo* thrombosis in mice regardless of genders. WD-induced weight gain, liver and visceral fat deposition was significantly reduced in the *Tymp*$^{-/-}$ male mice, but not in the female mice. TYMP deficiency significantly reduced WD-induced activation of p38, ERK1/2, and JNK2 in the livers, and NF-κB activation in white adipose tissue, and attenuated HFD-enhanced risk of thrombosis. Overexpression of TYMP in VSMCs induced constitutive NF-κB activation, which is further enhanced by Toll-like receptor (TLR) 1/2 agonist, Pam3CSK4. Treatment of WT mice with TPI dramatically inhibited *in vivo* thrombosis, and attenuated lipid deposition in the liver, and reduced atherosclerotic lesion of *Apoe*$^{-/-}$ mice on a WD. TPI treatment did not affect tail-bleeding time.

Conclusions: Our study demonstrated that TYMP participates in multiple cellular signaling pathways and plays a mechanistic role in regulating inflammation, atherogenesis and thrombosis. Targeted TYMP-inhibition may be a novel safe therapy for atherothrombotic diseases.

OC 02.2 | The Cyclooxygenase-1/Microsomal Prostaglandin E Synthase-1/Endothelial EP4 Receptor Pathway Constrains Myocardial Ischemia-reperfusion Injury: New Insight into Microcirculation

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Background: Use of nonsteroidal anti-inflammatory drugs that inhibit both cyclooxygenase (COX)-1 and COX-2 or selectively inhibit COX-2, is associated with increased risk of heart failure. It is unknown whether microsomal (m) prostaglandin (PG) E synthase (S)-1, a new therapeutic target downstream of COX, regulates myocardial (M) ischemia/reperfusion (I/R) injury, a key determinant of heart failure.

Aims: We aimed to examine the role of PGE synthetic cascade and responding pathway in MI/R injury.

Methods: Mice deficient in COX-1 or -2, mPGES-1, or endothelial PGE receptor-4 (EP4) were subjected to 30-min coronary artery occlusion, followed by 24-hour reperfusion.

Results: COX-1 deficiency reduced biosynthesis of PGE_2 and increased infarct size after MI/R. Deletion of mPGES-1 also depressed PGE_2 while exacerbating MI/R injury. Reciprocal bone-marrow transplantation showed that both vascular cells and bone marrow derived cells contributed to the enhanced MI/R injury resulting from global mPGES-1 deletion. Cardiac microvascular perfusion, as assessed by laser Doppler flow, was significantly impaired in mPGES-1 deficient mice following restoration of coronary blood flow. Consistent with these results, mPGES-1 deletion depressed the arteriolar dilatory response to I/R in vivo and to acetylcholine ex vivo, and enhanced leukocyte-endothelial cell interactions, which were mediated via EP4. Furthermore, endothelium-restricted deletion of EP4 (EC cKO) impaired microcirculatory perfusion and exacerbated cardiac injury in MI/R. EP4 activation suppressed MI/R injury, however, EC cKO mice still showed enhanced cardiac injury despite the EP4 agonism. By contrast, EP4 blockade increased infarct size similarly in EC cKOs and littermate controls. Treatment with misoprostol, a clinically available PGE analogue, improved cardiac microcirculation and protected against MI/R injury.

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Conclusions: mPGES-1 constrains MI/R injury and this effect is partially mediated via endothelial EP4. mPGES-1 is a key protector of the microcirculation in I/R. Inhibition of COX-1/mPGES-1-derived PGE_2 confers a risk of myocardial injury in the setting of MI/R.

OC 02.3 | Recombinant Factor VII Activating Protease (FSAP) Decreases Infarct Volume and Improves Neurological Outcome in two Different Mouse Models of Ischemic Stroke

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Background: The Factor VII activating protease (FSAP) promotes fibrinolysis by selective cleavage of fibrinogen. FSAP also activates protease activated receptor-1 (PAR-1) and inhibits apoptosis. Considering that the Marburg I (MI)-polymorphism, which produces an inactive protease, is a risk factor for stroke in humans; FSAP may have a therapeutic utility in the treatment of ischemic stroke.

Aims: We investigated the effects of recombinant serine protease domain (SPD) of wild type (WT) FSAP, in comparison to MI-SPD, in two mouse models of ischemic stroke.

Methods: In the transient middle cerebral artery occlusion (tMCAO) a filament was inserted in the MCA for 1h in male C57BL6J mice. In the thrombosis mediated stroke model (TMS), $FeCL_3$-soaked filter paper was applied on the common carotid artery (CCA) for 20 min. In the tMCAO model, 1h after induction of ischemic stroke, the effect of intravenous application of buffer was compared to tissue plasminogen activator (tPA, 10 mg/kg), WT- SPD or MI-SPD (5 mg/kg each) and a combination of tPA and both isoforms of FSAP. In the TMS model, the FSAP isoforms were tested only in combination with tPA (n=7 mice in every group).

Results: In the tMCAO model, WT-SPD but not MI-SPD or tPA reduced infarct volume and improved behavioral outcome significantly. The combination of tPA and WT-SPD was more effective than WT-SPD alone. In the TMS model, the reduction of infarct volume and behavioral outcome was observed in the case of WT-SPD and tPA treatment but not MI-SPD plus tPA or tPA alone. Cerebral blood flow was only corrected in the WT-SPD plus tPA treatment group whereas tPA application in all combinations increased arterial blood flow in the CCA and reduced thrombus size.

Conclusions: Through a combination of pro-fibrinolytic and neuroprotective effects, recombinant FSAP is an effective agent for the treatment of ischemic stroke in mouse models.

OC 02.4 | Patients with Atrial Fibrillation Overexpress Fibrin-binding Platelet Receptor GPVI-dimer. A Future Anti-thrombotic Target?

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Background: Platelet receptor glycoprotein (GP) VI-dimer, rather than its inactive monomer, activates platelets through binding collagen and fibrin. Patients who have had a cardioembolic ischaemic stroke (CES) exhibit higher GPVI-dimer ($P< 0.0001$) compared to healthy controls, yet it is unclear whether this is due to a pre-existing platelet phenotype or a consequence of acute thrombosis.

Aims: Because atrial fibrillation (AF) is the major risk factor for CES, we investigated whether GPVI expression and platelet function in AF patients, who have never had a stroke differed from CES and controls.

Methods: Patients with AF admitted acutely to general medicine (n=75) at Addenbrookes Hospital, Cambridge, were recruited. Whole blood flow cytometry analysed surface expression of total platelet GPVI (monomer and dimer), GPVI-dimer, and resting platelet P-selectin expression (platelet activation maker) using specific antibodies. Results were compared against a healthy non-AF population, n=299. Serum D-dimer, fibrinogen, brain-natriuretic peptide (BNP) and high-sensitivity C-reactive protein (hs-CRP) were measured separately.

Results: Median age (Q_1-Q_3) of the AF cohort was 74yrs (66-79); control population 60yrs (47-68) ($P< 0.0001$). 44% had paroxysmal AF and 75% were anticoagulated. Total GPVI was significantly lower ($P=0.04$), but GPVI-dimer was higher ($P=0.07$) in the AF group compared to controls. P-selectin expression was also higher in the AF group ($P< 0.0001$). No difference in platelet function or GPVI expression was seen in patients on anticoagulation. Paroxysmal AF patients exhibited significantly less total GPVI ($P=0.004$) but not GPVI-dimer, compared to permanent AF. Serum hs-CRP, BNP and fibrinogen levels were strongly correlated with total GPVI expression.

Conclusions: AF patients exhibit higher GPVI-dimer but less total GPVI expression compared to controls, which appears to be related to AF burden and systemic inflammation. CES patients have significantly higher GPVI-dimer expression compared to controls and AF patients. Therefore, GPVI-dimer inhibition represents a viable future pharmacological target in ischaemic stroke.

OC 02.5 | New Peptide-agonist of PAR1 Demonstrates Neuroprotective Effect at the Photothrombosis-induced Damage of Mouse Brain

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Background: Activated protein C is known as serine protease with neuroprotective and anti-inflammatory effects on cells through activation of PAR1. Recent studies have shown that peptide-analogs of the "tethered ligand" released by APC can also have protective properties.
Aims: The aim of this study is to investigate a neuroprotective role of the new peptide, AP9, in the mice model of photoinduced ischemia.
Methods: Injection AP9 (i/v) was carried out in two ways: once - 10 min before thrombosis and twice – 10 min before and 1 h after the induction of ischemia. Brain damage was assessed by the volume of the lesion (MRI), the BBB permeability, the number of damaged neurons and the number of GFAP+ and Iba1+ cells in the penumbra, and also by the level of motor deficiency in mice.
Results: It was found that the peptide 20 mg/kg significant decreased the lesion volume (30.1±8.2 % compared to the control group) at 24 h after thrombosis. At the same time, AP9 did not influence either the cell death or the sensorimotor dysfunction of mice. The repeated injection of AP9 (20 mg/kg) provided an even more pronounced protective effect in the form of a decrease in the damage volume by 44.2±9.5%, which persisted after 96 h. We also observed a significant improvement in the neurological status of the animals in the "Gridwalk" and the "Cylinder" tests.
Conclusions: Thus, at the first time we demonstrated the neuroprotective effect of AP9 *in vivo*.
The reported study was funded by RFBR according to the research projects № 18-34-00977 and 19-015-00529.

OC 03.1 | VTE-BLEED Score Predicts Major Bleeding in Patients with Atrial Fibrillation

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Background: Bleeding risk scores in atrial fibrillation (AF) are used to identify risk factors for bleeding but not to determine anticoagulant therapy since high bleeding risk strongly correlates to high risk of stroke. VTE-BLEED is a simple bleeding risk score (Klok FA Eur Respir J 2016) that predicts major bleeding (MB) in patients with venous thromboembolism, but has never been evaluated in AF.
Aims: To evaluate VTE-BLEED in AF and whether dabigatran dose reduction in VTE-BLEED high-risk patients would result in a lower incidence of MB and the composite endpoint of MB plus stroke/systemic embolism.
Methods: Assessment of VTE-BLEED in 18040 patients of the RE-LY trial that compared dabigatran (both 150mg BID and 110mg BID) to warfarin. The score was calibrated to fit the AF population. Hazard ratios (HR) were obtained for the VTE-BLEED high-risk patients randomized to dabigatran. The risk ratios for MB and the composite outcome of MB and stroke/systemic embolism between dabigatran 150mg and 110mg were calculated for the VTE-BLEED high-risk group.
Results: The adapted VTE-BLEED score (Table 1) classified 4060 patients (22.5%) as high-risk. A high score indeed predicted MB in patients treated with dabigatran 150mg BID or 110mg BID: HRs 2.48 (95%CI 1.96-3.13) and 2.61 (95%CI 2.04-3.33). In VTE-BLEED high-risk patients, the risk ratio between the two dabigatran doses were 0.53 (95%CI 0.35-0.78) for MB and 0.55 (95%CI 0.38-0.79) for the composite outcome, both in favor of dabigatran 110mg BID (Figure 1). Compared to the label, application of VTE-BLEED would change the dabigatran dose in 21% of patients.
Conclusions: VTE-BLEED was validated for AF. Our data suggest that dabigatran dose reduction in VTE-BLEED bleed high-risk

TABLE 1 Distribution of the adapted VTE-BLEED variables in the study population (original VTE-BLEED: Age ≥60, cut-off point ≥ 2)

Factor	Score	Number (%)	Hazard Ratio (95% CI), with low-risk as reference	Absolute risk during the whole study period, n (%)
Active cancer	2	1880 (10.4)	1.38 (1.15-1.65)	140 (7.4)
Male with uncontrolled hypertension	1	3621 (20.1)	0.81 (0.69-0.95)	175 (4.8)
Anaemia	1.5	2416 (13.4)	2.72 (2.37-3.13)	273 (11.3)
History of Bleeding	1.5	5435 (30.1)	1.12 (0.93-1.35)	318 (5.9)
Age ≥ 75 years	1.5	7205 (39.9)	2.18 (1.93-2.47)	582 (8.1)
Renal dysfunction (eGFR ≤60 ml/min)	1.5	6492 (36)	1.99 (1.76-2.26)	509 (7.8)
High-risk (adapted VTE-BLEED >3)	4060 (22.5)	2.13 (1.85-2.46)		
Low-risk (adapted VTE-BLEED ≤ 3)	13980 (77.5)	Reference		

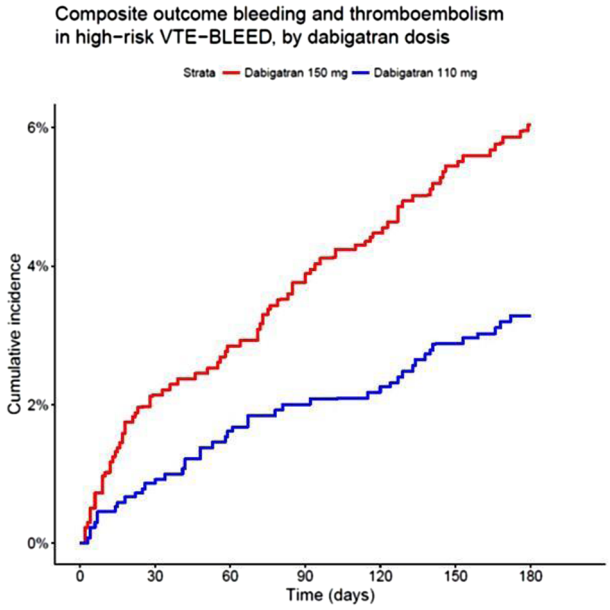

FIGURE 1 Cumulative incidence of the composite outcome in "high-risk" VTE-BLEED patients

patients -in addition to targeting individual modifiable risk factors for bleeding- may lower the risk of MB and improve patient outcome. This finding could have important clinical implications but should be confirmed in future studies.

OC 03.2 | Sex-Differences in Circulating Neutrophil Extracellular Traps (NETs) Levels and Stroke Risk: A Population-based Study

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Background: Stroke is a major worldwide health problem and an important cause of disability. We still do not fully understand the pathogenesis of stroke. An interesting possible mechanism for stroke are neutrophil extracellular traps (NETs).

Aims: The aim of this study is to determine the role of NETs in stroke risk.

Methods: In plasma from 5118 healthy individuals (aged ≥55 years) included in the Rotterdam Study, a prospective cohort study, NETs were quantified by measuring myeloperoxidase (MPO)-DNA complex levels by an enzyme-linked immunosorbent assay (ELISA). Cox proportional hazards regression was used to determine the association between NETs and ischemic and hemorrhagic stroke (adjusted for cardiovascular risk factors). Analyses were repeated for men and women separately. Since MPO-DNA levels were not normally distributed, four groups were created with MPO-DNA levels ≤0.05 AU/ml functioning as reference category. The remaining data were divided into tertiles.

Results: During a median follow up of 14.69 years [interquartile range (IQR) 9.15;15.66], a total of 548 events occurred; 385 (70.3%) ischemic strokes, 68 (12.4%) cases of intracerebral hemorrhage and 95 (17.3%) unspecified events. The median follow up from date of blood collection to stroke was 6.93 years [IQR 3.68;10.50]. A total of 245 (44.7%) stroke patients were male. MPO-DNA levels were not different between individuals with and without incident stroke (p=0.47) and between individuals with ischemic or hemorrhagic stroke (p=0.15). MPO-DNA levels were not associated with an increased stroke risk in the total population (Hazard Ratio (HR) 0.77 (95% confidence interval (CI) 0.28;2.12), p=0.62). However, subgroup analysis in men showed a decreased risk of ischemic stroke in the group with the highest MPO-DNA levels (HR 0.30, 95%CI 0.10;0.95).

Conclusions: High levels of MPO-DNA are associated with a 3.3-fold decrease in risk of acute ischemic stroke in men, suggesting a protective effect of NETs.

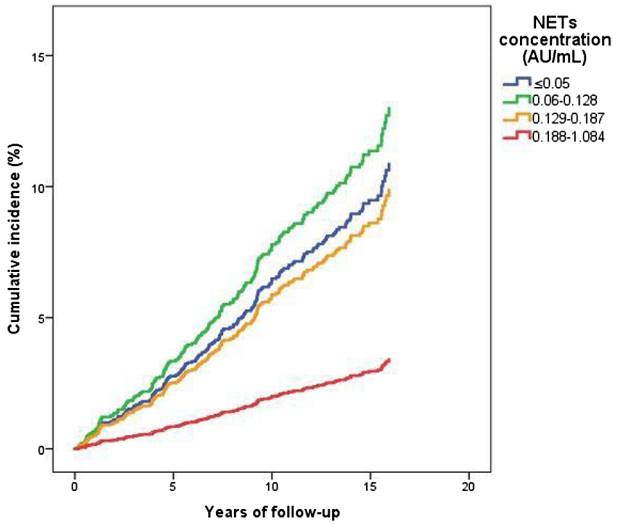

FIGURE 1 Cumulative incidence of ischemic stroke in male individuals

OC 03.3 | Evaluation of the Clinical Utility of Biomarkers for Stroke Risk Scoring in Non-anticoagulated Patients with Atrial Fibrillation – An ACTIVE-A and AVERROES Sub-analysis

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Background: High levels of biomarkers are independent predictors of ischemic events in patients with atrial fibrillation (AF). They could

help refine stroke risk prediction particularly in patients with an unclear benefit of oral anticoagulation (CHA2DS2-VASc 1-2).

Aims: We evaluated whether incorporation of biomarkers for stroke risk prediction leads to better stroke prevention.

Methods: Using data on 3578 patients with AF randomized to receive aspirin for stroke prevention in ACTIVE-A and AVERROES, we validated the association between baseline levels of NT-proBNP, Troponin-T and D-dimer and ischemic events in non-anticoagulated patients. Secondly, we assessed the incremental predictive value of these biomarkers added to the CHA2DS2-VASc score for stroke and systemic embolism. Finally, we applied a reclassification analysis to evaluate the clinical utility of biomarker-enriched risk scores in selecting patients for oral anticoagulation, compared with using CHA2DS2-VASc only according to the ESC2016 guideline. Rates of additional strokes prevented or major bleeds caused using oral anticoagulation were based on apixaban primary outcomes in AVERROES.

Results: NT-proBNP, Troponin-T and D-dimer independently predicted stroke or systemic embolism in non-anticoagulated AF patients. In a multivariable analysis with the CHA2DS2-VASc risk factors, NT-proBNP was the strongest predictor (HR 2.72; 95% CI 1.56-4.72), outperforming all clinical risk factors. Addition of NT-proBNP to CHA2DS2-VASc improved risk discrimination (C-statistic 0.699 vs 0.641), which was not further enhanced by Troponin T or D-dimer. However, using an NT-proBNP-enriched CHA2DS2-VASc risk score reclassifying patients for oral anticoagulation only marginally improved clinical net benefit (1.77) compared to using CHA2DS2-VASc >2 alone. A much larger clinical net benefit was achieved by giving oral anticoagulation using apixaban to all patients with a non-gender CHA2DS2-VASc score of >1 (2.34).

Conclusions: Although biomarkers, especially NT-proBNP, predict ischemic events and improve risk prediction in non-anticoagulated AF patients, their clinical utility to improve stroke prevention is limited and may not justify use on a population level.

OC 03.4 | Risk of Cardiac and Non-cardiac Adverse Events in Community-dwelling Older Patients with Atrial Fibrillation: A Prospective Cohort Study in the Netherlands

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Background: Patients with atrial fibrillation (AF) are at increased risk of adverse events, notably stroke. To prevent all adverse outcomes, integrated AF care is advocated though the potential domain for such multidisciplinary management is unclear. Therefore, insight in the systemic nature of AF and identifying patients at risk of adverse events after oral anticoagulation is needed.

Aims: The aim of this study was to describe the risk of hospitalization and mortality in community-dwelling older patients with AF using anticoagulants, and to assess the association between traditional cardiac risk factors for these outcomes.

Methods: This was a prospective cohort study in primary care, including 2,068 AF patients. All patients used oral anticoagulants for stroke prevention. We calculated incidence rates (IRs) of ischemic stroke, bleeding, hospitalizations and mortality, and compared risk factors using Cox regression between those with and without an adverse event, both for cardiac and non-cardiac causes.

Results: During a median follow-up of 2.7 (IQR 2.2-3.0) years, the IR per 100 person-years was 22.1 for hospitalizations and 6.7 for mortality. Non-cardiac events outnumbered cardiac events (IRs 15.7 vs 7.6 per 100 person-years for hospitalization, p< 0.001 and 5.0 vs 1.7, p< 0.001 for mortality). The IRs for stroke and major bleeding were 1.7 and 0.8 per 100 person years, respectively. In multivariate models, notably high age, heart failure and vascular disease were independently associated with all-cause hospitalization and all-cause mortality.

Conclusions: In anticoagulated community-dwelling patients with AF, stroke risk is effectively reduced, whereas risks of hospitalization and mortality remain high, importantly mainly for non-cardiac causes thereby indicating the systemic nature of AF. Notably high age, heart failure and vascular disease are predictive for such outcomes.

OC 12.1 | Pathological Features of Ruptured Atherosclerotic Plaque and Thrombus Interface in Acute Myocardial Infarction

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Background: Acute myocardial infarction is triggered by atherosclerotic plaque disruption and thrombus formation. It is considered that arterial thrombus formation is initiated by platelet adhesion on subendothelial matrix and platelet aggregation. However, the ruptured atherosclerotic plaque is characterized by less matrix proteins to support platelet adhesion. The scaffolds of platelet adhesion and thrombus formation on the disrupted atherosclerotic plaque remain unclear.

We aimed to characterize pathological findings of coronary ruptured plaque and thrombus interface.

Aims: We aimed to characterize pathological findings of coronary ruptured plaque and thrombus interface.

Methods: We examined 283 coronary aspiration thrombi within 24 hours of the onset of acute myocardial infarction. We assessed histological findings at plaque-thrombus interface with hematoxyline eosin, Azan, and Berlin blue stains (n=73, thrombi with plaque components), or cellular and thrombus components with immunohistochemistry (n=28).

Results: We found necrotic debris (95%), macrophages (95%), and cholesterin clefts (81%) at the plaque-thrombus interface, and fibrous matrix (34%), calcification (23%), extracellular DNA (15%) at the interface as small foci. Macrophages were heterogenous in cytoplasmic color; clear, yellow, or grey. One half of the grey macrophages had cytoplasmic iron deposition. We also found extracellular bilirubin/biliverdin deposition (23%) and iron deposited fibrous matrix (25%). Fibrin deposition was observed on and in the disrupted plaque. Fibrin immunopositive area was significantly larger or smaller than GPIIb/IIIa immunopositive area at the interface or thrombus, respectively. Tissue factor localized the necrotic debris and macrophages at the interface. Citrullinated histone H3 immunopositive area accounted for only 0.8 % of the interface area, and one-fourth of CD66b-positive neutrophil area.

Conclusions: Fibrin rather than matrix proteins on disrupted atherosclerotic plaque may contribute to scaffolds of platelet adhesion and further thrombus formation. Iron and bilirubin/biliverdin deposition suggest previous intraplaque hemorrhage and contribution of it to plaque instability. Neutrophils extracellular traps may play a minor role on thrombus formation.

OC 12.2 | Modeling the Formation and Mechanics of Platelet Thrombi at Extreme Shear

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Background: *In vitro* experiments suggest that formation of vWF-GPIb bonds between unactivated circulating platelets and the vessel wall or thrombus-bound platelets is essential for the formation of platelet thrombi under the very high shear conditions found in stenotic arteries[1].

Aims:

(1) To develop a spatial-temporal mathematical model of fluid dynamics, platelet and protein transport, and platelet receptor and bond dynamics;

(2) to experimentally validate the model; and

(3) to use it to investigate the conditions under which vWF-mediated bond formation and platelet activation can support occlusive thrombus formation.

Methods: A two-phase continuum model of platelet thrombus formation[2] is extended by including explicit treatment of vWF dynamics: flow-mediated transitions between globular and stretched conformations, self-association, adhesion to bound collagen and bound platelets, capture of unactivated platelets, and vWF-GPIb-mediated mechanosensitive activation of platelet integrin activation.

Results: In a model without vWF-mediated adhesion and activation of platelets[2], thrombus growth is severely limited for wall-shear rates above approximately 500/s because

(1) soluble agonist is carried downstream, and

(2) platelet attachment to the thrombus is limited by the requirement that a platelet be activated in order to cohere (Figure 1 Top). Simulations incorporating a shear-rate-dependent ability of unactivated platelets to attach to a thrombus and to become activated significantly raises the maximum shear rate under which substantial thrombus growth occurs (Figure 1 Bottom). Simulation-based characterization of how the various postulated vWF behaviors contribute to the ability of stable thrombi to grow under high shear is ongoing.

Conclusions: Mathematical modeling of the mechanical and biophysical processes occurring during arterial thrombosis elucidates whether hypothesized modes of platelet-protein interactions produce thrombus growth in accord with experiments.

Funding: NSF grant DMS-1716898.

[1] L. Casa and D. Ku, *Annual Review of Biomedical Engineering*, 2017, 19: 415-33.

[2] J. Du and A. Fogelson, *Mathematical Medicine and Biology*, 2018, 35: 225-256.

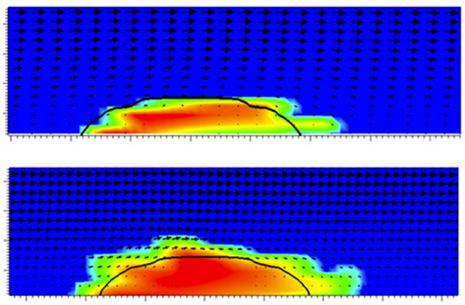

Figure 1: Snapshots at 25 sec from simulations of platelet deposition beginning in a specified injury zone (black contour) at initial wall shear rate 800/s and bulk platelet count ϕ_0 =250,000/μl. Vectors show fluid velocity. Colors show bound-platelet number density (green 10xϕ_0, red 180xϕ_0). Top: Unactivated platelets can be activated in injury zone or by ADP released by activated platelets. Only activated platelets can bind to platelets already bound in the thrombus. Bottom: In a preliminary step towards understanding the role of vWF, unactivated platelets can bind to platelets already bound in the thrombus whenever the local shear rate exceeds 1000/s.

FIGURE 1 Simulation Results

OC 12.3 | Association of Glycoprotein Ia Gene Polymorphisms with Pediatric Ischemic Stroke

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Background: Genetic risk factors for ischemic stroke (IS) in children are still incompletely characterized. Recent meta-analysis that included polymorphisms in glycoprotein Ia (GPIa) gene: dimorphism C807T and G873A, and human platelet antigen (HPA) -5, confirmed

association of GPIa 807T allele with increased risk for ischemic stroke in Asian adults (Liu H et al., 2017), but studies including children are lacking.

Aims: To study the association of individual polymorphisms HPA-5, GPIa C807T and G873A and their haplotypes with pediatric IS and its subtypes: arterial IS (AIS), including perinatal (PAIS) and childhood AIS (CAIS), and cerebral sinovenous thrombosis (CSVT).

Methods: The study group comprised 157 children with a confirmed diagnosis of IS (68 with PAIS, 70 with CAIS and 19 with CSVT) and 153 age- and sex-matched healthy children as a control group. Genotyping of HPA-5 was performed using real-time PCR with TaqMan probes (Ficko T et al., 2004). Genotyping of GPIa C807T and G873A was performed using real-time PCR followed by melting curve analysis (Morita H et al., 2001). The study was part of a project funded by Croatian Science Foundation (IP-2014-09-2047).

Results: The investigation of individual polymorphisms revealed that the presence of at least one HPA-5b allele was associated with a lower risk of IS (OR:0.59, 95% CI:0.31-1.10) but the difference was not statistically significant (P=0.096); however, a significant association was found for CAIS (OR:0.39, 95% CI:0.15-1.00) but not for PAIS (OR:0.83, 95% CI:0.38-1.79), or SVT (OR:0.51, 95% CI:0.11-2.34). Although lower but nonsignificant bCG haplotype (HPA-5/GPIa C807T/GPIa G873A) frequency (P=0.068) was calculated for IS (0.066) compared to controls (0.101), a 2.5-fold lower risk for CAIS (OR:0.37; 95% CI:0.14-0.93) was identified for the same haplotype carriers.

Conclusions: The obtained results suggest that a new, previously unreported single polymorphysm HPA-5 in GPIa is, together with the haplotype (HPA-5/GPIa C807T/GPIa G873A), protective against CAIS.

OC 12.4 | Role of Erythrocyte Gas Transport Function in the Destabilization of Atherosclerotic Plaque

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Background: One of the main clinical manifestations of atherosclerosis is the formation of atherosclerotic plaques (AP), the instability of which determines the risk of fatal cardiovascular events. It is important to develop new factors of atheroma destabilization that would have predictive value. Formation of unstable AP is affected by hypoxic conditions in different parts of the vessels and intravascular hemolysis that are regulated by oxygen exchange, which in its turn is limited by erythrocyte band 3 membrane proteins and erythrocyte structure.

Aims: To reveal erythrocyte parameters which deviations may contribute to the formation and progression of unstable atheroma.

Methods: Totally 15 patients and 20 donors were included in this study. They were divided into three groups, including patients with stable and unstable plaques and healthy donors. The stability of plaques, obtained from patients undergoing carotid endarterectomy, was determined by histological examination of AP microscopic sections by AxioCam MRc5. For erythrocyte analyses, the whole blood was taken by venipuncture. Characteristics of individual cells were determined from measurements carried out on scanning flow cytometer (fabricated by CytoNova Ltd., Novosibirsk, Russia, http://cyto.kinetics.nsc.ru). Standard diagnostic parameters, including assessment of lipid metabolism disorders and glycated hemoglobin level in blood, were also obtained for each donor.

Results: We investigated the following parameters: erythrocyte morphology (diameter, thicknesses, volume, surface area, sphericity index), functional membrane properties (elasticity, active and total anion exchangers) and hemoglobin content in individual cells. We combined these results with standard diagnostic parameters and compared them between three groups. It turns out that ratio between total and active anion exchangers differs: patients with unstable plaque have significant nonactive fraction of anion carriers (up to 80%).

Conclusions: Such deviations in gas transport function can lead to a risk of hypoxic conditions in places with increased oxygen demand, such as AP. Consequently, it can contribute to growth and destabilization of the AP.

OC 12.5 | Assessment of the Predictive Value of von Willebrand Factor Antigen and Activity Combined Detection in Patients with Acute Myocardial Infarction

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Background: Von Willebrand factor (VWF) acts as a bridge between platelet and the subendothelial matrix, and plays a vital role during thrombosis. Acute myocardial infarction (AMI) is an arterial thrombotic disease. Plasma VWF levels in AMI patients are not only elevated quantity, but also have qualitative change.

Aims: To analyze the levels of VWF antigen (VWF:Ag) and activity (VWF:GPIbR and VWF:CB) in AMI patients, then investigate the predictive value of combined detection for AMI.

Methods: Plasma levels of VWF:Ag, VWF:GPIbR and VWF:CB were measured in AMI patients (n=105) and healthy controls (n=101) by ELISA. Spearman correlation was chosen to analyze the correlation between VWF and AMI tests. The Receiver operating characteristic curve was used to evaluate the predictive value of VWF single detection and combined detection for AMI.

TABLE 1 Evaluation of VWF single and combined detection

Parameters	AUC	Youden Index	Sensitivity, %	Specificity, %	Cut-off value	95% CI	P
VWF:Ag	0.880	0.601	81.9	78.2	99.36%	0.835-0.925	<0.0001
VWF:GPIbR	0.967	0.874	94.3	93.1	123.55%	0.945-0.989	<0.0001
VWF:CB	0.828	0.522	82.9	69.3	81.30%	0.772-0.883	<0.0001
VWF:GPIbR/VWF:Ag	0.707	0.386	74.3	64.4	1.18	0.635-0.780	<0.0001
VWF:CB/VWF:Ag	0.563	0.233	87.6	35.6	0.59	0.482-0.645	0.116
VWF:Ag+VWF:GPIbR	0.967	0.874	94.3	93.1	–	0.945-0.989	<0.0001
VWF:Ag+VWF:CB	0.881	0.630	83.8	79.2	–	0.835-0.927	<0.0001
VWF:GPIbR+VWF:CB	0.967	0.864	94.3	92.1	–	0.945-0.989	<0.0001
VWF:Ag+VWF:GPIbR+VWF:CB	0.967	0.864	94.3	92.1	–	0.945-0.989	<0.0001

Results:

(1) Plasma VWF:Ag, VWF:GPIbR and VWF:CB levels in AMI patients were significantly higher than those in control group ($P< 0.0001$).

(2) VWF:GPIbR was positively correlated with platelet level in AMI patients, while VWF:Ag and VWF:CB were negatively correlated with APTT; Only plasma VWF:GPIbR level was positively correlated with creatine kinase in AMI patients.

(3) For single detection, the diagnostic efficacy of VWF:GPIbR was significantly higher than VWF:Ag and VWF:CB ($P< 0.0001$); For combined detection, VWF:Ag+VWF:CB has significantly lower diagnostic efficacy for AMI than VWF:Ag+VWF:GPIbR or VWF:GPIbR+VWF:CB ($P< 0.0001$); The three combined detection of VWF was also significantly higher than VWF:Ag+VWF:CB ($P< 0.0001$), and there was no difference between three combined detection and VWF:Ag+VWF:GPIbR or VWF:GPIbR+VWF:CB ($P>0.05$, Table 1).

Conclusions: Plasma VWF: Ag, VWF:GPIbR and VWF:CB in AMI patients were higher than those in controls. VWF:GPIbR had better predictive value for AMI than VWF:Ag and VWF:CB. Hence, the inclusion of VWF:GPIbR contributed to obtain higher predictive power, and the three combined tests cannot significantly improve the diagnostic value of AMI.

OC 21.1 | Adherence to Direct Oral Anticoagulant Treatment for Atrial Fibrillation in the Netherlands: A Surveillance StudyD

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Background: Adherence to direct oral anticoagulants (DOACs) in daily practice may be different from clinical trial settings.

Aims: To assess adherence to DOACs in patients with atrial fibrillation in daily practice and identify predictors for non-adherence.

Methods: Individually linked data of patients with atrial fibrillation who used a DOAC were obtained from the Foundation for Pharmaceutical Statistics covering the whole of the Netherlands between 2012-2016. Adherence to DOACs was defined as proportion of days covered (PDC) ≥80% and the association between clinical variables and adherence was assessed using logistic regression. Since the half-life of DOACs is < 24 hrs, we also defined adherence less leniently, using PDC ≥95% and ≥99%. We further determined non-persistence, i.e., patients who completely discontinued DOAC treatment during follow-up in relation to adherence.

TABLE 1 Adherence (PDC >80%) to DOAC in 77 333 atrial fibrillation patients

	Total, n	(%)	Odds ratio**	(95% CI)**
Apixaban	13 878	18	Reference	
Rivaroxaban	34 167	44	0.71	(0.67-0.75)
Dabigatran	29 288	38	0.52	(0.49-053)
VKA naïve	70 977	92	Reference	
VKA experienced	6356	8	1.72	(1.60-1.85)
Age < 60 years	12 889	17	Reference	
Age 60-75 years	39 885	52	1.40	(1.34-1.47)
Age >75 years	24 559	32	1.52	(1.43-1.60)
No concomitant drug use	10 982	14	Reference	
Concomitant drug use	66 351	86	2.81	(2.69-2.93)
Men	42 662	55	Reference	
Women	34 671	45	0.69	(0.67-0.71)
High dose DOAC	59 881	77	Reference	
Low dose DOAC	17 452	23	0.86	(0.82-0.90)
SES < 90% percentile	69 241	90	Reference	
SES > 90% percentile	7666	10	1.11	(1.05-1.17)

** Multivariable adjusted for previous VKA use, dosage, age sex, concomitant drug use socioeconomic status, DOAC type.

Results: A total of 77,333 patients were included with a mean age of 70 years, who were followed for a mean of 11 months (range 0,3-48 months). The proportion of adherent patients was 76% (n=58,995). Multivariable analyses revealed that younger age, no previous VKA use, no concomitant drug use and DOAC type (dabigatran and rivaroxaban) were the strongest predictors for non-adherence (Table 1). Adherence to DOAC defined as PDC ≥95% or PDC ≥99% was 55% and 36%, respectively. The proportion of non-adherent patients (PDC < 80%; n=10,745) who discontinued treatment was 95% (n=10,164), compared to 40% (n=12,934) of patients with PDC ≥80% (n=33,165).

Conclusions: Adherence to DOACs was poor in our study as at least 24% had a PDC below the accepted 80%. Exposures most strongly related to non-adherence included no previous VKA use, young age, no use of concomitant drugs and DOAC type. Non-adherent patients were more likely to discontinue their treatment.

OC 21.2 | Rivaroxaban Versus Warfarin and Renal Outcomes in Non-valvular Atrial Fibrillation Patients with Diabetes

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Background: Warfarin has been associated with renovascular calcification and worsening renal function; while rivaroxaban may provide renoprotection by decreasing vascular inflammation.

Aims: To compare the impact of rivaroxaban and warfarin on renal outcomes in diabetics with non-valvular atrial fibrillation (NVAF).

Methods: Using MarketScan data from 1/2011-12/2017, we identified adults with NVAF and diabetes, newly-initiated on rivaroxaban or warfarin with ≥12-months insurance coverage prior to anticoagulation initiation. Patients with stage 5 chronic kidney disease (CKD) or undergoing hemodialysis at baseline were excluded. Differences in baseline covariates between cohorts were adjusted using inverse probability-of-treatment weighting based on propensity-scores (standardized differences< 0.1 achieved for all after adjustment). Outcomes included rates (events/100-person-years [PYs]) of emergency department/hospital admissions for acute kidney injury (AKI) and the composite of the development of stage 5 CKD or need for hemodialysis. Patients were followed until an event, anticoagulant discontinuation/switch, insurance disenrollment or end-of-data.

Results: We assessed 10,017 rivaroxaban (22.6% received a reduced dose) and 11,665 warfarin users. Patients had a median (25%-75% range) age of 70 (62-79) years, CHA_2DS_2-VASc score of 3 (2-5), and a duration of available follow-up of 1.7 (0.76-3.2) years. Stages 3-4 CKD was present in 10.7% of patients at baseline. Upon Cox regression, rivaroxaban was associated with a 17% lower risk of AKI (rates/100PYs=7.70 versus 13.45) and an 18% lower risk of developing stage 5 CKD/hemodialysis (rates/100PYs=3.74 versus 6.03) versus warfarin (Figure). Sensitivity analysis using an intention-to-treat approach, excluding patients with AKI at baseline and limited to patients with >365 days of follow-up yielded consistent results. Subgroup analyses showed no interactions for either outcome (p-interaction≥0.17 for all), except for stage 3-4 CKD and the AKI outcome (p-interaction=0.01).

Conclusions: Rivaroxaban was associated with lower risks of undesirable renal outcomes versus warfarin in diabetic NVAF patients.

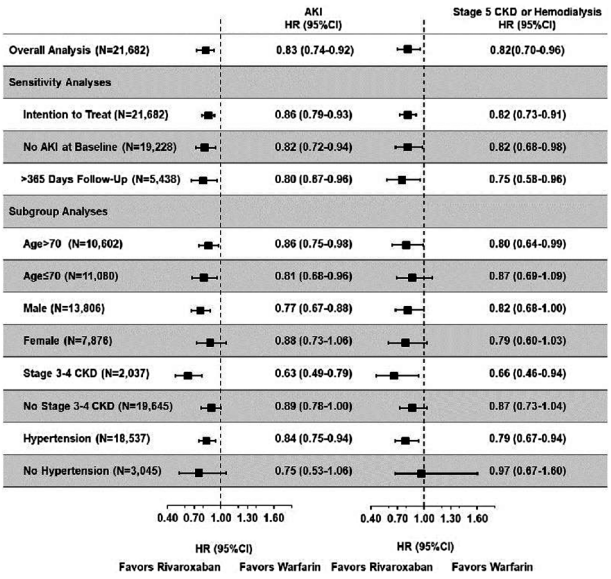

FIGURE 1 AKI and Stage 5 CKD/Hemodialysis Outcomes

OC 21.3 | Effectiveness and Safety of Rivaroxaban and Warfarin for Prevention of Major Adverse Cardiovascular or Limb Events in Nonvalvular Atrial Fibrillation Patients with Type 2 Diabetes

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Background: Individuals with type 2 diabetes mellitus (T2DM) are at an increased risk for peripheral artery disease (PAD) and those with PAD have a substantially higher risk of major adverse cardiovascular events (MACE) and major adverse limb events (MALE).

Aims: To assess the effectiveness and safety of rivaroxaban versus warfarin for the prevention of MACE or MALE in nonvalvular atrial fibrillation (NVAF) patients' with comorbid T2DM.

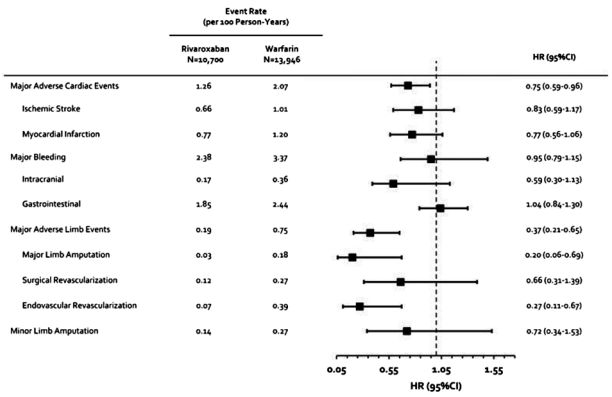
Methods: Using MarketScan data from 1/2012-12/2017, we identified oral anticoagulant (OAC)-naïve NVAF patients with comorbid T2DM and ≥12-months of insurance coverage prior to OAC initiation.

Differences in baseline covariates between cohorts were adjusted using inverse probability-of-treatment weights based on propensity scores calculated using generalized boosted models and 10,000 regression trees (absolute standardized differences < 0.1 achieved for all covariates after adjustment). Patients were followed until a MACE (ischemic stroke or myocardial infarction), MALE (major amputation or need for revascularization) or major bleeding event, OAC discontinuation or switch, insurance disenrollment or end of data availability. Hazard ratios (HRs) and 95% confidence intervals (CIs) comparing the cohorts were calculated using Cox regression.

Results: We identified 10,700 rivaroxaban (24.1% received a reduced dose) and 13,946 warfarin users. Median (25, 75% range) age was 70 (62, 79) years, CHA2DS2-VASc score was 4 (3, 5) and duration of available follow-up was 1.4 (0.6, 2.7) years. Eleven percent of patients had PAD, 1.4% had a prior MALE, including 0.3% with a history of major limb amputation at baseline. Rivaroxaban was associated with a 25% (95%CI=4-41%) reduction in the risk of MACE and 63% (95%CI=35-79%) reduction in the risk of MALE compared to warfarin (Figure). Major bleeding risk did not differ between cohorts.

Conclusions: Among NVAF patients with comorbid T2DM treated in routine practice, rivaroxaban was associated with lower risks of both MACE and MALE versus warfarin; with no difference in major bleeding.

TABLE 1 MACE and MALE Outcomes



OC 21.4 | The Influence of the Total Number of Concomitant Drugs Used on Major Bleeding Risk of Direct Acting Oral Anticoagulants Compared to Vitamin K Antagonists in Patients with Atrial Fibrillation Treated in Primary Care

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Background: Direct acting oral anticoagulants (DOACs) are preferred over vitamin K antagonists (VKAs) for stroke prevention in patients with atrial fibrillation (AF). Nevertheless, post-hoc analyses of DOAC-trials showed that, compared to VKAs, their benefits for reducing major bleeding risk diminished in patients with polypharmacy. These findings need verification in routine primary care, where the patient-mix is generally older and more frail.

Aims: To investigate whether the number of concomitant drugs used modifies major bleeding risk of DOACs compared to VKAs in primary care.

Methods: We performed a retrospective cohort study with primary care data from the United Kingdom Clinical Practice Research Datalink. Adult patients with AF and a first DOAC or VKA prescription between January 1st 2010 and July 1st 2018 were included. Incidence rates of major bleeding were calculated and multivariable Cox regression was used to account for time-varying exposures and confounding. Effect modification was assessed by stratification of the number of concomitant drugs used into 3 strata (0-5, 6-8 and ≥9 drugs), and by including it in the model as a continuous variable in an interaction term.

Results: In total 97,554 AF patients were included, with 322,125 person-years of follow-up of which 42,887 person-years of DOAC follow-up (48% rivaroxaban, 38% apixaban, 14% dabigatran). Median age was 76 years, median number of concomitant drugs used 7. Incidence rates for major bleeding were generally low and similar

TABLE 1 Stratified analysis of effect modification by the number of concomitant drugs used for the primary outcome major bleeding

	VKA	DOAC	DOAC versus VKA	
Number of concomitant drugs used	Incidence rate per 100 person-years (n events)	Incidence rate per 100 person-years (n events)	Crude HR (95% CI, p)	Adjusted HR* (95% CI, p)
0-5	0.94 (678)	1.13 (171)	1.41 (1.17-1.71, p<0.001)	1.40 (1.16-1.70, p<0.001)
6-8	0.59 (445)	0.80 (116)	1.62 (1.27-2.05, p<0.001)	1.57 (1.23-2.00, p<0.001)
9 or more	0.63 (408)	0.80 (104)	1.36 (1.06-1.75, p=0.015)	1.29 (1.01-1.67, p=0.045)

* Adjusted for age, sex, current smoking status, alcohol abuse, hypertension, eGFR<30 ml/min, liver failure or cirrhosis, history of stroke or transient ischemic attack (TIA), history of DVT, diabetes, congestive heart failure, history of coronary heart disease or acute coronary syndrome, peripheral artery disease, hypercholesterolemia, history of intracranial bleeding, history of major bleeding, history of gastrointestinal bleeding, gastritis/peptic ulcer disease, esophagitis, active cancer, previous use of a different oral anticoagulant, concomitant use of antiplatelet drugs, non-steroidal anti-inflammatory drugs (NSAIDs), oral corticosteroids, proton pump inhibitors (PPIs), Selective Serotonin Reuptake Inhibitors (SSRIs), diuretics, Renin-Angiotensin-Aldosterone System blockers, calcium channel blockers, digoxin, antidiabetic drugs, lipid lowering drugs, drugs possibly increasing plasma concentrations of DOAC (P-glycoprotein (P-gp) or cytochrome P450 (CYP)3A4 inhibitors) and drugs possibly decreasing plasma concentrations of DOAC (P-gp or CYP3A4 inducers).

across the three strata of the number of concomitant drugs used. The adjusted hazard ratio (HR) of major bleeding with DOAC versus VKA was 1.42 (95% CI 1.25-1.62), with no apparent differences across the 3 strata (p-value for interaction 0.76); see Table.

Conclusions: In this large retrospective dataset from routine primary care, DOACs increased the risk of major bleeding. The number of concomitant drugs used did not modify the effect of DOACs versus VKA on major bleeding.

OC 21.5 | Incidence and Risk of Major Bleeding in Patients with End-stage Renal Disease on Hemodialysis with and without Antithrombotic Therapy: Prospective Results of the Vienna InVestigation of AtriaL Fibrillation and Thromboembolism in HemoDIalysis Patients (VIVALDI)

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Background: Major bleeding is a severe complication of antithrombotic treatment in the population of patients with end-stage renal disease on hemodialysis (HD).

Aims: To assess the risk of major bleeding in HD patients associated with antithrombotic treatment.

Methods: VIVALDI is a prospective population-based cohort study in Vienna, Austria, investigating risk factors for stroke and bleeding in patients on HD with approval of the ethics committee and written consent from all patients. The outcome of interest of the present analysis was independently adjudicated occurrence of major bleeding according to the ISTH SSC definition. Risk factors were analyzed in a multivariable regression model considering death as a competing event. Patients receiving kidney transplantations (N=113, 18.1%) were censored.

Results: The study comprised 625 patients (229 women, 36.6%) with a median age of 66 years (25th to 75th percentile 54.5-75 years). Patients were followed for 908 days in median (410-1237 days). Eighty-nine (14.2%) patients suffered major bleeding events with an incidence-rate of 69.1 events per 1000 patient-years (Table 1) and 256 (41.0%) patients died during follow-up.

TABLE 1 Selected cohort characteristics

	Total (N=625)	Anticoagulation (N=237)	Antiplatelet therapy (N=234)	No antithrombotic therapy (N=154)
Female sex, N(%)	229 (36.6)	92 (38.8)	73 (31.2)	64 (41.6)
Age in years, median (25. to 75. percentile)	66 (54.5-75)	68 (58-76)	67 (58-76)	56 (42.75-70)
Diabetes, N(%)	237 (37.9)	102 (43.0)	106 (45.3)	29 (18.8)
Cardiovascular disease, N(%)	327 (52.3)	136 (57.4)	160 (68.4)	31 (20.1)
History of stroke, N(%)	129 (20.6)	64 (27.0)	51 (21.8)	14 (9.1)
History of venous thromboembolism, N(%)	61 (9.8)	39 (16.5)	15 (6.4)	7 (4.5)
Atrial fibrillation, N(%)	165 (26.4)	103 (43.5)	45 (19.2)	17 (11.0)
Major bleeding events, N(%)	89 (14.2)	41 (17.3)	41 (17.5)	7 (4.5)
Major bleeding incidence-rate per 1000 patient-years	69.1	80.2	85.5	23.6

TABLE 2 Univariable and multivariable risk regression models with consideration for competing risk of death

	Univariable hazard ratio for major bleeding (95% CI)	p	Multivariable hazard ratio for major bleeding (95% CI)	p
Female Sex	1.16 (0.76-1.77)	0.483	1.22 (0.79-1.88)	0.361
Age, years (median and 25. to 75. percentile)	1.00 (0.99-1.02)	0.667	0.99 (0.98-1.01)	0.481
Diabetes	0.74 (0.47-1.15)	0.176	0.55 (0.35-0.88)	0.011
Cardiovascular disease	1.85 (1.19-2.88)	0.007	**1.77 (1.06-2.95)**	**0.028**
History of stroke	1.21 (0.75-1.95)	0.438	1.07 (0.65-1.75)	0.802
History of venous thromboembolism	0.87 (0.42-1.78)	0.703	0.79 (0.38-1.63)	0.528
Atrial fibrillation	1.13 (0.72-1.77)	0.593	1.07 (0.66-1.71)	0.790
Anticoagulation treatment (compared to no antithrombotic)	3.36 (1.51-7.50)	0.003	**3.39 (1.46-8.03)**	**0.005**
Antiplatelet (compared to no antithrombotic)	3.49 (1.56-7.81)	0.002	**3.29 (1.38-7.86)**	**0.007**

Use of anticoagulants, i.e. vitamin-K-antagonists and low-molecular-weight heparins on non-HD days (N=237, 37.9%) and antiplatelet agents (N=234, 37.4%) were almost equally frequent, and 154 patients (24.6%) received no antithrombotic agents.

In a multivariable model, adjusted for sex, age, diabetes, hypertension, heart failure, atrial fibrillation, heart valves replacement, cardiovascular disease, history of stroke, and venous thromboembolism, use of anticoagulation agents (hazard ratio [HR] 3.39, 95% confidence interval [CI] 1.46-8.03) and use of antiplatelet agents (HR 3.29, 95%CI 1.38-7.86) were both independently associated with an increased risk of major bleeding (Table 2). Further, coronary artery disease was associated with increased risk of major bleeding (HR 1.77, 95%CI 1.06-2.95).

Conclusions: The incidence of major bleeding was high in patients on HD and the risk of major bleeding was equally increased in patents on anticoagulation and antiplatelet agents.

OC 25.1 | von Willebrand Factor as a Determinant of Silent Cerebral Micro-bleeds during Trans Aortic Valve Replacement in a Prospective Cerebral Magnetic Resonance Imaging Cohort

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Background: Cerebral microbleeds have been observed in "asymptomatic" elderly patients undergoing systematic MRIs and might be related with cognitive decline. The acute triggers of those microbleeds remains unknown. A defect in Von Willebrand factor (VWF) after Trans Aortic Valve Replacement (TAVR) enhanced bleeding risk and possibly contributes to vascular malformations.

Aims: To define the occurrence of "acute" microbleeds during TAVR and the clinical and procedural factors associated with this occurrence including a post-procedural defect in VWF.

Methods: A prospective cohort of patients with aortic stenosis referred for TAVR was included. A standardized neurological assessment, a cerebral magnetic resonance imaging (MRI) and a biological analysis including quantification of VWF multimers and PFA-CADP were performed before and after TAVR. Quantity and location of microbleeds were recorded independent from the Heart Team.

Results: Eighty-four patients completed the imaging protocol. On pre-procedural MRI, 22 patients (26%, 95%CI=17-37%) had at least one microbleeds. After TAVR, new microbleeds were observed in 19 (23%, 95%CI=14-33%) patients. The occurrence of new microbleeds was independent of the presence microbleeds at baseline. A lower HMW-multimer-ratio as measured immediately at the end of the procedure was associated with the occurrence of new microbleed detected on the MRI performed 3 days after the procedure (0.90±0.14 vs. 1.06±0.27, p=0.009). The same observation was made with a higher CT-ADP (165±72 vs. 127±57, p=0.02). A prolonged procedure (RR=1.21[1.01-1.170] for every 5 min of fluoroscopy time, p=0.04) and post-procedural acquired-VWF-defect (RR=1.42[1.08-1.89] for every lower "0.1 unit" of HMW-multimer-ratio, p=0.004) were the only factors associated with the occurrence of new post-procedural microbleed(s).

Conclusions: We report for the first time a high incidence (23%, 95%CI=14-33%) of new post-procedural cerebral microbleeds occurring during TAVR. Procedural management and persistence of acquired-VWF defect could play a major role in the occurrence of those new microbleeds.

OC 25.2 | Angiopoietin-like Protein 8 Overexpression Aggravates Lipid Metabolism and Promotes Atherogenesis in Mice

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Beijing An Zhen Hospital, Capital Medical University, Beijing, China

Background: Angiopoietin-like protein 8 (ANGPTL8), which is a novel hormone produced in liver and adipose tissue, is involved in regulating lipid metabolism. In our previous research, we found that patients with Coronary artery disease (CAD) have remarkably higher levels of ANGPTL8 than the controls. However, the role of ANGPTL8 in development of CAD remains unknown.

Aims: We examined the association between CAD and ANGPTL8 rs2278426 polymorphism and explored the role of ANGPTL8 in atherogenesis.

Methods: We performed a case-control study. The associations between ANGPTL8 rs2278426 polymorphism and CAD were determined by multivariate logistic regression analysis. The mechanisms were explored by using Adeno-associated virus serotype-9 of ANGPTL8 overexpression in AOPE-/- mice. Eight weeks old male APOE-/- mice were given a dose of 2E+11 viral genomes (N=15) or saline (N=15) by single tail vein injections. Mice were put on a Western type diet, which continued throughout the 12 weeks experiment. Oil red O staining was used to assess the size of the atherosclerotic lesion. Plasma lipid profiles including total triglycerides, total-, HDL- and LDL-cholesterol levels were measured with commercial kits.

Results: The frequency of ANGPTL8 rs2278426 T allele was significantly lower in subjects with CAD compared with the controls (16.67% Vs 24.88%; P=0.001) and subjects with T allele have a lower risk for developing CAD compared with subjects with C allele in additive (OR=2.253, 95%CI= 0.102-0.629, P=0.003), dominant

(OR=0.407, 95%CI=0.270-0.613, P=0.001) and recessive model test (OR=0.347, 95%ci=0.143-0.846, P=0.020). The atherosclerotic lesion area in ANGPTL8 overexpression group was 2.02-fold greater at 20 weeks than control group (*P*< 0.01) and ANGPTL8 overexpression could increase the levels of serum triglycerides, total-, HDL- and LDL-cholesterol.

Conclusions: Our data demonstrate that rs2278426 polymorphisms of ANGPTL8 gene significantly associated with CAD,ANGPTL8 promotes atherogenesis in ApoE −/− mice and ANGPTL8 may be a new therapeutic target.

OC 25.3 | Platelet Function in Non-severe Burn Injury

B. Johnson[1]; E. O'Halloran[2]; A. Stevenson[1]; F. Wood[3]; M. Fear[1]; M. Linden[1]

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Background: Burn injury initiates an acute thrombo-inflammatory response leading to susceptibility to venous thrombosis and pulmonary embolism. Despite increased understanding of the impact of burn injury on haemostasis in the acute phase, little is known about long-term consequences of burn injury on haemostasis and thrombosis. Recent Western Australian hospital data has shown that all burn survivors, regardless of severity, are at an increased life-long risk of cardiovascular morbidity.

Aims: Here we investigate whether non-severe burn injury is associated with long lasting changes in platelet function.

Methods: In mouse studies adolescent CD57/BL6 female mice were given an 8% full thickness thermal contact burn (n = 6) or sham control (n = 6) and blood tested 28 days later. In preliminary human studies, blood was collected from adults with a non-severe burn injury (n=12) at 2 weeks and 6 weeks after non-severe burn injury or age/sex-matched controls (n=10).

Results: Collagen related peptide (CRP) stimulated platelet P-selectin expression was 1.2 fold higher in burn vs sham mice. In humans platelet response to CRP was higher two weeks (1.6 fold PAC1 binding, 1.3 fold CD62P expression), and six weeks (2.0 fold PAC1 binding, 1.7 fold CD62P expression) post burn. Formation of monocyte-platelet aggregates in response to thrombin receptor stimulation was reduced at 6 weeks (0.8 fold), but not at 2 weeks.

Conclusions: Using mouse and human data we show preliminary data to suggest that platelets remain hyper-responsive to collagen stimulation for up to 6 weeks following a non-severe burn injury.

OC 25.4 | Dense Fibrin, von Willebrand Factor and Extracellular DNA Are Specific Structural Hallmarks of Platelet-rich Areas in Ischemic Stroke Thrombi

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Background: Ischemic stroke is caused by a thromboembolic occlusion of cerebral arteries. Treatment is focused on fast and efficient removal of the thrombus, either via intravenous thrombolysis using rt-PA or via thrombectomy. Recanalization, however, is not always successful and factors contributing to failure are not completely understood. Although being the primary target of acute treatment, little is known about the structure and composition of stroke thrombi.

Aims: The aim of this study was to analyze key structural hallmarks of ischemic stroke thrombi on the level of proteins, DNA and cells.

Methods: Thrombi (n=177) were collected from endovascular-treated ischemic stroke patients at Groeninge Hospital (Kortrijk, Belgium). All patients gave written informed consent (AZGS2015065). All thrombi were histologically analyzed using hematoxylin and eosin, martius scarlet blue staining (showing red blood cells (RBCs) and fibrin), Feulgen staining (showing DNA), and immunohistochemical/fluorescence analysis of von Willebrand factor (VWF), platelets (GPIb), fibrin and leukocytes (CD45). Multicolor immunofluorescent analysis was performed on randomly selected thrombi (n=8).

Results: Stroke thrombi are composed of two main types of regions: RBC-rich areas and platelet-rich areas (Figure 1). RBC-rich areas consist of RBCs that are entangled within a network of thin fibrin strands. Platelet-rich areas consist of denser fibrin structures

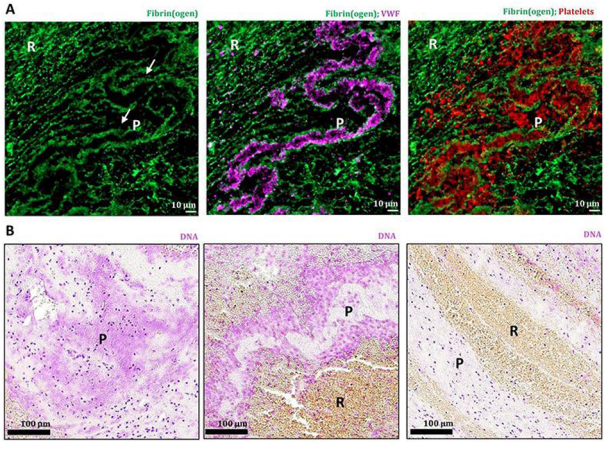

FIGURE 1 (A) Platelet-rich areas contain dense fibrin structures lined with VWF (B) and extracellular DNA

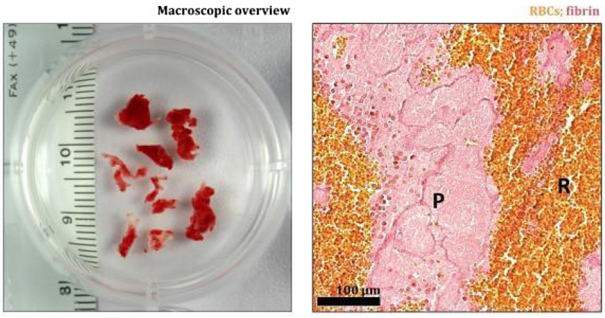

FIGURE 2 Stroke thrombi are mainly composed of RBC-rich areas (R) and platelet-rich areas (P)

(white arrows) that are lined with VWF and are filled with platelets (Figure 2A). Leukocytes typically accumulate on the interface between RBC-rich and platelet-rich areas. DNA-networks are seen throughout the majority of thrombi and are particularly present in and around the platelet-rich areas (Figure 2B).

Conclusions: RBC-rich areas have a limited complexity, but platelet-rich areas in stroke thrombi are characterized by dense fibrin structures aligned with VWF and abundant amounts of platelets, leukocytes and extracellular DNA. These findings can contribute to our understanding why platelet-rich thrombi are rt-PA resistant and difficult to retrieve via thrombectomy and are important to further improve acute ischemic stroke therapy.

OC 25.5 | Platelet Receptor Sensitivity is Associated with Acute Changes in Ambient Air Pollutant Concentrations in a Pilot Study of Patients Presenting with Acute Myocardial Infarction

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Background: We previously demonstrated that fine particulate matter $PM_{2.5}$ concentration triggered ST-Segment Elevation Myocardial Infarction (STEMI) but not Non-ST-Segment Elevation Myocardial Infarction (NSTEMI). We also showed that the platelet phenotype and proteomic profile is different in STEMI vs. NSTEMI patients.

Aims: We hypothesized that exposure to $PM_{2.5}$, ultrafine particles (UFP; < 10nm), black carbon, Delta-C (marker of wood smoke), SO_2, NO_2, CO, and O_3 may predict platelet activation through specific surface receptors during MI.

Methods: Platelets were isolated from patients with STEMI and NSTEMI prior to a $P2Y_{12}$ receptor antagonist load and before coronary angiography, then stimulated with agonists for the $P2Y_{12}$ receptor (ADP), thromboxane receptor (U46619) and the protease-activated receptor 1 [PAR1] (TRAP-6). Platelet activation was assessed by surface p-selectin (antibody) and quantified by flow cytometry. Using linear regression, we estimated the change in platelet receptor activity associated with each interquartile range increase in pollutant concentrations in the previous 1, 3, 12, 24, 48, 72, and 96 hours.

Results: In a pilot study of 85 patients with acute MI, receptor signaling ($p< 0.05$) was associated with increasing concentrations of multiple pollutants: $P2Y_{12}$ receptor with increased Delta-C, CO, and NO_2 in the previous 3 hrs, PAR1 signaling with increased Delta-C in the previous 1 and 3 hrs, CO in the previous 3 hrs, and thromboxane signaling with increased $PM_{2.5}$ in the previous 3 hrs and Delta-C in the previous 1 and 3 hrs.

Conclusions: For the first time, this pilot study shows exposure to air pollutants favors platelet activation through certain receptors, and suggests that a mechanism by which air pollution may trigger STEMI but not NSTEMI is platelet activation through specific surface receptors. This raises the possibility of personalizing anti-platelet therapy to the exposome in which a patient resides, for the purposes of preventing thrombotic events such as MI.

OC 41.5 | Prevalence and Relative Risk of Atrial Fibrillation in Patients with Cancer - A Nationwide Cohort Study

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Background: Atrial fibrillation (AF) is the most common cardiac arrhythmia, which increases with age and contributes to significant morbidity and mortality. An association between cancer and AF has been reported. However, the exact magnitude of this association is unclear.

Aims: To quantify the prevalence and relative risk of AF in patients with a history of or active cancer in a nationwide cohort study.

Methods: ICD-10 diagnosis codes of all persons (aged between 0 and 90 years) with a public insurance in Austria were extracted from the Austrian Association of Social Security Providers dataset covering the years 2006-2007 (n=8.306,244). Patients with a history of cancer or active cancer were defined as having at least one ICD-10 "C" diagnosis code, and patients with AF as having the diagnosis code I48.

Results: Among 158,675 subjects with cancer, 15,504 (9.77%) had a diagnosis of AF. In contrast, among 8.147,569 subjects without cancer, 97,323 (1.19%) had an AF diagnosis code. This corresponded to a random-effects relative risk (RR) of 10.45 (95%CI: 7.47 - 14.62, p< 0.001) for AF in patients with cancer, with increasing RR of AF in younger age categories (Figure 1). The association between AF and cancer was similar in women (RR: 8.65 [95%CI: 6.05-12.39]) and men (8.96 [6.33-12.69]).

As compared to patients without cancer or other cancers, the prevalence of AF was highest in patients with hematologic cancer (RR: 9.15 [95%CI: 8.78-9.53]) and lowest in patients with endocrine cancer (3.55 [3.04-4.14]; Figure 2).

Conclusions: The prevalence and relative risk of AF is significantly higher in persons with a diagnosis of cancer compared to those without cancer. Furthermore, the relative risk of AF differs across cancer types. Our population-level meta-data indicates a strong association between cancer and AF and supports the concept of shared risk factors and pathobiology between these diseases, which needs to be further investigated.

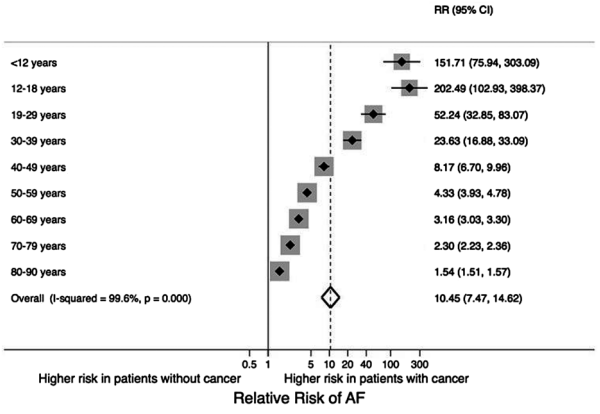

FIGURE 1 Relative risk of AF in subjects with and without a cancer diagnosis separated by age

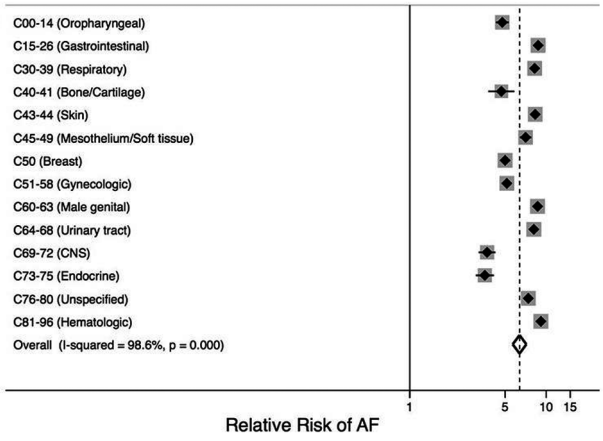

FIGURE 2 Relative risk of AF in subjects with a diagnosis of a cancer diagnosis separated by types of cancer

OC 50.1 | Effect of DOAC Type and DOAC Dose, and Residual DOAC Level on Risk for Perioperative Major Bleeding and Arterial Thromboembolism

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Background: The perioperative management of patients with atrial fibrillation (AF) who are taking a direct oral anticoagulant (DOAC) and need elective surgery/procedure is a common problem for which management is uncertain. The PAUSE study protocol provides a simple, standardized DOAC-specific management approach for patients on apixaban, dabigatran or rivaroxaban. However, the effect of DOAC type, DOAC dose, and residual level of anticoagulation at the time of surgery/procedure on adverse clinical outcomes is not known.

Aims: To determine the effect of the type of DOAC used by patients, the DOAC dose regimen, and residual pre-operative DOAC levels on perioperative rates of major bleeding (MB) and arterial thromboembolism (ATE).

Methods: We compared, using odds ratios (ORs) and associated 95% confidence intervals (CIs), 30-day postoperative rates of MB and ATE, and residual pre-operative DOAC levels (< 30, 30-49.9, ≥50 ng/mL) in these groups: apixaban (any dose, 5 and 2.5 mg BID); dabigatran (any dose, 150 and 110 mg BID); and rivaroxaban (any dose, 20 and 15 mg QD).

Results: We studied DOAC interruption for elective surgery/procedure in 1257 apixaban, 668 dabigatran and 1082 rivaroxaban patients. Compared to apixaban patients, there was no significant difference in MB rates vs. dabigatran patients (OR=0.66; CI: 0.24-1.60) or rivaroxaban patients (OR=1.37; CI: 0.72-2.67); and no significant difference in ATE rates vs. dabigatran patients (OR=3.78; CI: 0.74-27.3) or rivaroxaban patients (OR=2.33; CI: 0.45-16.8). In the Table below, the DOAC dose had no significant effect on MB and ATE; compared to no residual DOAC level (< 30 ng/mL), a minimal residual level (30-49.9 ng/mL) and elevated level (>50 ng/mL) had no significant effect on MB and ATE rates.

Conclusions: In patients with AF who require perioperative DOAC interruption for an elective surgery/procedure, the DOAC type and dose used, and residual DOAC level did not affect risks for perioperative MB and ATE.

TABLE 1 DOAC residual pre-operative levels and rates of MB and ATE. Legend: †1-sided; ‡patients with pre-operative DOAC levels measured; n/a, not applicable

DOAC Dose Regimen			Major bleeding n, % (95% CI) & OR (95% CI)† for both doses		Arterial Thromboembolism n, % (95% CI) n, % (95% CI) & OR (95% CI)† for both doses	
apixaban	5 mg BID	n=1004	14, 1.39 (0-2.15)	1.17 (0.32-6.42); p=1.0	0.46 (0.16-1.49); p=0.13	0.25 (0-19.7); p=0.36
	2.5 mg BID	n=252	3, 1.19 (0-2.94))		1, 0.4 (0-1.76)	
dabigatran	150 mg BID	n=420	4, 0.95 (0-2.1)	1.18 (0.17-13.16); p=1.0	3, 0.71 (0-1.77)	1.78 (0.14-93.6);p=1.0
	110 mg BID	n=248	2, 0.81 (0-2.41)		1, 0.4 (0-1.79)	
rivaroxaban	20 mg QD	n=900	14, 1.56 (0-2.39)	0.46 (0.16-1.49); p=0.13	3, 0.33 (0- 0.83)	0.6 (0.05-31.78); p=0.52
	15 mg QD	n=181	6, 3.31 (0-6.28)		1, 0.55 (0- 2.44)	
Residual DOAC Level (ng/mL):‡	<30	n=2020	27, 1.34 (0-1.83)	reference	9, 0.45 (0-0.76)	n/a
	30-49.9	n=363	6, 1.65 (0-3.16)	1.24 (0.46-2.83); p=0.64	0, 0 (0-0.74)	
	≥50	n=158	3, 1.9 (0-4.66)	1.43 (0.34-4.10); p=0.56	0, 0 (0-1.68)	

OC 50.2 | Retrospective Analysis of the Clinical Effectiveness of a Reduced Dose of Idarucizumab in Dabigatran Reversal

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Background: The recommended dose of idarucizumab, a specific reversal agent for dabigatran, is two sequential intravenous boluses of 2.5g. However, published data from the Re-VERSE AD study demonstrated biochemical reversal after the initial 2.5g dose.

Aims: To compare the clinical effectiveness of 2.5g and 5g doses of idarucizumab used in two tertiary hospitals in Auckland, New Zealand.

Methods: All patients receiving idarucizumab for dabigatran reversal between 1st April 2016 and 31st December 2018 were included. The primary outcome was the proportion of patients requiring a second dose of idarucizumab during their admission. Secondary outcomes included normalisation of coagulation profiles (activated partial thromboplastin time (APTT) and dilute thrombin clotting time (dTCT)), 30 day thrombotic events, and 30 day all-cause mortality.

Results: Of 159 patients, 118 received an upfront 2.5g dose and 41 received a 5g dose. Baseline characteristics were similar. There was no significant difference in the proportion of patients in the 2.5g and 5g groups requiring a second dose (4.2% vs 7.3%, p=0.437). By univariate analysis, the 2.5g dose was not associated with increased likelihood of requiring a second dose (HR 0.3, 95%CI 0.04-2.24). Coagulation profiles were similar between the two groups before, immediately after and 24-hours following reversal (Figures 1 and 2). A similar proportion in both groups achieved a normal APTT (78.9% vs 82.1%, p=0.709) and dTCT (97.3% vs 88.9%, p=0.117) immediately after reversal, and at 24-hours (APTT 70.2% vs 68.2%, p=0.864; dTCT 40.0% vs 50.0%, p=0.580). There were significantly fewer thrombotic events in the 2.5g group (0.8% vs 9.8%, p=0.005) but no difference in 30-day mortality rates (7.6% vs 9.8%, p=0.668).

Conclusions: This is the first published dataset evaluating the real-world use of a reduced dose of idarucizumab in dabigatran reversal. The 2.5g dose appears to be effective, with no increase in adverse clinical outcomes compared to the 5g dose.

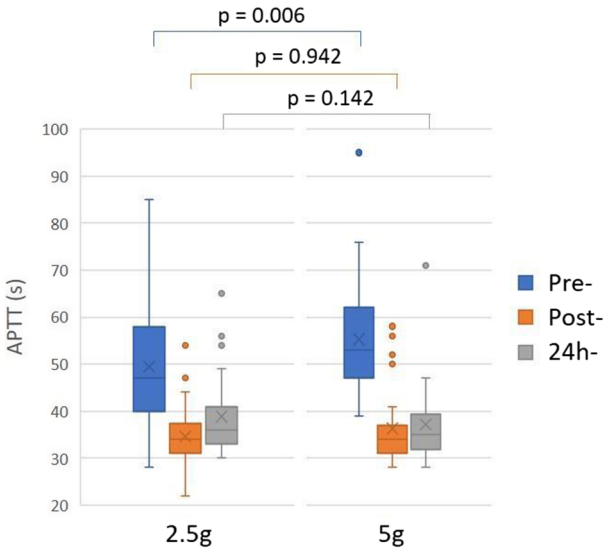

FIGURE 1

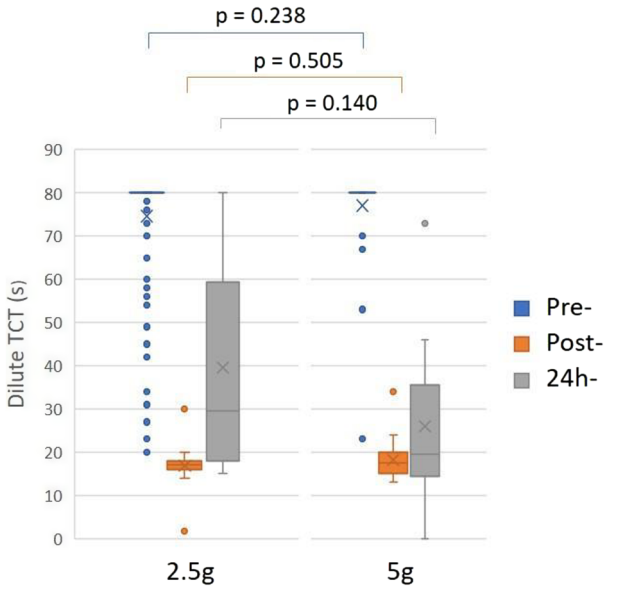

FIGURE 2

OC 50.3 | Neither a Structured Questionnaire at Pre-anesthesia Visit Nor the Prescription of Routine Hemostatic Tests Have Acceptable Performances to Detect Hemostatic Abnormalities before Surgery

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Background: Identifying patients with bleeding risk before surgery remains challenging. Several studies have demonstrated the very low efficacy of routine pre-operative PT, aPTT, platelet count (PC) for the prediction of bleeding risk. In 2011 several professional societies recommended AGAINST systematic prescription of routine hemostasis tests and advocated their replacement by a structured questionnaire.

Aims: To investigate diagnostic accuracy of a structured questionnaire at the pre-anesthesia visit in order to detect patients with bleeding risk requiring further hemostatic investigations.

Methods: We conducted a multicenter study in 7 French academic hospitals. Inclusion criteria were patients scheduled for surgical interventions. Exclusion criteria were previously documented risk of bleeding and anti-thrombotic medication.

All patients underwent hemostatic tests prescribed in the context of care (routine tests), completed the questionnaire and underwent systematically the gold standard strategy: PT, aPTT, PC, and vWF activity and antigen, factors VIII, IX and XI, Platelet Function Analyzer (PFA) and when required, factors II, V, X and VII, and hemostasis consultation. We compared the diagnostic performance of the two strategies: questionnaire versus routine hemostatic tests.

Results: From January 2015 to January 2018, we identified 16/1405 (1.14%) patients with hemostatic abnormalities potentially associated with bleeding risk: 11/1405 (0.78%) with von Willebrand disease, 2 with hepatic impairment, 1 with moderate FXI deficiency, 1 with moderate hemophilia A and 1 with platelet defect.

Sensitivity of the questionnaire (score≥2) was 50% (IC 95%:[25-75]) and specificity 87% (IC 95%:[85-89]). Sensitivity and specificity of the routine hemostatic tests strategy were 75% (IC 95%:[48-93]) and 51% (IC 95%:[48-54]), respectively. Concordance between the 2 strategies was very low (kappa=0.04).

Conclusions: In conclusion, prevalence of hemostatic abnormalities was of 1.14% among 1405 patients. The standardized questionnaire at the pre-anesthesia visit as well as the routine hemostatic tests strategy have poor performances to detect an increased risk of bleeding.

OC 50.4 | Retrospective Review of Outcomes and Anticoagulation use for Elderly Patients that Present with an Acute Hip Fracture at Three Tertiary Hospitals in Perth, Western Australia

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Background: Hip fractures are a worldwide public health issue in the elderly population. Delay to surgical intervention is associated with increased mortality and morbidity. Increasing use of direct oral anticoagulants (DOACs) within this population may delay surgery and increase rates of bleeding, transfusion and 30-day mortality risk.

Aims: To determine if anticoagulation with warfarin or DOAC therapy is associated with a clinically significant delay to surgery, and if anticoagulation and / or anti-platelet use is associated with worse post-operative outcomes and higher transfusion requirements.

Methods: A retrospective cohort study was undertaken to review surgical outcomes for all patients who presented with an acute hip fracture at the tertiary hospitals in Perth, Western Australia. Hip fracture registry data in addition to anticoagulant use, antiplatelet use, baseline DOAC level, change in haemoglobin and transfusion requirements was collected. Comparisons between groups across all outcome measures were performed.

Results: 1240 fractures (median age 84 years, 72% female) were identified in the study period. 149 patients (12%) were receiving anticoagulation, 369 (29.8%) were on antiplatelet therapy and 13 patients (1%) were receiving both. 25/82 (30.5%) patients on a DOAC had a drug level done within 12 hours of presentation with 15 (60%) having levels < 50 ng/mL. Anticoagulated patients had a significantly longer time to surgery than those not on anticoagulation; with those on a DOAC having a longer time to surgery compared to warfarin ($p < 0.05$). There was no significant difference in change in haemoglobin, transfusion requirements and 30-day mortality between the groups or site (Figure 1).

Conclusions: Anticoagulation is associated with a delay to theatre with no significant increase in bleeding or transfusion requirements. The frequent finding of DOAC levels < 50 ng/mL within 12 hours of presentation suggests there is potential to reduce theatre delay in many patients on DOAC therapy.

	Hospital A	Hospital B	Hospital C	TOTAL
Number of fractures	532 (43%)	407 (33%)	301 (24%)	1240
Age (median years, interquartile range)	84 (77, 89)	83 (73, 89)	86 (77, 91)	84 (76, 90)
Sex (% female)	405 (76%)	272 (67%)	213 (71 %)	890 (72%)
Number anticoagulated on admission (% at site)				
• Total	66 (12.4%)	43 (10.5%)	40 (13.2%)	149 (12%)
• Apixaban	19 (3.6%)	12 (2.9%)	13 (4.3%)	44 (3.6%)
• Dabigatran	5 (1%)	2 (0.5%)	1 (0.3%)	8 (0.6%)
• Rivaroxaban	10 (1.8%)	13 (3.2%)	6 (2%)	29 (2.3%)
• Edoxaban	0 (0%)	0 (0%)	1 (0.3%)	1 (0.1%)
• Warfarin	32 (6%)	16 (3.9%)	19 (6.3%)	67 (5.4%)
Number using anti-platelets on admission (% at site)				
• Total	175 (32.9%)	131 (32.2%)	63 (20.9%)	369 (29.8%)
• Aspirin	122 (22.9%)	86 (21.1%)	41 (13.6%)	249 (20.1%)
• Clopidogrel	33 (6.2%)	21 (5.2%)	12 (4%)	66 (5.3%)
• Ticagrelor	1 (0.2%)	0 (0%)	0 (0%)	1 (0.1%)
• Dual antiplatelet	19 (3.6%)	24 (5.9%)	10 (3.3%)	53 (4.3%)
Hours to surgery (median, interquartile range)[1]				
• Total	24 (18.0, 34.0)	30 (21.3, 45.9)	20 (14.6, 24.5)	24 (18.0, 38.9)
• DOAC	43 (37.3, 46.8)	63 (32.3, 70.2)	44 (31.4, 51.3)	44 (36.0, 55.3)
• Warfarin	25 (20.8, 35.3)	48 (28.4, 68.6)	26 (21.8, 38.6)	28 (22.0, 44.8)
• Antiplatelets	23 (16.5, 30.5)	27 (20.7, 43.5)	20 (14.9, 27.0)	23 (17.7, 35.6)
• Dual anticoagulation and antiplatelet	29 (23.8, 42.3)	30 (18.8, 41.2)	22	26 (21.2, 42.3)
• No anticoagulation or antiplatelet	24 (18.0, 30.0)	30 (21.3, 45.5)	18 (14.0, 22.2)	23 (17.3, 36.0)
Change in haemoglobin (g/L) from maximum level pre-operatively to minimum level within 72 hours of surgery (Mean, standard deviation)				
• Total	25.0 (12.9)	25.1 (14.4)	24.7 (13.5)	25.0 (13.5)
• DOAC	24.9 (12.4)	20.0 (13.6)	23.2 (11.3)	22.9 (12.5)
• Warfarin	22.6 (13.5)	23.4 (14.4)	22.1 (13.2)	22.7 (13.4)
• Antiplatelets	27.3 (13.8)	26.2 (14.5)	25.2 (12.5)	26.5 (13.8)
• Dual anticoagulation and antiplatelet	32.2 (10.9)	27.7 (16.4)	37.0	30.5 (13.1)
• No anticoagulation or antiplatelet	23.8 (12.3)	25.1 (14.4)	24.9 (14.1)	24.5 (13.5)
Number of patients' transfused packed red blood cell units 2 days pre-operatively to 3 days post-operatively (% at site)				
• Total	94 (18%)	102 (25%)	84 (28%)	280 (22.5%)
• DOAC	4 (0.7%)	4 (1%)	6 (2%)	14 (1.13%)
• Warfarin	5 (1%)	5 (1.2%)	5 (1.7%)	15 (1.2%)
• Antiplatelets	41 (8%)	48 (11.8%)	24 (8%)	113 (9.1%)
• Dual anticoagulation and antiplatelet	2 (0.3%)	1 (0.2%)	0 (0%)	3 (0.2%)
• No anticoagulation or antiplatelet	42 (8%)	44 (10.8%)	49 (16.3%)	135 (10.9%)
Mortality (number of patients who died within 30 days, % at site)				
• Total	42 (7.8%)	37 (9%)	22 (7.3%)	101 (8.1%)
• DOAC	1 (0.3%)	3 (0.75%)	1 (0.33%)	5 (0.4%)
• Warfarin	3 (0.6%)	1 (0.25%)	2 (0.63%)	6 (0.5%)
• Antiplatelets	12 (2%)	8 (2%)	1 (0.33%)	21 (1.7%)
• Dual anticoagulation and antiplatelet	0 (0%)	1 (<1%)	0 (0%)	1 (0.1%)
• No anticoagulation or antiplatelet	26 (4.9%)	23 (6%)	18 (6%)	67 (5.4%)

[1] A Kruskal-Wallis $\chi^2(4, n = 1191) = 68.004$, $p < 0.05$. Mann-Whitney U test post-hoc comparison with Bonferroni adjustment indicated time to surgery was greater in DOAC users compared to warfarin users $p = 0.004$.

FIGURE 1 Demographics and Results

OC 50.5 | Efficacy and Safety of Prothrombin Complex Concentrate in Patients with Intracranial Bleeding Treated with Factor Xa Inhibitors and Patients Treated with Vitamin K Antagonists

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Background: Intracranial haemorrhage (ICH) is the most feared complication of anticoagulant treatment, due to high mortality and morbidity. For reversal of factor Xa-inhibitor-related (FXaI) major bleeding no specific reversal agent is yet available and current international guidelines recommend administration of 25-50 IU/kg prothrombin complex concentrate (PCC). PCC is the specific reversal agent for vitamin K antagonists-related (VKA) bleeding with international normalized ratio (INR) and weight-based dosing.

Aims: To compare the efficacy and safety of PCC as reversal for FXaI-ICH with PCC as reversal for VKA-ICH.

Methods: Two contemporary cohorts were compared: consecutive patients receiving PCC for XaI-ICH were prospectively registered (2014-2018) and patients receiving PCC for VKA-ICH were retrospectively collected (2014-2015). Primary outcome was effective haemostasis after 24 hours, adapted from the ISTH definition (Khorsand, JTH, 2015). Herein, a stable neurological condition was classified as effective hemostasis and worsening of the neurological condition or cessation of treatment was classified as poor hemostasis. Safety outcomes were defined by venous and arterial thromboembolic complications and mortality within 30 days.

Results: Thirty-six patients with XaI-ICH and 41 patients with VKA-ICH were studied. Baseline characteristics (table 1) did not differ between the two groups, except for duration of oral anticoagulation and systolic blood pressure at presentation. Median PCC dose was 50 IU/kg for the FXaI-ICH and 25 IU/kg for the VKA-ICH. In the FXaI group, 63% achieved effective haemostasis compared to 59% in the VKA group (table 2). Mortality was 24% in the FXaI-ICH and 45% in the VKA-ICH (odds ratio [95%CI]: 0.38 [0.14-1.03]).

Conclusions: In spite of lack of a specific reversal agent for factor-Xa inhibitors, the prognosis of ICH managed with PCC does not seem worse for the XaI-ICH treated patients in comparison with the VKA-ICH treated patients.

TABLE 1 Baseline characteristics at presentation of the bleeding event

	Vitamin K antagonist (n=41)	Factor Xa inhibitor (n=36)	p-value
Age (years), mean (SD)	72.2 (11.6)	76.1 (8.9)	0.11
Male gender, n(%)	20 (49)	20 (56)	0.36
Weight (kg), mean (SD)	81.2 (17.5)	77.1 (14.3)	0.36
Duration OAC < 3 months, n (%)	1 (3)	8 (29)	0.01
Systolic blood pressure (mmHg), mean (SD)	139 (40)	162 (30)	0.01
INR at presentation, median (IQR)	3.3 (2.6-5.1)	N.A.	N.A
Dose of PCC (IU/kg), median (IQR)	25 (20-25.75)	50 (50-50)	<0.001
ICU admission, n (%)	13 (32.5)	11 (33.3)	0.57

TABLE 2 Primary and safety outcomes per treatment group in the bleeding population after administration of PCC

Primary outcome, % (95%CI)	VKA (n=41)	FXaI (n=36)
Effective hemostasis after 24 hours	59% (53-72)	67% (50-80)
Safety outcomes, % (95%CI)	VKA (n=40)	FXaI (n=34)
30-day thromboembolic rate	5% (1-7)	0
30-day mortality rate	45% (31-60)	24% (12-40)
Fatal bleeding rate	30% (18-45)	21% (10-37)

OC 65.1 | Development of a Novel Peptide Tracer for Detection of Cardiac Fibrosis and Atrial Fibrillation Using Mouse Models of Cardiomyopathies

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Background: Atrial fibrillation (AF), the most common cardiac arrhythmia, escalates in aging western societies and predisposes patients to heart failure (HF) and stroke. A solid link has been established between AF and atrial fibrosis, a scarring process leading to distorted atrial architecture and function. Current fibrosis imaging techniques (e.g. MRI) are limited by spatial resolution and image quality. We have developed a targeted peptide tracer ('T-peptide') with specific affinity towards MMP-degraded collagen IV, which could improve detection of cardiac fibrosis and AF monitoring.

Aims: We aimed to test whether the T-peptide can specifically bind fibrotic hearts using mouse models of AF and HF.

Methods: Cy5.5-labelled T-peptide (1 mg/kg), or the scrambled control 'S-peptide', were intravenously injected to the arrhythmic, double transgenic dominant negative PI3K-Mammalian sterile 20-like kinase 1 (Mst1) mice (AF+HF mice). Perfused hearts were collected 16 h later for histology or homogenisation, and near-infrared signals measured using the Odyssey scanner. Fresh frozen heart sections, also from transgenic mice overexpressing β2 adrenergic receptors (β2-AR) or undergoing experimental myocardial infarction (MI), were incubated with the tracer for microscopic examination.

Results: Interstitial fibrosis was demonstrated in AF+HF hearts by standard histological techniques. T-peptide robustly accumulated in transgenic hearts, with the atria absorbing ~5-fold higher tracer amounts than ventricles per tissue weight. S-peptide uptake was significantly lower than T-peptide. Heart section scans from the β2-AR and MI models detected patchy areas of enhanced staining of yet-to-be identified fibrillary structure.

Conclusions: These results demonstrate that the T-peptide tracer preferentially accumulates in atria, highlighting its potential for AF imaging. Yet, its affinity towards degraded collagen in cardiac pathology has to be examined further. Future *in vivo* molecular imaging experiments will reveal if the tracer can detect both robust and more subtle cardiac fibrosis as seen in the MI / β2-AR and the AF+HF models, respectively.

OC 65.2 | Possible Indicators for High Shear Thrombosis: An Enlarged Cell-free Layer and High Shear Rate

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Background: High shear rate thrombosis needs agents that form the thrombus, including platelets and von Willebrand factor, as well as a binding surface exposing collagen and high shear rate. It is not known what specific flow characteristics cause high shear thrombosis and determine the starting location of the thrombus

formation. Based on prior experimental work the important factors may be high shear rate (Kim et al. (2018)), a gradient in high shear rate (Nesbitt et. al. (2009)) or the elongational rate (Schneider et al. (2007)).

Aims: We preformed cell-based blood flow simulations to gain more insight in the starting location of a thrombus. The flow behavior on the cellular level is still unexplored and could provide additional valuable details.

Methods: A validated cell-based blood flow model by Zavodszky et. al. (2017) is used to simulate two experiments from literature: Nesbitt's (2009) bead assay experiment and Tovar-Lopez's (2010) microcontraction experiment. The bead assay experiment is simulated for two bead sizes and at two different velocities. We measured the platelet count, residence time and shear rate in the area around the bead. In the microcontraction experiment we measured the cell-free layer (CFL) as well as the shear rates and stresses.

Results: The cellular flow simulations showed a high platelet count on the sides of the bead as wells as a high shear rate (see Fig. 1). Surprisingly, an enlarged CFL was found at the location where a platelet aggregate was formed in Nesbitt's experiment (see Fig. 2). This enlarged CFL was also visible in the microcontraction experiment. In both experiments the shear rates were high enough to uncoil von Willebrand factor.

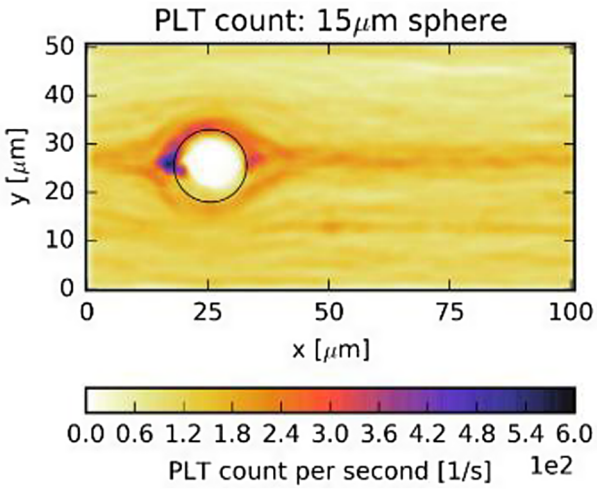

FIGURE 1 2D projection of platelet count for 15 μm bead simulation

FIGURE 2 Side view of simulation with CFL (green), red blood cells (red) and platelets (yellow)

Conclusions: We found that high shear together with an enlarged CFL can predict the location where a thrombus starts to from. We are working on an experiment to validate this finding.

OC 65.4 | *Slc44a2* Deficient Mice Exhibit Less Severity of Thrombosis in a Stenosis Model of DVT

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Background: Recent genome wide association studies (GWAS) identified *SLC44A2* as a novel susceptibility locus for venous thromboembolism (VTE), a region encoding the solute carrier 44A2 protein (SLC44A2). This is of interest as SLC44A2 is not linked to hemostasis or coagulation pathways and may therefore be a unique contributor to VTE.

Aims: Determine the importance of SLC44A2 in thrombotic disease by utilizing *Slc44a2* deficient mice (KO) in a murine representation of deep vein thrombosis (DVT).

Methods: Mice lacking *Slc44a2* were included in a model of DVT induced by flow restriction (stenosis) of the inferior vena cava (IVC). Subsequent measures of thrombosis were recorded and quantified at 48 and 6 hours post-stenosis.

Results: Following 48 hours, 10/10 WT and 12/15 KO mice developed an occlusive thrombus. Thrombi were significantly smaller both in length (p=0.0184) and weight (p=0.0413) in the KO animals. Immunohistochemical evaluation of the thrombi revealed no differences in von Willebrand Factor (VWF) or neutrophil (Ly6G) staining, however a significant reduction in platelet marker GPIb was recorded (p=0.0259). Interestingly, as expected, GPIb was significantly correlated with VWF (r=0.9039; p=0.0052) in WT thrombi, however this relationship was completely absent in KO mice (r=−0.3471; p=0.2956). To further delineate the contributions of SLC44A2 during initiation versus propagation, stenosis was performed and thrombosis measured at 6 hours. At this point, 6/6 WT and 5/8 KO developed thrombi, with KO thrombi being even more reduced in length (p=0.0040) and weight (p=0.0150). Furthermore, at the time of sacrifice, the WT mice had significantly lower levels of blood platelets as compared to KO (p=0.0061), which is a possible reflection of platelet consumption during thrombogenesis.

Conclusions: Based on these data, SLC44A2 may contribute to the activation of platelets (either directly or indirectly) during the initiation of VTE. Moreover, these findings substantiate the original GWAS data and indicate a role for SLC44A2 in thrombotic disease.

OC 65.5 | Prophylactic Inhibition of Factor XI Contact Activation Enhances Deep Vein Thrombosis Resolution in an in vivo Model of Inferior Vena Caval Constriction

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Background: Post-thrombotic syndrome (PTS) after deep vein thrombosis (DVT) remains highly prevalent despite modern medical therapy. Treatment of DVT using direct factor Xa or thrombin inhibitors have decreased major bleeding complications, but are still associated with significant bleeding. Components of contact activation, including factor XI (FXI), are promising targets for anticoagulation that could potentially lower the risk of bleeding, inflammatory response to DVT, and development of PTS. The role of contact activation in vascular homeostasis and mediation of inflammation during DVT resolution is unknown.

Aims: Since coagulation factor XI (FXI) supports contact activation that has no known contribution to hemostasis, we sought to determine the thrombogenic and inflammatory role of FXI activation in a mouse model of DVT resolution using an anti-FXI monoclonal antibody, 14E11, that inhibits contact activation of FXI. We tested whether experimental thrombus resolution is accelerated in 14E11-treated mice.

Methods: Adult male mice were treated with a SQ dose of 5 mg/kg 14E11 versus placebo prior to undergoing surgical constriction of the inferior vena cava below the renal veins. This 14E11 dose was expected to be effective for at least one week in mice.

Results: On post-operative day 3 of thrombus formation, there was no significant difference between thrombus weights among all groups. On post-operative day 7, during thrombus resolution, immunohistochemical analysis with a macrophage specific marker (Iba1) indicated that 14E11 treated mice had decreased macrophage contents in the vein walls and intra-thrombus compared to control mice. Additionally, the thrombus weights normalized to body weight were significantly decreased in 14E11 treated mice as compared to control mice.

Conclusions: Prophylactic inhibition of FXI contact activation did not alter initial thrombus formation, but did result in accelerated thrombus resolution. These findings identify the contact activation system of blood as a potential pharmacologic target for augmenting thrombus resolution in DVT.

OC 09.1 | Identification of the Interacting Domains between the Extracellular Domains of Tissue Factor and β1-intergin and their Influence on Cell Proliferation

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Background: Tissue Factor (TF) has been shown to interact with β1 integrin to induce signalling and influence cell proliferation.

Aims: This study aimed to identify the domains within TF and β1 integrin which are involved in the interaction of these two proteins.

Methods: MDA-MB-231 cells (10^4) were seeded in glass bottomed dishes and transfected with FLAG-HA-pcDNA3.1 plasmids encoding for peptide fragments corresponding to the first, second or combined fibronectin-like domains of TF, or a peptide corresponding to the EGF-4-βTD domains of β1 integrin. The ability of the expressed peptides to interact with either the native β1 integrin or TF respectively, was assessed using the proximity ligation assay (PLA). In addition, MDA-MB-231 cells (10^5) were seeded in 12-well plates and transfected as stated above. The cells were grown for 48 h after which the rate of cell proliferation were assess using the crystal violet staining procedure.

Results: The expression of the fragments corresponding to the first, second and combined fibronectin-like domains of TF reduced the rate of cell proliferation to the same extent, probably due to direct competition with endogenous TF. In addition, all three fragments were shown to be capable of interacting with β1 integrin on the surface of MDA-MB-231 cells, as determined by PLA. Furthermore, the fragment corresponding to the EGF-4-βTD domains within β1 integrin was capable of binding TF on MDA-MB-231 cell surface, but had no detectable influence on the rate of cell proliferation.

Conclusions: This study is the first to identify a direct interaction between these domains within TF and β1 integrin proteins *in situ*, and demonstrates the potential of the TF-β1 integrin interaction in the enhancement of the rate of cellular proliferation.

OC 09.2 | Deletion of CD248, a Novel Cofactor for Tissue Factor (TF), Confers Protection against Thrombosis in a Mouse Model

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Background: CD248 is a transmembrane glycoprotein expressed by activated cells of mesenchymal origin, including vascular smooth

muscle cells (VSMCs) and pericytes. Mice lacking CD248 (KO) are protected against arthritis, atherosclerosis and some tumors.

Aims: Due to its perivascular distribution similar to that of tissue factor (TF), we hypothesized that CD248 also participates in thrombosis.

Methods: We subjected wild-type (WT) and KO mice to the inferior vena cava (IVC) stenosis model of thrombosis.

Results: KO mice developed thrombi that were significantly smaller than those of WT mice. Levels of circulating coagulation factors, blood cells, prothrombin time, and activated partial thromboplastin time were similar in both groups. CD248 colocalized with TF at the VSMC layer and adventitia of IVCs from WT mice, consistent with proximity assays confirming their close association. Flow cytometry of whole blood from healthy humans revealed cell surface CD248 expression by monocytes and neutrophils. ROTEM studies with whole blood confirmed that membrane-anchored CD248 accelerates clotting and that the extrinsic TF pathway is primarily involved. ROTEM studies with plasma from KO and WT mice did not differ in clotting kinetics. VSMCs that express CD248, accelerated TF-factor VIIa mediated activation of factor X that was not related to decryption of TF or changes in expression levels of TF.

Conclusions: Overall, CD248, expressed by VSMCs and monocytes/neutrophils, is a newly uncovered cofactor for TF that accelerates clotting *in vitro* and *in vivo*. The findings reveal a potential new therapeutic target for hemostatic disorders.

OC 09.3 | Loss of Tissue Factor in Zebrafish Disrupts Hemostasis and Results in Synthetic Lethality, but Is Compatible with Embryonic Development

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Background: Tissue factor (TF) is responsible for initiation of the extrinsic coagulation pathway through binding to activated factor VII. TF-null mice are embryonic lethal at an earlier timepoint than other procoagulant knockouts, suggesting additional functions beyond coagulation, including hypotheses of abnormal angiogenesis and loss of perivascular support. External development and optical transparency make zebrafish a powerful tool to study coagulation and development. Zebrafish have two TF paralogs (TFa and TFb) with unknown spatial and functional differentiation.

Aims: To study the role of TF in embryonic development.

Methods: We used genome editing with CRISPR/Cas9 to produce null alleles for TFa and TFb. Laser-induced endothelial injury was used to assess thrombus formation. We tracked vascular development using a fluorescent endothelial reporter and angiography.

Results: TFb, but not TFa, is present in the zygotic transcriptome, indicating maternal deposition. It has been shown that transcription of both genes increases following gastrulation and we have confirmed transcript presence at the initiation of circulation. Single mutants survived well into adulthood with no gross phenotypes and were able to reproduce. However, double mutants died by 9 weeks of age due to hemorrhage. Loss of TFa resulted in delayed induced thrombus formation in the venous system at 3 days old while TFb had no observable effect. Double mutants demonstrated a complete inability to form thrombi. Fluorescent vascular studies demonstrated normal vasculogenesis, angiogenesis, and vascular integrity throughout embryonic development.

Conclusions: Overall, our data suggest that the hemostatic role of TF is conserved in fish while its loss is compatible with normal development, but early adult mortality. Interestingly, maternal deposition of TFb suggests a developmental function prior to vasculogenesis. Our results indicate that the duplication provides an additional layer of quantitative regulation, and creates a titratable TF level that will allow the interrogation of the role TF in perivascular development, cardiovascular stability, remodeling and regeneration.

OC 09.4 | Heterozygous Tissue Factor Deficiency is Associated with a Human Bleeding Disorder

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Background: Tissue factor (TF) initiates blood coagulation. TF deficiency in humans has never been described.

Aims: To evaluate the contribution of heterozygous TF deficiency to coagulation initiation in a woman with unexplained bleeding and in mice with a parallel genotype.

Methods: Clinical evaluation, routine laboratory assays, and whole genome sequencing were performed in humans with undiagnosed bleeding defects enrolled in the NIHR BioResource. A heterozygous defect in *F3* encoding TF was identified in a woman with familial bleeding characterized by menorrhagia, postpartum hemorrhage, and normal laboratory evaluation. The recombinant variant was expressed, purified, and evaluated in factor Xa- and thrombin generation assays, and in transduced endothelial cells. CRISPR-edited induced pluripotent stem cells (iPSCs) expressing the variant were differentiated into the endothelial and vascular smooth muscle lineage as confirmed by immunophenotype. Cells were analyzed by qPCR, Western blot, and in assays of TF-dependent coagulation initiation. Thrombus formation was evaluated in *F3*+/- mice and littermate controls via intravital microscopy following laser-induced cremasteric arteriole injury.

Results: A heterozygous *F3* frameshift causes premature termination (Ser117HisfsTer10) of TF in a woman with a mild bleeding diathesis. CRISPR-engineered iPSCs heterozygous for the Ser117HisfsTer10 allele were differentiated into vascular smooth muscle and endothelial-like cells that demonstrate haploinsufficiency of TF in factor Xa generation assays. The variant *F3* transcript increases upon cycloheximide treatment, indicating degradation by nonsense-mediated decay. Neither overexpression in cultured cells nor exogenous recombinant TF mutant inhibited factor Xa or thrombin generation, excluding a dominant negative mechanism. *F3*+/- mice replicating haploinsufficiency exhibit impaired fibrin generation and platelet accumulation following vascular injury. Analyzing *F3* null alleles in 141,456 humans, heterozygous TF deficiency has an incidence of 1 in 25,000.

Conclusions: Haploinsufficiency of TF is a modifier of coagulation initiation that is associated with clinical bleeding. Heterozygous TF deficiency should be considered in individuals with undiagnosed bleeding disorders with normal hemostatic evaluations.

OC 09.5 | Expression of Tissue Factor and Production of Extracellular Vesicle by Human Macrophages Requires Alternative Polarization

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Background: Macrophages are versatile cells that can be polarized by the tissue environment to fulfill required needs. Proinflammatory polarization is associated with increased tissue degradation and propagation of inflammation whereas alternative polarization within a Th2 cytokine environment is associated with wound healing and angiogenesis.

Aims: To understand if polarization of macrophages can lead to a procoagulant macrophage subset we polarized human monocyte derived macrophages to a proinflammatory and an alternative activation state.

Methods: Human macrophages were derived from monocytes by either MCSF or GMCSF treatment. Polarization of macrophages was achieved by LPS and IFN-gamma for proinflammatory macrophages and IL4 and IL13 for alternative polarized macrophages.

Results: We provide evidence that alternative polarization leads to a macrophage phenotype characterized by increased tissue factor (TF) production and release as well as an increase in extracellular vesicle production. In addition, also TF activity is enhanced in extracellular vesicles of alternatively polarized macrophages. This TF induction is dependent on STAT6 signaling and PARP activity. In contrast to monocytes, human macrophages do not show increased TF expression upon stimulation with LPS and interferon-γ. Also previous polarization to either a proinflammatory or an alternative activation subset does not change the subsequent stimulation of TF. The diminished response to LPS is due to an increase in TF promoter methylation and reversible when treating macrophages with a demethylation agent.

Conclusions: Proinflammatory polarization of macrophages does not lead to a procoagulatory environment whereas alternative polarization of macrophages leads to an increased expression of TF and increased availability of extracellular vesicles supporting the procoagulatory state of alternatively polarized macrophages.

OC 18.1 | Mechanisms Coupling Fibrinogen to Colorectal Adenocarcinoma Growth

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Background: Previous studies showed that fibrin(ogen) promotes metastasis across multiple cancers, whereas the role of fibrin(ogen) in primary tumor growth is context dependent. One cancer strongly dependent on fibrin(ogen) for tumor growth is colorectal cancer (CRC).

Aims: Our aim was to define the mechanisms coupling fibrin(ogen) to CRC growth.

Methods: We performed RNA-Seq analyses on murine CRC tumors grown in fibrin(ogen)-sufficient (Fib+) and -deficient (Fib-) mice, and confirmatory studies at the protein level.

Results: We observed significant fibrinogen-dependent differences in the expression of 226 genes regulating cellular proliferation, migration and metabolism. One gene highly expressed in tumors from Fib- mice relative to Fib+ mice was *Stratifin*, which encodes 14-3-3-σ. 14-3-3-σ stabilizes p53 and other negative cell cycle regulators, limiting growth. Consistent with this, independent analyses demonstrated increased expression of 14-3-3-σ and multiple negative cell cycle regulators (i.e., p53, p21 p27) at the protein level in tumors from Fib- mice relative to Fib+ mice. We also compared FAK activation and its downstream target MDM2, which together negatively regulate P53. Both FAK and MDM2 were inactive in tumors from Fib- mice, as indicated by phosphorylation specific antibodies for each protein. These data suggest that loss of fibrin(ogen) limits FAK activation and subsequent MDM2 activation, resulting in less P53 degradation, thereby limiting tumor growth.

Conclusions: These findings suggest that fibrin(ogen) in the CRC microenvironment promotes tumor growth through at least 2 mechanisms, diminished expression of 14-3-3-σ and increased activation of FAK. Both pathways result in downregulation of p53, p21 and p27, thereby driving tumor proliferation and survival. Given the potential of fibrin(ogen) to bind multiple receptors, it is possible that direct fibrin(ogen)-tumor cell interactions are responsible for these events, and could represent novel targets for treating CRC.

OC 18.2 | Lysophosphatidylcholine: A Pro-inflammatory Lipid of Platelet Microvesicles Promoting Platelet Activation, Vascular Inflammation and Indicating Atherosclerotic Plaque Instability

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Background: Microvesicles are cell blebs released from a variety of different cells and have gained attraction as pro-inflammatory circulating biological vectors. It is well known that microvesicles deliver RNAs, surface receptors and cell organelles to their target cell and thereby influence the target cell's phenotype. However, not much is known about the lipid composition of microparticles and whether these lipids have a biological effect on their target cells.

Aims: Determine whether platelet microvesicles contain distinct lipids that mediate vascular inflammation.

Methods: Lipids of microvesicles and of tissue were analysed with liquid chromatography electrospray ionization-tandem mass spectrometry. For lipid mapping of human atherosclerotic plaque, matrix-assisted laser desorption ionization (MALDI) imaging was used. The effect of LPC on platelets was investigated using flow cytometry and aggregometry. Gene regulation was quantified with qRT-PCR. Unstable plaques in mice were obtained with the tandems stenosis mouse model of atherosclerotic plaque instability.

Results: Lysophosphatidylcholine was one of the most abundant lipids of platelet-derived microvesicles. We demonstrate that LPC is a potent activator of platelets and induces platelet monocyte aggregates, spreading and aggregation of platelets as well as activation of endothelial dysfunction. We could also identify the G2A receptor as a target receptor of lysophosphatidylcholine on platelets. Using a murine model of plaque instability, circulating lysophosphatidylcholine was associated with plaque instability. Additionally, lysophosphatidylcholine was found to be highly abundant in vulnerable areas of human atherosclerotic plaques.

Conclusions: Lysophosphatidylcholine is abundant in platelet microvesicles and induces platelet activation and vascular inflammation most likely in a G2A receptor mediated way. Furthermore, circulating lysophosphatidylcholine is an indicator for plaque instability in mice and humans.

OC 18.3 | EPCR Signaling Regulates Activity of Hematopoietic Progenitor and Stem Cells

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Background: Hematopoietic stem cells (HSC) reversibly switch between dormancy and stress-induced proliferation, replenishing all differentiated blood cells. The endothelial cell protein C receptor (EPCR) is expressed by HSCs, but roles of EPCR signaling versus regulation of the anticoagulant pathway in HSC maintenance are still unknown.

Aims: We studied EPCR-C/S mice carrying a single point mutation abolishing intracellular palmitoylation implicated in raft localization. With normal protein expression and anticoagulant function of the mutated receptor, these mice provide a model to delineate the contribution of EPCR signaling to proliferation of hematopoietic progenitor and stem cells (HPSCs) in normal and malignant hematopoiesis.

Methods: We analyzed HSPC frequencies in the BM by multicolor flow cytometry at steady-state and after granulocyte colony stimulating factor (G-CSF) mobilization. Leukemia development was studied in the MLL-AF9-induced acute myeloid leukemia (AML) model.

Results: We found increased frequencies of circulating HSC in the blood of EPCR-C/S mice compared to controls, indicating that EPCR signaling mediates retention in the BM niche. $EPCR^+$ HSC from EPCR-C/S mice displayed lower ESAM expression, identifying the most primitive HSC subset, without increased cell cycle activity relative to strain-matched controls. Cell cycle activity in myeloid progenitor cells was increased at steady-state. G-CSF-induced myelopoiesis and mobilization resulted in an increased release of HSPC and myeloid cells into the peripheral blood of EPCR-C/S mice compared to controls. Consistent with increased myeloid progenitor proliferation, MLL-AF9 AML induced in EPCR--C/S BM cells resulted in a more aggressive disease with shortened survival times compared to controls. $MLL\text{-}AF9^+$ EPCR-C/S leukemia displayed an increased leukemic load in the peripheral blood and increased frequencies of $Ki67^+$ proliferative blasts in the BM relative to wild-type.

Conclusions: This novel mouse model revealed a critical role for EPCR signaling in regulating HSC dormancy, retention and HSPC proliferations in both normal and malignant hematopoiesis.

OC 18.4 | Effect of Combining Low Levels of Tissue Factor with Complete Deficiencies for Factor IX or Factor XI on Survival and Placental Development in Mice

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Background: The tissue factor (TF)-factor VIIa (FVIIa) complex triggers coagulation by activating FIX and FX leading to the generation of thrombin. FIX can also be activated by a positive feedback loop involving thrombin and FXI. FIX deficient mice and low TF mice are viable but exhibit hemostatic defects. Importantly, low TF placentas develop blood pools.

Aims: To determine the effect of combining low levels of TF with complete deficiency for FIX or FXI on survival of mice and placental development.

Methods: Crossing of male $mTF^{-/-}$;$hTF^{+/0}$; $FIX^{+/y}$ with female $mTF^{+/-}$;$hTF^{+/-}$;$FIX^{+/-}$ was used to generate male low TF;$FIX^{-/y}$ offspring. Mendelian inheritance was assessed at embryonic day (ED) 14.5, postnatal day 1 and postnatal day (PD) 28. Crossing of $mTF^{+/-}$;$hTF^{+/-}$;$FXI^{+/-}$ male and female mice was used to generate low TF;$FXI^{-/-}$. Mendelian inheritance was assessed at PD28. Placental blood pooling was assessed histologically at ED14.5-16.5.

Results: Low TF;$FIX^{-/y}$ male mice were present at the expected frequency at embryonic day 14.5. No low TF;$FIX^{-/y}$ offspring were observed at wean with a high proportion of pups dying within the acute postnatal period. In contrast, low TF;$FXI^{-/-}$ mice were generated at the expected frequency at wean with 6 month survival comparable to that of low TF controls. In complete contrast to the survival data, FXI deficiency, but not FIX deficiency, was associated with increased severity of blood pools in low TF placentas and some maternal death.

Conclusions: FIX but not FXI is required for hemostasis in low TF mice. Conversely, FXI but not FIX plays a role in placental development under conditions of low TF.

OC 18.5 | Distinct Spatiotemporal Thrombotic Mechanisms Promote Microvascular Obstruction during IR Injury

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Background: Microvascular obstruction is a common complication of ischemia reperfusion (IR) injury. Its pathogenesis is partly dependent on the loss of the natural anticoagulant and anti-inflammatory function of endothelial cells, resulted in platelet-endothelial and neutrophil-endothelial interactions that obstruct the microvasculature.

Aims: Investigate the sequence of pathogenic events underlying microvascular obstruction during ischemia insult and reperfusion injury.

Methods: A mouse model of gut IR injury and intravital confocal microscopy were used to monitor vaso-occlusive thrombotic mechanisms and their spatiotemporal association with endothelial changes during ischemia and reperfusion injury.

Results: Our studies revealed 3 distinct thrombotic mechanisms promoting microvascular obstruction, and their temporal and spatial association with endothelial changes during IR injury. The first thrombotic mechanism is triggered by ischemia and mediated by intravascular red blood cell (RBC) congestion. Irreversible RBC congestion occurs under conditions of blood flow stasis, predominantly in the gut mucosal (villus) microvasculature, at sites of ischemic endothelial injury, marked by the loss of endothelial junction proteins and the glycocalyx. The second mechanism occurs during early stage of reperfusion, is blood flow-dependent and primarily mediated by platelets. Extensive platelet deposition occurs in the villus as well as submucosal microvasculature, at sites of endothelial cell death (phosphatidylserine exposure). The third thrombotic mechanism involves neutrophil macroaggregates facilitated by dying platelets in the submucosal microvasculature during blood reperfusion. The vaso-occlusive neutrophil macro-aggregates trigger ischemic injury in remote organs, including the liver and lung.

Conclusions: These findings demonstrate 3 spatially and temporally distinct thrombotic mechanisms and associated phenotypic endothelial changes contributing to microvascular dysfunction during IR injury. They may have important implications for the timing and targets of specific antithrombotic therapies aimed at reducing microvascular obstruction.

OC 28.1 | The *Pseudonaja textilis* Factor V A2 Domain C-terminus Mediates Lipid-independent Cofactor Activity and Functional Activated Protein C Resistance

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Background: The heavy chain (A1-A2-domains) of coagulation factor Va (FVa) is characterized by an acidic region at its C-terminus that has been implicated in cofactor activity. Interestingly, the *Pseudonaja textilis* venom FV (ptFV) C-terminal A2-domain sequence (A2T) is significantly extended relative to human FV (hFV). We previously reported that ptFV displays cofactor function in the absence of anionic membranes and is functionally resistant to activated protein C (APC) despite extensive APC-mediated proteolysis. Here we

hypothesized that the ptFV A2T region contributes to these procoagulant characteristics.

Aims: Assess the functional role of the elongated C-terminal A2-domain region in ptFV.

Methods: The ptFV A2T was exchanged for the homologous human FV region, thereby generating ptFV-hA2T and hFV-ptA2T.

Results: Kinetic assessment of prothrombin conversion revealed that the FVa variants demonstrated a similar substrate conversion rate and comparable apparent binding affinities for prothrombin and the factor Xa (FXa) species relative to their wild-type counterpart. This indicates that the A2T does not significantly contribute to prothrombinase assembly nor activity. Remarkably, hFVa-ptA2T displayed lipid-independent cofactor activity similar to ptFVa, albeit in the presence of *P. textilis* venom FXa (ptFXa) only. Consistent with this, similar ptFXa-mediated prethrombin-1 conversion rates were observed for ptFVa and hFVa-ptA2T with and without anionic phospholipids. In contrast, hFVa or ptFVa-hA2T lacked lipid-independent cofactor activity. In addition, APC-treatment significantly reduced the cofactor activity of ptFVa-hA2T, while ptFVa retained full cofactor activity.

Conclusions: Together, our results demonstrate that the *P. textilis* FV A2T plays a crucial role in the unique procoagulant features of ptFV. Using this extended region, ptFV bypasses the need for membrane binding, presumably by mediating FXa and/or substrate binding. Additionally, this region was found to play a major role in the functional APC-resistance. Therefore, the ptFV A2T represents an exceptional structural element responsible for the enhanced functions which are at the basis of its potent toxicity.

OC 28.2 | A mathematical Model of Flow-mediated Coagulation Identifies Factor V as a Modifier of Thrombin Generation in Hemophilia A

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Background: The variability in bleeding patterns amongst individuals with hemophilia A, which have similar factor VIII (FVIII) levels, is significant and the origins are unknown.

Aims: The objective of this study was to use a mathematical model of flow-mediated coagulation as a screening tool to identify parameters that are most likely to enhance thrombin generation in the context of FVIII deficiency.

Methods: Using a mathematical model of flow-mediated coagulation, we performed a global sensitivity analysis to identify modifiers of thrombin generation; the sensitivity analysis was based on

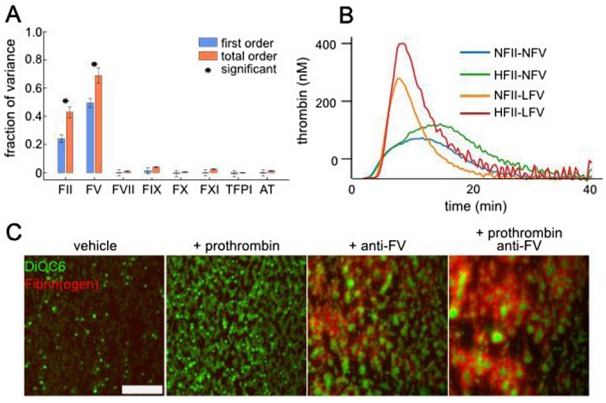

FIGURE 1 (A) Global sensitivity analysis of a mathematical model of flow-mediated coagulation. First (blue) and total (orange) order Sobel sensitivity indices plotted as mean +/- standard deviation show the fraction of variance in thrombin output when zymogens and anticoagulant levels are varied between 50-150% of normal values. (B) Calibrated automated thrombography (5 pM TF) in plasma containing varying levels of FII and FV in the presence of an anti-FVIII function blocking antibody. 'N', 'H', and 'L' correspond to normal (100%), high (150%), and low (50%) of the respective zymogen. (C) Flow assays with whole blood from an individual with FVIII deficiency treated with vehicle control, 50 µg/mL exogenous prothrombin, 100 µg/mL anti-FV, and both exogenous prothrombin and anti-FV. Representative images of DiOC6 labeled platelets (green) and Alexa Flor 555 labeled fibrin(ogen) (red) after 25 min perfusion over type collagen-TF surfaces at 100 1/s. Scale bar = 50 µm

results from 110,000 simulations in which coagulation factor levels varied by 50-150% of their normal values and FVIII was fixed at 1%. Candidates from the sensitivity analysis were confirmed by calibrated automated thrombography (CAT) and flow assays on collagen-TF surfaces at 100 s^{-1}. Blood from individuals with FVIII deficiencies was collected using protocols approved by the IRB of the University of Colorado. Factor V (FV) was partially inhibited with an anti-FV antibody (AHV-5101, Haematologic Tech.).

Results: Simulations identified low FV (50%) as the strongest candidate, with additional enhancement when combined with high prothrombin levels (150%) (Fig. 1A). Low FV zymogen levels (50%) or partial FV inhibition (60% activity) augmented thrombin generation in FVIII-inhibited (Fig. 1B) or FVIII-deficient plasma in CAT. Partial FV inhibition (60%) boosted fibrin deposition in flow assays performed with whole blood from individuals with mild and moderate FVIII deficiencies (Fig. 1C). These effects were amplified by high prothrombin levels in both experimental models.

Conclusions: These results show that FV levels on the low end of the normal range can enhance thrombin generation in hemophilia A. Further explorations with our mathematical model suggest a mechanism by which FV and FVIII compete to bind with factor Xa and initiate thrombin generation in low FV, FVIII-deficient blood.

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OC 28.3 | Probing Interactions between the Tissue Factor Exosite and the Light Chain of Factor X

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Background: A solvent-exposed surface ("exosite") near the C-terminus of tissue factor (TF) is thought to contribute to macromolecular substrate binding by the tissue factor-factor VIIa complex (TF-FVIIa). However, it is still unclear how the TF exosite interacts with factor X (FX).

Aims: We hypothesize that portions of the FX light chain bind to the TF exosite to facilitate substrate activation. Our approach is to analyze stable, membrane-bound complexes of TF-FVIIa and a FX mimetic (XK1) with increased affinity for TF-FVIIa. XK1 (Girard et al. Science 248:1421, 1990) is a hybrid protein consisting of the FX light chain and first Kunitz domain of tissue factor pathway inhibitor.

Methods: Membrane-anchored TF (wild-type and mutants), FVIIa and XK1 were assembled on Nanodiscs and TF-FVIIa-XK1 activity was tested via enzyme assays. The TF-FVIIa-XK1 structure was analyzed by both molecular dynamics (MD) simulations (600 ns) utilizing a specialized membrane model and cryo-electron microscopy (cryo-EM).

Results: XK1 inhibited TF-FVIIa with an IC_{50} of 40 pM. TF exosite mutants reduced the inhibitory effect of XK1 comparable to their reduction in the rate of FX activation. MD simulations predicted that negatively-charged residues in the aromatic stack of the FX light chain bind to TF exosite residues, K165 and K166. Initial structures of the TF-FVIIa-XK1 complex were obtained using cryo-EM at 6Å resolution. Preliminary structural analyses indicate potential changes in TF-FVIIa conformation upon XK1 binding, as well as interactions between TF exosite and FX light chain.

Conclusions: Enzyme activation assays show that XK1 interacts with the TF exosite in the same way wild-type FX does. MD simulations and cryo-EM data indicate that the TF exosite directly interacts with the FX light chain. We propose that this interaction mediates substrate recognition by the TF-FVIIa complex.

OC 28.4 | An Inducible Mouse Model of Factor X Deficiency to Test Novel FX Therapeutics

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Background: The crucial role of clotting Factor X (FX) in blood coagulation is demonstrated by the severe bleeding diathesis of patients with inherited FX deficiency, for whom therapeutic options are limited. The development of novel FX therapeutics has been hampered by the fact that a severe deficiency is rare, and by the observation that a full deficiency in mice is incompatible with life, limiting their utility in preclinical studies.

Aims: To generate a viable mouse model of FX deficiency to validate novel treatment options for inherited FX deficiency.

Methods: Liver-specific *F10* deficient mice were generated using the inducible Cre-Lox recombination system. Residual plasma FX activity was measured using chromogenic assays and standard *in vitro* coagulation assays. *In vivo* evaluation of hemostasis was assessed via a tail-clip bleeding assay.

Results: Induction of the Cre recombinase in floxed *F10* mice resulted in severely reduced FX activity levels in plasma ($F10^{low}$). Coagulation analyses using standard PT and aPTT assays revealed that *in vitro* clotting times were significantly prolonged in $F10^{low}$ mice compared to transgenic mice harboring only the floxed *F10* gene ($F10^{WT}$). In line with these findings, thrombin generation in platelet-poor plasma from $F10^{low}$ mice was almost completely abrogated. Although no spontaneous bleeding events were observed in $F10^{low}$ mice, bleeding time and blood loss were significantly increased compared to $F10^{WT}$ mice following tail transection. Complete correction of the bleeding phenotype was achieved following infusion of purified plasma-derived human FX (0.5mg/kg). Moreover, an unexpected partial correction of the bleeding diathesis was observed after infusion of recombinant activated factor VII (4mg/kg), which likely exerts its procoagulant effect through the presence of trace amounts of FX.

Conclusions: This is the first report on a viable mouse model of severe FX deficiency associated with a severe bleeding phenotype following hemostatic challenge, which makes it an excellent tool for studying novel FX therapeutics.

OC 28.5 | Whole Genome Sequencing in a Severe Thrombophilic Family Revealed a SERPINC1 Defect Impairing Antithrombin Activity that is Not Detected by Current Functional Methods

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Background: Venous Thrombosis (VT) is a complex common disease where both rare and common variants contribute together to modulate the individual disease risk. As for other diseases,

early-age-of-onset VT patients are enriched for rare disease variants that can be efficiently detected by next generation sequencing.

Aims: To identify the mutation(s) causing portal vein thrombosis in a patient aged 44 yrs without local or systemic risk factors (including a normal thrombophilia screening) and with a strong family history of VT.

Methods: Whole genome sequencing was performed to search for putative rare VT-causing variant(s). Sanger sequencing, family segregation study and experimental validation were further conducted on the candidate variant(s).

Results: The proband and two affected relatives were found to share a never reported likely deleterious p.Glu227Lys mutation in the *SERPINC1* gene. Routine functional antithrombin tests (anti-FXa and anti-FIIa) as well as antigenic measurements of plasma antithrombin revealed normal values for all carriers in different time points except in two patients, who showed low functional antithrombin during the acute phase, while under curative dose of heparin. Longer incubation times of plasma with the target proteases revealed an impaired anticoagulant activity in mutation carriers. Thrombin generation analysis showed increased endogenous thrombin potential in all carriers. Western blot analysis of plasma antithrombin revealed the presence of an abnormal variant protein in carriers. Recombinant expression of the variant in HEK-EBNA cells demonstrated that the mutation did not significantly impair secretion of the variant protein, but it has impaired anticoagulant activity.

Conclusions: This study identifies a novel *SERPINC1* variant that impairs the anticoagulant capacity of the protein and demonstrates that functional tests routinely used in clinic to diagnose antithrombin deficiency are imperfect. This work adds strong support for the molecular analysis of *SERPINC1* in VT patients independently of the results of the classical biological tests.

OC 35.1 | A Safe and Potent Antithrombotic Agent: Salvianolic Acid B Inhibits Platelet Aggregation and Blood Coagulation

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Background: *Salvia miltiorrhiza* root (danshen) extracts have been used for centuries to control cardiovascular diseases in Chinese medicine. Danshen depside salts are currently approved in China to treat coronary heart disease and angina; and are under evaluation in the USA in phase II clinical trials. Salvianolic acids have been identified as the active components of danshen. Salvianolic acid B (SAB), the most abundant salvianolic acid, has been previously shown to exhibit anti-platelet and anti-thrombotic properties in animal models. However, the mechanism of action has not been adequately explored.

Aims: Uncover the anti-platelet and anti-thrombotic mechanisms of SAB.

Methods: *In vitro* platelet/coagulation assays and *in vivo* intravital thrombosis models combined with biochemical kinetic and biophysical calorimetry assays, and *in silico* molecular modelling.

Results: We demonstrate that SAB attenuated ADP, collagen, and thrombin receptor activating peptide (TRAP)-induced human platelet aggregation. Interestingly, SAB inhibited thrombin-induced platelet aggregation far more potently than other agonists. Furthermore, using our intravital microscopy thrombosis models, we demonstrated that SAB significantly decreased thrombus growth *in vivo*. Using a series of *in vitro* coagulation assays, we found that SAB significantly reduced clot weight in human whole blood, and delayed coagulation in human cell-free blood plasma using thromboelastography. In addition, SAB significantly reduced the fibrin network density in cell-free blood plasma. Through structural analysis, we found that SAB contains structural similarities to the trisubstituted benzimidazole class of thrombin inhibitors, such as dabigatran. *In silico* molecular modeling predicts that SAB binds within the thrombin active site - interacting with similar residues as dabigatran. Using isothermal titration calorimetry and kinetic thrombin inhibition assays, we corroborate these findings and reveal SAB as a direct competitive thrombin inhibitor.

Conclusions: These data establish a novel mechanism of SAB in the inhibition of both platelet aggregation and blood coagulation. These unique characteristics position salvianolic acids as safe and potent herb-derived anti-thrombotic agents.

OC 35.2 | Comparative Study of CM352 versus Prothrombin Concentrate Complex in Experimental Models of Haemorrhage Associated with Oral Anticoagulants

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Background: Direct oral anticoagulants (DOACs) have decreased the risk of Intracranial haemorrhage (ICH) [HR=0.48 (0.39-0.59)] in different thrombotic settings. Matrix metalloproteinases (MMPs) are involved in the coagulation-fibrinolysis system. We have developed a MMPs inhibitor (CM-352), that controls bleeding in experimental models.

Aims: In this study we evaluated the effect of CM-352 in different experimental models of haemorrhage with anticoagulated mice and the role of MMP-10.

Methods: Two experimental models were performed (tail bleeding and collagenase induced-ICH) in C57Bl6J mice anticoagulated with warfarin or rivaroxaban. CM-352 or saline was given intravenously before the tail excision and bleeding time was measured. In the ICH model, CM-352 and Prothrombin Concentrate Complex (CCP) were administered after collagenase injection. Haemorrhage volume (diaminobencidine staining), motor activity and inflammation (plasmatic IL-6 and infiltrated neutrophils) were measured at 24h. Finally, the role of CM-352 on ICH was assessed in Mmp10-/- mice.

Results: CM-352 treatment diminished bleeding time in mice anticoagulated with rivaroxaban (75%, p< 0.001) and warfarin (60%, p< 0.01). In the ICH model, CM-352 effectively reduced the volume of haemorrhage (70%, p=0.001) and infiltrated neutrophils in rivaroxaban-treated mice. CCP decreased the volume of haemorrhage in warfarin and rivaroxaban-treated mice (50%, p< 0.01 and p< 0.05). Both CM-352 and CPP, prevented motor impairment at 24h (p< 0.05). Finally, Mmp10-/- mice showed lower haemorrhage volume (p=0.041) and IL-6 levels than wild type, not further modified by CM-352.

Conclusions: MMPs inhibition by CM-352 is effective in the control of bleeding associated with warfarin and rivaroxaban but highly effective in rivaroxaban associated-ICH. This beneficial effect is dependent of MMP10 inhibition, suggesting that MMP10 is a key factor in the control of ICH associated with rivaroxaban.

OC 35.3 | The Intestinal Microbiome Affects Thrombin Generation in Human Subjects Through Effects on Coagulation Related Plasma Protein Levels

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Background: The intestinal microbiome plays a versatile role in the etiology of arterial thrombosis. In venous thrombosis, driven chiefly by plasma coagulation, no such role has yet been established.

Aims: We hypothesized that the intestinal microbiome composition affects coagulation in humans and is affected by fecal microbiota transplant (FMT).

Methods: We used healthy donor FMT to experimentally change the microbiome composition in metabolic syndrome patients. The Institutional Review Board of the Academic Medical Center in Amsterdam, the Netherlands, approved the study and all subjects gave informed consent. 35 subjects were randomized in a blinded fashion to healthy donor FMT or autologous FMT as a control in a 2:1 ratio. We measured thrombin generation at baseline and after 6 weeks using automated calibrated thrombinography and plasma abundance of 43 coagulation related proteins using targeted mass spectrometry-based quantitative proteomics assay with heavy labeled internal standards. Values were expressed as deltas (6 weeks minus baseline).

Results: Healthy donor FMT prolonged the lag time in thrombinography (delta -0.14 vs. 0.28 min. p=0.039). The other thrombinography parameters showed similar trends of diminished thrombin generation, however deltas were not significantly different. Unsupervised cluster analysis of the protein abundance showed overall downregulation of coagulation related proteins in clusters containing almost exclusively healthy donor FMT treated subjects. The proteins showed no clustering according to an apparent biological grouping.

Conclusions: These results show that healthy donor FMT can suppress thrombin generation in metabolic syndrome patients, representing initial evidence that the intestinal microbiota composition affects the coagulation system. Plasma protein levels changed in parallel to the thrombin generation, suggesting the effect works through protein expression rather than enzymatic activity. The findings merit external validation as a role for gut intestinal microbiota in venous thrombosis can have clinically important implications.

OC 35.4 | Enhanced Potency of Recombinant Factor VIIa upon Increasing Affinity to Activated Platelets

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Background: Recombinant factor VIIa (rFVIIa) enhances thrombin generation in a platelet-dependent manner, however, rFVIIa binds activated platelets with relatively low affinity. Triggering receptor expressed on myeloid cells (TREM)-like transcript (TLT)-1 is expressed exclusively on activated platelets.

Aims: To enhance the potency of rFVIIa via binding to TLT-1.

Methods: rFVIIa was conjugated to a Fab binding TLT-1. The *in vitro* potency of this platelet-targeted rFVIIa (PT-rFVIIa) was evaluated using FX activation assays, and by measuring the viscoelastic changes in whole blood. *In vivo* potency was evaluated using a tail vein transection model in fully anaesthetised *F8*-/- mice expressing human TLT-1.

Results: PT-rFVIIa and rFVIIa had similar K_D values for binding to tissue factor (TF) and similar TF-dependent FX activation. However, PT-rFVIIa had increased affinity for activated platelets and increased catalytic efficiency on TLT-1 vesicles and activated platelets. The *in vitro* potency in normal human blood with antibody-induced

haemophilia A was dependent on assay conditions used. When platelets were maximally activated by adding PAR-1 and GPVI agonists, the EC_{50} for PT-rFVIIa clot time was 49-fold lower than for rFVIIa, while smaller differences in EC_{50} values for PT-rFVIIa and rFVIIa were observed when kaolin or TF were used to initiate the assay. When tested *in vivo* in the murine bleeding model, a 53-fold lower EC_{50} was observed for PT-rFVIIa relative to rFVIIa, supporting the relevance of the *in vitro* assay conditions with maximally activated platelets. *In vitro* analysis of blood from subjects with haemophilia A using maximally activated platelets confirmed the data obtained with normal blood.

Conclusions: Increasing the affinity of rFVIIa to activated platelets via TLT-1 resulted in approximately 50-fold increased potency *in vitro* and *in vivo*. The correlation of *in vivo* with *in vitro* data using maximally activated platelets supports that these assay conditions are relevant when evaluating platelet-targeted haemostatic concepts.

OC 35.5 | A Single Domain Antibody that Inhibits Factor VIIa in the Absence but Not Presence of Tissue Factor

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Background: Activated factor VII (FVIIa) is a serine protease that is important to the initiation of the coagulation cascade. Free FVIIa is relatively inefficient, and it requires binding to Tissue Factor (TF) to have physiologically relevant activity.

Aims: We aimed to generate a single domain antibody (VHH or nanobody) that recognizes free FVIIa rather than TF-bound FVIIa.

Methods: A llama-derived phage library was used to screen for anti-FVIIa VHH. One candidate, KB-FVIIa-004, was selected for further analysis using various in vitro activity assays.

Results: KB-FVIIa-004 bound to FVIIa, but not to its precursor FVII or to homologous proteins (prothrombin, factor X or their activated derivatives). FVIIa amidolytic activity was inhibited by KB-FVIIa-004 (Ki=0.5±0.1 μM) in a competitive manner. KB-FVIIa-004 also inhibited FVIIa-mediated FX activation, and half-maximal inhibition was obtained at a molar VHH/FVIIa ratio of 1.2±0.3. Noteworthy, inhibition of FVIIa by PPACK interfered with FVIIa binding to KB-FVIIa-004, suggesting that KB-FVIIa-004 binds in or near the active site. To test the effect of TF, we first measured the capacity of KB-FVIIa-004 to prolong the prothrombin time, observing that clotting times started to prolong at VHH-concentrations of 0.33 μM or higher, corresponding to a >300-fold molar excess over FVIIa concentrations generated in this assay. Furthermore, FVIIa/TF amidolytic activity or FVIIa/TF-mediated FX activation remained unaffected up to a 100-fold molar excess of VHH. Moreover, KB-FVIIa-004-mediated inhibition of FVIIa could by reversed by the addition of TF. Apparently, TF induces conformational changes within FVIIa that strongly diminish antibody binding.

Conclusions: We have developed a VHH that is specific for free FVIIa rather than TF-bound FVIIa. This antibody may be used to investigate physiological functions of free FVIIa relative to TF-bound FVIIa. This could be of particular interest to better understand FVIIa's mode of action during hemophilia A treatment, which is proposed to be predominantly TF-independent.

OC 42.4 | Patient Reported Outcome Data on Switch from Standard to Extended Half Life in Irish Adults with Haemophilia

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Background: From 2017 to the end of 2018, all people with Haemophilia B and the majority with Haemophilia A (PwH) in Ireland were switched to extended half-life FIX (rFIXFc) or FVIII (rFVIIIFc) concentrates.

Aims: The Irish Haemophilia Society surveyed measurable impact on their health-related quality of life

Methods: Utilising the Patient Reported Outcomes, Burdens and Experiences (PROBE) survey, the Society surveyed members of the organisation with Haemophilia A/B pre-change and a minimum of 10 months post product change. There were 12 paired responses from people with Haemophilia B and 6 with haemophilia A including 5 paired responses.

Results: Three patients switched from episodic treatment to regular prophylaxis (2-HB, 1-HA).

Post-switch, there were reductions in PWH reporting the use of mobility aids (67% to 53%), acute pain (78% to 47%), frequency of use of pain medications, activities of daily living (ADL) being affected, reduced range of motion (83% v 65%) and chronic pain in a target joint (patient defined) (67% to 13%).

There was a significant reduction in reported bleeds. Pre-switch 29% reported having greater than 8 bleeds/year and 41% having 1 or less bleed/year. Post-switch there we no responses greater than 8 bleeds/year and 59% having 1 or less bleed/year.

Overall, VAS (68 v 73), EQ-5D (0.71 v 0.77) and PROBE (0.72 v 0.78) score improved post-switch, which equates to ~5-6% increase in utility in a year. All dimensions of the EQ-5D showed improvement with the most significant changes seen in mobility, self-care and pain/discomfort.

Conclusions: From this initial data set, there is a demonstrated benefit to PWH switching to EHL products in reduced bleeding rates as well as in the physical and pain limitations that haemophilia imposes on daily life. Data collection and analysis are ongoing.

OC 47.1 | Using Site-directed PEGylation and the Insertion of Targeted Novel N-linked Glycans to Further Elucidate the Role Played by the A-Domains in Regulating VWF Clearance

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Background: Previous studies have demonstrated that the A1A2A3 domains of VWF are key in regulating VWF clearance *in-vivo*. Notably, the A1 domain has been shown to modulate LRP1-mediated macrophage clearance. Moreover, N-glycans within the A2 domain have also been shown to protect VWF against premature clearance. Importantly, a series of mutations associated with type 1C VWD are clustered within the A-domains. Critically, the biological mechanisms through which the A-domain modulate VWF clearance remains poorly understood. Additionally, it is unclear whether discrete regions within the A-domains may be of specific importance.

Aims: Based on the A-domain crystal structures, we investigated the role of the A-domains in regulating VWF clearance by site-directed PEGylation or introducing novel N-linked glycans (NLG) within A1A2A3-VWF.

Methods: Clearance of modifed A1A2A3-VWF variants was assessed in $VWF^{-/-}$ mice.

Results: Unsurprisingly, introduction of a 40kDa PEG moiety significantly extended the half-life of A1A2A3-VWF. However, the effects were site-specific. PEGylation at S1286 (A1 domain), V1803 and S1807 (A3 domain) inhibited *in-vivo* clearance of A1A2A3-VWF. Conversely, PEGylation at other positions even within the same domains (Q1353-A1 domain and M1545-A2 domain) had no significant effect. The role of A1A2A3 in regulating clearance was also studied by introducing novel NLG chains (3-4 kDa) at N1652 (A2 domain), N1803 and N1807 (A3 domain). Although these NLG were targeted at sites of interest identified by PEGylation studies, no significant extension in half-life was observed. To define the mechanism through which targeted PEGylation inhibited A1A2A3 clearance, binding to THP-1 macrophages and purified LRP1 receptor was examined. In keeping with their increased half-life, PEGylation at S1286, V1803 or S1807 ablated binding of A1A2A3-VWF to macrophages and LRP1 *in-vitro*.

Conclusions: These novel data support the hypothesis that the A1A2A3 domains play a critical role in regulating macrophage-mediated VWF clearance and that specific regions within the A-domains may be key in facilitating LRP1-mediated clearance.

OC 47.2 | Sec22b-Dependent Trafficking Is Essential for VWF Multimerization and Formation of Elongated WPBs

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Background: Von Willebrand factor (VWF) is a key hemostatic glycoprotein that is predominantly synthesized in endothelial cells. Upon synthesis, the pro-VWF precursor dimerizes in the ER and is subsequently transported to the Golgi where it undergoes proteolytical processing, multimerization and finally is stored in newly emerging secretory organelles, known as Weibel-Palade bodies (WPBs). Assembly of VWF into high molecular weight (HMW) multimers, packaging in elongated WPBs and regulated release to the extracellular enviroment as ultra-long VWF strings is crucial for its ability to recruit platelets to sites of vascular injury. The adhesive properties of VWF are strongly tied to the length of the secretory organelles in which it is contained, however, how endothelial cells control the dimensions of their WPBs is still unclear.

Aims: In this study, we investigated the crucial role of SNARE-mediated ER/Golgi trafficking in VWF maturation and WPB biogenesis.

Methods: We employed an shRNA screen of longin-containing SNAREs in primary endothelial cells. Protein expression was assessed by Western blot and the morphology of intracellular compartments was studied using confocal microscopy.

Results: We found that silencing of Sec22b resulted in the loss of the typically elongated morphology of WPBs. VWF was partially retained in the ER, resulting in a significantly higher ratio of proVWF:VWF. Decreased VWF influx to the Golgi affected the morphology of both WPBs, that were short and "stubby", as well as the *trans*-Golgi network (TGN). In addition, downmodulation of Sec22b expression resulted in the absence of HMW VWF multimers. Overexpression of a dominant-negative Sec22b variant lacking its SNARE domain confirmed these phenotypes, revealing that correct localization and/or function of Sec22B is important for VWF trafficking and WPB formation.

Conclusions: Our data suggest that Sec22b is a novel regulator of VWF trafficking and WPB biogenesis that modulates VWF multimerization and the elongated shape of WPBs by controlling ER/Golgi VWF influx.

OC 47.3 | A New Transgenic Mouse Model Tolerant to Human Recombinant von Willebrand Factor

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Background: Preclinical immunogenicity assessment of human recombinant von Willebrand factor (rVWF) preparations is challenging

because appropriate animal models are missing. Conventional mouse models recognize the human rVWF as a xenoantigen and mount an immune response after application of the human protein. To avoid this issue, we developed a new humanized VWF mouse model which is suitable for advanced preclinical immunogenicity assessment of preparations containing human VWF.

Aims: Generation and characterization of a new human VWF mouse model for preclinical immunogenicity testing.

Methods: Mice were generated via homologous recombination of human VWF cDNA into the murine VWF locus. The humanized VWF model was characterized for expression of human rVWF mRNA and human rVWF protein. Additionally, mice were tested for immunological tolerance against human rVWF by measuring anti-rVWF antibodies after eight immunizations on a weekly basis using 10µg and 50µg of rVWF.

Results: The mRNA analysis showed expression of human rVWF mRNA in all analyzed organs. Expression analysis of murine VWF revealed expression of some residual murine VWF mRNA. However, expression of a functional murine VWF protein could be excluded. To show expression of a functional human rVWF we performed a human VWF activity assay that is not crossreactive with murine VWF. Furthermore, analysis of the human VWF multimer pattern showed indications of the presence of human rVWF antigen in the human VWF strain but not in VWF k.o. mice and C57/BL/6 wildtype mice. Moreover, immunization with high and low doses of human rVWF revealed that human VWF knock-in mice are tolerant to human rVWF.

Conclusions: A new site-specific human VWF knock-in mouse strain that is tolerant to human rVWF was generated which can be used for advanced pre-clinical immunogenicity risk assessments of human VWF containing compounds.

OC 47.4 | Skeletal Muscle Myosin Enhances the Anticoagulant Activity of Activated Protein C and Protein S But Not Protein C Activation by Thrombin: Thrombomodulin

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Background: Skeletal muscle myosin promotes thrombus formation ex vivo in flowing human blood and supports thrombin generation in plasma and in purified mixtures containing prothrombin and factors (F) Xa and Va. Myosin binds FXa and FVa. The influence of myosin on protein C pathway reactions is unknown.

Aims: We assessed whether skeletal muscle myosin augments the anticoagulant actions of activated protein C (APC) and its cofactor, protein S (ProS), and whether myosin enhances protein C activation by thrombin.

Methods: Purified coagulation factors and protein C pathway components were used in standard activity and immunoblotting assays to monitor the effects of myosin on FVa inactivation by APC/ProS and on protein C activation by thrombin:thrombomodulin.

Results: FVa inactivation by APC was significantly enhanced by myosin compared to controls. Notably, FVa inactivation in the presence of 40 nM myosin was much greater than in the presence of 40 nM phospholipids (20%PS:80%PC) but was similar to the FVa inactivation rate in the presence of 25 µM phospholipids (Figure 1). ProS (≥25nM) enhanced myosin-stimulated inactivation of FVa by APC, especially at lower APC concentrations. Immunoblots showed that myosin enhanced Arg506 cleavage in FVa by APC alone and enhanced both Arg506 and Arg306 cleavages in the presence of ProS (Figure 2). Myosin did not enhance activation of protein C by thrombin in the presence or absence of thrombomodulin.

Conclusions: Myosin enhances protein C pathway-mediated anticoagulant actions in purified reaction mixtures. These findings complement the previous discovery that myosin enhances thrombin generation in vivo and in vitro such that myosin can initially promote thrombin generation and then subsequently provide negative feedback downregulation of thrombin generation by promoting FVa inactivation by APC:ProS. Further studies are needed to determine the molecular bases for these reactions and to assess the physiologic and pathologic relevance of these reaction mechanisms.

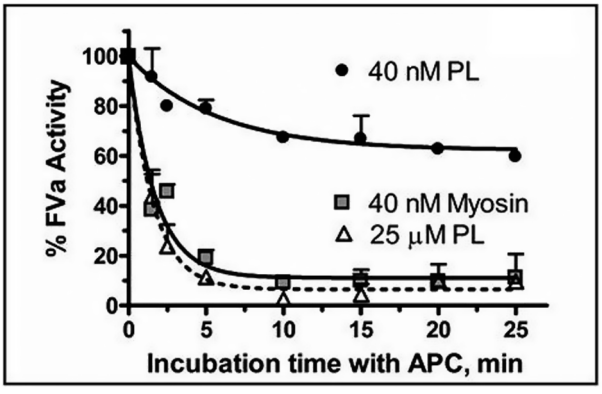

FIGURE 1

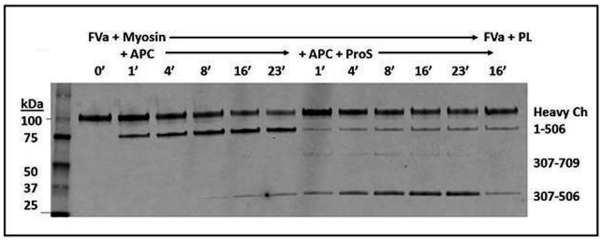

FIGURE 2

OC 47.5 | APC Receptor Expression Analysis in Myeloid Cells Reveals Agonist-inducible EPCR Expression Leading to Increased APC Binding

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Background: Activated protein C (APC) is an anticoagulant and anti-inflammatory enzyme with therapeutic activity in preclinical inflammatory disease models. APC is required to bind several cell surface receptors to exert its functions. These include endothelial protein C receptor (EPCR), thrombomodulin (TM), apolipoprotein E receptor 2 (ApoER2) and Mac-1. On macrophages, APC binds Mac-1 to induce signaling via protease-activated receptor 1, however, the potential role of other APC receptors in orchestrating APC anti-inflammatory activity on macrophages is poorly understood. Furthermore, it is not clear whether this activity is modified by macrophage phenotype or activation status.

Aims: To investigate the influence of local inflammatory signals on APC receptor expression and function on myeloid cells.

Methods: We performed a comprehensive analysis of receptor expression on human and murine myeloid cells using qPCR and flow cytometry. Macrophage-APC binding was monitored by flow cytometry and APC generation measured by protein C activation assay.

Results: All cells tested constitutively expressed TM and Mac-1. ApoER2 expression was only detectable on monocytes and EPCR expression was negligible on all cell types tested. Notably, murine bone marrow-derived macrophages stimulated with bacterial lipopolysaccharide (LPS), viral or fungal components prompted a dramatic elevation in EPCR gene and cell surface expression. Inflammatory 'M1' macrophages also exhibited elevated EPCR levels, whereas EPCR expression on pro-resolution 'M2' macrophages remained minimal. In parallel, fluorescently-labelled APC exhibited significantly enhanced interaction with LPS-treated $EPCR^{+}$ macrophages. Moreover, LPS-stimulated macrophages supported TM-dependent APC generation by thrombin, which was significantly improved by LPS-driven EPCR expression.

Conclusions: We have performed a systematic analysis of known APC receptor expression on human and murine myeloid cells. We demonstrate that the receptor repertoire available for APC signaling on macrophages is not fixed, but is instead responsive to environmental cues generated during infection. This study indicates APC signalling output on macrophages may vary based on macrophage phenotype and activation status.

OC 53.1 | Differential Production of Cytokines in Response to FVIII in Hemophilia Patients Undergoing ITI: A Preliminary Analysis of a Multicenter Longitudinal Study

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Background: Immune Tolerance Induction (ITI) via the long-term, intravenous administrations of high-dose FVIII is the only proven strategy to eradicate inhibitors and promote long-term immune tolerance towards FVIII but its mechanism of action is still poorly understood.

Aims: This study aims at understanding the process of activation and regulation of immune response to FVIII in patients undergoing ITI, and to compare the immunological events in patients who successfully eradicate anti-FVIII antibody to those occurring in patients failing to achieve FVIII tolerance.

Methods: This multicenter, observational, prospective cohort study enrolled severe hemophilia A patients who developed high-titer inhibitors, candidate to ITI. A blood sample has been collected before

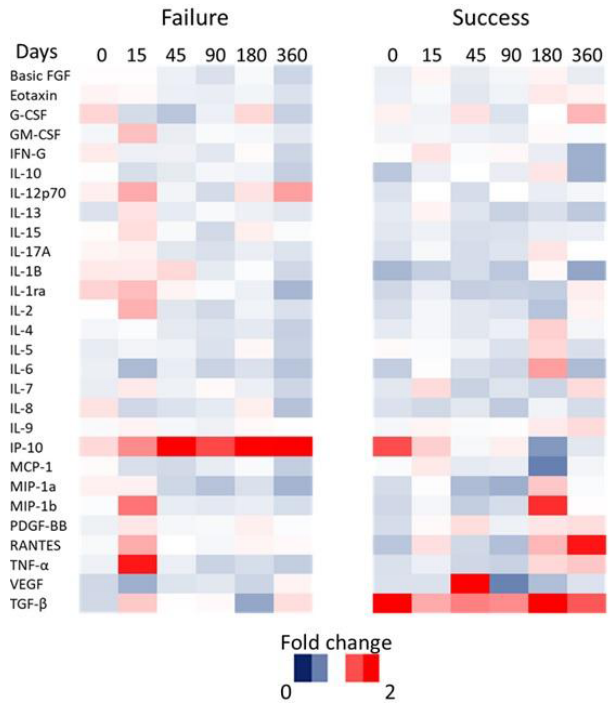

FIGURE 1 Heat map representation of cytokine responses in cell culture supernatants

starting and during the course of ITI, until ITI end. PBMCs have been used to establish cell cultures where cells were re-stimulated with the FVIII used during the ITI or cultured in medium alone as control. Cytokines were assessed in culture medium.

Results: Eighteen subjects have been enrolled. Among these, 3 patients reached the study endpoint of ITI success by study criteria while three were assessed as ITI failure. The evaluation of the cytokines in the supernatants from *in vitro* cultured cells showed a different response pattern in the two groups. We found an increased production of pro-inflammatory cytokines in response to FVIII in patients who continued to produce high-titer inhibitors, especially at the beginning of the treatment compared to those who eradicate inhibitors (Fig. 1). Interestingly, the tolerogenic cytokine TGF-b was more consistently induced in response to FVIII in successfully tolerized patients.

Conclusions: The results obtained so far in patients who have reached the study endpoint indicate a differential activation of the immune system in patients with sustained immune response to FVIII when compared to those who develop FVIII-specific tolerance.

OC 53.2 | Identification and Characterization of Murine FVIII-producing Cells *in vivo*

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Background: Coagulation factor VIII (FVIII) acts as a cofactor to generate intrinsic Xase complex in the coagulation pathway. Most coagulation factors are produced from hepatocytes; however, FVIII is secreted from endothelial cells. Nonetheless, primary tissues and cell species to produce FVIII are still controversial.

Aims: We tried to identify and characterize primary FVIII-producing organ and cell species using genetically engineered mice.

Methods: We generated *F8* knock-in mice, in which Cre recombinase induces the expression of the enhanced green fluorescent protein (EGFP) cDNA with the elimination of the *F8* gene. *F8* knock-in mice were crossed with CAG-Cre mice to detect FVIII-producing cells as EGFP-positive cells. EGFP-positive cells were detected using flow cytometry and microscopy.

Results: EGFP-positive FVIII-producing cells in adult *F8* knock-in mice were detected specifically in the liver, but not in other organs including lymph node, spleen, kidney, bone marrow, and lung. EGFP-positive cells in liver were characterized as $CD31^{high}$, $CD146^{high}$, $Lyve1^{+}$, and C-type lectin-like receptor (CLEC) 2^{+}, but they did not express the lymphatic endothelium marker podoplanin. Histological analysis revealed that EGFP-positive cells were observed in sinusoid endothelial layer expressing CD146, Lyve1, VWF, and CLEC2. In contrast, vascular endothelial cell in central vein highly expressed VWF and CD146, but not EGFP, Lyve1, and CLEC2. In embryogenesis, EGFP-positive cells in the fetal liver began to emerge at embryonic stage E14.5 and subsequently increased according with liver maturation. Finally, we confirmed that plasma FVIII could be abolished by crossing *F8* knock-in mice with Tie2-Cre and Lyve1-Cre mice, but not Alb-Cre mice.

Conclusions: These results indicate that FVIII is only produced from liver sinusoidal endothelial cells expressing CD31, CD146, Lyve1, and CLEC2. Our findings may contribute to develop a strategy to efficiently transduce FVIII-producing cells for future gene therapy and genome editing of hemophilia A.

OC 53.3 | Plasma-derived FVIII/VWF Combined with Emicizumab in Hemophilia A Plasma with and without Inhibitor Has a Non-additive Effect on *in vitro* Thrombin Generation

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Background: Emicizumab (Hemlibra®, Chugai-Roche) is a hemophilia A (HA) chronic treatment that may require concomitant acute hemostatic treatment (e.g., FVIII, rFVIIa, aPCC). Fatal thromboembolic events have been associated with the use of aPCC but not with rFVIIa and FVIII, although further studies are still needed.

Aims: To assess Thrombin Generation (TG) *in vitro* when pdFVIII/VWF was added to HA plasma with inhibitors (HAp-i) and without (HAp) containing emicizumab.

Methods: Individual HAp-i (5 BU) or pooled HAp, either alone or containing emicizumab (50-100µg/mL) was analyzed when combined with: aPCC 0.5U/ml or rFVIIa 0.5 to15µg/ml, as thrombogenic controls, or pdFVIII/VWF (Alphanate®/Fanhdi®, Grifols) 1 to 4.5IU/ml. Samples were analyzed for TG (tissue factor: PPP-Reagent Low, Stago). Results were calculated using CAT software.

Results: TG for thirteen individual healthy plasma was 47-104 nM for Thrombin Peak (TP) and 591-1419 nM.min for Endogenous Thrombin Potential (ETP). TG of both Hap/HAp-i, showed that, in presence of emicizumab 50µg/mL (Table 1), addition of aPCC 0.5U/ml had a synergic effect on TP and ETP, while rFVIIa moderately increased both parameters although within normal ranges. When pdFVIII/VWF was added to Hap/HAp-i, TG was similar to that observed without emicizumab, once achieved the normal TG levels. Similar results were observed with emicizumab 100µg/mL.

Conclusions: Our data support that pdFVIII/VWF has non-additive effects when combined with emicizumab. These results may suggest that emicizumab has a limited ability to promote FX activation in the presence of pdFVIII/VWF, thus reducing the risk of overdosing, in agreement with HAVEN 3 clinical trial.

TABLE 1 TG of HAp and HAp-i plus emicizumab 50 µg/mL (mean±SD)

		Emicizumab (µg/mL)							
		HAp without inhibitors				HAp with inhibitors (5 BU)			
		TP (nM)		ETP (nM·min)		TP (nM)		ETP (nM·min)	
Products	Dose	0	50	0	50	0	50	0	50
aPCC (U/mL)	0.5 (~25 U/Kg)	102 ± 6	436 ± 53	1435 ± 79	3220 ± 363	49 ± 5	303 ± 32	1008 ± 98	2491 ± 312
rFVIIa (µg/mL)	0.5 (~50 µg/Kg)	59 ± 2	79 ± 1	863 ± 68	1184 ± 13	25 ± 1	63 ± 0	655 ± 4	1452 ± 179
	7 (~700µg/Kg)	83 ± 6	169 ± 12	1118 ± 38	1507 ± 80	38 ± 4	109 ± 10	911 ± 87	1492 ± 124
pdFVIII/VWF (IU/mL)	1 (~50 IU/Kg)	127 ± 20	116 ± 14	1356 ± 133	1354 ± 85	25 ± 4	44 ± 4	669 ± 72	1092 ± 73
	2 (~100 IU/Kg)	186 ± 27	156 ± 25	1521 ± 80	1375 ± 192	60 ± 6	69 ± 9	1171 ± 71	1324 ± 79
	4-5 (~200 IU/Kg)	275 ± 34	244 ± 30	1713 ± 103	1597 ± 78	183 ± 16	165 ± 30	1737 ± 167	1638 ± 169

OC 53.4 | *In vivo* Enrichment of Genetically Manipulated Platelets for Murine Haemophilia B Gene Therapy

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Background: Our previous studies have demonstrated that platelet-targeted FIX gene therapy can introduce sustained levels of platelet-FIX expression in Haemophilia B (HB) mice.

Aims: In this study, we aimed to enhance platelet-FIX expression in HB mice under a nonmyeloablative preconditioning.

Methods: We constructed a novel lentiviral vector (2bF9/MGMT LV), which harbors dual genes, the FIX gene driven by the αIIb promoter (2bF9) and the MGMT P140K gene under the murine stem cell virus promoter. Platelet-FIX expression was introduced in HB mice after 2bF9/MGMT LV-mediated hematopoietic stem cell transduction and transplantation. Recipients were administered with O^6-benzylguanine/1,3-bis-2-chloroethyl-1-nitrosourea for 3 times. Animals were analyzed by PCR, quantitative PCR, FIX assays, inhibitor assays and tail bleeding test.

Results: 2bF9/MGMT LV-transduced cells were effectively enriched after each round of drug treatment. There was an approximately 2.9-fold higher FIX antigen and a 3.7-fold FIX activity in the platelets, respectively, post-treatment compared to pre-treatment. More than 95% of whole blood FIX was stored in the platelets in the treated animals. All recipients stopped bleeding within 6 hours with a remaining hemoglobin level of 65.1±4.9%, which were significantly different from those of the HB control mice. Notably, none of the recipients developed anti-FIX inhibitory antibodies as measured by modified Bethesda assay. Only one out of four recipients developed a low titer of inhibitors when challenged with recombinant human FIX in the presence of Incomplete Freund's adjuvant. In contrast, all of the HB controls developed inhibitors after the same challenge. ELISA results showed that anti-FIX IgG were barely detectable in the recipients. However, the antibody levels in the HB control mice under the same conditioning were 875-fold higher than those developed in the recipients.

Conclusions: Our data indicated that using MGMT-mediated drug-selection system in 2bF9 gene therapy can significantly enhance therapeutic platelet-FIX expression, resulting in sustained phenotypic correction and immune suppression in HB mice.

OC 53.5 | The Route of FVIII Endocytosis by Antigen-presenting Cells Determines the Pattern of FVIII Peptide-specific T Cells Activation

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Background: Neutralizing antibodies against factor VIII (FVIII) remain the major challenge in hemophilia A care. Why some patients develop neutralizing antibodies whereas others do not is not well understood. Previously, we presented data suggesting that von Willebrand Factor (VWF) may alter the sorting of FVIII into endolysosomal compartments of antigen-presenting cells (APCs, Lubich 2017), thereby modifying patterns of FVIII peptides presented on MHC-class II. Sorvillo et al. (2016) published data indicating that VWF modulates the internalization and presentation of FVIII-derived peptides on MHC-class II.

Aims: Based on these data, we asked if the uptake of FVIII by different receptors expressed on APC modulates the pattern of the subsequent activation of FVIII peptide-specific T cells.

Methods: FVIII-specific CD4+ T cell hybridoma libraries were generated using humanized HLA-DRB1*1501 (human MHC-class II) hemophilic mice (Steinitz 2012). APC were derived from the same mice. Several receptors (e.g. mannose receptor, asialoglycoprotein receptor, low-density lipoprotein receptor-related protein-1, heparan sulfate proteoglycans) previously associated with FVIII clearance were blocked individually prior to incubating APC with FVIII and subsequent co-incubation of APC with the CD4+ T cell hybridoma libraries. Stimulation of peptide-specific $CD4^+$ T-cells was assessed by IL-2 release into cell culture supernatants.

Results: Blocking mannose receptors or asialoglycoprotein receptors on APC did not influence the pattern of FVIII peptide-specific T cell activation. Blocking low-density lipoprotein receptor-related protein-1 or heparan sulfate proteoglycans on APC, however, resulted in a distinct change in the pattern of FVIII peptide-specific T cell activation. The activation of some peptide-specific T-cell hybridomas was significantly reduced whereas the activation of other peptide-specific T cell hybridomas was increased. This differential effect was specific for peptides derived from individual FVIII domains.

Conclusions: Our study suggests that the route of FVIII endocytosis in APC determines the pattern of FVIII peptide-specific T cell activation.

OC 65.3 | Investigation of Recanalization and Cerebral Perfusion with Adjunctive Thrombolytic Therapies in the iCAT Mouse Model of Ischemic Stroke

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Background: Thrombolytic therapy with recombinant tissue plasminogen activator (rtPA) remains the primary pharmacological therapy for ischemic stroke. Despite improvements in stroke outcomes, the extent of arterial recanalization attained with intravenous thrombolysis remains suboptimal in many patients. Moreover, recanalization with rtPA does not always achieve successful cerebral reperfusion, demonstrating the need for adjunctive therapies to enhance both large vessel recanalization and cerebral microvascular perfusion.

Aims: To investigate the effects of adjunctive therapies on rtPA-mediated large artery recanalization, sustained vessel patency and cerebral perfusion in a mouse model of stroke.

Methods: The *in situ* carotid artery thrombo(ly)sis (iCAT) model employs electrolytic injury to the common carotid artery to form a platelet and fibrin-rich occlusive thrombus. We have coupled this with transient stenosis (60mins) of the contralateral carotid artery to achieve controlled hypoperfusion and ipsilateral cerebral infarction.

Results: In the iCAT stroke model, rtPA therapy (10mg/kg/30mins bolus/infusion) induced transient carotid artery recanalization. Combining rtPA with the anticoagulant Argatroban (80ug/kg bolus;40ug/kg/min 24-hour infusion) enhanced recanalization (41.9% vs 22.2% rtPA alone) and significantly improved cerebral perfusion (laser speckle contrast imaging), leading to a moderate reduction in infarct volumes (TTC). Combination therapy resulted in a marked impairment of hemostasis (>75% incidence of surgical bleeding and haemorrhagic transformation in the brain), yet surprisingly, was associated with a high incidence (80%) of thrombotic reocclusion of recanalized carotid arteries. Histological analysis confirmed that reocclusion was due to the formation of platelet-rich thrombi. Consistent with this, rtPA combined with anti-platelet agent integrilin achieved (24-hour) carotid artery recanalization in up to 70% of treated mice.

Conclusions: The iCAT model is a useful preclinical model to examine the effects of adjunctive thrombolytic agents on large artery recanalization, microvascular cerebral perfusion and stroke outcomes. Our studies with Argatroban or integrilin indicate that platelet inhibition is more effective than thrombin inhibition at sustaining carotid artery recanalization induced by rtPA.

OC 66.1 | Identification of Amino Acids that are Crucial for FXIII-A Subunit Interactions and Stability

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Background: Coagulation factor XIII (FXIII) is the main stabilizer of the fibrin clot. It circulates in plasma as a tetramer of two A- and two B-subunits. Little is known about which amino acids are crucial for FXIII-A_2 dimer formation and stability of the FXIII molecule. Therefore, we designed a systematic approach to identify crucial amino acids for the expression and stability of FXIII-A_2.

Aims: To identify amino acids located at the interface between the two A-subunit monomers that are crucial for the expression and stability of the FXIII-A_2 dimer.

Methods: We identified amino acids that are located at the interface between the two A-subunit monomers within 8.5 Å from the other subunit and that are involved in the formation of intersubunit salt bridges and H-bonds. Mutations of these amino acids were designed to eliminate the intersubunit interactions without significantly changing the size of these amino acids using PDB Viewer. The mutated FXIII-A variants were stably expressed in CHO cells, and studied using Western Blot and ELISA.

Results: Twelve amino acids are associated with intersubunit salt bridges and 21 amino acids are associated with H-bond formation between two A-subunits. Ten of them are involved in both salt bridges and H bond formation, and form five particularly strong interactions between two A-subunits (Fig.1). Mutations of Lys257,

Lys113, Asp343, Glu401 and Asp404 completely abolished FXIII-A expression in CHO cells. Mutations of Glu111, Arg100 and Asn112 have no significant effect on FXIII-A expression (Fig. 2). This suggests that four intersubunit interactions (Arg11-Asp343, Lys113-Asp367, Lys257-Glu401 and Arg260-Asp404) are essential for the stability of FXIII-A_2.

Conclusions: We have identified eight amino acids involved in both H-bond and salt bridge formation between the two A-subunits of FXIII that are crucial for FXIII-A expression, FXIII-A_2 dimer formation and stabilization.

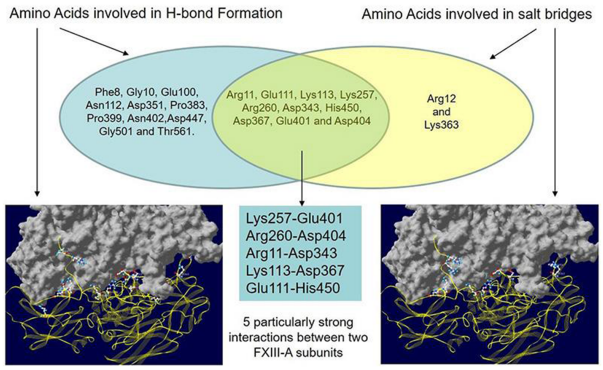

FIGURE 1

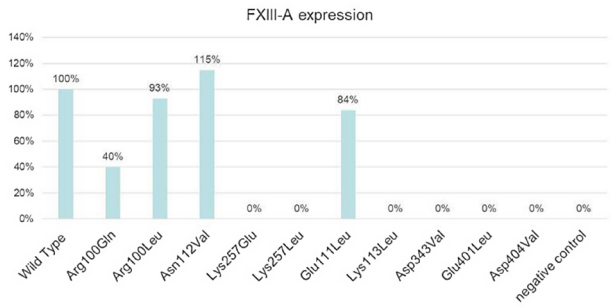

FIGURE 2

OC 66.2 | Potential Multifactorial Mechanisms for Hormone-induced Venous Thromboembolism

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Background: Hormone-induced venous thromboembolism (HI-VTE) affects thousands of women worldwide, causing significant morbidity and mortality. Estrogens pose the major thrombotic risk, while the impact of progestins is less clear. Although sex hormones alter expression of coagulation factors, the pathways that connect hormones to thrombosis are unknown. Mice do not develop HI-VTE and an animal model has been lacking. External development, the ability to generate thousands of offspring at low cost, and optical transparency all make zebrafish a powerful tool to study coagulation.

Aims: Dissect the mechanisms underlying HI-VTE.

Methods: 5-6 day old zebrafish were treated with therapeutics for 4 hours, evaluated for fluorescent thrombi in our transgenic line (which generates GFP-tagged fibrinogen that is incorporated into fibrin-rich thrombi) using standard and confocal microscopy, and scored by a blinded observer.

Results: Estradiol-treated zebrafish developed venous thrombosis within 4 hours. Pre-incubation with warfarin, rivaroxaban, and dabigatran inhibited estrogen-induced VTE by 50-80%. Fibrin deposition was reduced by 35% in a factor X-knockout background which has severely defective hemostasis. To evaluate the role of progestins, fish were treated with levonorgestrel, drospirenone, or norethindrone, alone and in combination with estradiol. Progestins alone did not cause thrombosis, but increased estrogen-induced VTE by ~20%. Estrogen-induced VTE was decreased by fulvestrant, an estrogen receptor (ESR) antagonist. Treatment of *ESR1*, *ESR2a*, and *ESR2b* knockout fish, demonstrated reduced, unchanged, and increased estrogen-induced VTE respectively.

Conclusions: Our data are consistent with human HI-VTE, although the rapidity of thrombosis development was surprising. We found that progestins do not cause thrombosis, but exacerbate this phenomenon, consistent with human data. The inability to completely inhibit HI-VTE through genetic/pharmacologic anticoagulation or estrogen disruption suggests that the mechanisms mediating HI-VTE are multifactorial. The zebrafish model is uniquely suited to dissect such complex pathways. Results of further studies could lead to novel therapeutic targets and ascertain patients at higher risk for thrombosis.

OC 66.3 | DNAse-dependent but NETs-independent Pathway of Thrombus Formation in vivo

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Background: The contribution of NETs in thrombus formation has been intensively documented in both arterial and venous thrombosis in mice. These observations were, however, mainly based on the inhibition of thrombus formation following the treatment of mice by DNAse-1 or using deficient mice, in which neutrophil activation could be affected. We previously demonstrated that neutrophils play a key role in the initiation of the TF-dependent activation of the coagulation cascade leading to thrombus formation following a laser-induced injury. In this model, thrombus formation is independent of GPVI. Thrombin, ADP and ATP constitute the main platelet and neutrophil agonists.

Aims: The goal of our study was to determine the contribution of NETs in thrombus formation following a laser-induced injury.

Methods: The contribution of NETs in vivo was determined by treatment of mice with DNAse-1 using intravital microscopy. Thrombi were analyzed by transmission electron microscopy.

Results: Treatment of mice with 100U of DNAse-1 significantly inhibited thrombus formation induced by a dye laser. In vivo, DNAse-1 reduced thrombus formation in the seconds following the injury. In vitro, formation of NETs occurred in 3h following activation of neutrophils by TNF-alpha or LPS. Electron microscopy of the thrombus formed revealed that neutrophils present at the site of the laser-induced injury did not formed NETs. To understand this discrepancy, we determined if DNAse-1, that cleaves phosphodiester linkages, could hydrolyze ATP and ADP in vitro. As suspected, addition of 25U of DNAse-1 was sufficient enough to cleave ATP and ADP, decreasing the concentration of ATP by 25% in 5 minutes. Aggregation of platelets by ADP and activation of neutrophils by ATP were also significantly reduced when DNAse-1 was added.

Conclusions: Following a laser-induced injury, neutrophils but not NETs are involved in thrombus formation. Treatment by DNAse-1 induces the hydrolysis of ATP and ADP, leading to the inhibition of thrombus formation in vivo.

OC 66.4 | Identification of a Possible Allosteric Disulfide in the αIIb Subunit of Platelet αIIbβ3 Integrin

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Background: Platelet aggregation is mediated by cross-linking of platelet αIIbβ3 integrin receptors by circulating fibrinogen. Numerous studies indicate that αIIbβ3 integrin function is controlled by vascular thiol isomerases. Five different vascular thiol isomerases are produced by activated platelets and endothelial cells and are essential for normal thrombosis in mice. We recently elucidated a function of one of the vascular thiol isomerases, ERp5. The Cys177-Cys184 disulfide bond in the β3 subunit of αIIbβ3 integrin is cleaved by ERp5, which triggers release of fibrinogen from the activated integrin. We now report a functional role for an αIIb subunit disulfide.

Aims: To identify allosteric disulfide(s) in the αIIb subunit of platelet αIIbβ3 integrin.

Methods: The redox state of 7 of the 8 disulfide bonds in the αIIb subunit of purified human platelet αIIbβ3 was quantified by differential cysteine alkylation and mass spectrometry. To mimic the reduced disulfide, cysteines of interest were mutated to alanine and the integrin expressed on the surface of BHK cells. Activation of wild-type and disulfide mutant αIIbβ3 and binding to fibrinogen was measured.

Results: Two of the 7 disulfide bonds in the thigh domain of the αIIb subunit of the platelet integrin were 20-25% reduced in the purified protein. Analysis of αIIb disulfide bond mutants revealed a function for the Cys490-Cys545 disulfide in activation of the integrin and binding to fibrinogen. Expression of αIIb C490A or C545A mutant integrins was comparable to wild-type but the disulfide mutant integrin was insensitive to activation by Mn^{2+} and binding to immobilized or soluble fibrinogen was markedly impaired.

Conclusions: We postulate that the redox state of the Cys490-Cys545 disulfide bond in the thigh domain of αIIb has a role in the conformational change associated with αIIbβ3 integrin activation. We are currently characterizing possible control of this disulfide by the vascular thiol isomerases.

OC 66.5 | Platelet but Not Plasma Protein S Limitsthrombus Growthin Large Veinsbut Not in Large Arteriesby Controlling Coagulation *in situ*

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Background: Protein S (PS) is a coagulation inhibitor acting as cofactor for activated protein C (APC) and tissue factor pathway inhibitor (TFPI). Although the majority of PS circulates in plasma, 2.5% is stored in platelets (pltPS) and released upon activation. Biochemically comparable to plasmatic PS, pltPS role and impact has never been assessed *in vivo*.

Aims: Biochemically comparable to plasmatic PS, pltPS role and impact has never been assessed *in vivo*.

Methods: Conditional PS knockout mice in the megakaryocyte lineage were generated using the Pf4-Cre transgene ($Pros1^{lox/lox}$Pf4-Cre^{+}).

Results: Antigenic assays confirmed the absence of PS in $Pros1^{lox/lox}$Pf4-Cre^{+}mice, whereas PS plasma concentration was comparable in $Pros1^{lox/lox}$Pf4-$Cre^{+}$and $Pros1^{lox/lox}$Pf4-Cre- mice. As expected, *ex vivo*TF-initiated thrombin generation demonstrated an increased thrombin potential in platelet-rich plasma in line with the loss of APC- and TFPI-dependent activities in $Pros1^{lox/lox}$Pf4-Cre^{+}mice. Nevertheless, platelet function tests were comparable in both genotypes. $Pros1^{lox/lox}$Pf4-Cre^{+}mice did not display disseminated intravascular coagulation. They showed reduced blood loss (P=0.01). In the tissue factor (TF)-induced venous thromboembolism model, their survival rate was reduced by 50% (P=0.047). They developped occlusive thrombosis in mesenteric arterioles ($FeCl_3$-model) twice faster and their thrombi were more extended than in $Pros1^{lox/lox}$Pf4-Cre^{-}mice. More diffuse factor Xa and thrombin formation was detected by a fluorogenic substrate in thrombi from $Pros1^{lox/lox}$Pf4-Cre^{+}than from $Pros1^{lox/lox}$Pf4-Cre^{-}mice. Consistently, $Pros1^{lox/lox}$Pf4-Cre^{+}thrombi showed an intense fibrin staining all over the vessel while fibrin staining remained localized to the injury site in $Pros1^{lox/lox}$Pf4-Cre^{-}mice. In the vena cava $FeCl_3$-injury model, thrombus volume was more than three

times larger in Pros1$^{lox/lox}$Pf4-Cre^{+}than in Pros1$^{lox/lox}$Pf4-Cre^{-}mice (P=0.0007). Fibrin and PS colocalized in both genotypes. However, PS staining was strong in Pros1$^{lox/lox}$Pf4-Cre^{-}thrombi and faint in Pros1$^{lox/lox}$Pf4-Cre^{+}thrombi. In contrast, there was no difference between the two genotypes in the carotid artery $FeCl_3$-injury model.

Conclusions: pltPS, by controlling coagulation in situ, limits thrombus growth in large veins but not in large arteries.

OC 67.1 | Knockout of von Willebrand Factor in Zebrafish by CRISPR/Cas9 Mutagenesis

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Background: von Willebrand factor (VWF) is a multimeric glycoprotein that plays critical role in hemostasis. Each subunit contains domains arranged as D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-CK. The D'-D3 interacts with and stabilizes coagulation FVIII. The A1 and C1 bind to GPIb-V-IX and GPIIbIIIa respectively. A1 and A3 bind to collagen. vwf is conserved in zebrafish and expressed in endothelium and thrombocytes . Currently zebrafish vwf knockout model is not available. Generating such model should complement other VWD animal models.

Aims: This work aims to generate the zebrafish VWD model.

Methods: We used CRISPR/Cas9 mutagenesis to knockout zebrafish *vwf* gene. PCR and sequencing were used to identify indels. Various assays such as laser thrombosis, kPTT, mechanical injury assay, and ristocetin mediated agglutination assay were used to characterize the zebrafish.

Results: To generate zebrafish VWD model we targeted exon29 encoding A3 domain. We injected Cas9 RNA and gRNA into one-cell stage embryos and raised them to adulthood and analyzed tail DNAs for indels. We found a founder fish showed transmission of an insertion. We raised heterozygotes and obtained homozygous larvae that gave using laser thrombosis an arterial time to occlusion of >120 seconds suggesting an autosomal recessive type 3 VWD. Sequencing of the homozygotes showed 55 bp insertion in exon29 resulting in a frameshift generating a termination codon. mRNA sequencing also confirmed this result. Subsequently, wild-types, heterozygotes, and homozygotes and six fish of each category were used in the following assays kPTT assay, mechanical injury assay, ristocetin-mediated agglutination assay. All the assays showed that the homozygotes showed VWD phenotype of prolonged kPTT, greater bleeding and prolonged time to agglutination. The results were analyzed by ANOVA which showed p< .001 in all the above experiments.

Conclusions: In conclusion, we generated vwf knockout zebrafish that may be useful to study vwf´s role in angiogenesis and may uncover unknown pathologies in the VWD.

OC 67.2 | The Crystal Structure of Monomeric D'D3 Reveals Principles of VWF Concatemer Formation and its Interaction with FVIII

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Background: Von Willebrand factor (VWF) is a large, cysteine-rich glycoprotein that multimerizes to form long concatemers which promote arteriolar hemostasis. These concatemers are formed via a unique biosynthetic pathway. First, interchain disulfides form between C-terminal cysteine knot domains in the alkaline endoplasmic reticulum (ER) followed by interchain disulfides between N-terminal D'D3 assemblies in the acidic Golgi. Mechanistic details of this process are unknown. Furthermore, D'D3 constitutes the FVIII-binding site on VWF. VWF increases FVIII half-life in the bloodstream and restricts interaction of FVIII with phospholipids until thrombin activation triggers its release. High-resolution structural information of D'D3 and its interaction with FVIII are lacking.

Aims: To determine the three-dimensional structure of monomeric D'D3 and address questions relating to D'D3 dimerization and interaction with FVIII.

Methods: D1D2D'D3 (C1099A/C1142A) was expressed as a thrombin-cleavable Fc-fusion to facilitate purification. D1D2 was removed via intracellular processing of a native furin site, while Fc was removed during purification. The resulting D'D3 monomer was crystallized at an ER-like pH and its structure solved using anomalous dispersion.

Results: In the D'D3 structure refined to 2.5 Å, D' projects away from the wedge-shaped D3 assembly. All 25 disulfide bonds within D'D3 are unambiguously assigned and an unexpected, conserved Ca^{2+}-binding site was identified in D3. While residues implicated in D'D3 dimerization lie near the solvent accessible surface, they are buried in hydrophobic pockets.

Conclusions: The monomeric D'D3 structure suggests C1099 and C1142 are protected from dimerization in the ER and pH-dependent structural rearrangements are required for dimerization in the Golgi. We propose that, after secretion into blood, D'D3 at concatemer ends also adopts this structure to prevent spurious disulfide formation with other proteins. Orientation of D' relative to D3 supports direct interaction between VWF and the a3 region of FVIII while mapping VWD Type 2N mutations onto D'D3 provides additional insights into the VWF-FVIII interaction.

OC 67.3 | von Willebrand Factor Induces Human Vascular Smooth Muscle Cells Proliferatio through Low Density Lipoprotein-related Receptor Protein 4 and αvβ3 Integrin

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Background: Von Willebrand factor (VWF) has been reported to induce proliferation of vascular smooth muscle cells (VSMCs). However, the underlying mechanisms and the pathophysiological relevance remain unknown.

Aims: We addressed how VWF modulates VSMCs proliferation and identified the molecular determinants involved.

Methods: In vitro cell proliferation was assessed by incubating human or murine aortic VSMCs with VWF or recombinant VWF-fragments in the presence or absence of various treatments (siRNA, antibodies, peptides...). In vivo, a femoral artery wire injury (FWI) was applied to VWF+/+ and -/- mice and intimal hyperplasia (IH) was measured 4 weeks after injury.

Results: We showed that 1 nM VWF induced VSMC proliferation equivalent to 0.2 nM PDGF treatment. Concentration-dependent VWF-induced proliferation was accompanied by VSMC dedifferentiation. Treatment of cells with a siRNA against $\alpha_v\beta_3$ integrin or a RGT-peptide blocking $\alpha_v\beta_3$ signaling abolished proliferation. However, VWF did not bind to $\alpha_v\beta_3$ on VSMCs through its RGD site. Rather, we identified the VWF A2 domain as the region mediating binding to the cells. We hypothesized the involvement of a member of the low density lipoprotein-related receptor protein (LRP) family due to their known ability to act as co-receptors. Using the universal LRP-inhibitor RAP (receptor-associated protein), we confirmed LRP-mediated VSMC proliferation. SiRNA experiments and duolink staining identified LRP4 as the VWF-counterreceptor on VSMCs and showed co-localization between $\alpha_v\beta_3$and LRP4. In vivo, the number of VWF-/- mice developing IH following FWI was reduced compared to VWF+/+ mice (30% vs 71%, p=0.038).

Conclusions: In conclusion, VWF is able to mediate VSMC proliferation through a mechanism involving A2 domain binding to the LRP4 receptor and integrin $\alpha_v\beta_3$ signaling. The decreased IH following FWI suggests that the well-known negative effects of endothelial cells on VSMC proliferation involve the VWF/LRP4/$\alpha_v\beta_3$circuit. Altogether, targeting VWF-LRP4 interactions using specific inhibitors may have clinical utility in preventing unresolved vascular remodeling.

OC 67.4 | Mapping the Interaction with Vwf and Fxii and Its Potential as a New Anti-coagulant Target

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Background: Both Von Willebrand Factor (VWF) and Factor XII (FXII) have well established roles in haemostasis and coagulation. While VWF is a mediator of platelet capture to sites of vascular injury under high shear stress, FXII initiates the intrinsic coagulation pathway. Interestingly, studies have shown that the absence or inhibition of FXII does not impair haemostatic function but has a thrombo-protective effect.

Aims: In this study, we investigated a novel binding interaction between VWF and FXII.

Methods: Substrate based activation assays were used in order to test activation of FXII by VWF. Specific binding interactions between VWF and FXII were demonstrated using static plate binding assays and surface plasmon resonance (SPR). Full-length VWF and isolated VWF A1 domain mutants were generated by site-directed mutagenesis and physiological relevance of the VWF/FXII interaction was examined using plate based thrombolysis assays.

Results: Results of the activation assays showed that VWF is a potent, previously unrecognised activator of FXII. Binding analysis demonstrated that inactive FXII and activated FXII (FXIIa) bound to plasma derived VWF ($K_{D(app)}$ = 4.1nM for FXIIa and 26.2nM for FXII) but not to VWF lacking the A1 domain suggesting that the FXII binding site lies within the VWF A1 domain. Further binding studies with isolated VWF A1 domain and full-length VWF mutants allowed for the identification of amino acids E1452 and aspartic D1459 on the α-6 helix of the A1 domain as the FXII binding site in VWF ($K_{D(app)}$ = 564nM to FXIIa in SPR). Thrombus lysis time was significantly reduced when either VWF, FXIIa or activity of both proteins was blocked and interference with FXII/VWF binding had a prohibitive effect on thrombus formation.

Conclusions: Based on these results, we hypothesise that inhibition of the interaction between FXII and VWF might be a novel and safe anticoagulant target without increasing the risk of bleeding side effects.

OC 67.5 | VWFA1 Interacts with Scavenger Receptor LRP1 via Lysine 1408

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Background: VWF is cleared by macrophage lectins & scavenger receptors. Fragment A1A2A3 is cleared at the same rate as full length

VWF. Shear or misfolding mutations in VWF are required for LRP1 binding. Proteins bind to LRP1 via Lysine residues; heparin binds via Lysine & Arginine. A1 binds to LRP1 but not A2 nor A3. N-linked glycans in VWF A2 domain modulate clearance by LRP1.

Aims: Understand how shear or misfolding increase binding of VWF to LRP1. Understand how N glycans in A2 affect interactions of A1 to LRP1.

Methods: HEK293T-produced VWFA1A2A3 fragments are infused in C57BL6 $vwf^{-/-}$ mice. ELISA binding of VWF fragments to LRP1-ClusterIV was performed.

Results: Unfractionated & fractionated heparins dose-dependently inhibit A1 binding to LRP1-ClusterIV. A1 with Lys 1408 mutated to Glu or Ala showed reduced binding (5.5-fold). K1408E mutation introduced in fragment A1A2A3 changed fast clearance of A1A2A3-N1515Q back to normal & decreased fast clearance of A1A2A3-N1574Q beyond normal in mice. K1408E mutation did not affect clearance in the absence of glycan mutations. Binding of A1A2A3 fragment to LRP1-clusterIV coated plate is maximal for double mutant N1515Q-N1574Q (100%); in comparison single glycan mutants N1515Q & N1574Q showed respectively 33.8 % & 31.2% binding. VWF type2B V1316M mutation has been described to be cleared via LRP1. Introducing K1408E mutation in A1A2A3-V1316M did not correct fast clearance. At maximal heparins inhibition, A1-V1316M still binds 1.9-fold more than A1 wt to LRP1-ClusterIV.

Conclusions: Lys1408 in A1 is a critical binding site for LRP1. In absence of mutation, N-glycans in A2 prevent interaction of Lys1408 to LRP1. In absence of glycan N1515 or N1574, A1A2A3 is removed quickly from plasma via LRP1 unless Lys1408 is mutated. Lys1408 does not have a role in clearance of VWD Type2B V1316M. Heparin inhibitions suggest mutation V1316M exposes other Lys for LRP1 binding.

OC 70.4 | Spontaneous Bleeding and Poor Bleeding Response with Extended Half-life Factor IX Products: A Survey of Select US Hemophilia Treatment Centers

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Background: Factor IX (FIX) has significant distribution to the extravascular space and binding to type IV collagen which is important in hemostasis, but not quantifiable in clinical practice for patients receiving FIX replacement. Among the pivotal trials of extended half-life factor IX products (EHL-FIX), FIX plasma trough activity were widely ranging with a narrow range of bleeding rates suggesting trough value alone may not predict bleeding.

Aims: To characterize the use and performance of EHL-FIX in clinical practice at four U.S. hemophilia treatment centers (HTCs).

Methods: An electronic survey regarding center specific use of EHL-FIX amongst patients with severe hemophilia B (SHB) was sent in fall 2018. Providers were asked if patients utilizing EHL-FIX had experienced 1) spontaneous/minimally traumatic bleeding events at factor levels >10% or 2) poorly controlled bleeding events requiring more frequent/higher doses of EHL-FIX than anticipated in addition to patterns of EHL-FIX product switching.

Results: Surveyed HTCs cared for 71 patients with SHB including 55 (77%) who utilized EHL-FIX, 24 (34%) recombinant factor IX (FIX) albumin fusion protein (rFIX-FP) and 31 (44%) recombinant factor IX Fc fusion protein. No patients received glycopegylated recombinant FIX. Unexpected spontaneous/minimally traumatic bleeding and poorly controlled bleeding were reported in 13 patients, all using rFIX-FP prophylaxis (Table 1). Six patients have discontinued use of EHL-FIX based on shared decision making with providers (Table 2).

Conclusions: Although plasma FIX activity levels have driven prophylaxis and bleed management decisions, clinical experience suggests novel properties of EHL-FIX may impact hemostasis. Successful bleed prevention or control in SHB may be predicted by the distribution of FIX in circulation and extravascular space, and the presence of FIX in tissues at time of injury. These data demonstrate the importance of real-world monitoring of efficacy of new FIX products and suggest the need for more robust mechanisms to understand the hemostatic performance of products.

TABLE 1 Center-specific data regarding use of EHL-FIX products amongst patients with SHB

	Center 1	Center 2	Center 3	Center 4
Total patients with severe hemophilia B	19	18	24	10
Patients who have received EHLs				
rFIX-FP	8	4	7	5
rFIXFc	7	4	18	2
vrFIX-GP	0	0	0	0
Patients with unexpected bleeding or poorly controlled bleeding events				
rFIX-FP	4	3	4	2
rFIXFc	0	0	0	0
rFIX-GP	n/a	n/a	n/a	n/a

TABLE 2 Patterns of product switching amongst patients receiving EHL-FIX

	Center 1	Center 2	Center 3	Center 4
rFIX-FP to different EHL	2	0	3	0
rFIX-FP to a SHL	2	3	0	1
rFIXFc to a different EHL	0	1	0	0
rFIXFc to a SHL	0	0	0	0
rFIX-GP to a different EHL	n/a	n/a	n/a	n/a
rFIX-GP to a SHL	n/a	n/a	n/a	n/a

OC 71.1 | Isolation of Silica-free DNA and Studies of its Effects on Organ Damage in Mice

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Background: Neutrophil extracellular traps (NETs) are an important interface between innate immunity and coagulation. The major structural components of NETs are nucleosomes (DNA-histone complexes). Although nucleosomes do not modulate coagulation, there are conditions where DNA and histones dissociate from each other in circulation (e.g. in the presence of heparan sulfate or therapeutic heparin). Previous studies have shown that DNA (when not in complex with histones) activates coagulation and impairs fibrinolysis. However, these effects have been questioned due to silica contamination when DNA is isolated using common silica-based kits.

Aims:

(1) To identify and characterize alternative methods of isolating silica-free DNA and

(2) to determine the impact of elevated cell-free DNA (CFDNA) concentrations in circulation in mice.

Methods: DNA was isolated from whole blood using the QIAGEN PAXgene Blood DNA kit (PAX-DNA), and QuickGene DNA kit (DBL-DNA). The procoagulant properties of DNA were assessed by thrombin generation and plasma clotting assays. C57Bl/6 mice (8-14 weeks) were intravenously administered 200uL of PAX-DNA (200ug) or saline. Blood and organs were collected at 0, 15, 30, and 60 minutes post-injection. CFDNA was quantified from plasma at each time-point. Lungs, livers, and kidneys were stained with H&E and blindly scored for thrombus formation.

Results: PAX-DNA and DBL-DNA yielded silica-free DNA that increased thrombin generation and accelerated clotting in a dose-dependent manner, which was neutralized by DNaseI. The circulating half-life of PAX-DNA based on CFDNA quantitation in mice was ~10min. Lung and liver harvested post-DNA injection possessed thrombi/emboli, as evidenced by the presence of fibrin and platelet aggregation.

Conclusions: We identified and characterized two methods of isolating silica-free DNA. These studies are the first to investigate the effects of silica-free DNA *in vivo*. These studies may shed light into the functional role of elevated CFDNA in the pathogenesis of thrombosis in vascular diseases.

OC 71.2 | Coagulation Activation Induced by Platelet-Derived Microvesicles is Mediated by Kallikrein-Dependent Activation of Factor IX

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Background: Platelet transfusion may be associated with thrombosis, inflammation and increased mortality. The mechanisms underlying these adverse outcomes are unclear.

Aims: To elucidate how microvesicles(MVs) isolated from platelet concentrates(PLTC-MVs) activate coagulation.

Methods: PLTC-MVs were isolated from discarded platelet apheresis units by double centrifugation(2500g;15 minutes). PLTC-MVs were visualized, sized and counted using electron microscopy and nanoparticle tracking analysis. Phosphatidylserine exposure was also measured. To assess their ability to initiate thrombin generation(TG), PLTC-MVs were re-suspended in re-calcified MV-free normal pooled plasma(MVfNPP), or in MV-free plasmas deficient in factor XII(MVfDef-XII), XI(MVfDef-XI), IX(MVfDef-IX) or VII(MVfDef-VII). Antibodies against tissue factor(antiTF-Ab), corn trypsin inhibitor(CTI) or soybean trypsin inhibitor(STI) were used to block TF, FXIIa or kallikrein, respectively. FXII, prekallikrein (+high molecular weight kininogen(HK)) or FXI(+HK) activation was detected in buffer using fluorogenic substrate sensitive to FXIIa, FXIa or kallikrein. FIX activation in buffer was assessed using an ELISA that detects FIXa-antithrombin complexes(FIXa-AT).

Results: The mean concentration of PLTC-MVs was 9.66 x 10^{12} particles/ml; mean diameter was 73.7nm with 90% of particles ≤100nm(Fig1B); mean phosphatidylserine activity was 428 nM(Fig1C). PLTC-MVs triggered TG in MVfNPP despite the presence of inhibitory antiTF-Abs, and in MVfDef-VII(Fig1D). No TF activity was detected on PLTC-MVs(Fig1E). PLTC-MV-induced TG was unchanged in MVfDef-XII and MVfDef-XI, but was abolished in MVfDefIX(Fig1F). PLTC-MVs directly activated FXII(Fig1G) and PK(Fig1H), but not FXI(Fig1I) in buffer. PLTC-MVs activated FIX in buffer in a PK(+HK)-dependent manner(Fig2A). Purified kallikrein similarly activated FIX in buffer(Fig2B) and in plasma(Fig2C). In contrast to kaolin(Fig2D-F), PLTC-MV-induced(Fig2G-I) TG was insensitive to CTI, FXII or FXI deficiency, but was abolished by STI.

Conclusions: PLTC-MVs activate the contact system leading to TG via a noncanonical kallikrein-FIX pathway. These results should be confirmed by using molecules that block kallikrein more specifically than STI. Our findings support that PLTC-MVs may support hemostasis, but also promote thrombotic and inflammatory reactions to platelet transfusion.

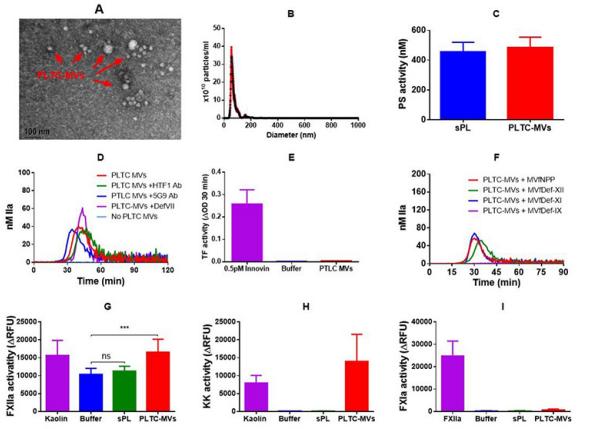

Figure 1: Small size phosphatidylserine-positive PLTC-MVs trigger thrombin generation in plasma via contact activation. (A) Negative stain electron microscopy of PLTC-MVs. (B) enumeration of PLTC-MVs according to size as measured by nanoparticle tracking analysis. (C) Phosphatidylserine (PS) activity on synthetic lipid vesicles (sPL; 4µM; 44%/41%/15% PC/PE/PS)or PLTC-MVs. (D) TG in MV-free factor VII deficient (DefVII) or normal pool plasma in the presence of inhibitory anti-TF (HTF1 Ab) or anti-TF-VIIa complex (5G9 Ab).(E) TF activity on Innovin™ or PLTC-MVs. (F) TG induced by PLTC-MVs in MV-free plasma deficient in FXII, FXI or FIX. (G) Activation of factor XII (G), prekallikrein (+HK) (H) or factor XI (+HK) (I) by sPL or PLTC-MVs. RFU: relative fluorescence unit. MVfNPP (Def-): microvesicle-free normal pool (or deficient) plasma.

FIGURE 1

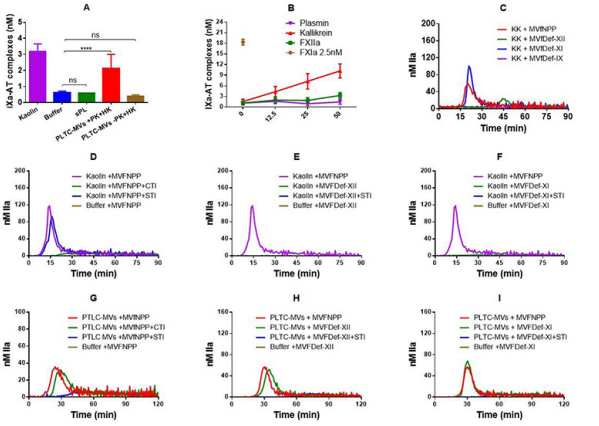

Figure 2: PLTC-MVS trigger thrombin generation (TG) in a kallikrein-dependent manner. (A): Activation of human factor IX(FIX) by kaolin, synthetic lipid vesicles (sPL; 4µM; 44%/41%/15% PE/PC/PS) or PLTC-MVs in the presence of PK and HK in buffer. (B): Activation of human FIX by purified human kallikrein, FXIIa, plasmin or FXIa in buffer. (C) TG triggered in plasma by 50nM KK in MV-free normal pool and factor deficient plasmas. Effect of CTI or STI on TG triggered by kaolin in normal pool (D), DefXII (E) and DefXI (F) plasmas. Effect of CTI or STI on TG triggered by PLTC-MVs in normal pool (G), DefXII (H) and DefXI (I) plasmas. PLTC-MV: microvesicles isolated from platelet concentrates; MVfNPP: microvesicle-free normal pool plasma. MVfDef-XII(-XI)(-IX): microvesicle-free plasma FXII, (-XI), (-IX). PK: prekallikrein; KK: kallikrein; HK: high molecular weight kininogen; CTI: corn trypsin inhibitor; STI: soybean trypsin inhibitor

FIGURE 2

OC 71.3 | Endothelial Plasminogen Activator Inhibitor-1 Blocks the Intrinsic Pathway of Coagulation, Inducing the Clearance and Degradation of Activated Factor XI

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Background: The activation of coagulation factor (F) XI by activated coagulation FXII (FXIIa) is a prothrombotic process. The endothelium is known to play an antithrombotic role by limiting thrombin generation and platelet activation. It is unknown whether the antithrombotic role of the endothelium includes sequestration of FXIa activity.

Aims: Determine the regulation of the intrinsic pathway of coagulation by endothelial cells.

Methods: To measure FXIa activity in the presence of human umbilical vein endothelial cells (ECs) we used a FXIa chromogenic assay. To detect and identify FXIa-ECs inhibitor complex we used western blot and mass spectrometry (MS) analysis. Immunofluorescence was used to measure FXIa binding to ECs and internalization. FXIa- plasminogen activator inhibitor-1 (PAI-1) complexes in baboons challenged with heat-inactivated *Staphylococcus aureus* (S. aureus) were detected by ELISA.

Results: ECs selectively block FXIa activity, while permitting kallikrein and FXIIa activity. FXIa formed a covalent bond with a potential inhibitor present on ECs. MS analysis revealed that FXIa forms a complex with PAI-1. Incubation of ECs with a blocking anti-PAI-1 antibody increased the cleavage of a chromogenic substrate by FXIa and the capacity of FXIa to promote fibrin formation in recalcified plasma. We observed that after incubation of FXIa with ECs, FXIa-PAI-1 complexes were detected 1) in the supernatant and 2) in ECs localized with early and late endosomes and lysosomes. When baboons were challenged with S.aureus, a significant increase in FXIa-PAI-1 complexes was detected in the circulation within the first 2-8 hours post-challenge.

Conclusions: PAI-1 forms a complex with FXIa on ECs blocking its procoagulant activity and inducing the clearance and degradation of FXIa. FXIa-PAI-1 complexes were detected in the circulation in a baboon model of S.aureus sepsis. While ECs support kallikrein and FXIIa activity, inhibition of FXIa by the endothelium may promote the clearance of thrombogenic FXIa from the circulation.

OC 71.4 | Factor XII-W268R, a Human Mutation Causing Autoinflammation, Accelerates Activation through Exposure of the Activation Loop

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Background: Coagulation factor XII (FXII) drives production of the inflammatory peptide bradykinin. This causes acute tissue swelling in hereditary angioedema (HAE) that can result from mutations in FXII. We here describe a new mutation in FXII (W268R). Instead of HAE, this mutation causes cold-inducible urticarial-rash, headache, arthralgia, and fatigue (FACAS; FXII-associated cold autoinflammatory syndrome).

Aims: To unravel the disease mechanism of FXII-W268R.

Methods: We expressed wild type FXII (FXII-WT), mutants FXII-T309R (causes HAE), FXII-W268R, and other FXII variants in HEK293 freestyle cells. We analyzed cell culture supernatants, cell lysates, patient plasmas and purified proteins for FXII activation by western blotting, chromogenic assays and ELISA.

Results: All 4 FXII-W268R carriers were symptomatic. Their plasma contains high levels of FXIIa-C1-inhibitor (C1-INH) complex, plasma kallikrein-C1-INH complex and cleaved kininogen, unlike their healthy relatives. This suggests constitutive bradykinin production.

Recombinant FXII-W268R (but not FXII-WT or FXII-T309R) forms cleavage products that are present intracellularly, as well as in cell culture medium (Fig.1A). These fragments display enzymatic activity (Fig.1B).

Active-site incapacitated mutant FXII-W268R-S544A demonstrates that fragmentation is largely dependent on FXII auto-activation. In addition, intracellular inhibition of proprotein convertases significantly attenuates FXII-W268R activity, suggesting that these enzymes are involved in the intracellular processing of FXII-W268R. Purified FXII-W268R is more sensitive to activation by both plasma kallikrein (Fig.1C) and plasmin compared to FXII-WT or FXII-T309R. Furthermore, binding studies demonstrate that FXII-W268R exposes a plasminogen binding site that is cryptic in FXII-WT. Our findings suggest that the FXII-W268R mutation alters protein conformation, exposing its activation loop, which is normally concealed in FXII-WT (Fig.1D).

Conclusions: FXII-W268R accelerates activation both in the intra- and extracellular milieu. Continuous low-grade secretion of active FXII helps to explain the systemic autoinflammatory phenotype of FXII-W268R carriers.

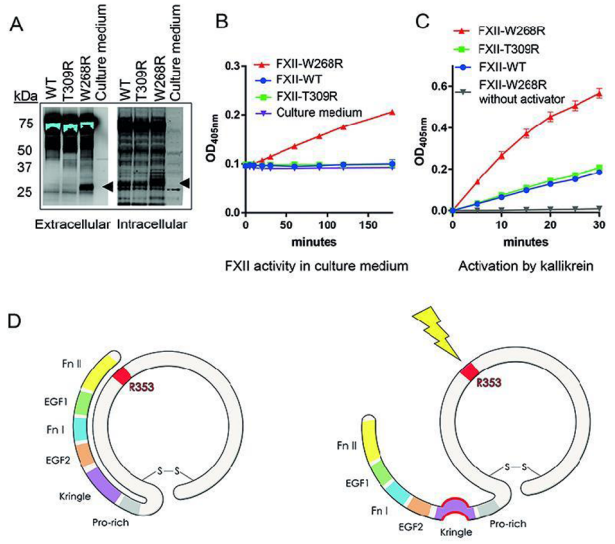

FIGURE 1 Mutation W268R accelerates FXII activation. A) Western Blot B,C) Activity assays D) Overview

OC 71.5 | High-molecular-Weight Kininogen Is a Critical Component of Host Defense against Gram-negative Bacteria: Evidence from a Humanized HK Mouse Model

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Background: Patients with gram-negative sepsis had an increase in activation of plasma contact activation system (CAS) and cleaved fragment of high-molecular-weight kininogen (HK). Injection of lipopolysaccharide (LPS) to healthy human subjects activated plasma CAS and induced the cleavage of HK. However, the physiological and pathophysiological relevance of these observations remains unknown.

Aims: To determine the role of plasma CAS and HK in host response to gram-negative bacterial infection.

Methods: Phenotypes of the mice lacking Factor XII, prekallikrein, and HK were compared in cecal ligation puncture (CLP) and peritoneal injection of *E.coli* models. Humanized HK ($hKng1^{+/+}$/$mKng1^{-/-}$) mice, whose Kng1 gene was replaced with human gene using CRISPR-Cas9 strategy, were used to evaluate the role of human HK.

Results: In both CLP and peritoneal injection of *E.coli* models, compared with wild-type mice, survival rate of $Kng1^{-/-}$ mice lacking HK was significantly decreased, but that of other CAS factors-deficient mice remained comparable, suggesting the selective role of HK in mice. To determine the role of human HK, $hKng1^{+/+}$/$mKng1^{-/-}$mice received peritoneal injection of *E.coli*. Compared with $Kng1^{-/-}$mice, $hKng1^{+/+}$/$mKng1^{-/-}$ mice showed a significant increase in survival rate, accompanied with lower plasma bacterial load and proinflammatory cytokines, and less lung and liver injury. I^{125}- labeled human HK was intravenously injected to $Kng1^{-/-}$ mice bearing *E.coli* infection, it was rapidly recruited into the site of infection, shown by autoradiography. Human HK bound to *E.coli* dose-dependently, which was significantly blocked by LPS. The binding site of human HK to LPS was mapped to DHG15 a.a. in the domain 5. The DHG15 peptide had anti-bacterial effect in vitro and in vivo. In patients with gram-negative sepsis, the cleaved form of HK was increased in plasma, in consistence with an increase in circulating LPS levels.

Conclusions: Human HK is critical in host defense against gram-negative bacterial infection as a patter-recognition molecule and anti-bacterial protein.

OC 76.1 | Unexpected Enhancement of FVIII Immunogenicity by Endothelial Expression in Lentivirus-transduced and Transgenic Mice

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Background: Endothelial cells (ECs) provide the physiologic site for FVIII production, However, the potential for therapeutic expression of FVIII from ECs remains uncertainty and the effect on development of inhibitory antibodies (inhibitors) remains unknown.

Aims: To explore the potential role of endothelial cells for *in situ* engineered FVIII expression and the impact on FVIII immunogenicity.

Methods: FVIII expression was introduced by lentivirus-mediated *in situ* transduction or embryonic stem cell-mediated transgenesis. FVIII activity and inhibitor titers were determined by standard techniques. A T cell proliferation assay was used to examine the antigen presenting cell (APC) functions of ECs. Confocal microscopy was used to examine FVIII uptake by ECs.

Results: All HA mice unexpectedly developed inhibitors after *in situ* T2F8 lentivirus-mediated EC transduction. Inhibitors, but not FVIII activity, were detected in plasma. In contrast lentivirus-mediated bone marrow transduction, producing FVIII in platelets led to tolerization. T2F8 transgenic ($T2F8^{Tg}$) mice, in which FVIII expression is driven by the EC-specific Tie 2 promoter normalized plasma FVIII levels but contributed to an unexpectedly robust immune response. A single FVIII - incomplete Freund's adjuvant injection led high titers of inhibitors, reducing plasma-FVIII to undetectable levels. Titers were significantly higher than in wild-type C57BL/6 controls and in platelet-targeted FVIII transgenic mice. Because EC's are putative non-hematopoietic, semi-professional-APCs we asked whether EC's might directly influence the FVIII immune response. T cell proliferation assays confirmed that microvascular ECs that were conditioned with inflammatory cytokines could take up exogenous FVIII and stimulate T follicular helper (Tfh) cells to FVIII-specific proliferation. Imaging studies confirmed that these ECs indeed bind and take up FVIII protein.

Conclusions: Our results demonstrate unanticipated antigenicity of endothelial-expressed FVIII and a possible pathway for immune enhancement of FVIII immunogenicity through EC function as auxiliary APCs for Tfh cells.

OC 76.2 | Suppression of FVIII-specific Memory B Cells by Regulatory T Cells Expressing BARs

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Background: Anti-FVIII neutralizing antibody (inhibitor) formation poses significant obstacles for optimal treatment of hemophilia A. We have recently shown that human regulatory T cells (Tregs) expressing a FVIII-domain as a chimeric B-cell antibody receptor (BAR) prevented anti-FVIII antibody formation in hemophilic mice by directly suppressing B cells (Zhang AH, J Immunol. 201:1434-1441, 2018).

Aims: In this study, we sought to determine whether FVIII-specific memory B cells could be suppressed by the FVIII BAR Tregs.

Methods: To test this *in vitro*, we used plasmablast-depleted splenocytes from FVIII immunized hemophilia mice to serve as the source of FVIII-specific memory B cells.

Results: Co-culture of $CD138^{-}$ splenocytes with FVIII antigen in the presence of FVIII A2-domain BAR human Tregs completely blocked the development of FVIII-specific antibody forming cells (ASC). In addition, FVIII A2-BAR human Tregs efficiently suppressed FVIII-specific ASC formation in the presence of anti-FVIII antibodies *in vitro*. Similar results were obtained when BAR-expressing murine Tregs were used.

Conclusions: In conclusion, we demonstrated *in vitro* that FVIII BAR Tregs suppress FVIII-specific memory B cells activity. The efficacy of FVIII BAR Tregs on FVIII-specific memory B cells will be further tested *in vivo* by adoptive transfer of FVIII-specific memory B cells in syngeneic hosts. Supported by a Bayer Hemophilia Grant [AHZ] and NIH HL126727 [DWS].

OC 76.3 | Characterization of Factor VIII Binding Sites on the Low-density Lipoprotein Receptor-related Protein 1 (LRP) Suggests a Dynamic Bivalent Mode of this Interaction

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Background: Current replacement therapy of Hemophilia A is limited by a relatively short plasma half-life of Factor VIII (FVIII) or its modified variants (12-19 h). Previous studies demonstrated the involvement of hepatic low-density lipoprotein receptor-related protein 1 (LRP) in FVIII clearance. Such studies also characterized FVIII-binding regions on LRP (*Meijer et al, 2007*) and proposed a bivalent mode for this interaction (*Young et al, 2016*). In the present study, we further investigated these mechanisms.

Aims: To map the LRP site(s) for binding FVIII using several *in vitro* approaches.

Methods: Recombinant LRP fragments were produced in a baculovirus expression system and purified by chromatography. The fragments were tested for interactions with FVIII by surface plasmon resonance (SPR) and cell-based flow cytometry assay for LRP-mediated uptake of FVIII by human hepatic Huh-7 cells. Correctness of the overall folding of selected LRP fragments was verified by circular dichroism spectroscopy.

Results: Several LRP fragments comprising specific complement-type repeats (CRs) were found to bind FVIII with affinity similar to that of LRP (K_D = 50-100 nM) by SPR. The same fragments consistently inhibited LRP-mediated internalization of FVIII in the tissue culture model. Specificity of these interactions was confirmed by silencing LRP expression and using, as competitors,

(i)an anti-FVIII antibody fragment,
(ii)receptor-associated protein (RAP) and
(iii)mutated variants of the LRP fragments.

Conclusions: We identified specific CR-doublets of LRP forming primary sites for interaction with a specific region on FVIII. These results support the bivalent nature of interaction between FVIII and LRP, and indicate that the bivalent sites of the receptor interact with FVIII alternatively in a dynamic mode. These data further contribute to understanding FVIII clearance mechanisms and may be useful for generation of new FVIII variants with extended half-life in the circulation.

OC 76.4 | Variants from Patients with Hemophilia Impair Proper Carboxylation of Factor IX

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Background: γ-carboxylation of the GLA domain of Factor IX (FIX) is required for its activation and participation in the coagulation cascade. Previous work has shown that A37T, which lies in the carboxylation recognition motif, leads to warfarin sensitivity. Furthermore, many variants within the GLA domain have been identified in patients with hemophilia B, but the mechanism of dysfunction remains unknown. The presumed processive nature of the carboxylation process leads us to hypothesize that variants within and around the GLA domain may directly interfere with γ-carboxylation and thus lead to disease.

Aims: To determine whether and how variants from patients with hemophilia B impact carboxylation.

Methods: A cell-based reporter consisting of a Tet-inducible promoter, prothrombin signaling peptide, the FIX GLA domain, and a transmembrane domain was genomically integrated into HEK293T cells using Bxb1 recombinase. After integration, cells expressing variant or wildtype FIX GLA domain at their cell surface were incubated with 50nM vitamin K +/- 100 μM warfarin, stained with a APC-conjugated FIX carboxylation-specific antibody, and then analyzed using flow cytometry in triplicate. For variant comparison, the median of each fluorescence measurement was taken and scaled such that warfarin-treated cells were zero and wildtype cells were one.

Results: Cells expressing GLA domain variants from patients with hemophilia B showed complete or near complete loss of carboxylation. Variant E79A, isolated from a patient with moderate hemophilia, showed the highest γ-carboxylation of any variant, approximately 25% of wildtype. Another variant from a patient with severe hemophilia at the same site, E79K was indistinguishable from warfarin-treated FIX.

Conclusions: We employed a reporter assay for FIX variants to test their effect on the degree of carboxylation, and by proxy, pro-coagulant activity. Variants from patients with hemophilia B show a quantitative loss in the degree of carboxylation that appears to correlate with disease severity, indicating a potential loss of function mechanism.

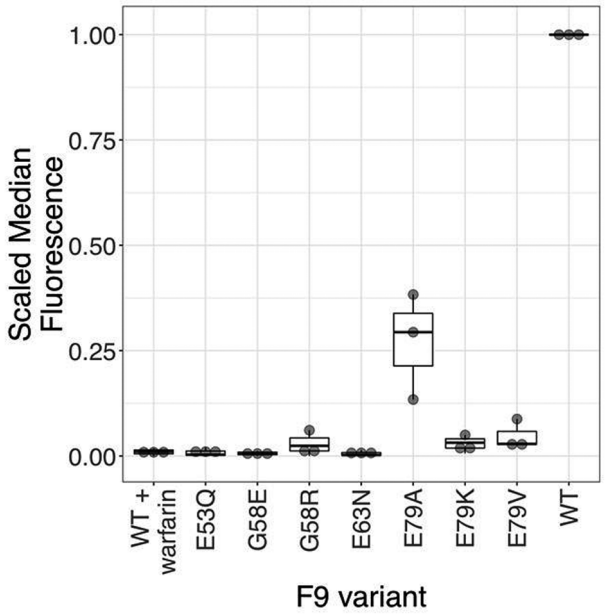

FIGURE 1 Scaled median fluorescence of Factor IX variants

OC 76.5 | Factor VIII (FVIII)-Haplotype Mismatch Increases Risk of Inhibitor Development in the Treatment of Hemophilia A (HA)

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Background: The FVIII haplotype mismatch hypothesis proposes that HA patients administered therapeutic FVIII proteins (tFVIIIs)—which currently represent the two most common haplotypes designated H1 and H2—mismatched against the FVIII encoded by their mutant FVIII gene (*F8*) have increased risk for developing neutralizing anti-FVIII-antibodies termed "inhibitors". Rigorous evaluation of this hypothesis requires accounting for *F8*-causal-mutation and heritable genetic effects on inhibitor risk while modeling a mismatch effect.

Aims: We advocate a variance components model for inhibitor status (Inh) that accounts for *F8*-causal-mutations and heritable genetic variation as random effects while modeling FVIII haplotype mismatch as a regression variable.

Methods: For our study, 442 North American HA patients (237 Whites & 205 Blacks; 88% severely affected) were:

1) Immuno-Chip genotyped at ~167,000 single-nucleotide polymorphisms (SNPs) in genes implicated in autoimmune disease risk;
2) Evaluated by Sanger DNA sequencing and assays for the recurrent intron (I)1- and I22-inversions to identify their *F8*-causal-mutations; and
3) Tested with the Nijmegen-modified Bethesda assay to determine their Inh status.

Results: The Immuno-Chip genotypes were used to construct a genetic-relationship matrix, and the *F8* sequence data along with results from the I1- and I22-inversion assays were used to construct a shared *F8*-mutation matrix. These matrices were used to estimate the heritable genetic and shared *F8*-mutation effects. Importantly, modeling a *F8*-mutation effect has the added advantage of accounting for the mutational heterogeneity in *F8*-mutations. We found that heritability and *F8*-mutation effects respectively accounted for 50% and 23% of the phenotypic variance in Inh (both $p < 0.0001$).

Conclusions: While accounting for age and race effects, we found a significant effect for the FVIII haplotype mismatch variable ($p=0.003$) such that inhibitor prevalence increased from 29% in the overall sample to 46% in those receiving mismatched tFVIIIs (Figure 1). These results provide further support for a personalized approach to FVIII replacement therapy in patients with HA.

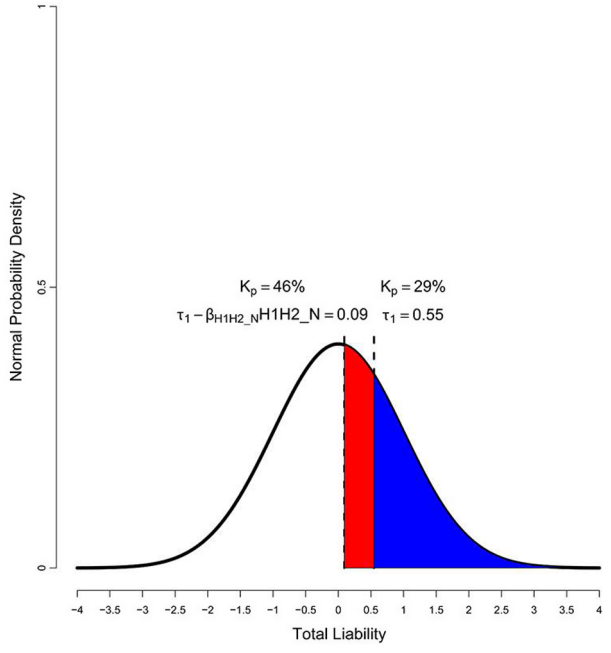

FIGURE 1 FVIII-haplotype-mismatch effect on risk of inhibitor development (Inh)

DIAGNOSTICS AND OMICS

OC 07.1 | Genotypical Classification of Patients with von Willebrand Disease

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Background: Von Willebrand disease (vWD) is a bleeding disorder caused by inherited defects in the concentration, structure or function of Von Willebrand Factor (vWF). The classification of vWD remains difficult, and is not always conclusive. The various vWD types warrant specific treatment and thus require improved diagnostic measurements.

Aims: To improve diagnostic classification by direct sequencing of *vWF*.

Methods: Targeted gene analysis was performed on 703 patients suspected to have vWD, either by classical Sanger sequencing or by ion semiconductor next generation sequencing technology (IonTorrent PGM™). All coding regions including intron-exon boundaries were sequenced. Large deletions and duplications in the *VWF* gene were detected by MLPA. As our center served as a nation wide service center for genotypic classification of VWD, samples were sent from all nation-wide Hemophilia Treatment Centers and abroad.

Results: Of 703 patients 438 patients had a *VWF* genetic abnormality. The mutations found were 14 nonsense, 405 missense and 20 splice-site mutations, 29 small deletions, 8 duplications and 24 large deletions detected by MLPA. Of all mutations detected 431 were reported previously and 69 were novel (3 nonsense, 38 missense and 9 splice-site mutations, 4 duplications, 14 small deletions and 1 large deletion detected by MLPA). 113 patients were carrying the D1472H polymorphism affecting the von Willebrand Factor Ristocetin cofactor activity.
Conclusions: Our systematic targeted genotypic approach resulted in confirmation[SS1] of 62% of the cases in vWD. Furthermore, we were able to classify 88%. Since the overall yield to detect (likely) pathogenic variants was only 62% and bleeding scores do not always match the type of mutation, we implemented whole exome sequencing (WES). WES has the ability to detect combinations of *VWF* variants with other autosomal recessive abnormalities in genes involved in hemostatic disorders. A prospective evaluation of WES is currently under investigation.

OC 07.2 | Quantitation of One-stage and Two-stage Discrepancies in Monitoring of Enhanced Half-life Factor IX Products

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Background: With the introduction of enhanced half-life (EHL) factor IX (FIX) products, the monitoring of hemophilia therapy has become cumbersome. All approved coagulation products rely on potency labelling based on two-stage chromogenic assays, while most laboratories employ one-stage assays. Due to differences in activation, intrinsic cascade in one-stage assays and FIX directly in chromogenic assays, discrepancies between both approaches have been observed.
Aims: In this study, those discrepancies are quantified for three EHL and one recombinant FIX product in direct comparison. Confounding variables are minimized by using identical assay setups between all FIX products as well as identical reagents and analyzer systems.
Methods: The concentration-dependent recovery of FIX in deficient plasma spiked with three EHL FIX products (albutrepenonacog alfa, eftrenonacog alfa, nonacog beta pegol) and one recombinant FIX (nonacog alfa) were determined using one-stage clotting and two-stage chromogenic assays. Actin FS, Actin FSL and Pathromtin SL (Siemens Healthineers), aPTT SP, SynthaFax and SynthaSil (Werfen), Cephascreen (Stago), Cephen 5 (Coachrom), Dapttin TC (Haemochrom) were used in the one-stage assays (ACLTOP 500, Werfen), while Rox FIX (Rossix) and Biophen FIX (Hyphen Biomed) were applied for two-stage assays (BCS, Siemens Healthineers). Results were calibrated against plasma derived FIX (Berinin P). Assay discrepancies were calculated using regression analysis plotting the one-stage assay result over the two-stage assay result.
Results: Depending on reagent and FIX product, large discrepancies between one- and two-stage assays can be observed ranging from about one-fifth up to twenty-fold recovery (see table).
Conclusions: This is the largest study so far comparing the discrepancies between one- and two-stage assays in monitoring EHL FIX products using almost all currently used test reagents. Since sample handling was identical for all products and test runs, confounding variables on the assay results are minimized. The results underscore the need for test standardization in order to better streamline treatment.

OC 07.3 | Genotypic Abnormalities in Phenotypic Confirmed Patients with a Rare Bleeding Disorder

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Background: Patients with a Rare Bleeding Disorder (RBD) have a heterogenous clinical phenotype in which it is difficult to predict bleeding, in contast to the bleeding diathesis in hemophilia and vWD

TABLE 1 Discrepancies between FIX concentrations in 1-stage assays and a 2-stage assay (Rox FIX); linear regression analysis, 95% CI

	albutrepenonacog alfa	eftrenonacog alfa	nonacog alfa	nonacog beta pegol
Actin FS	0.22 (0,16 - 0,30)	1,21 (1,02-1,44)	1,12 (0,98-1,28)	0,27 (0,16-0,44)
Actin FSL	0.33 (0.29-0.38)	0.97 (0.81-1.15)	1.21 (1.07-1.38)	0.39 (0.32-0.47)
Cephascreen	0.85 (0.77-0.95)	0.68 (0.54-0.86)	1.11 (0.97-1.28)	0.46 (0.38-0.56)
SynthaFax	0.98 (0.89-1.08)	1.34 (1.12-1.60)	0.82 (0.73-0.93)	1.06 (0.90-1.25)
aPTT SP	0.48 (0.44-0.53)	0.78 (0.69-0.87)	1.20 (1.07-1.35)	12.0 (6.83-21.0)
Cephen 5	0.30 (0.27-0.33)	0.65 (0.56-0.75)	1.13 (1.00-1.27)	0.21 (0.18-0.25)
Pathromtin SL	0.60 (0.56-0.64)	0.91 (0.79-1.04)	1.16 (1.08-1.24)	19.6 (12.1-32.0)
SynthasIL	0.44 (0.42-0.46)	1.20 (1.05-1.36)	1.57 (1.41-1.74)	0.47 (0.40-0.56)
Dapttin TC	0.30 (0.22-0.39)	1.88 (1.47-2.41)	1.68 (1.40-2.02)	0.38 (0.26-0.55)

patients. In most cases the disease is diagnosed by laboratory phenotypic approaches in combination with clinical symptoms.

Aims: To confirm the laboratory-based phenotypic diagnosis by a targeted gene approach.

Methods: 289 patients with a laboratory-phenotypic confirmed RBD were evaluated by Sanger sequencing or by ion semiconductor next generation sequencing technology (IonTorrent PGM™). All coding regions including intron-exon boundaries were sequenced. Two different classes of hemostatic genes were analysed: procoagulant genes and platelet specific genes.

Results: The overall yield of our targeted single gene approach to detect pathogenic mutations was 31%. For cases with abnormalities in primary hemostasis the yield was only 16%. Only 5 out of 58 patients had a (likely) pathogenic mutations and 4 patients a variant of unknown significance (VUS). The success rate in secondary haemostatic abnormalities was higher (36%). Of the 231 patients tested for abnormalities in the procoagulant genes, 71 patients were carriers of a (likely) pathogenic mutation, and 14 carrier of a VUS.

Conclusions: The patients' bleeding phenotype, including bleeding assessment score and laboratory analysis, is an important driver to start targeted single gene approach to detect genotypic abnormalities in RBD patients. The overall yield in our center, as nation-wide reference center for RBD genotyping, is only 31%. To increase the yield, we recently implemented whole exome sequencing (WES) as a diagnostic genotyping test. WES allows analysis of all genes in a single test making it time and cost efficient. Our first approach is to target only proven haemostatic related genes using a filter with 145 genes involved in primary and secondary hemostasis and fibrinolysis. If the diagnostic panel reveals a negative result, exome wide analysis will be performed.

OC 07.4 | Analytical Performance Evaluation of a Dedicated Calibrator and Controls for Emicizumab Quantification

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Background: A modified one-stage FVIII assay for the quantification of emicizumab plasma concentration was developed using an on-board 1:8 plasma sample pre-dilution followed by the standard one-stage FVIII test.

Aims: To evaluate the performance of a dedicated calibrator and controls (r^2 Diagnostics) for emicizumab quantification carried out on the BCS® XP analyzer (Siemens).

Methods: *Reagents:* Actin FSL/FVIII deficient plasma (Siemens). *Samples:* Emicizumab spiked, commercially sourced, frozen, citrated plasma from hemophilia A patients. *Precision:* Within-lab assay precision was assessed in a 20-day study, 2 runs per day, 3 replicates per run using 7 spiked samples. Between-lab assay precision was assessed in a 5-day study, 2 runs per day, 3 replicates per run. Within-lab and between-lab calibrator (100µg/mL) and controls (L1=25µg/mL; L2=75µg/mL) precisions were also assessed. Analysis was performed via nested ANOVA. *Analytical Sensitivity*: LoB/D/Q were determined using spiked and non-spiked samples. *Analytical Specificity:* Samples were spiked with 4 levels of potential endogenous and exogenous interferents and emicizumab recovery compared with interferent-free samples.

Results: *Precision*: Assay within- and between-lab precision results are presented in Tables 1 & 2. Calibrator, L1 and L2 total %CVs within-lab: 6.7%, 6.0% and 6.9%; between-lab: 9.5%, 6.9% and 6.1%. *Analytical Sensitivity*: The LoB/D/Q were 1.8µg/mL, 3.2µg/mL and 10µg/mL respectively. *Analytical Specificity:* The assay was insensitive to the following interferents up to the specified levels: hemoglobin (500mg/dL); bilirubin (20mg/dL); triglycerides (2000mg/dL); FVIII inhibitor (108BU/mL); lupus anticoagulant (73.2Δsec STAClot LA); heparin (1.0U/mL). The assay was not sensitive to NovoSeven (5µg/mL) but sensitive to levels of FEIBA, Advate and Obizur within each drug's dosage guidelines.

Conclusions: The modified one-stage FVIII assay using dedicated emicizumab calibrator and controls demonstrated excellent precision and analytical sensitivity. The assay was insensitive to endogenous interferents and NovoSeven but demonstrated sensitivity to FVIII replacement drugs and FEIBA.

TABLE 1 Assay Within-Lab Precision (3 reagent lots / 1 site)

All Lots Combined			Within Run		Between Run		Between Day		Between Calibration		Between Lot		Total Within Site	
Sample	N	Mean	SD	%CV	SD	%CV	SD	%CV	SD	%CV	SD	%CV	SD	%CV
1	360	12.2	0.4	3.4%	0.4	3.0%	0.4	3.4%	0.3	2.3%	0.6	4.9%	1.0	7.8%
2	360	28.0	1.0	3.7%	1.5	5.4%	1.3	4.6%	0.0	0.0%	1.3	4.6%	2.6	9.2%
3	360	55.5	2.0	3.6%	1.1	2.0%	2.9	5.2%	1.0	1.8%	0.0	0.0%	3.8	6.9%
4	360	83.0	3.1	3.7%	1.7	2.0%	3.5	4.2%	1.3	1.6%	1.8	2.2%	5.4	6.6%
5	360	106.7	4.3	4.0%	5.1	4.7%	3.9	3.6%	3.2	3.0%	2.5	2.3%	8.7	8.1%
6	360	149.5	5.7	3.8%	10.4	7.0%	7.5	5.0%	6.0	4.0%	0.0	0.0%	15.3	10.2%
7	360	202.3	7.7	3.8%	5.0	2.5%	6.1	3.0%	4.4	2.2%	4.5	2.2%	12.7	6.3%

TABLE 2 Assay Between-Lab Precision (1 reagent lot / 3 sites)

All Sites			Within-Run		Between-Run		Between-Day		Between-Site		Total	
Sample	N	Mean	SD	%CV	SD	%CV	SD	%CV	SD	%CV	SD	%CV
1	90	11.9	0.8	6.3%	0.1	0.8%	0.1	0.9%	0.4	3.4%	0.9	7.4%
2	90	28.3	2.0	6.9%	0.4	1.4%	0.4	1.5%	0.7	2.4%	2.2	7.7%
3	90	56.2	3.4	6.0%	0.4	0.8%	1.2	2.2%	0.6	1.0%	3.4	6.0%
4	90	83.6	4.3	5.1%	0.5	0.6%	1.1	1.3%	0.7	0.9%	4.6	5.5%
5	90	103.4	9.3	9.0%	1.2	1.1%	2.5	2.4%	3.7	3.6%	10.5	10.2%
6	90	157.4	12.5	7.9%	1.5	0.9%	2.0	1.2%	2.1	1.3%	13.0	8.2%
7	90	200.4	14.5	7.2%	1.8	0.9%	2.2	1.1%	5.3	2.7%	15.8	7.9%

OC 07.5 | Comparison between One-stage and Chromogenic Testing of FIX Extended Half-life Product Measurements in External Quality Control Assessment Programs

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Background: Patients with severe Haemophilia B are treated with extended half-life (EHL) FIX products (rFIXFP, rFIXFc and N9-GP). For the laboratory, the introduction of these EHL products presents a major challenge, since these products each have different behavior in one-stage (OSA) and chromogenic assays (CA). To elucidate

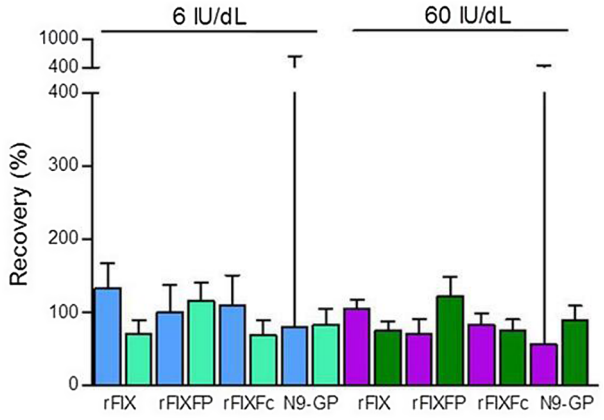

FIGURE 1 Median (SD) Recovery of FIX activity in samples spiked with EHL FIX products. Blue/purple are results with the OSA, green/dark green with CA

the differences for the different drugs, these two methods need to be directly compared by diagnostic laboratories in a real life setting.

Aims: Compare FIX activity levels measured with OSA and CA assays in a global external quality control assessment (EQA) survey.

Methods: Three EQA programs (ECAT, UKNEQAS and the RCPAQAP) collaborated and organized a global survey with FIX measurements. Factor IX deficient plasma was spiked with rFIXFP, rFIXFc and N9-GP to levels of 6 IU/dL and 60 IU/dL, based on potency label. Participants in the external quality control programs were asked to use their routine protocols.

Results: One hundred and forty-four participating laboratories reported results with OSA and 28 laboratories with CA. Recoveries in the samples spiked with 6 IU/dL for the OSA were 133%, 100%, 110% and 82% for standard half-life recombinant FIX (rFIX), rFIXFP, rFIXFc and N9-GP, respectively. For CA these were 70%, 117%, 70% and 83%, respectively. For the samples spiked with 60 IU/dL the recoveries were 105%, 71%, 85% and 87% for OSA and 75%, 122%, 75% and 89% for CA, respectively. We also observed that within a type of assay (both OSA and CA, the reported values showed a large variation (figure).

Conclusions: The data of our survey show that both for OSA and CA a wide range of recoveries were observed.

OC 10.1 | Plasma Levels of Activated Factor VII-Antithrombin Complex Predict Mortality in Subjects with Liver and Colon Cancer Undergoing Curative Surgical Intervention

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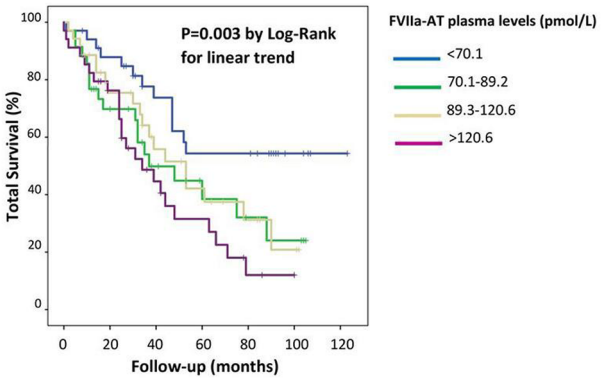

FIGURE 1 Kaplan-Meier survival curves in patients with liver and colon cancer according to quartiles of FVIIa-AT plasma levels

Background: Cancer is well known to be characterized by a hemostatic imbalance, usually shifted to a procoagulant diathesis. Tissue factor (TF) is one of the key initiators of the coagulation cascade through the binding and activation of factor VII (FVII). TF plays a role in cancer progression/diffusion and has been investigated as potential target in the treatment of malignancies. Plasma levels of activated FVII-antithrombin (FVIIa-AT) complex, a biomarker of prothrombotic diathesis reflecting FVIIa-tissue factor (TF) interaction, have been shown to predict mortality in patients with cardiovascular diseases.

Aims: To evaluate the association between FVIIa-AT and mortality in a cohort of subjects with liver and colon cancer undergoing surgical intervention with a curative intent.

Methods: One hundred and thirty-eight neoplastic patients (67.4% males, mean age 67.4±9.6 years, 37.6% hepatocellular carcinoma, 31.2% cholangiocarcinoma, and 31.2% colon cancer) were enrolled. Samples of venous blood were drawn at the time of enrollment before surgery. FVIIa-AT levels were measured by ELISA.

Results: After a 34-month median follow-up, 75 subjects (54.3%) died. Baseline FVIIa-AT plasma concentrations were higher in subjects who died than in survivors (99.6 with 95%CI 90.9-109.2 *versus* 83.4 with 95%CI 75.6-91.9 pmol/L, P=0.009). Stratifying the study population according FVIIa-AT levels, mortality rate increased progressively and significantly from the lowest to the highest quartile (38.2-->54.3-->54.3-->70.6%, P=0.003 by Log Rank for linear trend). The subjects within the highest quartile had a greater risk of mortality than those in the lowest (HR 2.95 with 95%CI 1.49-5.84 by Cox Regression). This association was confirmed after adjustment for sex, age, type of cancer, BMI, albumin, and creatinine levels (HR 3.07 with 95%CI 1.42-6.61) and even after excluding subjects who died perioperatively (HR 3.00 with 95%CI 1.47-6.11).

Conclusions: This preliminary study suggests that plasma concentration of FVIIa-AT may predict mortality in patients with liver and colon cancer undergoing surgical intervention with a curative intent.

OC 10.3 | Apoptosis Inhibition: A Promising Approach for Cold Storage of Apheresis Platelet Concentrates

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Background: Apheresis-derived platelet concentrates (APCs) is a standard therapy to treat bleeding. However, bacterial infection caused by storage at room temperature (RT) still remains a drawback. Recently, we showed that cold-stored APCs are associated with better PLT functionality but with shorter survival (Haematologica 2018, PMID: 30115655). Cold-induced apoptosis was identified as a potential mechanism of the accelerated PLT clearance.

Aims: To investigate the protective effect of apoptotic inhibitors during cold storage of APCs.

Methods: APCs were collected and stored at RT and 4°C in the presence or in the absence of caspase-3 inhibitor. The phosphatidylserine exposure and the mitochondrial membrane potential (MMP) (tetramethylrhodamine ethyl ester perchlorate [TMRE] staining) were measured using flow cytometry. The protein expression was quantified by western blot.

Results: A higher expression of the apoptotic marker phosphatidylserine was detected in cold-stored APC compared to RT (% apoptotic events mean±SEM: 13±1% vs. 5±1% p=0.018). To investigate if the apoptotic signal, observed with phosphatidylserine, specifically involved the intrinsic pathway, the MMP was analyzed as a marker of alive cells. Interestingly, after cold storage a decrease of the MMP was observed compared to RT indicating the activation of the intrinsic pathway (Mean fluorescence intensity TMRE Mean±SEM: 6.13±1.89 vs. 18.53±3.64, p=0.038). Accordingly, a decrease of the procaspase-3 level after cold storage was detected by western blot. However, when PLTs were stored in the presence of caspase-3 inhibitor a significant rescue of the cold-stored cells viability was detected (TMRE staining: % alive cells mean±SEM: 74±3% vs. 22±3%, caspase 3 inhibitor vs. ionomycin, p=0.005). This indicates that the cold-induced apoptosis can be prevented by caspase inhibitor.

Conclusions: Our results show that the reduction of cold-stored PLT viability can be prevented by a specific caspase inhibitor. Consequently, cold storage, associated with a better PLT functionality, may become an efficient strategy for APC storage in combination with apoptotic inhibitors.

OC 10.4 | The Effect of a Viscoelastic-based Bleeding Algorithm Implementation on Blood Products Use in Adult Liver Transplant Patients: A Before-after Study

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Background: Perioperative blood products transfusion is correlated with increased morbidity and mortality in patients undergoing liver transplantation.

Aims: To assess whether the use of a standardized viscoelastic test (VET)-based bleeding algorithm impacts transfusion rates of hemocomponents in adult liver transplant patients.

Methods: In this retrospective observational before-after study, adult patients undergoing liver transplantation before (2013) and after (2018) the implementation of a VET-based bleeding algorithm were compared. VET, factor concentrates and blood products were available in both periods, the only difference being the use of the VET-based bleeding algorithm in the intervention phase (2018). The primary outcome was the transfusion of blood products. The local ethics committee approved the study and waived the informed consent due to the retrospective nature of the study.

TABLE 1 Baseline characteristics of the patients and intraoperative bleeding

	Unmatched cohort (n=139)			Matched cohort (n=96)		
Parameter	**Control (n=91)**	**Intervention (n=48)**	**p value**	**Control (n=48)**	**Intervention (n=48)**	**p value**
Age (years)	54 (47;59)	51 (37; 57)	0.051	49.5 (41.25; 56.75)	51 (37; 57)	0.988
Male sex	49/91(53.84%)	30/48 (62.5%)	0.327	24/48(50%)	30/48(62.5%)	0.217
MELD score	18 (±6.72)	17.5 (±7.63)	0.703	17.9 (±7.03)	17.5 (±7.63)	0.792
Preoperative platelet count (/µl)	65000 (44000; 88000)	75500 (46000; 103500)	0.25	65500(43250; 99500)	75500 (46000; 103500)	0.387
Preoperative Hb (g/dl)	10.41 (±2.05)	10.92 (±2.33)	0.209	10.9 (±2.13)	10.9 (±2.33)	0.967
Preoperative fibrinogen level (mg/dl)	169 (122;200)	198.5 (134; 297.5)	0.005	182.5 (181; 219)	198.5 (134; 297.5)	0.248
Preoperative INR	1.5 (1.32; 1.76)	1.48 (1.26; 1.86)	0.639	1.59 (1.29; 1.9)	1.48 (1.26; 1.86)	0.373
Intraoperative Bleeding (ml)	4000 (2000;7000)	5000 (2500; 8700)	0.088	4000 (2000;7000)	5000 (2500; 8700)	0.153

TABLE 2 Intraoperative blood products use, factor concentrate use and postoperative ICU LOS

	Unmatched cohort (n=139)			Matched cohort (n=96)		
Parameter	Control (n=91)	Intervention (n=48)	p value	Control (n=48)	Intervention (n=48)	p value
Any Blood product use	90/91 (98.9%)	41/48 (85.4%)	0.001*	47/48 (97.9%)	41/48 (85.4%)	0.027*
Red Blood Cells (units)	5 (2; 8)	3 (1; 5)	0.019*	5 (1.25; 8.75)	3 (1; 5)	0.131
Fresh Frozen Plasma (units)	12 (8; 17)	0 (0;0)	<0.001*	11.5 (6.25; 16)	0 (0; 0)	<0.001*
Cryoprecipitate (units)	0 (0; 5)	0 (0; 0)	0.001*	0 (0; 5)	0 (0; 0)	0.006*
Platelet concentrate (units)	0 (0; 4)	0 (0; 5.5)	0.884	0 (0; 3.25)	0 (0; 5.5)	0.857
Fibrinogen concentrate use	18/91 (19.78%)	32/48 (66.66%)	<0.001*	10/48 (20.83%)	32/48 (66.66%)	<0.001*
Prothrombin complex concentrate use	16/91 (17.58%)	9/48 (18.75)	0.865	8/48 (16.6%)	9/48 (18.75%)	0.789
ICU LOS (days)	7 (6; 10)	6.5 (4; 10.75)	0.237	7 (5; 9)	6.5 (4; 10.75)	0.78

Results: A total of 91 patients were included in the control (without algorithm use) and 48 in the intervention phase (with algorithm use). The characteristics of the patients are reported in Table 1. After propensity score matching, the proportion of patients receiving any transfusion of hemocomponents was lower in the intervention phase. Patients in the intervention phase received less fresh frozen plasma, less cryoprecipitate and more fibrinogen concentrate, while the transfusion of red blood cells and platelets was similar (Table 2). There were no differences between the groups in the use of prothrombin complex concentrate or the ICU length of stay (Table 2). In postoperative day1, the levels of haemoglobin and platelets were similar (p=0.058 and 0.356), while patients in the intervention group had lower fibrinogen levels and higher INR (p< 0.001) compared to the control group. Postoperative complications and mortality during ICU stay (p=0.9) were similar between the groups.

Conclusions: The use of a VET-based bleeding algorithm resulted in decreased blood products transfusion with similar intraoperative bleeding and short-term outcomes. Further studies are needed to study long-term outcomes.

OC 10.5 | Coagulation Function Changes after CD19 CAR T Cells Therapy for B-ALL

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Background: Chimeric antigen receptor T cells (CAR-Ts) targeting CD19 have resulted in unprecedented durable remissions for patients with relapsed and refractory B-ALL. Cytokine release syndrome (CRS), resulting from rapid immune activation induced by CAR-Ts , is the most significant treatment-related toxicity. The clinical signs of CRS correlate with T cell activation and high levels of cytokines including IL-6 and IL-10. However, the changes of coagulation function in CAR-Ts treatment were not explored.

Aims: This study aims to detect the levels of cytokines of IL-6,IL-10,IFN-γ,TFNα,IL-2,IL-4 and IL-17A in patients of B-ALL before and after CD19 CAR-Ts therapy. Meanwhile, we examined the prothrombin time (PT), activated partial thromboplastin time (APTT), D-dimer, and fibrinogen in patients before and after CAR-Ts infusion.

Methods: The expression of IL-6, IL-10,IFN-γ,TFN-α,IL-2,IL-4 and IL-17A were analyzed using flow cytometry in B-ALL patients before and after treated with CAR-Ts and in healthy controls (HCs). All these coagulation indicators(PT,APTT,D-dimer,Fg) were analyzed by automatic blood coagulation analyser.The patients´ clinical parameters were detected, and the severity of CRS was graded according to consensus criteria.

Results: The levels of cytokines of IL-6 and IL-10 in patients of B-ALL after CD19 CAR-Ts therapy were significantly higher (P< 0.05) in patients compares with controls and before therapy(p< 0.05); correlation analysis showed that the IL-6 were positively correlated with the CRS grade. The patients with grade ≤3 CRS had normal or mildly changed PT, APTT, D-dimer, and fibrinogen. In contrast, those with grade ≥4 CRS developed early prolongation of the PT and APTT((p< 0.05), increasing D-dimer(p< 0.05) and falling fibrinogen. Moreover, the increase of D-dimer was positively correlated with the increase of IL-6((p< 0.05).

Conclusions: These data demonstrate that cytokines of IL-6 may play a role in patients of CD19 CAR-Ts therapy. The different levels of CRS patients have different changes in coagulation function.

OC 15.1 | Different Protein Spectrum in Patients with Venous Thrombosis after Lower Leg Plaster Cast Application or Knee Arthroscopy than in Controls

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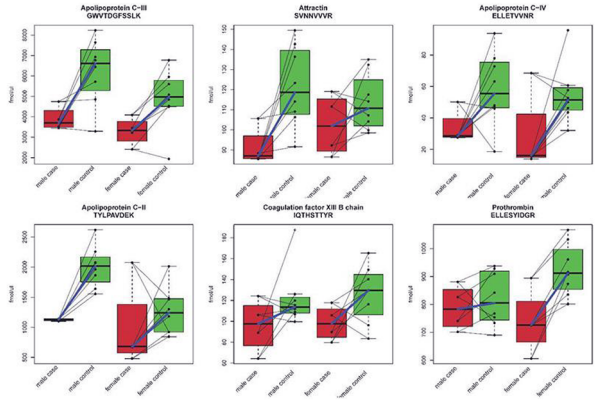

FIGURE 1 The 6 discriminating proteins in the POT-KAST samples used for logistic regression

Background: Patients with lower-leg cast immobilization or who undergo knee arthroscopy have an increased risk of developing venous thrombosis (VT). Prediction scores have been developed to predict VT risk in these patients but have limited performance. Biomarker protein data are a potential valuable addition to such models.

Aims: To use targeted quantification of coagulation and other biomarker proteins in plasma to discriminate patients who develop VT after lower-leg trauma or knee arthroscopy from those who did not.

Methods: For this case-control study we used blood samples from the POT-CAST (Prevention Of Thrombosis after Lower Leg Cast; blood sampling shortly after trauma) and POT-KAST (Prevention Of Thrombosis after Knee Arthroscopy; blood sampling before procedure) trials. We included all patients who developed VT (23 POT-CAST and 8 POT-KAST cases) and matched them to two patients who did not develop VT (46 POT-CAST and 16 POT-KAST controls). We used a validated multiplexed targeted proteomics LC-MRM/MS assay with heavy-labelled internal standards to determine concentrations of 270 proteins in a blinded way. Gender-specific profiles were caught so we analysed data separately for female and male subjects. We used LASSO, random forest, multiple regression and non-parametric Wilcoxon tests to select discriminating features between subjects with and without VT.

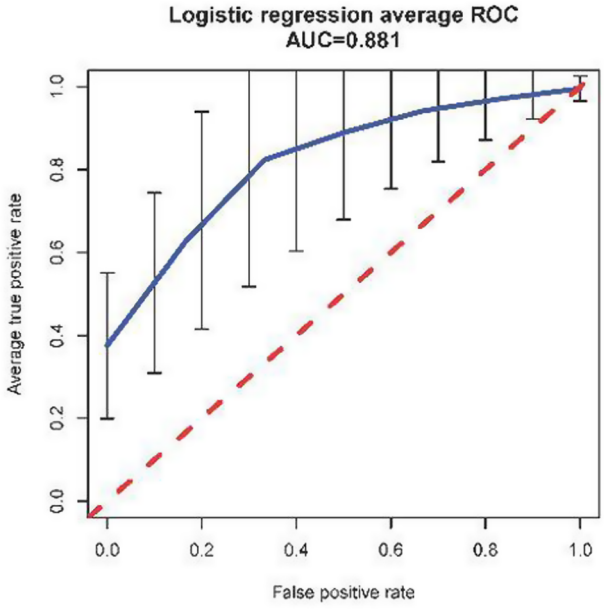

FIGURE 2 ROC curve with 95% confidence intervals for the model using the 6 discriminating proteins

Results: Hierarchical clustering based on selected features showed a very good separation between cases and controls in both genders. When considering paired samples, clustering produced even better discrimination. Logistic regression based on the top discriminating protein abundances from both genders combined resulted in AUCs of 88% and 78% for POT-KAST (figures) and POT-CAST respectively.

Conclusions: Using our simple yet novel approach of LC-MRM/MS multiplexed assays to screen plasma proteins in an unbiased way showed insightful results allowing prediction of VT risk based on six proteins. However, due to the small sample size, validation in a second cohort is mandatory.

OC 15.2 | Evaluation of ADAMTS-13 Activity as Risk Factor for Deep Vein Thrombosis

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Background: Deep vein thrombosis (DVT) is a multifactorial disease which includes several known risk factors such as high levels of factor (F)VIII and von Willebrand factor (VWF), whereas the role of ADAMTS-13 is still unclear.

Aims: To evaluate the association between ADAMTS-13 activity levels and DVT.

Methods: Plasma ADAMTS-13 activity was measured in 365 Italian cases and 291 age and sex matched controls using FRETS-VWF73 assay. VWF antigen (VWF:Ag) and FVIII coagulant activity (FVIII:C)

TABLE 1 Characteristics of the study population

	Cases (n=365)	Controls (n=291)
Age (years), median (range)	48 (5-88)	47 (15-86)
Female Sex, n (%)	184 (50.4%)	152 (52.2 %)
ADAMTS13 activity (%), median (range)	94 (42-154)	98 (57-186)
FVIII:C (%), median (range)	148 (60-298)	113 (52-295)
VWF:Ag (%), median (range)	168 (47-768)	115 (39-262)

TABLE 2 DVT risk for strata of ADAMTS-13 activity

ADAMTS-13 activity	Cases (n)	Controls (n)	OR (95% CI)
1st quartile (<86%)	129	76	1.60 (1.03-2.50)
2nd quartile (86-98%)	92	78	1.15 (0.73-1.80)
3rd quartile (98-111%)	72	65	1.08 (0.68-1.73)
4th quartile (>111%)	72	72	1 (reference)

were measured with immunoassay and one-stage assay on ACL TOP analyzer (Instrumentation Laboratory Italy), respectively. Quartile analysis, using the highest quartile of the controls values distribution as reference, was performed to asses the association between ADAMTS-13 activity levels and DVT. Odds ratios (ORs) were estimated using a logistic regression model after adjusting for age and sex.

Results: The characteristics of the study population are shown in Table 1. As expected, both FVIII:C and VWF:Ag were higher in DVT patients when compared to controls. Conversely, ADAMTS-13 activity was lower in DVT patients (94% versus 98% of control group). ADAMTS-13 activity levels in the lowest quartiles (< 86%) were associated with a 1.60-fold increased risk of DVT (95%CI 1.03-2.50), with a decreasing trend for the other quartiles as shown in Table 2.

Conclusions: We found that ADAMTS-13 activity levels < 86% are associated with a moderately increased risk of DVT.

OC 15.3 | Prognostic Value of Elevated D-Dimer Level after 1 Month of Anticoagulant Therapy in Patients with Venous Thromboembolism: Results of the 10-year Follow-up

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Background: Our previous study showed that elevated D-dimer (D-D) level after 1 month of anticoagulant therapy (ACT) was an independent predictor of deep vein thrombosis (DVT) recurrences and combined endpoint (DVT recurrence or death from any causes) during 18 months. Prognostic value of elevated D-D after 1 month of ACT for long-term venous thromboembolism (VTE) outcomes is unknown.

Aims: To estimate the influence of elevated D-D after 1 month of ACT on the 10-year prognosis in VTE pts.

Methods: One hundred and twelve pts (77 men) aged 18-76 (mean 54±14) years with DVT and/or pulmonary embolism were included in the study. Pts received unfractionated or low molecular weight heparin for at least 5 days followed by long-term warfarin therapy (INR 2,0-3,0). D-D was measured after 1 month from the start of ACT by a quantitative assay with an estimated cut-off level of 0,5 ug/ml. The follow-up period was 10 years. The primary endpoint was death from any causes.

Results: Median of follow-up was 7,16 years (min 2 weeks, max 11,61 years, IQR 1,50 to 10,60 years). Seventy (62,5%) pts had ended the 10-year follow-up completely or achieved the endpoint. In these pts, median of follow-up was 10,41 years (IQR 9,57 to 10,75 years). Forty-two cases were censored. During 10 years, 14 pts (12,5%) died. Kaplan-Meier analysis showed that pts with elevated D-D had higher 10-year total mortality (chi-square=9,7; p=0,002). Cox regression confirmed that elevated D-D after 1 month of ACT was associated with a 4,8-fold increase in the 10-year total mortality risk (HR 4,84; 95% CI 1,62-14,51; p=0,005) compared pts with normal D-D.

Conclusions: In VTE pts, the rate of total mortality during 10 years was 12,5% or 1,25%/year. Elevated (>0,5 ug/ml) D-D after 1 month of ACT had a prognostic value and was associated with a 4,8-fold increase in the 10-year total mortality risk.

OC 15.5 | A Clinical-genetic Risk Score to Predict Cancer-associated Venous Thromboembolism: A Development and Validation Study in Two Independent Prospective Cohorts

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Background: Venous thromboembolism (VTE) is a leading cause of death among patients with cancer. The Khorana score has been developed to assess VTE risk in outpatients with cancer. However, it has been questioned whether this score is accurate in identifying patients who should receive thromboprophylaxis.

Aims: The aim of our study was to develop and validate a clinical-genetic score to improve the assessment of individual risk of VTE in oncology patients.

Methods: The ONCOTHROMB project, including 406 Spanish outpatients with cancer, has been used to develop the Clinical-Genetic Risk Score to predict VTE in patients with cancer. 483 patients from the Vienna Cancer and Thrombosis Study (CATS) served for external validation.

ONCOTHROMB patients were genotyped for the 53 genetic variants known to be associated to VTE. At the time of diagnosis, we

also collected clinical data associated with VTE: primary tumor site, tumor node metastasis stage, body mass index, use of tobacco, age, sex, family history of VTE, diabetes, hypertension, and blood cholesterol level, Khorana score, previous surgery, number of platelets and leukocytes, and immobilization. Multivariate logistic regression analysis was performed to determine the weight of each genetic and clinical variable to build a clinical-genetic risk score (TiC-ONCO). The Khorana and the TiC-ONCO scores were compared using the c-statistic (area under the receiver operating curve -AUC).

Results: Eight genetic variants, tumor site risk, TNM and BMI >25 kg/m2 were associated with VTE and used to build the TiC-ONCO score. The TiC-ONCO risk score was significantly better for prediction of VTE than the Khorana score in both, the development and the validation cohort (Table 1).

Conclusions: We developed and externally validated a clinical-genetic risk score (TiC-ONCO) that performed better than Khorana score in identifying cancer patients at high risk of VTE, who would benefit from thromboprophylaxis.

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OC 44.1 | Hemolysis Induces a Prothrombotic State in Human Plasma

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Background: Although reliable evidence has been provided that hemolytic anemias (e.g., sickle cell disease, autoimmune hemolytic anemia, thalassemias and other unstable hemoglobin disorders) are associated with enhanced thrombotic risk, little is known on the underlying biological mechanisms.

Aims: This study was aimed to explore whether hemolysis of human blood may influence thrombin generation in plasma.

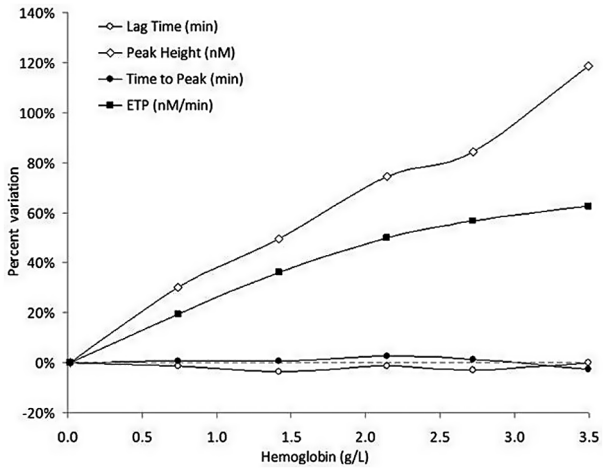

FIGURE 1 Hemolysis

Methods: A pool of whole blood samples anticoagulated with 3.2% sodium citrate was divided in two aliquots. Hemolysis was generated in one of these two aliquots by mechanical injury. Briefly, whole blood was aspirated with a 1 mL syringe equipped with a small-gauge needle (30 gauge, 0.3×8 mm). Scalar amounts of hemolyzed blood were added to the non-hemolyzed pool, to obtain gradually increasing values of cell-free hemoglobin, finally comprised between 0.02 g/L (i.e., non-hemolyzed blood) and 3.5 g/L. The hemolysis degree was assayed as hemolysis index (H-index) on Roche Cobas 8000 (Roche Diagnostics, Risch-Rotkreuz, Switzerland). The thrombin generation in the different hemolyzed aliquots was evaluated with the innovative and fully-automated thrombin generation analyzer ST Genesia (Diagnostica Stago, Asnières sur Seine, Cedex, France). The following parameters were measured: lag time (LT), time to peak (TP), peak height (PH), and endogenous thrombin potential (ETP). The results obtained on hemolyzed plasma aliquots were reported as percent (%) variation from the non-hemolyzed pool.

Results: A highly significant increase of both PH and ETP was observed in hemolyzed samples, whereby values increased in parallel with hemolysis, reaching the peak in the plasma aliquot with 3.5 g/L cell-free hemoglobin (+119% and +63%, respectively). The LG and TP were almost unchanged (i.e., comprised within the intra-assay imprecision) in all hemolyzed aliquots.

Conclusions: Hemolysis of human blood generates a prothrombotic-like state in plasma, thus potentially explaining the enhanced risk of thrombosis observed in patients with hemolytic anemias.

OC 44.2 | A Hemodynamically Controlled Micropump/mixer Applied to Anti-platelet Drug Screening

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Background: Microfluidic technologies offer unique improvements in preclinical drug discovery. However, these systems are often incapable of rapid on-chip reagent delivery and reagent-blood mixing due to low Reynolds number Stokes flow. Effective on-chip micromixing that allows for control of drug bolus concentration and incubation times is a key requirement for high-throughput hematological drug screening.

Aims: This study describes a modular hemocompatible elastomeric micropump-mixer and demonstrates its utility in rapid anti-platelet drug screening.

Methods: We fabricated a microbioreactor incorporating a pneumatically-actuated reciprocating elastomeric micropump-mixer (Fig. 1). FITC-PEG drug analogues of molecular weights 350Da,

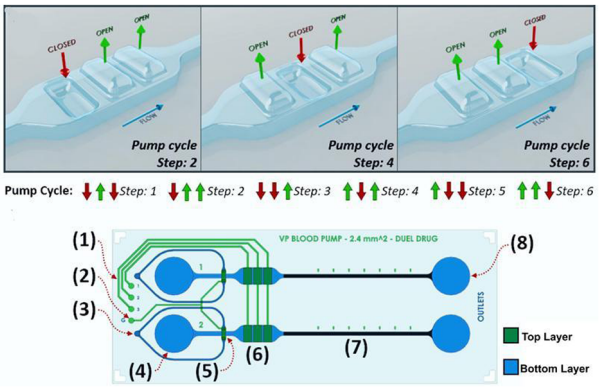

FIGURE 1 Micropump schematic

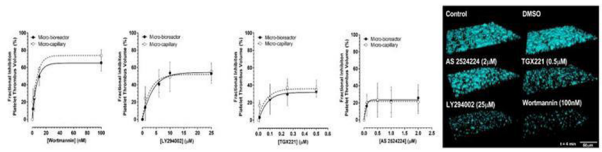

FIGURE 2 Micropump-reactor application to platelet thrombus dynamics and drug screening

2000Da, and 20kDa were injected via drug injection ports into the microbioreactor. Reagent-blood mixing was assessed via scanning confocal microscopy, as the standard deviation in pixel intensities. Micron-resolution Particle Image Velocimetry (μPIV) results were correlated with platelet distribution and activation. The utility of the system as a rapid anti-platelet drug screen was determined through measurement of the efficacy of small molecule platelet PI3-Kinase inhibitors in inhibiting thrombus growth, and assessment of platelet thrombus dynamics on type 1 collagen (Fig. 2).

Results: The μPIV results demonstrated that dynamic operation of the micropump-mixer induced complex fluidic phenomena that led to good mixing efficiency. Homogenous mixing of all molecular weight analogues was achieved following a single pass through the system. On-chip drug treatment with PI3-Kinase inhibitors demonstrated a reduction in collagen-induced thrombus volume equivalent to the literature at a rate ~55-fold faster than the benchmark micro-capillary assay.

Conclusions: We demonstrate a hemocompatible in-line micropump-mixer that acts as a rapid reagent-blood delivery system and allows for the assessment of fast (-11 sec) drug pharmacokinetics. The micropump-mixer can homogenously mix species across a wide molecular weight range and therefore demonstrates broad utility for rapid pharmacokinetic analyses.

This modular scalable system can be multiplexed into complex microfluidics and offers the basis for high-content screening systems for new anti-platelet therapies.

OC 44.3 | Effects of Edoxaban on Routine Tests of Haemostasis as Determined with a Full Range of Reagents: Results of a UK NEQAS Exercise

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Background: British Society for Haematology guidelines recommend that laboratories be aware of the sensitivity of their coagulation screening tests to the presence of direct oral anticoagulants (DOACs).

Aims: Data are needed on effect of this drug on the full range of PT/APTT reagents in routine use.To assist with this we assessed the effects of a range of edoxaban concentrations including likely peak, trough and accumulation levels on prothrombin time (PT), activated partial thromboplastin time (APTT), fibrinogen and thrombin time (TT).

Methods: The same pooled normal plasma was spiked with 0, 39, 241, 469 and 897 ng/ml edoxaban (a kind gift of Daiichi Sankyo UK Ltd), lyophilised in 1 ml aliquots and despatched to participants in the UK National External Quality Assessment Scheme (NEQAS) for Blood Coagulation, The median result for each reagent group was calculated (for PT, APTT and TT as test/mid-point normal range).

Results: Results were received from >500 centres (~65% UK and 35% non UK). Table 1 shows the median results for the most widely used PT and APTT reagents at each concentration of edoxaban in plasma. At 39ng/ml, PT was increased by an average 8% and APTT by 15% compared to the 0ng/ml sample. At 897ng/ml, the PT was

TABLE 1 Edoxaban concentration in plasma and PT/APTT results

PT (ratio)	n	0 ng/ml	39 ng/ml	241 ng/ml	469 ng/ml	897 ng/ml
Werfen HemosIL Recombiplastin 2G	177	1.09	1.18	1.60	2.11	3.06
Siemens Innovin	132	1.13	1.19	1.44	1.71	2.23
Stago STA Neoplastine R	46	1.11	1.25	1.85	2.51	3.86
All	439	1.10	1.19	1.58	2.08	3.00
APTT (ratio)	n	0 ng/ml	39 ng/ml	241 ng/ml	469 ng/ml	897 ng/ml
Werfen HemosIL Synthasil	174	1.07	1.25	1.53	1.82	2.29
Siemens Actin FS	129	1.05	1.18	1.56	1.93	2.53
Stago STA Cephascreen	55	1.18	1.33	1.71	2.07	2.62
All	425	1.07	1.24	1.57	1.90	2.44

prolonged by an average 2.7x and the APTT by an average 2.3x. At 241ng/ml, all PT and APTT results were at or above the upper limit of local reference ranges. TT was minimally affected by edoxaban, and fibrinogen levels remained unchanged across the range of edoxaban concentrations.

Conclusions: Our data show that edoxaban can prolong PT and APTT at trough levels of drug and therefore interfere with screening tests on patients receiving this therapy.

OC 44.4 | A Novel Whole Blood Thrombin Generation Model to Study the Involvement of Blood Cells in Coagulation

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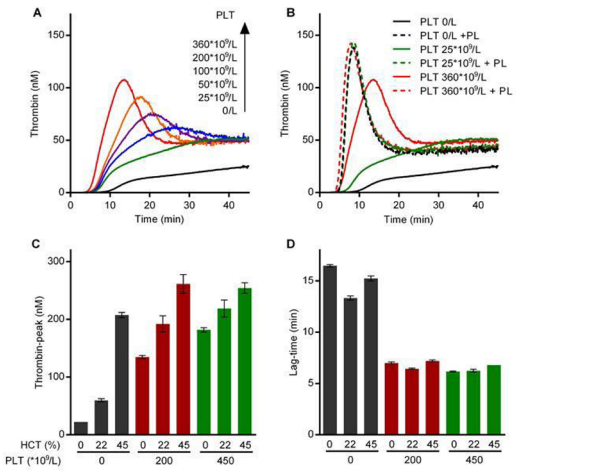
Background: Whole blood thrombin generation (WB-TG) reaches one step closer to physiology than plasma-TG because it involves the intrinsic blood cells instead of synthetic phospholipids. Red blood cells (RBCs) seriously cause variable quenching of the fluorescence signal. We have solved this issue by continuously mixing the sample during the whole measurement course.

Aims: To develop a new WB-TG model.

Methods: This WB-TG model was validated by evaluating the reproducibility and comparing with a previously reported filter paper-based model. Reconstitution experiments were performed to study the effects of RBCs and platelet numbers on WB-TG. WB samples from 119 healthy donors were tested with this novel model and multivariate linear regression analysis was done to analyze the effect of cell counts, age, sex and oral contraceptives (OC) use on WB-TG.

Results: Under optimized assay conditions, a reproducible light transmission was established. The intra- and inter-assay variations of the WB-TG parameters were all below 7%. This model showed good correlation with the previous model (Pearson coefficients were 0.82, 0.79 and 0.75 for ETP, lagtime and time-to-peak, respectively, $P < 0.02$ for all), and the lagtime from the old model was significantly shorter than the new model ($P < 0.0001$). Reconstitution experiments showed that: (1) thrombin peak increased with ascending numbers of RBCs or platelets, and RBCs addition augmented peak even at high platelet numbers (Fig 1A, C); (2) the addition of platelets into PPP markedly shortened the lag-time, but RBC addition did not (Fig 1B, D). Regression analysis showed that peak was significantly affected by RBC count, age and OC use ($P < 0.02$ for all).

Conclusions: We have developed a novel WB-TG model, which was less influenced by contact activation than the previous model. Our model showed that RBCs (and platelets) significantly augment the peak, while platelets are essential for a fast TG initiation.



Note:
Human whole blood was separated into RBCs, platelets and PPP and then reconstituted into the indicated levels.
TG was measured in reconstituted PRP with varying platelet (PLT) count in the absence (A) or presence (B) of 4 μM phospholipid (PL).
(C, D), TG was measured in reconstituted samples with varying platelet counts and hematocrits (HCT) as indicated in the figures.
The thrombin-peak and lagtime are shown. Fluorescence signals were corrected using the H transform when necessary.

FIGURE 1 Reconstitution experiments to study the effect of cell counts on WB-TG

OC 44.5 | Determination of Sensitivity and Specificity of Mixing Studies to Aid in Lupus Anticoagulant Confirmation in a High Volume Reference Lab Setting

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Background: Automated lupus anticoagulant (LA) assays are a mainstay of antiphospholipid syndrome (aPS) diagnosis. While many studies focusing on performance and robustness of various LA tests and mixing study protocols have been published, few studies provide quantitative real world endpoints such as test sensitivity (Se), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV). Such data could assist with result interpretation and follow up testing.

Aims: First, determination of mixing studies Se, Sp, PPV, NPV, prevalence, and cutoffs using available data were calculated. Second, post-test probability analysis was performed to establish positive result prevalence correlated to patient symptomology.

Methods: STA Staclot DRVV Screen/Confirm (reported as simple ratio), STA PTT A, Staclot LA, PTT-LA, Platelet Neutralization Procedure and HemosiL RecombiPlasTin 2G (Instrumentation Laboratory, Bedford, MA) were performed on a STA-R Max coagulation analyzer (Diagnostica Stago, Inc., Parsippany, NJ, USA). PTT and Platelet Poor Plasma (Precision Biologic, Nova Scotia, CA) were used for 0 and 1 hour 37°C incubated mixing studies. Data from 279 negative (41% male/59% female, median age 50) and 499 positive anonymized patients (32% male/68% female, median age 58) were retrospectively analyzed. Tests were validated based on

TABLE 1 Results

		Rosner Index Cutoff						
Patient category	**Calculated Data Point**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
All	Se	0.93	0.91	0.89	0.86	0.84	080	0.77
All	Sp	0.58	0.68	0.75	0.80	0.85	0.89	0.90
All	PPV	0.80	0.84	0.86	0.89	0.91	0.93	0.93
All	NPV	0.83	0.81	0.79	0.76	0.74	0.72	0.69
Healthy Individuals (1-5%)	1 hour mix post-test probability	2-8%	2-10%	2-12%	3-14%	4-18%	5-20%	6-24%
Venous Thrombosis (1-16%)	1 hour mix post-test probability	2-25%	2-28%	2-32%	3-38%	4-44%	5-48%	6-53%
Arterial Thrombosis (4-18%)	1 hour mix post-test probability	7-28%	8-31%	10-36%	12-41%	15-47%	17-52%	20-57%
Pregnancy loss (7-12%)	1 hour mix post-test probability	12-19%	14-22%	16-26%	19-30%	24-36%	27-40%	31-45%

manufacturer recommendations and local protocols. Rosner index cutoffs were calculated as shown previously (1).

Results: Please see Results table.

Conclusions: By calculating sensitivity, specificity, PPV, and NPV values, assay robustness has been demonstrated. In addition, post-test probability and prevalence have been shown at various cutoffs and patient populations. Mixing studies are often performed in high volume laboratories where determination of these parameters would greatly assist with algorithmic-based interpretation by lab staff and clinical decision support for ordering clinicians. Further improvements would be gained if the calculations were performed onboard the instrument or in the laboratory information system (LIS).

References: 1. Rosner E et al. Detection and quantitative evaluation of lupus circulating anticoagulant activity. Thromb Haemost 1987;57:144-147.

OC 54.1 | The Rare MAST2 Arg89Gln Mutation Causes Venous Thrombosis in a French Family with Unexplained Thrombophilia: The Breizh MAST2 Mutation

P.-E. Morange[1]; F. Peiretti[1]; L. Gourhant[2]; C. Proust[3]; G. Varma-Saripella[4]; M.-C. Alessi[1]; D.-A. Tregouet[3]; F. Couturaud[2]

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Background: Rare mutations outside the classical coagulation cascade have been proposed to cause severe rare inherited thrombosis, the identification of which being facilitated by the development of whole exome/genome sequencing technologies.

Aims: To identify the mutation(s) causing venous thromboembolism (VTE) in a French family with multiple relatives affected with unprovoked VTE in which no thrombophilia defects had been identified.

Methods: Whole exome sequencing was performed in 4 VTE-affected and 2 unaffected relatives to search for putative rare VTE-causing variant(s). Sanger sequencing, family segregation study and experimental validation were further conducted on the candidate variant(s).

Results: We identified a never reported missense Arg to Gln mutation (Mast2R89Q) in exon 2 of the Microtubule Associated Serine/Threonine Kinase 2 (*MAST2*) gene that completely segregates with unexplained thrombophilia in the family. Extensive coagulation screening revealed that free-tissue factor pathway inhibitor (TFPI) plasma levels were significantly (p = 0.01) decreased in affected family members compared to healthy relatives (6.6 +/- 1.9 ng/mL vs 17.4 +/-1.2 ng/mL). Conversely, plasma levels of plasminogen activator inhibitor-1 (PAI-1) were significantly (p = 0.01) higher in affected members than in healthy relatives (21.7 +/- 6.1 IU/mL vs 3.3 +/- 0.7 IU/mL). In an endothelial cell model (ECV304 cells), a drastic Mast2 knockdown (around 80 % reduction of mRNA levels) obtained by the cotransfection of two Mast2 specific siRNA significantly increased the mRNA levels of TFPI (+52%) and reduced those of PAI-1 (-64%), respectively. In HEK293 cells overexpressing Mast2R89Q, TFPI and PAI-1 promoter activities were respectively lower (- 20%) and higher (+ 70%) than in cells overexpressing wild type Mast2.

Conclusions: This study identifies a novel mutation located in the *MAST2*gene that causes VTE in a thrombophilia family likely by interfering with TFPI and PAI-1's regulations.

OC 54.2 | Development and Validation of a Plasma micro-RNAs Profile Associated with Venous Thrombosis

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Background: Venous thrombosis (VT) is a complex disease in which genetic variants only explain 15% of the heritability. There

is great interest in studying novel biomarkers associated with VT. Accordingly, micro-RNAs (miRNAs) are small non-coding RNAs that regulate gene expression and are being studied as potential biomarkers of diseases. However, few studies, with small sample sizes, have been performed to establish a miRNA signature in VT and none of them have been validated.

Aims: To identify a plasma miRNA profile asscoiated with VT.

Methods: We investigated the plasma miRNA profile associated with VT in the 'Genetic Analysis of Idiopathic Thrombophilia' (GAIT2) project, composed by 935 individuals of 35 Spanish families. We quantified by qPCR (Exiqon) plasma miRNAs in two steps: a discovery step where we interrogated 752 miRNAs in 52 cases and 52 controls, not genetically related; and an internal validation based on an extension step where the set of those miRNAs statistically associated with VT or intermediate phenotypes of VT were quantified in all the GAIT2 population (N=935).

Results: The results of the discovery step (752 miRNAs , N=104) allowed us to select 16 miRNAs with potential interest in VT. This set of 16 miRNAs was quantified in the whole GAIT2 population (N=935). Interestingly, we found that 3 miRNAs were significantly associated with VT in both the discovery and validation phases: hsa-miR-885-5p, hsa-miR-192-5p and hsa-miR-194-5p. Moreover, we found that these 3 miRNAs were significantly associated with plasma levels of intermediate phenotypes of VT, for instance, FVIIc (hsa-miR-885-5p; p-val= $1x10^{-4}$) or Protein S (hsa-miR-194-5p; p-val=$1.5x10^{-4}$).

Conclusions: In conclusion, we obtained great evidences of the implication of 3 miRNAs in VT. Further studies to elucidate their role in the disease and their possible use as biomarkers are guaranteed.

OC 54.3 | Generating Plasma Protein Signatures for Murine Venous Thrombosis and *Slc44a2* Deficiency Using a Multiplex Targeted Proteomics Approach

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Background: Previously we demonstrated that targeted proteomics using stable-isotope-labeled internal standards can be used to rapidly and precisely quantify coagulation- and fibrinolysis-related proteins in multiplex in human plasma. Moreover, this approach was able to distinguish protein signatures for patients with venous thrombosis (VT), and particularly VT and cancer, which was not apparent from single measurements using the conventional assays.

Aims: Apply quantitative proteomics to murine VT research by investigating the dynamics of plasma proteins in mice following the onset of VT and in mice lacking the novel VT-susceptibility gene *Slc44a2*.

Methods: We used our recently developed panel of 375 validated multiplexed mouse protein assays measured with mass spectrometry in a total of 12.5µl plasma per mouse. First, we applied this technique to a murine model where VT is induced by RNA interference targeting the natural anticoagulants antithrombin and protein C. Next, we determined the plasma protein levels for *Slc44a2* knockout mice to detect possible pathways affected by this gene.

Results: Mice with VT displayed changes in numerous plasma proteins and thereby we could establish a strong signature. This signature allows clear discrimination between VT their control and consists of multiple proteins which are going both directions in abundance. Amongst others, the discriminating proteins include proteins linked to complement and inflammatory pathways. Furthermore, sex had a major effect leading to a stronger discrimination than *Slc44a2* deficiency. Amongst the signature for *Slc44a2* deficiency were glycosylation-dependent cell adhesion molecule-1 and thrombospondin-1 both for males and females. Noteworthy, also apolipoprotein-IV, a protein recently identified as potent inhibitor of thrombosis, was part of the *Slc44a2* signature albeit only in males.

Conclusions: Quantitative proteomics can be applied to mouse plasma to generate protein signatures for VT models, sex and VT-related genes. These signatures increase our knowledge of VT and form a foundation for further exploration into the underlying mechanisms.

OC 54.4 | Proteomic Analysis Reveals Age-Specific Global Changes in Platelet, Plasma and Endothelial Haemostatic Proteins

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Background: Developmental haemostasis is a well-established concept that describes changes in the haemostatic system across the age-spectrum from neonates to adults. Studies in this field to date have focused on age-specific changes in the quantity or function of individual proteins only, not the haemostatic proteome as a whole. Considering that bleeding and clotting remain a serious complication in hospitalised children, understanding the global changes in all haemostatic proteins at any age, is crucial.

Aims: This study aimed to utilise a proteomics approach to characterise age-specific changes in plasma proteins associated with

haemostasis, including platelet and endothelial components. By quantifying the haemostatic proteome of healthy children, we aim to increase understanding of bleeding and clotting in diseased children, and support a holistic understanding of developmental haemostasis.

Methods: Plasma from healthy neonates, infants under 1 year, children 1 to 5 years, and adults were analysed using SWATH-Mass Spectrometry. This created a dataset of plasma protein expression, which was mined for differentially expressed proteins associated with haemostasis. Written informed consent was obtained from parents of children or adult participants, and the study was approved by the Royal Children´s Hospital Ethics in Human Research Committee (#20031).

Results: We identified 27 haemostatic proteins that change in an age-specific manner. These were associated with coagulation, or serve as markers of platelet activation or endothelial involvement. While we found strong demarcations between each of the age groups, it was evident that the haemostatic profile of neonates is heterogeneous and clearly distinct from other groups. Pathway analysis yielded overlap with acute inflammation and the complement system, indicating a central role of haemostasis in developmental physiology.

Conclusions: The dynamic nature of haemostasis means that proteomic analysis yields new insight into all aspects of coagulation and sets a new standard for plasma research. Appreciating developmental haemostasis through proteomics will yield precise and personal medical treatments.

OC 54.5 | Curating Disease-causing Genes for Bleeding, Thrombotic and Platelet Disorders, and Application to the ThromboGenomics Gene Panel Test

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Background: Genetic testing for inherited bleeding, thrombotic and platelet disorders (BTPD) provides a significant opportunity to improve patient care. Multiple gene panel tests have been implemented to diagnose BTPD patients, however all differ in gene content. While considering an exhaustive list of genes is desirable, best practice suggest genes should only be tested with appropriate evidences, to provide clinical actionable findings.

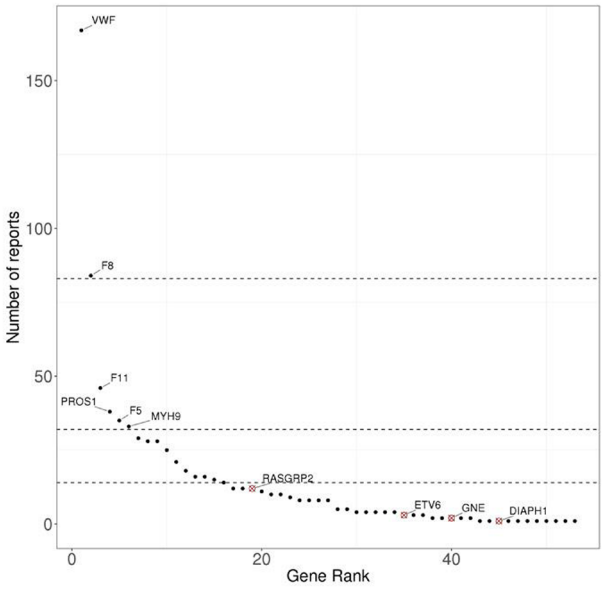

FIGURE 1 Gene ranking according to the number of reports

Aims: To generate a curated reference list of gene-disease pairs causal of BTPD.

Methods: Genes were included if the gene-disease association had been reported in at least 3 independent pedigrees, or < 3 pedigrees and supported by specific functional data and/or a mouse model. A reference transcript was selected for each gene based on community usage (HGMD, ClinVar), transcript and protein lengths (Ensembl), level and tissue of expression (BLUEPRINT data), and cap analysis gene expression data to identify the most common transcription start site. Genes were included on the ThromboGenomics gene panel test and nearly 1,700 patients with BTPD were sequenced.

Results: We curated 91 genes and their transcripts, and classified them into platelet (61), bleeding (21) and thrombotic (9) -disease causing. Twenty-one (23%) have been discovered or had a new mode of inheritance identified since 2015, showing the field is fast evolving. Of the 1,696 patients, 50.4% received a molecular diagnosis in 61 genes. Over half of the referred cases were explained by causal variants in only 6 genes (*VWF*, *F8*, *PROS1*, *F11*, *F5*, *MYH9*) and 17 cases were explained by variants in recently discovered genes (*RASGRP2*, *ETV6*, *GNE*, *DIAPH1*) (Figure 1). The curated gene and transcript list is available at https://www.isth.org/page/GeneList2019.

Conclusions: A curated, evidence-based, gene list is fundamental for reliable genetic diagnosis of BTPD.

OC 64.1 | CLOTSref: A Mobile Application for Standardized Reference Ranges in Developmental Haemostasis

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Background: Developmental haemostasis is known as the evolution with age of the coagulation system in neonates and children, as characterized by marked physiological differences in the concentration of coagulation factors and hemostatic proteins. Reference ranges for hemostatic parameters have important clinical implications for the definition of healthy children, the diagnosis of disease states and the monitoring of anticoagulant therapy. However, reference ranges are analyzer and reagent dependent; and it is estimated that only a small number of laboratories worldwide use adequately established age-, reagent- and analyzer-specific reference ranges. Inappropriate clinical use of reference ranges leads to both over diagnosis and missed diagnosis of the child.

Aims: The objective was to create a platform for practitioners to compare their patients' results with appropriate age-, reagent- and analyer-specific reference ranges. In addition, a platform for clinicians to contribute data towards a worldwide harmonized reference ranges for hemostatic parameters was created.

Methods: Data on developmental haemostasis from the literature was collected and pooled into age-, reagent- and analyer-specific reference ranges. A mobile application was created as a platform to present the data conveniently. In addition, a user-friendly form was embedded for clinicians to contribute data towards a worldwide harmonized reference ranges for hemostatic parameters.

Results: One hemostatic protein, antithrombin was chosen to be preliminary analyzed. Data from 9 identified articles in the literature was extracted and presented in the developing mobile application. Inadequate sample size of each age group and differences in analyzer use were noticed. Overall, limited data demonstrated an expected increasing trend with age in infants. Link to app website: https://www.pwastore.com/w/clotsref/

Conclusions: The CLOTSref mobile application is promising to provide a platform for practicioners, especially pediatricians to provide the best possible standard of care based on appropriate reference ranges. In addition, it allows clinicians to contribute data towards a worldwide harmonized reference ranges for hemostatic parameters.

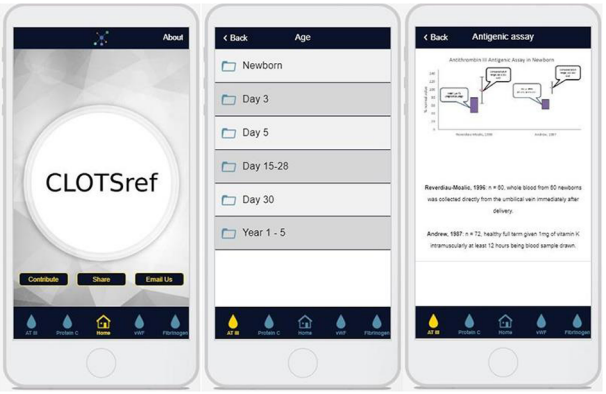

FIGURE 1 The CLOTSref mobile application user interface

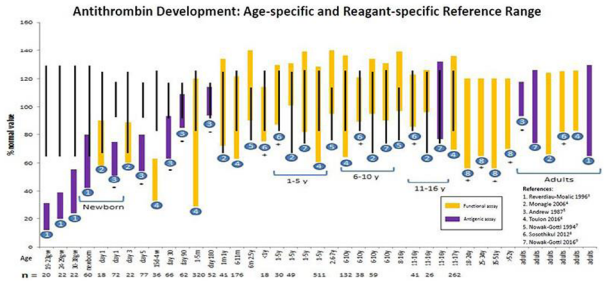

FIGURE 2 Antithrombin development across lifespan in healthy individuals

OC 64.2 | Mepacrine Uptake Can Be Used in the Diagnosis of Platelet Dense Granule Deficiency

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[1]Center for Circulatory Health, Department of Clinical Chemistry and Haematology, University Medical Center Utrecht, Utrecht University, Utrecht, the Netherlands, [2]Van Creveldkliniek, University Medical Center Utrecht, Utrecht University, Utrecht, the Netherlands, [3]Van Creveld Laboratory, University Medical Center Utrecht, Utrecht University, Utrecht, the Netherlands

Background: Dense granule deficiency (DGD) is a bleeding disorder characterized by a reduced number of platelet dense granules and the diagnosis depends on the measurement of platelet ADP content, but this test is time consuming, requires a relatively large blood volume and cannot be performed in samples obtained from patients with thrombocytopenia. Flow cytometric analysis of platelet mepacrine uptake is a potential alternative, but this approach lacks validation.

Aims: To validate mepacrine uptake measured on the flow cytometer as a diagnostic tool in patients with a bleeding tendency and a suspected platelet function disorder.

Methods: As proof of concept, mepacrine uptake was measured with flow cytometry in 20 healthy controls and 7 patients with previously diagnosed DGD. The diagnostic value of mepacrine uptake was subsequently determined in 156 patients with a suspected platelet function disorder without previous diagnosis and compared with platelet ADP content as reference test.

Results: Mepacrine uptake was in perfect agreement (κ=1) and mepacrine release was in good agreement (κ=0.93) with platelet ADP content in 7 patients with previously diagnosed DGD and 20 healthy controls, providing proof of concept that the test can discriminate DGD from healthy individuals. In the validation cohort of patients with a suspected platelet function disorder, the discriminative ability of mepacrine uptake was good (area under the Receiver Operating Curve (ROC): 0.87), as was the discriminative ability of mepacrine release (Area under ROC: 0.80). The sensitivity of mepacrine uptake for DGD was 76%, whereas the specificity was 83%. In our tertiary reference center, this test showed high negative predictive value (97%) with low positive predictive value (35%).

Conclusions: Mepacrine uptake is inferior to platelet ADP content in diagnosing DGD. However, flow cytometric mepacrine uptake can be used when rapid exclusion of DGD is needed, when limited blood is available, or in case of thrombocytopenia.

OC 64.3 | Fibrinolysis in Patients with Chemotherapy Induced Thrombocytopenia and the Effect of Platelet Transfusion

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Background: Bleeding events in chemotherapy induced thrombocytopenic (CIT) patients with similar platelet counts might be influenced by changes in clot lysis potential.

Aims: In this observational study we investigated thromboelastographic lysis parameters, alterations in clot strength and susceptibility to clot lysis in CIT patients and identified factors associated with fibrinolytic profiles. Furthermore, the effects of platelet transfusions were evaluated.

Methods: Independent determinants of tPA-ROTEM lysis parameters were identified with multivariable linear regression. Clot formation, strength and lysis parameters were compared with results of healthy individuals. Characteristics of CIT patients with and without hyperfibrinolytic profiles were compared. tPA-ROTEM results before, 1 hour and 24 hours after platelet transfusion were compared.

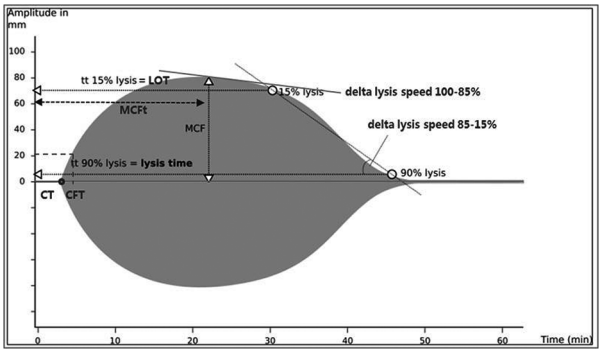

FIGURE 1 Schematic representation of tPA-ROTEM assay

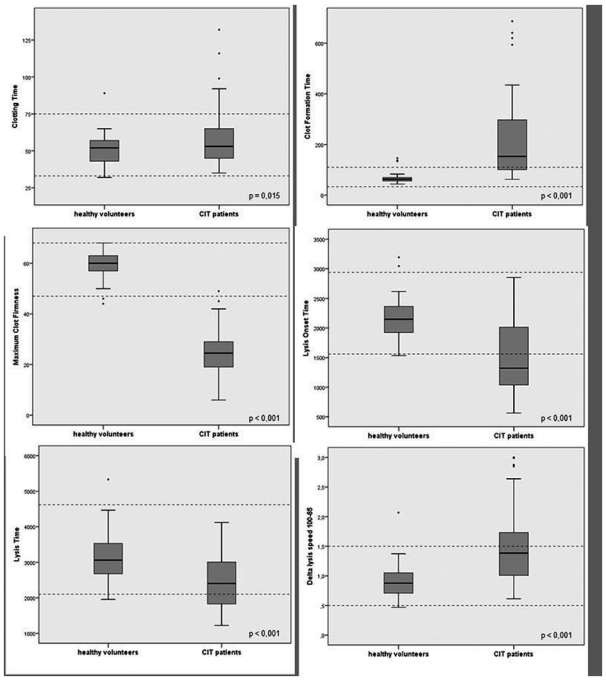

FIGURE 2 tPA-ROTEM results of healthy individuals compared to CIT patients

Results: A total of 72 consecutive CIT patients were included. tPA-ROTEM lysis parameters correlated with changes in fibrinolytic proteins. Compared to healthy individuals, clot formation time was longer, maximum clot firmness was weaker and lysis times were shorter. CIT patients had low PAI-1 and TAFI levels and forty percent exhibited hyperfibrinolytic profiles. Platelet transfusions resulted in less hyperfibrinolytic profiles in many, but not all CIT patients. Patients without hyperfibrinolytic profiles had higher fibrinogen, FVIII and a2-AP levels.

Conclusions: tPA-ROTEM can be used as a fast and reliable assay to detect hyperfibrinolytic profiles in CIT patients. CIT patients have weaker clots which are more susceptible to clot lysis compared to healthy individuals. Besides platelets, other factors are likely to influence clot susceptibility to fibrinolysis in CIT patients. The impact of a hyperfibrinolytic tPA-ROTEM profile on bleeding remains to be investigated.

OC 64.4 | High-throughput Sorting of Platelet Aggregates by Intelligent Image-activated Cell Sorting

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University of Tokyo, Tokyo, Japan

Background: Platelet activation in circulating blood is involved in atherothrombosis. Although flow cytometry has been performed as an assay to detect platelet activation/aggregates, this method is not employed in clinical settings partly because platelets are sensitive to chemical and mechanical stimulation *in vitro*. We have developed intelligent image-activated cell sorting (IACS), a machine intelligence technology that enables wide clinical applications including the detection and isolation of platelet aggregates (Nitta N, Cell 2018).

Aims: To establish a new method for high-throughput detection and isolation of platelet aggregates as a marker for platelet activation by intelligent IACS.

Methods: We used intelligent IACS to quantify and sort platelet aggregates in human healthy citrated blood stimulated with a thrombin receptor activator peptide-6 (TRAP-6). This study was approved by the Institutional Ethics Committee of Faculty of Medicine, the University of Tokyo (No. 11049-5).

Results: As shown in Figure 1, intelligent IACS was able to image large platelet aggregates, small platelet aggregates, single platelets, and leukocytes with a high throughput of ~100 events per second and to clearly distinguish them. Furthermore, applying progressive gating to the probabilities yielded high specificity and sensitivity of 99.0% and 82.0%, respectively, in detecting platelet aggregates.

As shown in Figure 2, intelligent IACS identified and sorted 23.1% of the cell population in a blood sample stimulated with TRAP-6. Our microscopic enumeration verified a high purity of 79.5% in sorting of platelet aggregates using this new technology.

Conclusions: The method for high-throughput detection and isolation of platelet aggregates, reflecting activated platelets by intelligent IACS possesses high sensitivity and specificity. A further investigation of the sorted platelets may identify key factors that may be related to the determination of the sensitivity of platelet activation, leading to the risk assessment of atherothrombosis and monitoring of anti-platelet therapy, and the elucidation of pathogenesis of this disorder.

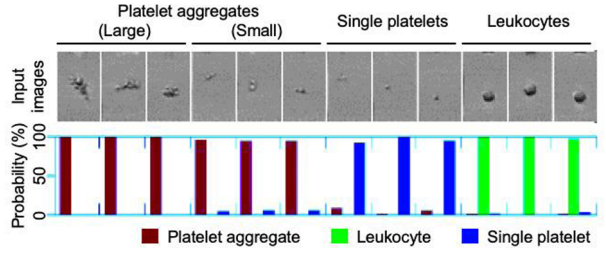

FIGURE 1 Probability distributions of each blood cells

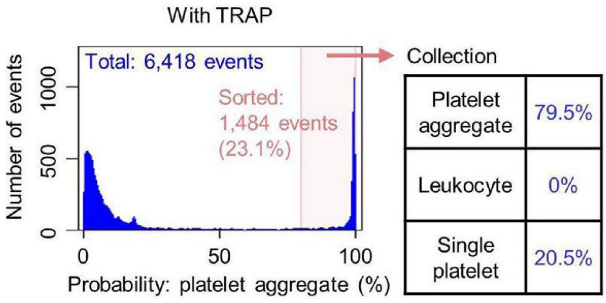

FIGURE 2 Isolation of platelet aggregations in human blood

OC 64.5 | The Prevalence of Giant Platelets in Children without Platelet Disorders on Screening Blood Counts

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[1]Einstein University, Pediatrics, Philadelphia, United States, [2]The Children's Hospital of Philadelphia, Philadelphia, United States

Background: Current technologies for laboratory evaluation of a complete blood count (CBC) have evolved to include automated analysis of the peripheral smear, which may detect rare abnormalities. We have received calls recently to evaluate children for giant platelets noted in otherwise healthy children. We were unable to find a published literature on the prevalence of giant platelets in otherwise healthy children, in fact the literature suggests that giant platelets are associated with primary platelet disorders.

Aims: Determine the prevalence of giant platelets on routine testing.

Methods: We conducted a retrospective cohort study. CBCs were performed by a Sysmex XN analyzer. The electronic medical record (EMR) was used to identify CBCs obtained during well child evaluations in children without concomitant diagnosis seen at our primary care practices over the last 4 years. CBCs for children aged 9 months to 2 years were chosen as those were within the American Academy of Pediatrics standard window for screening CBC for lead poisoning and iron deficiency and therefore enriched for well children. Chart reviews were conducted for all subjects with identified giant platelets.

Results: 5511 CBC results were retrieved of which 949 CBCs were excluded due to abnormal platelet counts, leaving 4562 results included in the study. 59 were noted to have giant platelets; the prevalence of giant platelets was 1.2%. The average platelet count was 336±150x10^9/L for those with giant platelets with a mean MPV of 10.2±0.9fL. None of the children had an underlying platelet disorder or a history of abnormal bleeding. 15(25%) had viral symptoms, fever or otitis media at time phlebotomy, while 8.4%(5) had neutropenia and 10.16%(6) had slow weight gain.

Conclusions: Giant platelets are seen in healthy children without platelet disorders. Improved sensitivity of current analyzers makes detection of these platelets more common. Further study will determine prevalence of this finding in healthy populations.

FIBRINOLYSIS AND PROTEOLYSIS

OC 10.2 | Platelet and Plasma Factor XIII Levels after Replacement Therapy in Severe Congenital Factor XIII Deficiency: Origin of Platelets Factor XIII

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Background: Factor XIII (FXIII), the fibrin stabilizing factor, is present both in plasma and platelets. Megakaryocytes can synthesize FXIIIA but it is still unclear whether platelet FXIIIA may derive from endocytosis of exogenous FXIII by megakaryocytes.

Aims: To study the possible mechanisms of endocytosis and/or synthesis of FXIIIA by healthy megakaryocytes. To evaluate plasma and platelets FXIII levels in a patient with severe FXIII deficiency under replacement therapy with recombinant (r)FXIIIA. To investigate the possible *in vitro* uptake of FXIIIA by patient megakaryocytes.

Methods: Synthesis and/or uptake of FXIII by healthy or patient megakaryocytes culture were studied by immunofluorescence technique. Patient plasma and platelets levels of FXIII were measured before and after replacement therapy.

Results: Megakaryocytes from healthy donors revealed high expression of FXIIIA and, if cultured in the presence of fluorescein labeled exogenous rFXIIIA, were able to make uptake of rFXIIIA. Moreover, megakaryocytes from the patient were negative for the expression of FXIIIA and became positive only after the addition of rFXIIIA to the culture medium. Patient plasma FXIII activity and antigen levels were 5% and < 1%, respectively. These values increased about 100% after administration of rFXIIIA and, subsequently, a progressive reduction of both levels up to 35% were seen at day 14 after infusion. Platelet FXIIIA antigen levels were < 1% before therapy and reached 6% within 14 days after infusion and remained at 3 % until day 35.

Conclusions: In normal megakaryocytes the two systems of FXIIIA endocytosis and synthesis coexists. Moreover, patient megakaryocytes revealed that these cells, which were unable to synthesize FXIIIA, can endocytose exogenous rFXIIIA and possibly produce FXIII-containing platelets. Administration of rFXIIIA in severe FXIII deficient patients results in restoration of plasma and platelets FXIII. The role of platelet FXIIIA still remains to be fully elucidated.

OC 17.1 | The Use of in vitro Clot Lysis Assay for Predicting Outcomes in Acute Ischemic Stroke Patients Undergoing Intravenous Thrombolysis

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[1]*University of Debrecen, Faculty of Medicine, Division of Clinical Laboratory Sciences, Debrecen, Hungary,* [2]*University of Debrecen, Faculty of Medicine, Department of Neurology, Debrecen, Hungary,* [3]*MTA-DE Cerebrovascular and Neurodegenerative Research Group, Debrecen, Hungary*

Background: The outcome of intravenous thrombolysis using recombinant tissue plasminogen activator (rtPA) is favorable in only 33-35% of acute ischemic stroke (AIS) patients.

Aims: We tested whether an in vitro clot-lysis assay performed before thrombolysis might predict therapy outcomes.

Methods: In this observational study, blood samples of 175 consecutive AIS patients, all undergoing i.v. thrombolysis by rtPA within 4.5 h of their symptom onset, were taken before thrombolysis. Platelet-poor-plasma was clotted using recombinant tissue factor, lysis was induced with rtPA. Clot formation and lysis was monitored by turbidimetry. In order to test the impact of neutrophil extracellular traps on lysis, assays were also performed in the presence of cell-free-DNA and histones. Curve analysis was performed by the ShinyApp software. Stroke severity was determined by NIHSS on admission. Therapy-associated intracerebral hemorrhage was classified according to ECASSII criteria. Short- and long-term outcomes were defined at 7 days and 3 months post-event according to the change in NIHSS and by the modified Rankin Scale, respectively. All patients or their relatives provided written informed consent.

Results: The median time to reach 50% lysis (50%CLT) was 47.3 (IQR:35.0-64.0) min in the total cohort, and became significantly prolonged in the presence of cell-free-DNA and histones. A dose-response relationship was observed between stroke severity and 50%CLT with or without DNA and histones. Patients with favorable short-term outcome had significantly shorter 50%CLT in the presence of DNA and histones as compared to patients with poor outcomes (median: 50.6 (IQR:36.8-70.5) min vs. 62.0 (IQR:45.0-74.7) min; p< 0.05). Long-term outcomes and occurrence of post-lysis hemorrhage showed no association with clot-lysis assay results.

Conclusions: Clot-lysis assay using pre-thrombolysis plasma of AIS patients might be useful to predict short-term outcomes, particularly when the assay is supplemented with DNA and histones. Clot lysis assay however showed no association with hemorrhagic complications and long-term outcomes.

Funding: GINOP-2.3.2-15-2016-00043, NKFI-K120042

OC 17.2 | Targeted Drug Therapy: Effective and Localised Anticoagulation without Bleeding Risks

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[1]Baker Heart and Diabetes Institute, Melbourne, Australia, [2]Monash University, Melbourne, Australia, [3]Harvard Medical School, Boston, United States

Background: Myocardial infarction and ischaemic stroke remain the leading cause of mortality both in Australia and worldwide. Whilst therapeutic advances using anti-platelet and anti-coagulation drugs have been made to address these statistics, they have significant drawbacks, including bleeding complications. Therefore, their use is highly restricted leaving many patients untreated.

Aims: Our aim was to develop a targeted anti-thrombotic therapy to deliver a therapeutic payload directly to the site of the thrombus, thereby preventing bleeding side effect.

Methods: We have genetically designed and engineered a recombinant fusion protein, Targ-TAP, which consists of a single-chain antibody targeted towards the activated platelet GPIIb/IIIa integrin, and tick anticoagulant peptide (TAP), which is a potent therapeutic inhibitor of Factor Xa. As a control, a non-binding single-chain antibody fused with an active TAP (Mut-TAP) was constructed. Using in vitro assays, as well as an in vivo model of thrombosis, the specificity and functionality of Targ-TAP as a novel anti-thrombotic drug was assessed.

Results: Flow cytometry assays using both human and mouse blood demonstrated the binding affinity of Targ-TAP specifically to platelets that were activated using ADP, CRP or TRAP, while no binding was observed with Mut-TAP ($p< 0.05$). Light transmission aggregometry demonstrated strong platelet inhibition using Targ-Tap but no inhibition using Mut-TAP ($p< 0.05$). Flow chamber assays using bright field microscopy demonstrated the potent anticoagulant capacity of Targ-TAP, as well as its specificity toward aggregated platelets ($p< 0.05$). Furthermore, Targ-TAP significantly inhibit thrombosis in murine models of both arterial and venous thrombosis. More importantly, Targ-TAP does not increase bleeding time nor blood volume loss.

Conclusions: Targ-TAP allows for the enrichment of the anticoagulant effect directly at the site of the developing thrombus, whilst ensuring low systemic concentrations. Targ-TAP represents a novel, effective and safe anticoagulant drug candidate.

OC 17.3 | Inhibition of Complement C5a Rreceptor Ameliorates Tissue Plasminogen Activator (t-PA)-mediated Increase in Blood Brain Barrier (BBB) Permeability

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[1]Monash University, Australian Centre for Blood Diseases, Melbourne, Australia, [2]University of Queensland, School of Biomedical Sciences, Brisbane, Australia, [3]Monash Health, Pharmacy, Melbourne, Australia, [4]Monash Health, Haematology, Melbourne, Australia, [5]Monash University, Epidemiology and Preventative Medicine, Melbourne, Australia

Background: t-PA can increase BBB permeability and trigger the infiltration of immune cells into the central nervous system. Few plasmin-dependent and -independent pathways participate in this phenomenon, for example, activation of Rho-Kinase 2 and platelet derived growth factor C, respectively. Since plasmin also possesses pro-inflammatory properties, including C3 and C5 convertase activity, we hypothesized that complement activation plays an additional role in this process.

Aims: We aimed to evaluate the effect of complement inhibition on t-PA- and plasminogen (plg)-mediated opening of the BBB using an established *in vitro* model with C5 and C5a-receptor 1 (C5aR1) inhibitors.

Methods: An *in vitro* model of the BBB was assembled on porous membranes of Transwell inserts as a co-culture system of human brain endothelial cells on the luminal surface and human immortalized astrocytes on the abluminal surface. The luminal compartment was stimulated with t-PA and plasminogen in the presence of PMX205 (non-competitive inhibitor of C5aR1), Avacopan (C5aR1 antagonist) or eculizumab (humanised monoclonal inhibitor of human C5).

BBB permeability was assessed at both 4hr and 24hr post-stimulation by evaluation of fluorescent albumin passage from the luminal to the abluminal chambers over 1hr. Permeability changes were calculated relative to non-treated controls.

Results: PMX205 and Avacopan decreased t-PA mediated increase in BBB permeability by almost 50%, at both the 4 and 24hr time points. Interestingly, our preliminary results indicate that eculizumab was without any significant effect.

Conclusions: t-PA- and plasmin-mediated increase in BBB permeability is partly driven by C5a receptor activation. Inhibition of C5 or C5aR1 may have therapeutic implications in traumatic brain injury and stroke thrombolysis, where either endogenous or administered t-PA can compromise the BBB, leading to secondary insults.

OC 17.4 | Fibrin clot Resistance to Thrombolytic Therapy is Controlled by Bypassing Flow through Collateral Vessels, Clot Permeability and PAI-1 Level

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[1]Center for Theoretical Problems of Physicochemical Pharmacology RAS, Moscow, Russian Federation, [2]University of Geneva, Computer Science Department, Geneva, Switzerland, [3]University of Amsterdam, Faculty of Science, Amsterdam, the Netherlands, [4]Dmitry Rogachev National Research Center of Pediatric Hematology, Oncology and Immunology, Moscow, Russian Federation, [5]M.V. Lomonosov Moscow State University, Faculty of Physics, Moscow, Russian Federation

Background: The fibrinolytic system is a biochemical reaction cascade in blood that physiologically dissolves fibrin clots to restore blood flow. Malfunction of clot formation or lysis may lead to thrombosis treated by administration of lysis activators. Yet, standard treatment has a success rate of less than 50%. It is not clear why some clots turn out to be resistant to lysis.

Aims: We used computational systems biology methods to understand the mechanisms of spatiotemporal clot lysis regulation and get clues for the improvement of treatment efficiency.

Methods: A mechanism-driven two-compartment computational model of tissue plasminogen activator (TPA) initiated spatial fibrin clot lysis was developed to investigate the regulation of this process by chemical reactions, diffusion and advection during thrombolytic therapy. TPA administration was simulated with the pharmacokinetic model, which described a bolus and continuous infusions protocol. To evaluate TPA perfusion inside the clot, we employed a 3-dimensional model with two collateral vessels, one of which was clogged with a fibrin clot, and another was free.

Results: We showed that endogenous lysis inhibitor PAI-1 dramatically decreased lysis efficiency even at concentrations that were below those of TPA because of its interaction with the oncoming diffusive front of TPA, and that this effect is decreased with the increase of clot permeability. We found that lysis onset depended on the ratio of PAI-1 and TPA concentrations in a threshold manner. When flow can bypass the occluded vessel via collateral, TPA concentration inside the clot is decreased and can be not sufficient to initiate lysis

Conclusions: Clot lysis is controlled by fluxes of TPA, the chemical, diffusional and advectional. Our findings may explain low success rate of standard thrombolytic therapy, as presence of bypassing flow through the collateral vessels, low clot permeability or elevated PAI-1 level can decrease lysis efficiency individually, and being combined they can prevent clot dissolution.

OC 17.5 | Thrombus Composition in Acute Ischemic Stroke: FXIII Content Impairs Complete Revascularization

M. Navarro-Oviedo[1]; J. Marta-Enguita[2]; M. Belzunce[1]; J.A. Rodriguez[1,3]; J.A. Paramo[3,4]; I. Blanco-Luquin[5]; R. Muñoz[2]; J. Orbe[1,3]

[1]University of Navarra, Center for Applied Medical Research CIMA, IdiSNA, Instituto de Investigación Sanitaria de Navarra, Atherothrombosis laboratory, Pamplona, Spain, [2]Complejo Hospitalario de Navarra, Department of Neurology, Pamplona, Spain, [3]CIBER Cardiovascular CIBERCV, Instituto de Salud Carlos III, Pamplona, Spain, [4]Clínica Universidad de Navarra, Universidad de Navarra, Department of Haematology, Pamplona, Spain, [5]Navarrabiomed-Navarra Institute for Health Research IdiSNA, Neuroepigenetics Laboratory, Pamplona, Spain

Background: The implementation of endovascular mechanical therapies has enabled the histological analysis of thrombi retrieved from acute ischemic stroke (IS) patients. Neutrophil extracellular traps (NETs) have recently been identified as key players involved in the formation of thrombi of various origins. Moreover, it has been suggested that thrombus NETs content may be responsible for reperfusion resistance in IS patients. Other factors related to clot stabilization and fibrinolysis could be present in cerebral thrombi and participate in arterial revascularization.

Aims: This study aimed to analyse the thrombus composition assessing the presence of NETs (citrullinated histone H3 [H3Cit] and extracellular DNA), thrombin activatable fibrinolysis (TAFI) and FXIII and their relationship with successful revascularization after mechanical thrombectomy in IS.

Methods: Forty-five thrombi were retrieved from IS patients by endovascular procedures and their content in NETs, TAFI and FXIII were characterized by immunohistochemical analysis. Clinical data was integrated and analysed with the data collected from the thrombi.

Results: 18% (8/45) of IS patients did not achieve complete revascularization after mechanical thrombectomy. NETs and TAFI were detected in almost all thrombi, but its content was not associated with successful recanalization (Thrombolysis in Cerebral Infarction [TICI] 2b and 3). However, FXIII within the thrombus was increased in patients that failed to recanalyse after mechanical thrombectomy ($p= 0.03$). Quantitative measurement of thrombus FXIII content correlated with NETs ($r=0.32$, $p< 0.05$). From receiver operating characteristic curves, we established a cut-off value for FXIII and complete recanalization (2.7, sensitivity= 71% and specificity= 81%). On binomial logistic regression analyses, this FXIII cut-off value was independently related with unsuccessful recanalization (OR= 9, IC 95%= 1.25-65.7, $p= 0.03$) after adjustment for potential confounders.

Conclusions: FXIII is an important constituent of cerebral thrombi associated with failure in complete recanalization. Therefore, strategies targeting FXIII might represent a novel revascularization approach in IS.

OC 37.2 | Both Carboxypeptidase B2 and Carboxypeptidase N are Responsible for Inactivation of Bradykinin

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Background: Bradykinin (BK) is a vasodilator and inflammatory mediator whose activity is dependent on a C-terminal Arg. Inactivation occurs by removal of that Arg by the plasma basic carboxypeptidases, carboxypeptidase B2 (CPB2, AKA TAFIa) and carboxypeptidase N (CPN).

Aims: To compare the importance of CPB2 and CPN in inactivating BK in a mouse model of vascular permeability..

Methods: The responses of mice to a skin irritation challenge were compared between mice deficient in CPB2 (*Cpb2*$^{-/-}$), CPN (*Cpn*$^{-/-}$) or both carboxypeptidases (*Cpb2*$^{-/-}$/*Cpn*$^{-/-}$) and wild type (WT) mice. The mice were injected with Evans blue dye and the ear treated with mustard oil. To measure vascular permeability, the amount of dye extracted from the treated ear was compared to the contralateral control ear by determining absorbance. Data was analyzed by ANOVA followed by Bonferroni correction.

Results: Treated ears always had more vascular permeability than the control contralateral ears. There was no difference in vascular permeability of the control ears between genotypes. The lowest vascular permeability was observed in WT mice, the next lowest in *Cpn*$^{-/-}$ mice followed by the *Cpb2*$^{-/-}$ mice and the *Cpb2*$^{-/-}$/*Cpn*$^{-/-}$ mice had the highest vascular permeability (Figure 1).

Conclusions: As there was no difference in vascular permeability between the genotypes in the control ears,neither CPB2 or CPN are critical for inactivating the small background amount of BK continuously produced. In treated ears, both enzymes play a role in BK inactivation, but CPB2 plays a more important role than CPN. CPB2 is activated from its circulating precursor, proCPB2 in this non-thrombotic model, but the mechanism of activation is unknown. BK is shown here to be a physiological substrate for CPB2.

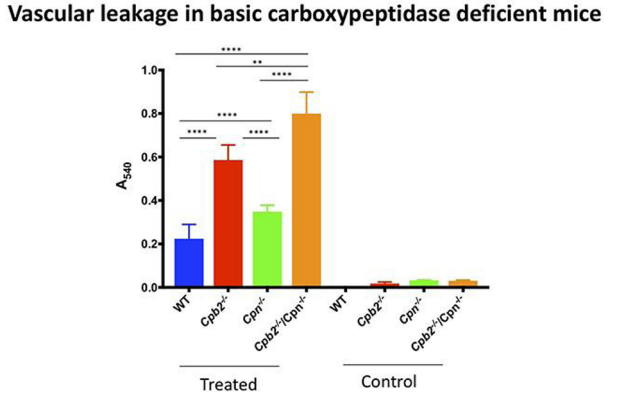

FIGURE 1 Vascuclar permeability in basic carboxypeptidase deficient mice

OC 37.3 | Fibrin(ogen) Protects Neutrophils from Membrane Damage by Extracellular Histones and Ionomycin

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Background: Neutrophils are pivotal players in immune defence and deploy an arsenal of antimicrobial mechanisms to combat pathogens including the release of histones and DNA in Neutrophil Extracellular Traps (NETs). Unfortunately, histones while toxic to invading pathogens can kill host cells, including neutrophils. Bacteria have also evolved mechanisms to escape neutrophils, including the secretion of pore-forming toxins.

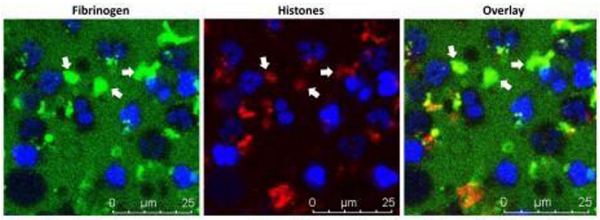
Aims: To determine how the local plasma protein environment, particularly fibrinogen and fibrin, influences neutrophil response to extracellular histones and membrane-damaging agents.

Methods: Live-cell confocal microscopy with cellular dyes and fluorescently labelled proteins, and plate-based biochemical and cellular assays were used to monitor neutrophil survival and histone neutralisation.

Results: Fibrinogen/fibrin-histone aggregates form rapidly to block histone binding to cell membranes and maintain neutrophil viability (Figure 1). Native fibrinogen, fibrin, or isolated fibrinogen domains (E domain > D domain) were effective in neutralising histones. Arginine-rich core histones (H3 and H4) had greater cytotoxicity than H1, and removal of histone arginine through citrullination by peptidyl arginine deiminase 4 (PAD4), or digestion by activated protein C (aPC), reduced cytotoxicity. Turbidimetric studies revealed charge-dependent fibrinogen gelation by histones, which also accelerated fibrin formation by thrombin, to give a structurally altered clot.

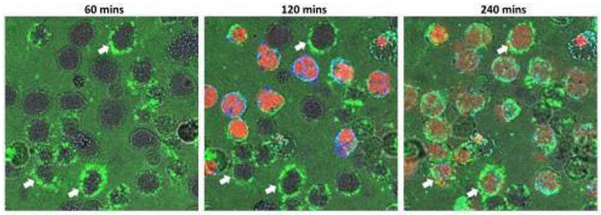
Membrane damage by ionomycin (acting analogously to bacterial pore-forming toxins) resulted in rapid intracellular calcium rises and cell death. Neutrophil survival was improved by the presence of fibrinogen, which could bind the surface of ionomycin-stimulated cells to limit cytotoxicity (Figure 2).

Conclusions: Fibrin(ogen) protects neutrophils from membrane damage through multiple mechanisms: binding/sequestering histones (Figure 1) and binding the cell membrane to improve survival (Figure 2). Fibrinogen depletion/consumption, such as in trauma,



Live-cell confocal microscopy showing accumulation of fibrinogen (Alexa Fluor 488, green) and histones (Alexa Fluor 647, red) in aggregates away from the neutrophil cell surface (white arrows). Nuclei are stained blue.

FIGURE 1



Live-cell confocal microscopy showing binding of fibrinogen (green) to the surface of ionomycin-stimulated neutrophils over 4 hours. Cells that bind fibrinogen early (white arrows) have prolonged survival, and delayed externalisation of phosphatidyl serine (annexin V, blue) and subsequent cell death (propidium iodide, red).

FIGURE 2

sepsis and disseminated intravascular coagulation (DIC), could allow histones to circulate unchecked, accelerating their damaging effects. Our findings emphasise the under-appreciated requirement to involve plasma proteins, and particularly fibrinogen and fibrin, in studies of neutrophil function ex vivo.

OC 37.4 | Molecular Mechanisms Underlying the Fibrin-VLDL Receptor-dependent Pathway of Transendothelial Migration of Leukocytes and the Inhibitory Function of Fibrin-derived β15-42 Fragment

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Background: According to the current view, fibrin degradation product E_1 fragment promotes transendothelial migration of leukocytes and thereby inflammation by bridging leukocytes to the endothelium through the interaction with endothelial VE-cadherin, and the fibrin-derived β15-42 peptide (fragment) reduces leukocyte transmigration by inhibiting such bridging and, in addition, by signaling through the Src family kinase member Fyn. However, the very low affinity of β15-42 to VE-cadherin raised a question about its ability to inhibit E_1-VE-cadherin interaction. Further, our previous study, which revealed that fibrin promotes leukocyte transmigration through a VLDL receptor-dependent pathway, suggested a possible link between the inhibitory properties of β15-42 and this pathway.

Aims: The major aims of our study were to test such a link and to assess which of the two proposed inhibitory mechanisms account for inhibition by the β15-42 fragment.

Methods: We performed *in vitro* experiments using ELISA and leukocyte transendothelial migration and permeability assays, and *in vivo* studies with wild-type and VLDL receptor-deficient mice using a mouse model of peritonitis.

Results: Competition ELISA experiments revealed that β15-42 cannot inhibit the proposed E_1-mediated bridging at the concentrations used in the previous *in vivo* studies leaving the other proposed signaling mechanism as a viable explanation for the inhibitory effect of β15-42. While testing this signaling mechanism, we confirmed that Fyn plays a critical role in controlling fibrin-induced transendothelial migration of leukocytes and found that signaling through the fibrin-induced VLDL receptor-dependent pathway results in inhibition of Fyn thereby increasing leukocyte transmigration. Furthermore, our *in vivo* experiments revealed that β15-42-containing fragments inhibit this pathway thereby preventing inhibition of Fyn and reducing leukocyte transmigration.

Conclusions: The present study clarifies the molecular mechanism underlying the VLDL receptor-dependent pathway of leukocyte transmigration and reveals that this pathway is a target for β15-42-containing fragments.

OC 37.5 | Fibrin is a Critical Regulator of Immunopathology at the Oral Mucosal Barrier

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Background: Fibrin plays an important role in protective physiological processes, such as hemostasis and tissue remodeling, however, due to its proinflammatory properties, excessive fibrin deposition causes chronic inflammation and severe tissue damage. Fibrin removal, fibrinolysis, is achieved by the proteolytic activity of plasmin. The critical role of defective fibrinolysis becomes evident in patients with type I plasminogen deficiency, which present with severe immunopathology at barrier surfaces, including the oral mucosa, eye, lung and GI tract. At the oral barrier, Plg-deficient patients display excessive mucosal fibrin deposition and severe periodontitis (destruction of tooth-supporting gingiva, bone and premature tooth loss).

Aims: Herein we aim to understand if persistent fibrin accumulation plays a role in developing periodontal immunopathology and to delineate the mechanism underlying fibrin-mediated inflammation.

Methods: The μCT method was used to measure bone loss in related mouse models. Multi-color flow cytometry, immunofluorescence microscopy and RNA-Seq approaches were employed for characterization of the gingival lesions.

Results: Consistent with observations from Plg-deficient patients, mice with defects in fibrinolysis (Plg-deficient, inactive plasmin

expression or urokinase and tissue-type plasminogen activator-deficient) displayed significant fibrin accumulation and spontaneous periodontitis. Characterization of the inflammatory gingival lesions revealed a neutrophil-dominated infiltrate in close proximity to the accumulated fibrin. To investigate the role of fibrin-neutrophil-mediated immunopathology, we employed mice that express a mutant form of fibrinogen lacking the β2 myeloid integrin, CD11b, binding site (Fibγ390-396A) preventing fibrin-myeloid cell binding. Crossing Fibγ390-396A mice with Plg-deficient mice rescued Plg deficiency-associated periodontitis, suggesting a critical role for fibrin-myeloid cell interactions in inducing inflammation at the oral barrier.

Conclusions: Collectively, our data highlight the role of fibrin as a critical immune-regulator of oral barrier homeostasis and provide opportunities towards understanding the role of fibrin in neutrophil activation and tissue immunity.

OC 45.1 | Tumor-derived Factor VII Associates with Survival and Regulates Tumor Progression in Breast Cancer

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Background: The presence of a procoagulant microenvironment promotes cancer progression, but anticoagulation has not yet been proven to be a successful anticancer strategy. Tissue Factor (TF), the initiator of coagulation, is expressed on cancer cells. Binding of Factor VII (FVII), produced in the liver, to TF induces angiogenesis and tumor growth via protease activated receptors (PARs). Numerous studies have shown that, apart from the liver, cancer cells can also produce FVII.

Aims: To study how FVII associates with survival and to determine whether it regulates breast tumor progression.

Methods: Analyses on metastasis-free survival and clinical variables were performed in a cohort of 574 breast cancer patients. NOD/SCID/$\gamma_c^{-/-}$ (NSG) mice were orthotopically injected with triple negative breast cancer (TNBC) cell lines (MDA-MB-231, MDA-MB-453 or MDA-MB-468). Tumor-derived FVII was inhibited using blocking antibody 3G12 or FVII specific shRNA. Liver-derived FVII was inhibited by siRNA approach. Tumor volume was measured over time and metastasis was determined in various organs. *In vitro* effects of FVII on tumor cells were studied by migration and invasion assays. The pro-metastatic cellular profile was studied by qPCR and western blot.

Results: FVII expression in breast cancer specimens is associated with tumor progression and metastasis to the liver. In TNBC cell lines, tumor-derived FVII drives tumor cell invasion and expression of the pro-metastatic genes Slug and Sox9. *In vivo*, tumor-derived FVII enhances tumor growth and formation of liver metastasis, while liver-derived FVII appears to inhibit metastasis. Finally, tumor-derived FVII induces pro-metastatic gene expression independent of TF, requiring a functional endothelial protein C receptor (EPCR), while recombinant FVIIa acts via the canonical TF:PAR2 pathway and inhibits expression of pro-metastatic genes.

Conclusions: We propose that tumor-derived FVII and liver-derived FVII have opposing effects on metastasis in TNBC. Our results may also explain why anticoagulation is ineffective in stemming tumor progression.

OC 45.2 | Tissue Factor Pathway Inhibitor (TFPI) Enhances Trans-Endothelial Migration of Chronic Lymphocytic Leukemia Cells through CXCL12/CXCR7

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Background: Chronic lymphocytic leukemia (CLL) is the most common leukemia in the western world. Cell trafficking and migration of CLL cells play a critical role in organ infiltration and contribute to the clinical course of CLL. Tissue factor pathway inhibitor (TFPI) is a biological inhibitor of coagulation and studies have shown that TFPI is involved in cell migration in solid tumors. However, the role of TFPI in the progression of solid tumors is still controversial and the effect of TFPI on leukemia progression has not been investigated.

Aims: To assess the effect of TFPI on trans-endothelial migration (TEM) of leukemic cells from CLL patients.

Methods: After written informed consent from patients, CLL cells were negatively isolated from the peripheral blood mononuclear cells (PBMC) of patients. Trans-endothelial migration (TEM) assays were performed in transwell insert plates with an endothelial monolayer and the migrated cells were counted by flow cytometry. qPCR and Western blot were used to detect the expression of CXCR7, CXCR4 and β-catenin. Immunofluorescence and co-immunoprecipitation were used to examine the binding between TFPI and glypican-3.

Results: Full-length recombinant TFPI (rTFPI) dose-dependently increased CXCL12-mediated TEM of CLL cells after 24 h treatment. The expression of CXCR7 which is a receptor of CXCL12 was increased dose-dependently by rTFPI treatment. Wnt/β-catenin signaling was activated 15 min after rTFPI treatment. Inhibition of Wnt/β-catenin signaling by IWP4 decreased CXCR7 expression and impaired the TFPI-induced TEM of CLL cells. Moreover, TFPI bound to glypican-3 on the surface of leukemic cells. The Glypican-3 inhibitor HS20 abolished the effect of TFPI on the TEM of CLL cells.

Conclusions: In this study, we have shown that TFPI enhances CLL cell migration through binding to glypican-3, activating the

Wnt/β-catenin signaling pathway and upregulating the expression of CXCR7 receptor. Our findings suggest a novel role of TFPI in regulating the trafficking of leukemic cells.

OC 45.3 | Blood Coagulation Initiation Complex,Tissue Factor-Factor FVIIa Mediated Breast Cancer Progression Via Microvesicles

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Background: Apart from blood coagulation, coagulation proteases are involved inextricably in cancer progression/propagation via intra-/inter-cellular signaling by activating protease-activated receptors (PARs). Microvesicles (MVs), has recently been identified as an essential contributor in human breast cancer progression.
Aims: The objective of this study is to investigate the mechanism of coagulation protease-mediated PAR2 induced MVs production and cancer induction to the recipient cells.
Methods: Collected PAR2 induced MVs from aggressive breast cancer cells MDA MB231 were fused with nonaggressive breast cancer MCF7. MVs biogenesis and mechanism the MV-induced transformation has been studied using western blot and RT PCR. Induced aggressive nature of MCF7 has been examined by in vitro migration and invasion assays and in vivo xenograft and metastasis assays.
Results: Here, we elicited that FVIIa and trypsin activate PAR2 and enhances MVs shedding from MDAMB231 cells. The elevation in MVs occurs through the activation of three independent (MAPK, P38, and Rho) signaling cascades. MAPK signals through activating MLCK followed by MLC phosphorylation to alter myosin organization whereas, P38 effects actin dynamics by activating MK2 and HSP27 sequentially. RhoA-dependent ROCKII activation also contributes to remodeling myosin II activity. Further, both our in vitro and in vivo analyses showed that these MVs potentiate invasive and migratory property to the recipient cells. Breast cancer patients blood also show an elevation of TF-bearing, pro-metastatic MVs than normal. We further found that MiR221 is transported via MDAMB231-derived MVs that fuses with MCF7 leading to enhanced recipient cell proliferation and metastasis both in vitro and in vivo via the induction of Epithelial to Mesenchymal Transition (EMT).
Conclusions: These findings give an insight about the detailed signaling mechanism PAR2 induced MV biogenesis and possible mechanism that how MVs induces cancerous property to the recipient cells. Understanding these mechanistic details will certainly provide crucial targets for therapeutic interventions in breast cancer progression

OC 55.1 | Platelet-dependent Activation of Thrombin-activatable Fibrinolysis Inhibitor (TAFI)

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Background: We recently reported that soluble thrombomodulin (TM) attenuated plasma clot lysis initiated from the activated platelet surface. The inhibition of thrombin-activatable fibrinolysis inhibitor (TAFI) reversed the attenuation of lysis after restoring the plasminogen accumulation on the surface of activated platelets. Thus, TAFI appeared a key molecule to regulate coagulation-dependent fibrinolysis.
Aims: We examined spatiotemporal regulatory mechanisms of TAFI activation and its function on activated platelets.
Methods:

1) Employing tissue-type plasminogen activator (tPA) induced clot lysis time (CLT) assay of platelet-containing plasma, effects of neutralizing antibody of TM (TM-Ab) and activated TAFI inhibitor (TAFIaI) were analyzed.
2) Fibrin network formation and its lysis, as well as the localization of the accumulation of fluorescence labeled plasminogen (see figure) and TAFI were analyzed by confocal microscopy.

3) The effect of TAFIaI was evaluated employing a microchip-based flow chamber system (Total Thrombus-formation Analysis System [T-TAS]) using tPA supplemented whole blood.

Results:

1) 1 Supplementation of platelets (~16×10^7/mL) prolonged CLT of normal plasma in a platelet concentration-dependent manner, which were abolished by either TM-Ab or TAFIaI. Such prolongation by normal platelets was not observed when TAFI deficient plasma was employed, suggesting that plasma TAFI was essential for platelet-dependent prolongation of CLT. 1)-2 TAFIaI also shortened CLT of FXIII deficient plasma, suggesting that cross linkage of TAFI to fibrin is not necessary for its function.
2) Plasminogen accumulation was accelerated by either TM-Ab or TAFIaI. Labeled TAFI also accumulated on both fibrin fiber and activated platelet surface, in which the former was Lys-binding site dependent whereas the latter was independent.

3) TAFIaI significantly prolonged the occlusion time of tPA containing whole blood in flow chamber, suggesting that TAFI attenuated tPA-dependent prolongation of clot formation under flow.

Conclusions: TAFI appeared to attenuate fibrinolysis on the activated platelet surface, which seems to also contribute to the thrombogenicity under flow condition.

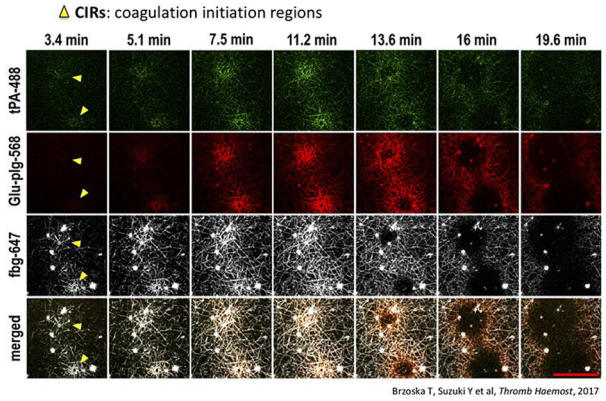

FIGURE 1 Platelet-dependent initiation of coagulation and fibrinolysis

OC 55.2 | The Imbalanced Hepatic Production of PAI1 and tPA Contributes to Decreased Fibrinolysis in Obesity

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Background: Fibrinolysis is initiated by tissue plasminogen activator (tPA) and inhibited by plasminogen activator inhibitor 1 (PAI1). The balance of tPA and PAI1 is critical for preventing excessive clotting without compromising hemostasis. Despite an increase in plasma tPA protein, obesity lowers blood tPA activity and fibrinolysis, but the mechanism is unknown. We showed that hepatocytes contribute substantially to basal plasma tPA and fibrinolysis.

Aims: To explore the regulation of tPA and PAI1 expression in hepatocytes, with the goal of understanding tPA-PAI1 imbalance and defective fibrinolysis in obesity.

Methods: Palmitate-treated human primary hepatocytes were treated with the indicated siRNAs, and diet-induced obese mice were treated with the indicated AAV8-H1-shRNAs, which silence genes specifically in hepatocytes.

Results: Both tPA and PAI1 mRNAs were increased in the livers of obese mice vs. lean mice. However, the fold increase in PAI1 mRNA was greater than that of tPA, and liver and plasma tPA activities were lower in obese mice. We uncovered a new PAI1 . tPA "compensatory" pathway: silencing PAI1 lowered tPA expression in hepatocytes, and, conversely, PAI1 treatment of hepatocytes induced tPA mRNA. Furthermore, we found that PAI1 activated CREB1, the transcription activator of tPA expression. Most importantly, hepatocyte-CREB1 silencing further lowered plasma tPA and fibrinolytic activity in obese mice. Finally, we explored the counterbalancing negative regulation of hepatic tPA by a transcription co-repressor DACH1.

Conclusions: Imbalance of hepatocyte PAI1 and tPA contributes to decreased systemic fibrinolysis in obesity. This imbalance is promoted by an obesity-induced increase in hepatic PAI1 mRNA and, through DACH1, limitation of the expression of hepatic tPA mRNA. However, the fibrinolysis defect in obesity is limited by a novel compensatory pathway in which PAI1, through CREB1, increases tPA mRNA. Therapeutic boosting of this compensatory pathway, and/or inhibition of the DACH1-tPA repression pathway may provide novel means to restore fibrinolytic balance in obesity.

OC 55.3 | Novel Plasmin Generation Assay Reveals a Plasminogen Activator Inhibitor-1-Independent Mechanism That Contributes to the Hemostatic Imbalance in Experimental Obesity

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Background: Obesity is a major risk factor for thrombosis. Multiple mechanisms likely drive this pathophysiology, including enhanced plasma procoagulant activity and increased expression of fibrinolysis inhibitors (e.g., PAI-1). The molecular underpinnings of the imbalance between procoagulant and fibrinolytic potential in obesity remain unclear, partly due to the lack of a sensitive and specific assay for monitoring plasmin generation (PG) kinetics in plasma.

Aims: Determine effects of obesity on plasma procoagulant and fibrinolytic activity.

Methods: Thrombin generation (TG) and PG were measured with specific fluorogenic substrates and fibrin formation/lysis by turbidity in plasmas from mice fed control diet (CD) or high fat diet (HFD) for 12 weeks.

Results: The novel PG assay reveals kinetic and quantitative information about plasmin formation. PG is sensitive to tissue plasminogen activator, plasminogen, α_2-antiplasmin, and active PAI-1. PG kinetics were mediated by fibrin formation, but not crosslinking. HFD-challenged mice developed marked weight gain (29.8±2.8 vs 38.9±4.8g, CD vs HFD, respectively) and steatohepatitis. HFD did not alter the TG lagtime or time-to-peak, but increased the rate and peak. In contrast, HFD delayed the PG lagtime and time-to-peak, and reduced the PG rate. Delayed PG in HFD-challenged mice was not due to changes in plasminogen, α_2-antiplasmin, or fibrinogen concentrations, as these did not differ in CD- or HFD-fed mice. Delayed PG in HFD-challenged mice also cannot be completely explained by elevated plasma PAI-1 (0.35±0.19 vs 0.88±0.41 ng/mL, CD vs HFD, respectively), since the concentration of active PAI-1

needed to inhibit PG was 100-fold higher than concentrations detected in HFD-fed mice. Effects of HFD on TG and PG led to normal fibrin formation kinetics, but delayed clot lysis.

Conclusions: The novel PG assay described here suggests a PAI-1-independent mechanism delays PG in plasma from obese mice. Combining TG and PG measurements may reveal synergistic effects of abnormal procoagulant and fibrinolytic activity in thrombotic disease.

OC 55.4 | Targeting Thrombolytics for Thrombotic Thrombocytopenic Purpura

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Background: Patients with thrombotic thrombocytopenic purpura (TTP) experience attacks of microvascular thrombosis, when platelets complex to von Willebrand Factor (VWF). This is caused by ADAMTS13 deficiency, which normally regulates the thrombogenicity of VWF. Current therapies aim to restore ADAMTS13 activity. This is hampered by persistent autoantibodies. We previously reported that systemic plasminogen activation (with streptokinase) was therapeutic in a TTP mouse model, suggesting that plasmin can act as an alternative to ADAMTS13. Although plasmin(ogen) can directly bind to VWF in microthrombi, natural plasminogen activators (tPA, uPA) cannot. For optimal therapeutic efficacy and safety, it is wishful to localize plasminogen activation to sites of microvascular occlusion.

Aims: Development of fusion proteins for targeted delivery of plasminogen activators to platelet-VWF complexes.

Methods: We fused the catalytic domain of urokinase (Mini-urokianse; mUPA) to nanobodies directed against VWF or platelet glycoprotein 1b (GP1b). As a control, we generated a non-targeting nanobody-uPA fusion protein (Neg-mUPA).

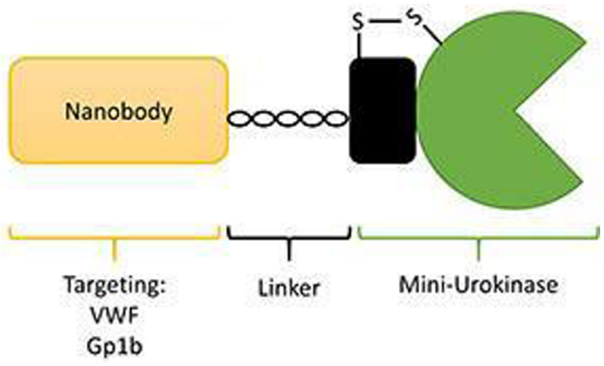

FIGURE 1 Nanobody-UPA fusion protein

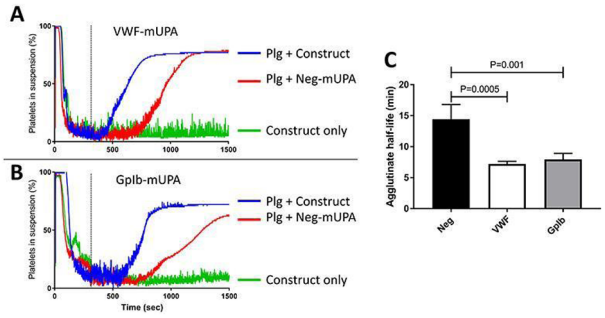

FIGURE 2 Targeting fusion proteins accelerate microthrombus breakdown

Results: Proteins were expressed in HEK293 cells and showed no spontaneous activity after isolation. We confirmed binding to VWF by ELISA (affinities of 30 pM, 330 pM and 12.8 nM for three constructs) and binding to GPIb by FACS. All constructs were enzymatically functional. VWF acts as a scaffold for accelerated plasminogen activation by VWF-targeting fusion proteins (6-fold increase). In VWF-platelet agglutination experiments, we found that targeting fusion proteins accelerate destruction of microthrombi (Fig.2; "Construct"), as compared to non-targeting controls (Fig.2; "Neg-mUPA"). At equimolar concentrations, targeting fusion proteins degrade microthrombi 7-fold more rapidly than a bivalent humanized nanobody sequence that is currently under clinical development. Under flow, targeting fusion proteins accelerate the removal of VWF-platelet strings from the endothelial surface in ADAMTS13 deficient plasma.

Conclusions: Targeted delivery of plasminogen activators to microthrombi is an attractive approach for the treatment of TTP.

OC 55.5 | Activated Thrombin Activable Fibrinolysis Inhibitor (TAFIa) Regulates Plasmin Generation on Thrombin-activated Platelets

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Background: Platelets contribute to fibrinolysis under flow conditions. However, the molecular mechanisms responsible for promoting and regulating fibrinolysis on the activated platelet surface are unknown.

Aims: To better understand platelet-dependent fibrinolysis by (a) characterizing the binding of plasminogen and tissue plasminogen activator (tPA) to activated platelets, (b) determining the effect of such binding on plasmin generation, and (c) examining whether

activated thrombin-activable fibrinolysis inhibitor (TAFIa) modulates platelet-dependent fibrinolysis.

Methods: Human and mouse platelets (isolated from wild-type or fibrinogen-deficient mice) were activated by thrombin or by protease activated receptor (PAR) 1 or 4 activating peptides (AP). In the presence or absence of TAFIa, binding of fluorescently labeled plasminogen or tPA was quantified using flow cytometry and plasminogen activation was quantified using a plasmin-specific chromogenic substrate.

Results: Plasminogen and tPA only bound thrombin-activated platelets in a fibrin-dependent manner, but not resting platelets. Plasminogen binding was lysine-dependent while tPA binding was both lysine-dependent and independent. Low concentrations of tPA enhanced plasminogen binding, whereas high concentrations decreased it consistent with the formation of ternary complexes with fibrin on the activated platelet surface. TAFIa attenuated plasminogen and tPA binding by 70% and 33%, respectively, and reduced plasmin generation by 57%. Activation of platelets with PAR 1/4-AP plus batroxobin (to induce fibrin polymerization) enhanced the binding of tPA but not plasminogen, suggesting that thrombin-mediated fibrin modification other than fibrinopeptide removal is essential. Mass spectrometry identified lysine 556 on the alpha-chain of fibrinogen as a newly generated C-terminal lysine, which is a target for plasminogen binding and TAFIa-mediated release.

Conclusions: Thrombin-activated platelets support the coordinated binding of plasminogen and tPA in a fibrin-dependent manner, and promote plasmin generation; a process attenuated by TAFIa. These studies provide new insights into the role of platelets in fibrinolysis.

OC 74.1 | Novel Murine Venous Thromboembolism Model to Evaluate Role of Factor XIII in Pulmonary Embolism Risk

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Background: Venous thrombosis and pulmonary embolism (PE), collectively venous thromboembolism, cause high morbidity and mortality. Factor XIII (FXIII) crosslinks fibrin and promotes red blood cell (RBC) retention in contracted thrombi, increasing thrombus size and stability. FXIII-mediated fibrin stabilization may also influence PE risk. Elucidating FXIII's role in PE is limited by the lack of models that recapitulate human PE characteristics.

Aims: Develop a model that permits embolization of fibrin- and RBC-rich thrombi formed in static blood. Determine the contribution of FXIII to PE risk.

Methods: We characterized the composition of human PEs and compared these to wild-type mouse venous thrombi (electrolytic, ferric chloride, inferior vena cava [IVC] stasis, and IVC stenosis), and mouse PEs generated by intravenous thrombin infusion (TI) or IVC stasis with subsequent ligature removal to permit embolization (LR model). We determined PE incidence in $F13a^{+/+}$, $F13a^{+/-}$, and $F13a^{-/-}$ mice subjected to the TI or LR models.

Results: Femoral vein electrolytic and ferric chloride injury rapidly produced small thrombi with few RBCs (5% and 4%, respectively), whereas IVC stasis and stenosis produced slower-forming, large thrombi with higher RBC content (68% and 76%, respectively), similar to human PEs (68%). Although $F13a^{+/+}$, $F13a^{+/-}$, and $F13a^{-/-}$ mice had similar PE incidence (60%, 67%, and 70%, respectively) in the TI model, PE were small, with low RBC content (≤ 7%), unlike human PEs. The LR model permitted embolization of existing RBC-rich thrombi, resulting in large PEs. In the LR model, compared to $F13a^{+/+}$ (13%), $F13a^{+/-}$ and $F13a^{-/-}$ mice had similar or increased PE incidence (17% and 64%, respectively).

Conclusions: The TI model produces microthrombi histologically-dissimilar to human PEs. The LR model produces fibrin- and RBC-rich PEs similar to human PEs. Complete FXIII deficiency increases PE incidence, but partial deficiency does not. Partial FXIII reduction may beneficially decrease VT burden without increasing PE.

OC 74.2 | Understanding the Chromatin Regulatory Landscape Controlling Fibrinogen Gene Expression

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Background: The human fibrinogen locus can adopt a looped chromatin configuration that contributes to fibrinogen expression. Within this loop are the three fibrinogen genes (*FGA*, *FGB* and *FGG*), their proximal promoters, and four liver cell enhancers. The functional importance of these enhancers and their regulatory hierarchy is unclear.

Aims: To understand the regulatory element hierarchy that coordinates expression of the fibrinogen gene cluster.

Methods: We used a CRISPR-Cas9-based method to direct an active regulatory element histone modification, acetylation of histone H3 lysine 27 (H3K27ac), to fibrinogen promoters and enhancers in non-fibrinogen-expressing HEK-293T cells. Transfection of combinations of plasmids for expression of single guide RNAs (sgRNAs) and a nuclease-deficient Cas9 nuclease fused to the histone acetyltransferase core domain of p300 (dCas9-p300), were used to direct p300 catalytic activity and H3K27ac to specific chromatin sites. Fibrinogen expression was measured by qRT-PCR.

Results: *FGA* and *FGB* transcripts were not detected in HEK-293T cells, *FGG* is expressed near the lower limit of qRT-PCR detection. dCas9-p300 and sgRNAs directed to the *FGA*, *FGB* and *FGG* proximal promoters induced expression of *FGA* and *FGB* and increased *FGG*

expression, respectively. The *FGG* gene, with pre-existing basal expression, showed increased expression when targeting dCas9-p300 to any of the gene cluster enhancers. However, increased expression of the *FGA* and *FGB* genes, by directing dCas9-p300 to enhancers, was only measured when their proximal promoters were also targeted. By concurrently targeting a single fibrinogen promoter and individual enhancers for H3K27ac we measured similar gene expression increases for each enhancer on each gene, suggesting functional redundancy among the fibrinogen locus enhancer elements.

Conclusions: Active enhancer elements of the fibrinogen gene cluster can act on any of the fibrinogen genes, provided the proximal gene promoters are in a transcriptionally active state. This redundancy in enhancer activity can now be investigated by sequential deletion of enhancers in fibrinogen-expressing cells.

OC 74.3 | The Fibrin αC Region is Critical for Mechanical Strength, Resistance to Fibrinolysis and the Formation of a Stable Whole Blood Clot

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Background: The fibrinogen αC-region contains FXIII, α2-antiplasmin and plasminogen binding sites, and is comprised of a globular domain (αC-domain) and connector region. We previously showed that truncations of the αC-region changed fibrin clot structure in purified systems. The relative contributions of the connector and the αC-domain to alterations in whole blood clot structure and function are unknown.

Aims: To understand the influence of the αC-domain and connector on clot lysis, mechanics and contraction.

Methods: Recombinant fibrinogens wild type (WT), α390 (lacking αC-domain) and α220 (deficient in αC-region) were produced in CHO cells. Fibrinolysis was studied through turbidity and clot mechanics by magnetic microrheology. Ex-vivo investigations of clot contraction, rotational thromboelastometry (ROTEM) and electron microscopy (SEM) were performed using whole blood from fibrinogen$^{-/-}$ mice reconstituted with recombinant fibrinogens. Results are shown as mean±SD.

Results: WT clots showed a 50% lysis time of 74min (±4.78,n=4), which was similar for α390 (69 ±11.97min,n=4), but α220 lysed significantly faster (18±3.63min,n=4). Clot mechanics for α220 could not be studied without FXIII, as clots were exceptionally weak. With FXIII, α220 clots showed markedly reduced storage modulus (G') (0.021±0.071Pa,n=4) compared to WT (2.4±0.357Pa,n=4), while α390 was slightly elevated (3.5Pa±0.737Pa,n=4). No difference in clot weight, contraction or platelet incorporation was observed comparing WT to α390, while α220 showed no platelet-driven contraction, and no visible clots after 120min. ROTEM displayed reduced clot firmness for both truncations, and increased clotting time for α220. SEM of α220 after ROTEM showed clots mainly composed of activated platelets with limited fibrin present.

Conclusions: Our results show that without αC-domains and connector, fibrin is extremely weak, loses resistance to fibrinolysis and does not support stable clot formation in whole blood. These data indicate that the fibrinogen αC-connector provides critical mechanical strength, stabilises the fibrin network and supports normal clot formation in whole blood.

OC 74.4 | Venous Thrombosis and Thrombocyte Activity in Zebrafish Models of Quantitative and Qualitative Human Fibrinogen Disorders

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Background: Afibrinogenemia and dysfibrinogenemia are caused by mutations in the fibrinogen genes and typically lead to a bleeding tendency and the possibility of thromboembolic complications. Bleeding can be explained by deficiency in the major blood clotting protein substrate, but the pathophysiology of thromboembolic events in fibrinogen deficiency has not been extensively studied.

Aims: Using zebrafish with a deletion in *fga* (*fga*$^{-/-}$), and a mutant designed to mimic a human dysfibrinogenemia mutation (Aα R35C) lacking a functional Aα chain N-terminal thrombin cleavage site, our study aimed to demonstrate the impact of afibrinogenemia and dysfibrinogenemia on hemostasis upon vascular challenge.

Methods: Zebrafish mutants were prepared using genome-editing of *fga* exon 2. Fibrinogen susceptibility to thrombin cleavage was assessed in plasma. Embryonic vascular injuries were made using a laser targeting the caudal vein. Time-to-occlusion (TTO) was measured as a global assessment of hemostasis and thrombosis. Thrombocyte adhesion and aggregation were recorded by monitoring fluorescent thrombocytes at the injury site.

Results: 3-day-old *fga*$^{-/-}$ zebrafish failed to support venous thrombosis (n=26) after laser injury whereas >90% of wild-type (n=29) and heterozygous (n=43) siblings showed vessel occlusion. Thrombocyte adhesion and aggregation were reduced in 5-day-old afibrinogenemic fish, compared to controls, and embolic cell aggregates were observed. The R28C mutation in *fga* lead to in-frame exon 2 skipping of its mRNA. The encoded Aα chain lacks a thrombin cleavage site, resembling the Aα chain in hypodysfibrinogenemia patients with an *FGA* splicing mutation. Fibrinogen Aα from *fga*$^{R28C/R28C}$ plasma was insensitive to thrombin cleavage. Prolonged TTO and reduced thrombocyte adhesion and aggregation were measured in *fga*$^{+/R28C}$ (n=42) and *fga*$^{R28C/R28C}$ (n=16) fish, compared to wild-types (n=17).

Conclusions: Zebrafish with afibrinogenemia cannot support venous thrombosis or sustained thrombocyte aggregation, whereas those with a mutated fibrinogen Aα chain thrombin cleavage site show prolonged thrombosis times and reduced thrombocyte aggregation, even in heterozygosity.

OC 74.5 | Detection and Analysis of Gene Mutations in Patients with Fibrinogen Deficiency by Next Generation Sequencing

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Background: Fibrinogen deficiency can be quantitative (afibrinogenemia, hypofibrinogenemia) or functional (dysfibrinognemia). Congenital fibrinogen disorders can lead to the development of bleeding or thromboembolic events. When striving for applicability in routine clinical practice, it soon becomes clear that population Sanger sequencing is too labor-intensive and time-consuming.

Aims: In this study, we aimed to assess FGA, FGB, FGG gene mutations through next generation sequencing (NGS) in diagnosed patients of Fibrinogen deficiency.

Methods: A cohort of 19 patients with clinical symptoms who underwent laboratory were enrolled for molecular genetic analysis by NGS. Sample DNA was amplified using Ampliseq primer panel, and libraries were prepared following the manufacturer´s Ion Ampliseq Library Preparation protocol. Individual samples were barcoded, pooled, templated, and sequenced on the Ion Torrent Proton Sequencer. Raw sequencing reads generated by the Ion Torrent sequencer were quality and adaptor trimmed by Ion Torrent Suite and then aligned to the hg19 reference sequence.

Results: We identified 16 pathogenic mutations, including 9 novel mutations, from all the 19 patients. Amon those mutations, mutations in FGA genes were p.L506fs, p.G36S, p.R743Q, p.M11T, p.R38G, p.L28P, p.R35C and p.R35H; mutations in FGB gene were p.N190fs and p.R285C; mutations in FGG gene were p.D167G, p.R301H, p.N351D, p.C365G, p.S404F, p.R301C. Compound heterozygous mutations were identified in 2 patients, 1 patients with FGA: p.R743Q and p.M11T, and 1 patient with FGB: p.N190fs and p.R285C.

Conclusions: NGS technology is systematic and efficient, and may be a useful method to detect the mutations in Fibrinogen deficiency patients.

HEMOPHILIA AND BLEEDING (INCLUDING TRANSFUSION)

OC 01.1 | AMT-061 (AAV5-Padua hFIX variant) an Enhanced Vector for Gene Transfer in Adults with Severe or Moderate-Severe Hemophilia B: Follow-up up to 9 Months in a Phase 2b Trial

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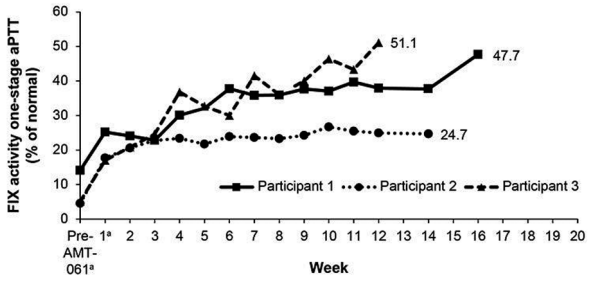
Background: Gene therapy for hemophilia offers the potential of sustained-disease amelioration with a single treatment. AMT-061 is an investigational gene therapy for hemophilia B comprised of an adeno-associated virus serotype 5 (AAV5) vector containing a codon-optimized Padua variant human factor IX (FIX) gene with liver-specific promoter.

Aims: Confirm that a single dose of AMT-061 will provide a minimum-therapeutic response of FIX activity 6-weeks post-dose in participants with severe or moderate-severe hemophilia B.

Methods: Phase 2b, open-label, multi-center trial (NCT03489291) in adult males requiring FIX prophylaxis and without active hepatitis or uncontrolled HIV. Participants were not excluded based on neutralizing antibodies to AAV5. Participants received a single intravenous dose of AMT-061 ($2x10^{13}$ gc/kg) and will be followed for 5-years. The primary endpoint was FIX activity at Week 6. Secondary endpoints include e-diary recordings of bleeds and FIX concentrate use, laboratory parameters, joint health, patient-reported outcomes, and adverse events (AEs).

Results: All participants had FIX < 1% (severe FIX deficiency) and had neutralizing activity to AAV5 at baseline. Following AMT-061 treatment, FIX activity increased rapidly (Figure) and achieved a mean of 31% at Week 6. FIX activity increased further to a mean of 38% at Week 12. As of 13 Dec 2018, FIX activity was 48% at Week 16, 25% at Week 14, and 51% at Week 12 in participants 1-3, respectively. There were no bleeds post-treatment and no requirement for FIX replacement. Alanine aminotransferase levels did not rise above normal limits after dosing for all participants. One participant experienced 2 mild AEs possibly related to treatment (self-limiting headache and slightly elevated CRP). Updated results to 26-39 weeks of follow-up will be presented.



aPTT, activated partial thromboplastin time; FIX, Factor IX. No immunosuppression required.
[a]May include activity from exogenous FIX replacement

FIGURE 1 FIX activity over time

Conclusions: Sustained therapeutic responses of FIX activity were observed 12-weeks after treatment with AMT-061. AMT-061 was well-tolerated with no requirement for immunosuppression. These data support the ongoing Phase 3 study.

OC 01.2 | Initial Results of the Alta Study, a Phase 1/2, Open Label, Adaptive, Dose-ranging Study to Assess the Safety and Tolerability of SB-525 Gene Therapy in Adult Subjects with Severe Hemophilia A

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Background: Hemophilia A is a rare blood disorder caused by a *F8* variant resulting in insufficient activity of Factor VIII.
Aims: Preliminary results of an ongoing gene therapy study in subjects with severe hemophilia A are presented.
Methods: The Alta study is a dose-ranging, single-dose study using SB-525 gene therapy, a recombinant adeno-associated virus (rAAV6) vector carrying a Factor VIII gene. SB-525 was injected into 8 subjects in 4 cohorts of 2 patients each over 4 ascending doses starting at e11 vg/kg. Endpoints included safety events, changes in circulating FVIII activity, FVIII replacement therapy usage, and frequency and severity of bleeding. Informed consent was obtained for each subject and the trial was performed according to the Declaration of Helsinki and principles of Good Clinical Practice.
Results: After the initial 2 cohorts, a single infusion of SB-525 resulted in stable and clinically relevant increases in Factor VIII activity, and a marked reduction in factor replacement therapy. The highest dose cohort reached Factor VIII levels within the normal range. One subject reported a treatment-related serious adverse event of hypotension and fever, with symptoms of headache and tachycardia, which occurred after the vector infusion and resolved with treatment within 24 hours. In the three first cohorts, no ALT elevation requiring more than 7 days of corticosteroid treatment was observed. The fourth cohort is ongoing, and additional analyses of the trial data including Factor VIII levels, bleeding rate and usage of factor replacement therapy will be collected and presented as available.
Conclusions: To date, treatment with a single infusion of SB-525 gene therapy resulted in dose-dependent, sustained increased FVIII levels, with a substantial decrease in FVIII usage. No transaminitis longer than 7 days was observed in the first three cohorts. The study is ongoing, and these results support further development of SB-525 for the treatment of severe Hemophilia A.

OC 01.3 | Health-related Quality of Life Improvements in Adult Haemophilia B Patients at One Year Follow-up after Receiving *SPK-9001* Gene Transfer

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Background: Factor replacement therapy in haemophilia is related to treatment burden and impacts patients' health-related quality of life (HRQoL). Gene therapy in haemophilia B patients demonstrates sustained factor IX activity following gene transfer resulting in eliminated or reduced bleeding events in all subjects.
Aims: Evaluation of HRQoL improvements at one year follow-up after gene transfer.
Methods: Fifteen adult haemophilia B patients (baseline FIX:C ≤2 IU/dL) with a mean age of 38.6±14.5 years (range 18-61) received a single 5x1011 vg/kg infusion of *SPK-9001*, an adeno-associated virus (AAV) factor IX gene transfer product. HRQoL was assessed with the EQ-5D/EQ-VAS and Haem-A-QoL questionnaires pre-vector (baseline) and post-vector (weeks 4, 12, 26 and 52) infusion. Differences were calculated from baseline (B) to post-vector (PV: week 52). Minimal clinically important differences (MCID) evaluating subject-level HRQoL improvements and HRQoL responder definitions (RDs) were estimated based on the one-half standard deviation criterion at baseline.

Results: Data from 11 of 15 subjects with 52 weeks follow-up after gene transfer are available. At PV they had a median of 0 bleeds (range 0-4) and 0 infusions (range 0-10). All participants reported good HRQoL in the EQ-VAS with significant improvement (B: M=79.4±5.4 vs. PV: M=88.1±5.3; p< 0.001). Significant improvements were found also for Haem-A-QoL domains 'Future' (p< 0.004), 'Treatment' (p< 0.004), 'View' (p< .007), 'Sport' (p< .017), 'Feeling' (p< .049) and 'Total Score' (p< 0.001). At baseline, 63.7% 'always/often' "felt dependent on factor concentrates" compared to 9.3% at PV (p< 0.0001). All patients (100%) were HRQoL responders according to the RDs of the Haem-A-QoL 'Total Score' (3.9-point decrease) and 72.7% according to the RDs of the EQ-VAS (4.2-point increase).
Conclusions: Improvements in Haem-A-QoL domains and the EQ-VAS after a single infusion of *SPK-9001* resulted in meaningful HRQoL improvements. These prospective data represent the first assessment of HRQoL measures in haemophilia B patients following gene transfer.

OC 01.4 | Stable Expression of FIX and Maintained Reductions in Bleeding and Factor IX Consumption Following AMT-060 Gene Therapy with up to 3.5 Years of Follow Up in Adults with Severe or Moderate-Severe Hemophilia B

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Background: Gene therapy aims to provide a long-term therapeutic effect from a single administration, yet knowledge on durability is limited. AMT-060 is an adeno-associated virus serotype 5 (AAV5) vector with a codon-optimized wildtype human factor IX (FIX) gene and liver-specific promoter studied in 10 participants with severe/moderate-severe hemophilia B (Phase 1/2 study, NCT02396342). We previously reported up short-term follow-up.
Aims: To describe follow-up data for up to 3.5-years post-AMT-060.
Methods: Adult males with FIX activity ≤2% and a severe bleeding phenotype received a single intravenous infusion of AMT-060 ($5x10^{12}$gc/kg, Cohort 1, n=5) or ($2×10^{13}$ gc/kg, Cohort 2, n=5). Assessments included FIX activity, FIX replacement use, annualized bleeding rate (ABR), treatment-related adverse events (TRAE), vector shedding, immunological and inflammatory biomarkers up to 3.5 years (Cohort 1) and 3 years (Cohort 2).
Results: As of 15 May 2018, mean FIX activity was 4.9% and 7.4% over 2.5- and 2-years follow-up for Cohorts 1 and 2, respectively. Eight of 9 participants using prophylaxis at baseline were able to discontinue use. Mean ABR decreased from 14.4 pre-AMT-060 to 5.4 over 2.5-years follow-up (Cohort 1) and from 4.0 pre-AMT-060 to 1.0 over 2-years follow up (Cohort 2). No participants developed FIX inhibitors or signs of sustained AAV5 capsid-specific T-cell activation. TRAE were only reported in the first 3.5-months after treatment, including three participants who experienced transient mild elevations in alanine aminotransferase as previously described, but no TRAE were observed over the subsequent follow-up to 2.5-years. Updated data up to 3.5-years of follow-up will be presented.
Conclusions: Long-term stable endogenous FIX activity and reductions in ABR and FIX replacement use were observed following a single treatment with AMT-060, with no additional safety concerns. These findings support the ongoing Phase III study of the enhanced construct, AMT-061, which encodes the highly-active Padua FIX variant.

OC 05.1 | Common and Rare Genetic Determinants of von Willebrand Factor Clearance and Factor VIII-binding Modify Factor VIII Pharmacokinetics in a Pediatric Hemophilia A Population

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Background: FVIII pharmacokinetics show significant intra-patient consistency and high inter-patient variability in hemophilia A patients, although genetic factors beyond ABO blood group that mediate these effects are largely uncharacterized. We have previously demonstrated that FVIII pharmacokinetics associate with plasma VWF:Ag, VWFpp/VWF:Ag ratio, VWF:FVIII binding activity and ABO blood group status in a pediatric population of severe hemophilia A patients.
Aims: In this study, we describe genetic variants that modify FVIII pharmacokinetics by influencing VWF-FVIII clearance, or VWF-FVIII binding activity.
Methods: 43 male patients (age 6 - 17.7 years) with severe hemophilia A were dosed with recombinant FVIII and sampled in accordance with an ISTH-recommended pharmacokinetic schedule. FVIII pharmacokinetic parameters were evaluated using TCIWorks. Informed consent was obtained for patient participation. The *VWF*

gene FVIII-binding D'D3 region and variants in the *CLEC4M*, *SCARA5* and *STAB2* loci, which modify VWF clearance, were genotyped.

Results: The FVIII-binding activity of VWF positively correlated with FVIII half-life (r=0.5179, p=0.001) and the heterozygous inheritance of the rare or low frequency, non-synonymous VWF variants p.(R826K) (n=1) and p.(R852Q) (n=4) were associated with reduced VWF:FVIIIB (p=0.05) but not plasma VWF:Ag (p=0.159). The *VWF* variants rs1063856/rs1063857 (p=0.05) and the *CLEC4M* variant rs868875 (p=0.039) associated with the rate of FVIII clearance while the *STAB2* variant rs4981022 associated with FVIII half-life (p=0.007), and VWF:Ag levels (p=0.015) by regression analysis. The *CLEC4M* variable number of tandem repeat (VNTR) genotype associated with FVIII clearance (p=0.002) and half-life (p=0.019) as determined by one-way ANOVA. When compared to patients in the quartile with the shortest FVIII half-life (n=11), the patients in the quartile with the longest FVIII half-life (n=11) had an increased frequency of non-O blood group (p=0.024), the *STAB2* rs12229292 variant (p=0.045), and the *CLEC4M* 9-VNTR allele (p=0.049).

Conclusions: Genetic variants that modify VWF-FVIII binding and VWF-FVIII clearance associate with FVIII pharmacokinetics in pediatric patients.

OC 05.2 | Development and Characterization of an ELISA Combined Chromogenic Assay for Selective Measurement of Human FVIII Activity in Animal Plasma Matrix

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Background: During the non-clinical development of human coagulation factor VIII (FVIII) drug candidates or the evaluation of alternative administration routes, it is often required to test them in animal models having normal levels of endogenous FVIII. This however challenges bioanalytical measurements to quantify human FVIII and differentiate it with sufficient accuracy and precision from FVIII of the animal.

Aims: Here, we describe the development and qualification of a simple method to specifically determine the hemostatic activity of human FVIII in animal plasma matrix.

Methods: The assay´s principle is based on capturing human FVIII specifically by anti-human FVIII antibodies immobilized on the wells of a microtiter plate. After incubation, a subsequent washing step removes any unbound components including animal FVIII. Then, reagents of a chromogenic assay for FVIII are added which include FIXa, FX, calcium, phospholipids and thrombin allowing FVIIIa to act as a cofactor for FIXa-mediated FXa generation. Finally, FXa generated is detected by a specific substrate for FXa and FVIII chromogenic activity is determined by comparison with a FVIII reference preparation.

Results: Several anti-FVIII antibodies for capturing human FVIII had been evaluated allowing a subsequent determination of FVIII activity. While a polyclonal anti-human FVIII antibody interfered with activity measurement, an anti-human FVIII B domain antibody was compatible. Spiking of human recombinant FVIII (hrFVIII) to canine and porcine normal plasma confirmed the selectivity of the assay. While a linear dose-response relationship in a concentration range between 0.04 and 0.625 IU/mL was observed for hrFVIII, animal plasma alone gave no signal above the buffer blank value. Checking the assay performance revealed acceptable intermediate precision and accuracy well within the expected ranges of biological assays.

Conclusions: A method allowing the selective quantification of hemostatic activity of human FVIII in animal plasma matrix was successfully developed. This new method is a valuable bioanalytical tool supporting nonclinical development studies.

OC 05.3 | FXII Activation Leading to Kallikrein and Bradykinin Formation in vitro Induced by Some Plasma Derived FVIII Concentrates, But Not by Recombinant FVIII

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Background: Alegre et al. described 1986 the activation of the kinin prekallikrein system after infusion of FVIII concentrates in haemophilia patients.

Aims: As current plasma derived FVIII concentrates contain quite high amounts of misfolded proteins and activates both platelets and neutrophils it is very likely that they might either directly and/or indirectly activate FXII.

Methods: Five pdFVIII products (Octanate®, HaemateP®, Immunate, Haemoctin® and Beriate®) and two rFVIII products (Kovaltry® and Advate®) were studied. NanoSight tracking analysis (NTA) was used for visualization of nanoparticles and protein aggregates. FXII-Prekallikrein activating activity was measured in the presence of various concentrations of FVIII products using the chromogenic substrate S-2302. S-2302 cleavage by autoactivation without FXII as used as negative control. Platelet activation releasate was used as positive control. For measurement of bradykinin formation by kallikrein, single chain high molecular weight kininogen (HMWK) was added to the reaction mixture without chromogenic kallikrein substrate and incubated for 10 min. Unreacted HMWK was removed after 30 minutes and bradykinin was quantified by a competitive ELISA.

Results: The tested pdFVIII products contained significantly more protein nanoparticles than the tested recombinant FVIII products Kovaltry

and Advate. Immunate, and Haemate induced a significant activation of FXII/kallikrein pathway, while this effect was not observed with Beriate, Advate and Kovaltry. While bradykinin formation induced by Kovaltry, Advate and Beriate did not differ from the negative control, Immunate and Haemate induced a significant bradykinin formation.

Conclusions: As the FXII/kallikrein pathway is proinflammatory and as its product bradykinin activates signaling pathways resulting in increased vasodilation, vascular permeability, edema formation and chemotaxis of neutrophils as well as inducing pain, our findings might be of clinical interest. Further research aims to investigate the effect of factor VIII products ex vivo. This work was supported by a grant of Bayer Vital GmbH, 51368 Leverkusen, Germany

OC 05.4 | LPS-induced Release of Monocyte Microvesicle-associated Tissue Factor Procoagulant Activity (MV TF PCA) Correlates with Markers of Systemic Inflammation in Patients with Hemophilia A and B

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Background: In congenital hemophilia, deficiency in FVIII or FIX results in a severely impaired intrinsic amplification loop of blood coagulation. At sites of vascular injury, thrombin generation and fibrin deposition are thus critically dependent on the extrinsic, TF-driven coagulation pathway. Although cytokine-mediated activation of monocytes/macrophages is a hallmark of blood-induced synovitis, no previous study has systematically investigated the link between inflammatory markers and monocyte TF expression in patients with hemophilia A and B.

Aims: To study the expression and release of monocyte TF in hemophilia patients under inflammatory conditions using an ex-vivo endotoxinemia model.

Methods: Monocyte TF antigen in citrate-anticoagulated whole blood was analyzed by two-color flow cytometry directly after blood draw (baseline) and after incubation with buffer (PBS) or LPS for 4 hours at 37°C and expressed as TF-specific mean fluorescence intensity (MFI). In addition, TF PCA of isolated plasma MVs was measured by a chromogenic Xa generation assay.

Results: We studied 43 patients (34 ± 13 years) with moderate or severe hemophilia A (n=34) or B (n=9) in comparison to 23 age- and sex-matched controls. While essentially no monocyte TF antigen and MV TF PCA was detectable in baseline and PBS-treated samples from both groups, stimulation with LPS increased TF MFI (patients vs. controls) to 5.9 ± 3.1 vs. 4.6 ± 2.5 arbitrary units (AU) (mean ± SD; P=0.08) and MV TF PCA to 10.2, 0.2-123.2 vs. 4.6, 1.0-15.0 AU (median, range; P=0.02). IL-6 and hs-CRP levels in baseline serum samples were significantly increased in patients compared to controls and correlated with LPS-induced MV TF PCA in the patient cohort (P< 0.01).

Conclusions: Our findings indicate a novel link between systemic inflammation and inducible monocyte TF expression and release in patients with congenital hemophilia, which could be of pathophysiological relevance in the development and progression of hemophilic arthropathy.

OC 05.5 | Identification of the Origin of de Novo Mutation in Hemophilia A without Prior Family History and Categorization of the Event how the de Novo Mutation Occurrs

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Background: The origin and occurrence of *de novo* mutation are distinct events, a person designated as origin of a *de novo* mutation

TABLE 1 Summary of the origin and occurrence of de novo inversion mutations of the hemophilia A gene in patients without prior family history

Mutation Type	Family file No.	Origin of mutation	Occurrence of de novo mutation	How the de novo mutation occurred?
Intron 22 Inversion	16, 64, 129, 138, 140, 142, 168, NTUH-1, NTUH-2, NTUH-3	MGFs+	Mothers	Single germ cell (male meiosis) mutation occurred in MGF causing heterozygous mutation in mothers, most likely
Intron 22 Inversion	174	MGM+	Mother	Mutation occurs in zygote within first few cell divisions occurred in the mother, very likely
Intron 1 inversion	NTUH-4	MGF	Motehr	Single germ cell (male meiosis) mutation occurred in MGF causing heterozygous mutation in the mother, most likely
Intron 22 Inversion	20	MGGM+ and MGM are carriers	-	inherited
Intron 22 Inversion	104	MGM is a carrier	-	inherited

+MGF: maternal grandfather MGM: maternal grandmother MGGM: maternal great grandmother

TABLE 2 Summary of the origin and occurrence of de novo noninversion mutations of the hemophilia A gene in patients without prior family history

Family file No.	Amino acid substitution	Origin of mutation	Occurrence of de novo mutation	How the de novo mutation occurred?
74, 31, 189	p.R2016G, p.W274C, p.R550C	Mothers	Patients	Mutation occurs in zygote within first few cell divisions in patients, most likely
78, 131	p.I1213F fs*5, splicing site mutation	MGMs+	Mothers	Mutation occurs in zygote within first few cell divisions in mothers, most likely
55	Exon25 deletion	Either MGF+ or MGM	Mother	Mutation occurs in zygote within first few cell divisions in the mother, most likely
153	p.R509*	MGM	Mother	Mutation occurs early in postzygotic embryogenesis causing somatic mosaicism and germline mutation (or germline mosaicism) in the mother, most likely
130	p.R546W	MGF+	Mother	Mutation occurs early in postzygotic embryogenesis causing somatic mosaicism and germline mutation (or germline mosaicism) in the mother, most likely
105	p.S62*	Either MGF or MGM	Mother	Mutation occurs early in postzygotic embryogenesis causing somatic mosaicism and germline mutation (or germline mosaicism) in the mother, most likely
149, NTUH	p.I1213F fs*5, p.617Sfs*7	MGGMs+	MGFs	Mutation occurs early in postzygotic embryogenesis causing somatic mosaicism and germline mutation (or germline mosaicism) in MGFs, most likely
155	p.Y1781*	Either MGGM++ or MGGF++	MGM	Mutation occurs early in postzygotic embryogenesis causing somatic mosaicism and germline mutation (or germline mosaicism) in the MGM, most likely

+ MGF: maternal grandfather, MGM: maternal grandmother, MGGM: maternal great grandmother, MGGF: maternal great grandfather. ++ Deceased

should be normal without that mutation. How the *de novo* mutation occurred were not well studied.

Aims: We want to study the origin and occurrence of *de novo* mutation in hemophilia A without prior family history.

Methods: Linkage analysis using intragenic and extragenic markers of factor VIII gene was done to identify the possible origin of *de novo* mutation. A technique named amplification refractory mutation system-quantitative polymerase chain reaction was used to determine the percent noninversion mutant cells in blood cells and buccal cells and to detect the real origin of mutation and the presence of mosaic mutation. How the *de novo* mutation occurred was analyzed according to the categorization of *de novo* mutation described by Freed D et al (Genes 2014).

Results: As shown in table 1, 10 intron 22 inversions and one intron 1 inversion originated from maternal grandfather (MGF) and most likely occurred in a single sperm cell during meiosis causing heterozygous mutation in mothers. The one intron 22 inversion originated from maternal grandmother (MGM) very likely occurred in zygote within first few cell divisions in the mother. As shown in table 2, among the 12 *de novo* noninversion mutations, 3 originated from mothers, 6 from maternal grandparents and 3 from maternal great grandparents; they occurred most likely in zygote in first few cell divisions in patients and mothers as well as in early postzygotic embryogenesis causing somatic and germline mosaicism in mothers, MGFs and MGM, respectively.

Conclusions: In hemophilia A, the *de novo* inversion mutation originated almost exclusively in male (MGF) and occurred in a single sperm cell. *De novo* noninversion mutations originated frequently in females (8/9, 88.9%) and somatic and germline mosaic mutations were frequently observed (6/12, 50%).

OC 11.1 | BIVV001: The First Investigational Factor VIII (FVIII) Therapy to Break through the von Willebrand Factor (VWF) Ceiling, with Potential for More Optimal, Extended Protection in Hemophilia A

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Background: Severe hemophilia A treatment goals are expanding to higher sustained FVIII levels that prevent joint bleeds (eg, ≥15%; Uijl

TABLE 1 PK parameters[a,b]

	25 IU/kg cohort			65 IU/kg cohort		
	BIVV001 (n=6)[c]	rFVIII (n=7)	Mean ratio	BIVV001 (n=8)[d]	rFVIII (n=9)	Mean ratio
$t_{1/2}$, h	37.61 (33.28-42.50)	9.12 (6.24-13.33)	4.13 (2.94-5.79) *P* <0.001	42.54 (39.72-45.56)	13.15 (10.89-15.87)	3.24 (2.76-3.79) *P* <0.001
C_{max}, IU/dL	70.1 (49.7-98.9)	51.8 (43.3-61.9)	1.35 (1.04-1.77) *P*=0.032	161 (142-183)	138 (117-162)	1.17 (1.09-1.25) *P* <0.001
$AUC_{0\text{-inf}}$, h × IU/dL	4470 (3280-6080)	638 (495-822)	7.00 (5.78-8.48) *P* <0.001	12,800 (11,100-14,900)	1960 (1670-2310)	6.54 (5.89-7.27) *P* <0.001
MRT, h	56.93 (49.19-65.89)	12.54 (9.82-16.01)	4.54 (3.64-5.66) *P* <0.001	67.66 (62.59-73.14)	15.66 (14.06-17.45)	4.32 (3.96-4.72) *P* <0.001
CL, mL/h/kg	0.56 (0.41-0.76)	3.91 (3.05-5.02)	0.14 (0.12-0.17) *P* <0.001	0.51 (0.44-0.59)	3.31 (2.81-3.88)	0.15 (0.14-0.17) *P* <0.001
IR, IU/dL per IU/kg	2.72 (1.95-3.80)	2.00 (1.60-2.50)	1.36 (0.98-1.89) *P*=0.063	2.48 (2.18-2.82)	2.11 (1.79-2.49)	1.18 (1.10-1.26) *P* <0.001

$AUC_{0\text{-inf}}$, total area under the curve from time zero to infinity; CL, clearance; C_{max}, maximum concentration; IR, incremental recovery; MRT, mean residence time; PK, pharmacokinetic; rFVIII, recombinant factor VIII; $t_{1/2}$, elimination half-life.

[a] Factor VIII activity determined by the one-stage activated partial thromboplastin time clotting assay.

[b] Values are geometric means (95% confidence interval).

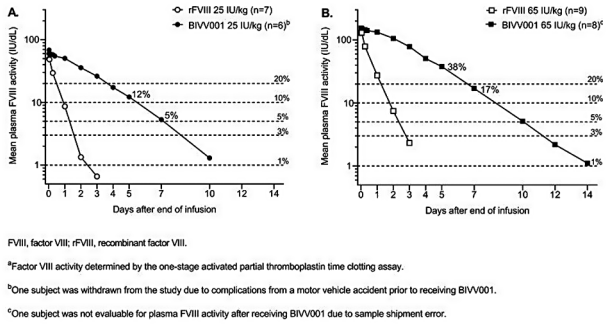
[c] One subject was withdrawn from the study due to complications from a motor vehicle accident prior to receiving BIVV001 and was not evaluable for BIVV001 PK parameters.

[d] One subject was not evaluable for BIVV001 PK parameters due to sample shipment error.

D, et al. *Haemophilia* 2011) and provide long-term joint protection. Endogenous VWF binds to FVIII and, for extended half-life ($t_{1/2}$) recombinant FVIII (rFVIII) products, imposes a $t_{1/2}$ ceiling and limits bleed protection. BIVV001 (rFVIIIFc-VWF-XTEN), a novel fusion of rFVIII Fc protein, XTEN polypeptides, and the FVIII-binding VWF D′D3 domain, circulates independently of VWF.

Aims: We assessed safety, tolerability, and pharmacokinetics (PK) of single-dose BIVV001 in the ethics committee-approved EXTEN-A (NCT03205163) Phase 1/2a trial.

Methods: Previously treated adults with severe hemophilia A (< 1 IU/dL [< 1%] endogenous FVIII activity), ≥150 FVIII product exposure days, and no inhibitors provided informed consent and were assigned to 25 IU/kg (n=7) or 65 IU/kg (n=9) cohorts. Subjects received single-dose rFVIII and, ≥3 days later, single-dose BIVV001. Blood samples were collected for PK and 14- and 28-day post-BIVV001 inhibitor testing. Adverse events and PK parameters were assessed.



FVIII, factor VIII; rFVIII, recombinant factor VIII.

[a]Factor VIII activity determined by the one-stage activated partial thromboplastin time clotting assay.

[b]One subject was withdrawn from the study due to complications from a motor vehicle accident prior to receiving BIVV001.

[c]One subject was not evaluable for plasma FVIII activity after receiving BIVV001 due to sample shipment error.

FIGURE 1 Mean plasma FVIII activity over time for the 25 IU/kg cohort (A) and the 65 IU/kg cohort (B)a

Results: Most subjects were white (13/16 [81.3%]) and age ranged from 19 to 63 years. Six of 7 subjects in the 25 IU/kg cohort and all 9 subjects in the 65 IU/kg cohort received BIVV001. BIVV001 was well tolerated; no inhibitors were detected through 28 days after BIVV001 dosing. For the 25 IU/kg and 65 IU/kg cohorts, mean $t_{1/2}$ for BIVV001 was longer than for rFVIII (37.6 vs 9.1 h and 42.5 vs 13.2 h, respectively; *P*< 0.001) (Table; Figure). Average FVIII activities at 5 and 7 days post 65 IU/kg BIVV001 were 38% and 17%, respectively.

Conclusions: BIVV001 was well tolerated; no subject developed an inhibitor to FVIII. BIVV001 $t_{1/2}$ was 3-4-fold higher than rFVIII, demonstrating a breakthrough in the VWF-imposed $t_{1/2}$ ceiling. Sustained, high FVIII activity has the potential to provide extended protection against all bleed types.

OC 11.2 | A Phase 1b/2 Study of the Safety, Tolerability, Pharmacokinetics, Pharmacodynamics, and Efficacy of PF-06741086, an anti-TFPI Monoclonal Antibody, in Patients with Severe Hemophilia A or B

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Background: PF-06741086, a monoclonal antibody, targets tissue factor pathway inhibitor (TFPI) to augment clotting activity.

Aims: Evaluate safety/tolerability of PF-06741086, administered subcutaneously (SC), for 3 months prophylactically in hemophilia A (hemA) and B (hemB). Efficacy, pharmacokinetics (PK), pharmacodynamics (PD), and immunogenicity were also assessed.

Methods: This open-label, phase 1b/2, ascending dose study was approved by ethics committees and, with consent, enrolled patients with severe (FVIII or FIX ≤1%) hemA or hemB (± inhibitors), aged 18-64y, receiving on-demand treatment, with ≥6 bleeding episodes during the prior 6 months. Patients were assigned to PF-06741086 cohorts (C): C1, 300 mg SC once weekly (QW); C2, 300 mg SC loading dose, 150 mg SC QW; C3, 450 mg SC QW; and C4 (inhibitor patients), 300 mg SC QW. Safety parameters included adverse events (AEs), laboratory assessments, and vitals. Efficacy assessments used the annualized bleeding rate (ABR). A negative binomial model-based ABR was compared with historic controls.

Results: Twenty-six patients were enrolled: C1, n=7 (6 hemA, 1 hemB); C2, n=6 (5 hemA, 1 hemB); C3, n=6 (5 hemA, 1 hemB); and C4, n=7 (7 hemA, with inhibitors). All received ≥1 PF-06741086 dose. Four serious AEs unrelated to treatment occurred. No thrombotic events occurred. Three patients discontinued because of AEs. Four subjects had grade 3 AEs; all other AEs were grade ≤2. Most common treatment-related AEs were injection site reactions. Model-based ABR was 4.2 (C1), 1.5 (C2), 4.2 (C3), and 0.7 (C4). Percent reduction versus historical controls (ABR=27.6) was 85% (C1), 95% (C2), 85% (C3), and 98% (C4). Across all cohorts, exposure levels were as predicted. Three patients had antidrug antibodies, with no impact on safety, PK, or PD. There were no confirmed neutralizing antibodies.

Conclusions: PF-06741086 was safe and efficacious in hemA and hemB patients, with or without inhibitors.

OC 11.3 | Fitusiran, an RNAi Therapeutic Targeting Antithrombin to Restore Hemostatic Balance in Hemophilia: Interim Analysis from the Open-label Extension Study

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Background: Fitusiran is a once-monthly subcutaneously (SC) administered investigational RNA interference therapeutic targeting antithrombin (AT) to improve thrombin generation (TG) and rebalance hemostasis in patients with hemophilia A or B with or without inhibitors. The Phase 1 study results and Phase 2 open-label extension (OLE) interim analysis showed that fitusiran was generally well tolerated and led to dose-dependent AT lowering, increased TG, and decreased bleeding frequency.

Aims: To report interim safety and efficacy results from the Phase 2 OLE study of fitusiran prophylaxis (NCT02554773).

Methods: The Phase 2 OLE study includes patients with hemophilia A or B with or without inhibitors who completed the Phase 1 study. Patients received monthly fixed doses of fitusiran 50 mg or 80 mg SC.

Results: Thirty-four patients (hemophilia A, n=27; hemophilia B, n=7) enrolled in the Phase 2 OLE study were treated for a maximum of over 3 years, with a median exposure of approximately 2.3 years at the time of data cut. In 33 patients examined, once-monthly SC dosing of fitusiran prophylaxis achieved sustained AT lowering in the background of hemophilia, leading to TG levels approaching the lower end of the range seen in healthy volunteers. Exploratory analysis showed an overall median annualized bleeding rate (ABR) of 1.5. No antibodies against fitusiran developed in any patients during the study. Replacement factors or bypassing agents were used only during breakthrough bleeds, which were successfully managed with revised guidelines for reduced doses. Updated safety and efficacy data will be presented.

Conclusions: Fitusiran prophylaxis provided sustained AT lowering in the context of hemophilia, resulting in low ABR over an extended period of time, and fitusiran was generally well tolerated. These data suggest that fitusiran is a promising investigational approach for rebalancing hemostasis in treating patients with hemophilia A or B, with or without inhibitors.

Study funded by Sanofi Genzyme and Alnylam.

OC 11.4 | Phase 2/3 Trial of Subcutaneous Engineered FVIIa Marzeptacog alfa (activated) in Hemophilia A or B with Inhibitors: Pharmacokinetics, Efficacy and Safety

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Background: Hemostasis in hemophilia with inhibitors requires bypass agents, or emicizumab that only treats hemophilia A. Marzeptacog alfa (activated) (MarzAA), a variant recombinant

FVIIa has increased catalytic activity and prolonged half-life allowing subcutaneous dosing.

Aims: Determine if subcutaneous delivery of MarzAA in subjects with inhibitors provides effective prophylaxis (NTC03407651).

Methods: MAA-201 was approved by ethics committees and enrolled 13 subjects with inhibitors and annual bleeding rate (ABR) ≥12 who provided consent (NTC03407651). Intravenous 18µg/kg and subcutaneous 30µg/kg MarzAA pharmacokinetics and coagulation were measured. Bioavailability was calculated. Subcutaneous 30µg/kg MarzAA was injected for 50 days. If spontaneous bleeding occurred then the dose could be escalated to 60, 90 or 120µg/kg for 50 days without bleeding. Subjects were tested for anti-drug antibodies to MarzAA and FVII weekly and had safety follow up 3 weeks after last dose.

Results: Mean baseline ABR was 19.0; Range 12.2-27.7. Seven subjects have completed the trial with clinically significant reduction of pretreatment to on-treatment ABR, and proportion of days of bleeding (paired t-test p-value 0.016).

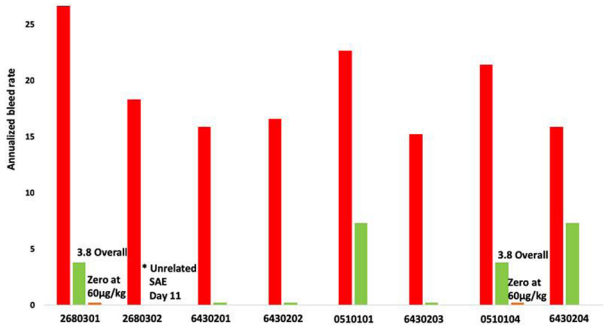

FIGURE 1 Significant reduction in annualized bleed rate with subcutaneous MarzAA prophylaxis

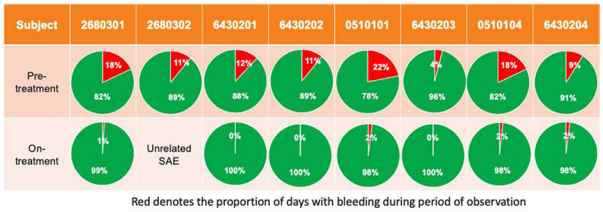

FIGURE 2 Statistically significant reduction in days with bleeding with subcutaneous MarzAA prophylaxis

Five subjects had no bleeding for 50 days at their final dose level, three at 30µg/kg and two at 60µg/kg MarzAA. One revoked consent after intravenous dosing. A fatal hemorrhagic stroke, not related to study drug, occurred on Day 11 in a subject with untreated hypertension.

Subcutaneous half-life was 13.1 hours compared with intravenous of 3.9 hours; bioavailability 22%. Cmax 5 hours after SQ injection were similar to IV levels at 6 hrs. After >450 exposure days, only 5 mild or moderate localized injection site reactions, and no anti-drug antibodies have been detected to date.

Conclusions: An individualized dose of daily subcutaneous MarzAA can provide effective prophylaxis in hemophilia patients with inhibitors by significantly decreasing bleed frequency and the duration of bleeding episodes.

OC 11.5 | Immunogenicity, Safety, Pharmacokinetics and Preliminary Efficacy Results from the First Human Subcutaneous Factor VIII Trial (Subcutaneous Turoctocog Alfa Pegol, SC N8-GP) in Previously Treated Patients with Hemophilia A (Alleviate 1)

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Background: alleviate 1 is the first clinical trial to investigate turoctocog alfa pegol, an extended half-life glycoPEGylated recombinant factor VIII (rFVIII), administered subcutaneously (SC N8-GP), for prophylactic use in patients with severe hemophilia A. The pathfinder™ clinical trial program previously demonstrated the safety and efficacy of turoctocog alfa pegol administered intravenously in >250 children and adults with severe hemophilia A.

Aims: To evaluate the safety, pharmacokinetics (PK), preliminary efficacy and immunogenicity of SC N8-GP in previously treated patients (PTPs) with severe hemophilia A.

Methods: This phase 1, multicenter, double-blinded trial (NCT02994407) comprised two parts. In part A, four patient cohorts (n=6 per cohort) received a single dose of SC N8-GP 12.5-100 IU/kg. In part B, patients received a fixed daily dose of SC N8-GP 2000 or 4000 IU for 3 months. The primary endpoint was frequency of adverse events (AEs). Secondary endpoints included PK endpoints, incidence of FVIII inhibitors (≥0.6 BU), injection-site reactions and number of treatment-requiring bleeds.

Results: Part A: 24 patients completed treatment. Ten patients reported 15 treatment-emergent AEs (TEAEs), including 11 injection-site reactions. No FVIII inhibitors were detected. PK parameters and mean PK profiles supported a linear dose response for SC N8-GP (Table 1).

Part B: 26 patients completed the trial (90.4 mean exposure days [EDs]), including one patient who discontinued treatment due to a low-titre FVIII inhibitor. Seventeen patients reported 40 TEAEs, including two serious AEs. Five patients, including the patient with detectable inhibitor, developed anti-drug-binding antibodies (42-91

TABLE 1 Pharmacokinetic parameters in part A of the alleviate 1 trial

	Part A			
PK parameter	**12.5 IU/kg**	**25 IU/kg**	**50 IU/kg**	**100 IU/kg**
C_{max} (IU/dL)				
Geometric mean (CV%)	1.3 (106.9)	2.5 (62.8)	4.6 (32.6)	15.2 (75.6)
Min.; Max.	0.5; 3.0	0.9; 4.7	2.9; 6.5	4.5; 26.1
$t_{½}$ (h)				
Geometric mean (CV%)	74.0 (25.5)	29.3 (27.4)	20.6 (13.3)	21.6 (12.0)
Min.; Max.	44.7; 86.0	21.8; 48.4	17.6; 24.4	18.3; 25.7

C_{max}, maximum concentration; CV, coefficient of variation; PK, pharmacokinetic; $t_{½}$, half-life.

TABLE 2 Pharmacokinetic parameters in part B of the alleviate 1 trial

	Part B		
PK parameter	**Visit 2 SD**	**Visit 3 SS**	**Visit 8 SS**
C_{max} (IU/dL)			
Geometric mean (CV%)	5.7 (90.0)	13.9 (64.4)	11.9 (80.2)
Min.; Max.	1.0; 17.1	4.1; 46.9	2.9; 46.5
C_{avg} (IU/dL)			
Geometric mean (CV%)	-	12.2 (62.7)	9.8 (85.5)
Min.; Max.	-	3.7; 41.0	2.4; 35.4

C_{avg}, average concentration at steady state; C_{max}, maximum concentration; CV, coefficient of variation; PK, pharmacokinetic; SD, single dose; SS, steady state.

EDs). Overall, five patients reported nine bleeding episodes; none were severe. PK results are shown in Table 2.

Conclusions: SC N8-GP demonstrated clinically relevant FVIII levels with dose linearity in PTPs with severe hemophilia A. The immunogenicity findings in part B may relate to the subcutaneous route of administration because no immunogenicity concerns were reported with N8-GP administered intravenously in previous studies.

OC 13.1 | Diagnosis of VWD Based on a Single Low VWF:RCo in the Zimmerman Program Comparative Effectiveness Study

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Background: VWD is a common inherited bleeding disorder, however diagnosis is complicated due to subjective bleeding history and issues with current assays.

Aims: In the Comparative Effectiveness in the Diagnosis of VWD study, we hypothesized that re-testing is critical for diagnosing VWD. We sought to determine how often type 1/Low VWF diagnosis was based on a single low VWF:RCo due to the common variant p.D1472H or an assay issue, if that low value was corrected by VWF:GPIbM, and if re-testing confirmed original levels.

Methods: Subjects being evaluated for bleeding were enrolled. Diagnosis of VWD was made by the local center. Central testing was performed at Blood Research Institute including VWF:Ag, VWF:GPIbM and *VWF* sequencing.

Results: Of 462 subjects diagnosed with type 1/Low VWF, 18% had low local VWF:RCo (mean 43), but normal VWF:Ag (64) and normal central testing (VWF:GPIbM=71 ($p< 0.0001$); VWF:Ag=65). This group had ISTH bleeding score (BS) of 3 that was not different from BS in subjects without VWD. 70% of the single low VWF:RCo group had no potentially pathogenic SV identified, however 33% carried p.D1472H. 24 low VWF:RCo subjects had follow-ups (FU) including local testing within 2 years. 10 had p.D1472H where FU testing confirmed low VWF:RCo (44) and low VWF:RCo/VWF:Ag (0.7) compared to normal VWF:GPIbM (65) and ratio of 1.1. The other 14 subjects had mean FU VWF:RCo=60 (VWF:GPIbM=75) compared to 44 at baseline, resulting in 57% with normal levels upon re-testing.

Conclusions: 18% of our cohort had a VWD diagnosis based on a single low VWF:RCo but had normal VWF:GPIbM activity. The discrepancy between VWF:RCo and VWF:Ag was often due to p.D1472H or variability in VWF:RCo that was eliminated with VWF:GPIbM. Reliance on VWF:RCo alone for diagnostic purposes may be an error in those with p.D1472H. Repeat VWF:RCo or performing VWF:GPIbM testing can be valuable to avoid an erroneous diagnosis.

OC 13.2 | Evaluation of the von Willebrand Factor (VWF) Inhibitor in a Large Cohort of European and Iranian Patients Previously Diagnosed with Type 3 von Willebrand Disease (VWD3) Enrolled into the 3WINTER-IPS Project

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Background: Patients with VWD3 have virtually complete deficiency of VWF and a very severe bleeding phenotype. A severe complication in VWD3 patients is the development of an inhibitor against VWF. This event not only complicates patients' therapy, but also exposes patients to the risk of a life-threatening anaphylactic shock.

Aims: Our aim was to evaluate the presence of inhibitors against VWF in a cohort of European and Iranian patients with previously diagnosed VWD3 enrolled in the 3WINTERS-IPS project.

Methods: Among the 260 VWD3 patients enrolled in the project 205 had plasma VWF antigen (VWF:Ag) < 5% and in 151 of these the VWF:Ag was undetectable (< 1%). The Bethesda method was used to test all patients for the presence of inhibitors using the VWF collagen binding (VWF:CB) assay. Patients with VWF:CB inhibitors were also evaluated by Bethesda method using the VWF:Ag and an automated gain-of-function mutant glycoprotein Ib binding (VWF:GPIbM) assay.

TABLE 1

Patient ID	VWF:Ag	Mutation	VWF:CB Inhibitors	VWF:Ag Inhibitors	VWF:GPIbM Inhibitors
IR05-01-I-04	<1%	c.311_312delAG/ c.311_312delAG;	56 BU	7.6 BU	70 BU
IR03-04-II-15	<1%	p.Gln1346Ter/unknown	23 BU	1.5 BU	52 BU
IT01-F19-I-P01	<1%	del ex 1-52/del ex 1-52	15 BU	0.7 BU	5.9 BU
IR06-F11-I-P13	<1%	c.4309delG / c.4309delG ;	13 BU	1.7 BU	28 BU
IT04-F04-II-P05	<1%	del ex 1-52/del ex 1-52	10 BU	2 BU	3.8 BU
FI01-6-I-6	<1%	p.Arg1659Ter/p.Arg1659Ter	5 BU	0.3 BU	2.8 BU
IT05-F01-I-P04	<1%	c.6182delT/ c.6182delT	3.8 BU	<0.3 BU	0.4 BU
NL02-F03-I-P01	<1%	p.Asn2546Tyr/p.Asn2546Tyr	1.8 BU	<0.3 BU	<0.3 BU
FR03-F02-II-P01	<1%	del ex 1-52/del ex 1-52	1.8 BU	0.1 BU(<0.3)	0.17 BU (<0.3)
IT04-F04-II-P08	<1%	del ex 1-52/del ex 1-52	1.3 BU	<0.3 BU	0.7 BU
IR01-F07-I-P07	1.4 %	del ex 35-52 /del ex 35-52	1.2 BU	<0.3 BU	0.5 BU
IT04-F01-II-P02	<1.5 %	c.8155+1G>T/c.8155+1G>T	0.4 BU	<0.3 BU	<0.3 BU
IT04-F04-II-P07	<1%	del ex 1-52/del ex 1-52	0.3 BU	<0.3 BU	0.3 BU

The inhibitors were considered to be present for values ≥ 0.3 Bethesda units (BU)

Results: Neutralizing antibodies for VWF:CB were identified in 13 out of 205 (6%) patients and most of them have the VWF:Ag < 1% (Table). Among them, 10 out 13 (77%) were positive to the Bethesda VWF:GPIbM and 6 out 13 (46%) for Bethesda VWF:Ag assays. All patients with inhibitors present mutations that results in null alleles with one exception, a case who appears to be homozygous for a missense mutation.

Conclusions: The development of inhibitors in VWD3 patients is confirmed to be a rare event, only 6% of our patients were found to be positive. High-titer antibodies anti-VWF have been reported to be able to precipitate VWF in normal plasma, this would explain the positive results obtained in six of our patients using the VWF:Ag Bethesda assay. Development of inhibitors is mainly restricted to patients with null alleles and nearly half of them carry homozygous large VWF gene deletions.

OC 13.3 | Mixed Phenotype von Willebrand Disease Caused by a Novel Cysteine Variant that Results in Both Qualitative and Quantitative von Willebrand Factor Defects

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Background: Von Willebrand factor (VWF) cysteine residues are essential for maintaining conformation and multimer structure. Indeed loss of cysteine residues has been associated with both type 1 and type 2 VWD.

Aims: We report a novel cysteine variant (c.3251G>A, p.Cys1084Tyr) that is associated with both qualitative and quantitative VWF defects and results in an unusual form of mixed phenotype VWD.

Methods: Study approval was obtained from the Research Ethics Board of the Hospital for Sick Children, Toronto, Canada. All study participants gave written informed consent. Bleeding phenotype was assessed using the ISTH BAT. Hemostatic values were measured using standard laboratory assays.

Results: Family phenotypic values are outlined in table 1. Heterozygosity for p.(Cys1084Tyr) was associated with qualitative VWF defects. Normal VWF:Ag levels but significantly reduced VWF:GPIbM and VWF:CB, as well as loss of high molecular weight multimers were observed. Interestingly, homozygosity for this variant was further associated with significantly reduced FVIII-binding and an additional quantitative defect (VWF:Ag < 0.40IU/mL). Post-DDAVP survival of VWF:Ag, VWF:GPIbM and FVIII:C was also reduced. Quantitative *in vitro* expression studies revealed impaired synthesis/secretion of p.(Cys1084Tyr) for both the heterozygous and homozygous states (58.4±5.2% and 25.2±7.0% of wild-type VWF respectively, p< 0.0001) and subsequent imaging studies

TABLE 1 Laboratory and clinical phenotypic data for heterozygous and homozygous family members with the (c.3251G>A, p.Cys1084Tyr) variant

	I-1	I-2	II-1 (IC)	II-2	II-3
Sex	M	F	F	F	F
Genotype	c.[3251G>A]+[=]	c.[3251G>A]+[=]	c.[3251G>A]	c.[3251G>A]	c.[3251G>A]+[=]
BAT Score	0	8	4	4	0
VWF:Ag (U/mL)	0.94	0.63	0.23	0.38	0.64
VWF:GPIbM (U/mL)	0.41	0.32	0.11	0.15	0.29
VWF:CB (U/mL)	0.34	0.26	0.09	0.13	0.3
VWF:pp (U/mL)	1.13	0.84	0.56	0.76	0.91
FVIII:C (U/mL)	0.65	0.83	0.05	0.10	0.72
VWF:Ag t1/2 (hours)	-	-	2.0	3.6	-

demonstrated qualitative differences in pseudo-Weibel Palade body formation.

Analysis of the crystal structure of the D'D3 assembly revealed the close proximity of the C1084-C1060 disulfide to C1099, the cysteine involved in inter-subunit disulfide formation. Loss of C1084 and generation of a novel free cysteine at C1060 likely interferes with D'D3 structural conformation and multimerization, leading to both the quantitative and qualitative defects observed.

Conclusions: The novel p.(Cys1084Tyr) variant causes codominant inheritance of a complex, mixed phenotype VWD associated with reduced VWF:Ag levels due to accelerated VWF clearance and impaired VWF synthesis, packaging and secretion, as well as deficiencies in VWF:GPIbM and FVIII-binding.

OC 13.4 | Management of Patients with Severe VWD Type 3 and Centrally Confirmed Anti-VWF Neutralizing Antibodies: Results of the 24-month Prospective Observation in 10 Cases Enrolled into the 3Winters-Ips

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Background: Rare patients with type 3 von Willebrand disease (VWD3) may develop anti-VWF inhibitor (VWD3-INH) after VWF concentrates (VWF-C). To better characterize VWD3-INH, a large cohort of patients with VWD3 has been enrolled into the type ***3*** Von ***W***illebrand ***Inter***national ***R***egistrie***S*** ***I***nhibitor ***P***rospective ***S***tudy (*3Winters-Ips*), an investigators initiated, multicenter, European-Iranian (EU-IR) observational study.

Aims: Assess efficacy/safety of different therapeutic approaches used to manage bleeds in VWD3-INH.

Methods: 260 cases with centrally confirmed VWD3 diagnosis were enrolled and 201/260 (77%) were included into the 24-months clinical observation (*NCT02460458*, *Clinical-Trials.gov*). Data were reported into an online database.

Results: Among 179/201 (89%) VWD3 who completed the 24-month prospective observation, 10/179(5.6%) VWD3-INH identified at EU (n=7) or IR (n=3) centres were characterized by deletions [large(n=5)/small(n=2)] or mutations [missense(n=1)/nonsense(n=1)/splice]. Mean (range) data at enrolment were: sex (M/F)=3/7; age=36.2(2-63); BS=16.2(5-33); BU-anti-CB-INH=10.4(0.4-56); FVIII:C(IU/dL))=3.1(1.2-5.1). Local investigators reported previous anaphylactic or allergic reactions in most adult (n=8) cases and treated on demand (OD) only severe bleeds (n=12) such as gastrointestinal (n=6), abdominal (n=3), major surgeries (n=2), uterus (n=1). Minor bleeds were managed with local therapies (anti-fibrinolytic agents, estrogens). VWF-C such as HAEMATE-P or WILATE with annual VWF:RCo IU/patient of 45.600 (10.200-175.500) were used successfully in 4 cases with anti-CB-INH< 5BU. Patients with anti-CB-INH>5BU were management with recombinant activated factor VII (n=4) or recombinant B domain deleted FVIII (n=1) using frequent injections. VWF-C were effective without major

side effects (anaphylactic or allergic reactions) when used together with high-dose intravenous Ig in 2 cases with anti-CB-INH>5BU.

Conclusions: Based on these data collected from the largest cohort of VWD3, we could confirm that the rare VWD3-INH patients are treated OD only for severe bleeds. Attempts of immune tolerance have been used in single cases. Further analyses with longer prospective observation proposed by *3Winters-Ips-Extended* is required to determine their best therapeutic approach.

OC 13.5 | Population Pharmacokinetic Modelling of Factor VIII Plasma Levels during Perioperative Dosing of a VWF/FVIII Concentrate in von Willebrand Disease Patients

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Background: During surgery, we have reported that dosing patients with von Willebrand Disease (VWD) with VWF/FVIII concentrate (ratio 2.4:1) according to factor VIII (FVIII) target levels leads to unnecessarily high levels of Von Willebrand factor, resulting in higher costs than necessary and a hypothetical risk of thrombosis.

Aims: Develop a population pharmacokinetic (PK) model based on factor FVIII (FVIII) plasma concentrations to allow for more accurate perioperative dosing of von Willebrand Factor (VWF)/FVIII concentrates in VWD patients.

Methods: VWD patients undergoing surgery in one of five hemophilia treatment centers in the Netherlands between 2000-2018 treated with VWF/FVIII concentrate were included in this study. Population PK (popPK) modelling was performed based on obtained FVIII levels using nonlinear mixed-effects modelling (NONMEM).

Results: The popPK model is based on the inclusion of 732 FVIII plasma concentrations of 104 patients, aged 0 to 82 years, undergoing 149 surgerical procedures. The PK profiles were best described using a one-compartment model. Typical values for the volume of distribution and clearance were 3.22 L/70 kg and 0.0401 L/h/70 kg, with corresponding inter-individual variability of 30.6% and 84.2%, respectively. Increasing surgery duration and lower physical status (ASA class >2) were associated with a clearance decrease. All types of VWD were included, although the type of VWD had no significant influence on the PK. Validation of the popPK model with independent clinical data from 20 additional VWD patients undergoing 31 surgeries resulted in an acceptable prediction bias of -2.0% (95% CI: -7.0% - 3.0%) and a inaccuracy of 13.3%. Bootstrap results and predictive check analysis confirmed the robustness of the popPK model.

Conclusions: FVIII plasma levels after perioperative VWF/FVIII concentrate administration are adequately described by the developed popPK model. This model facilitates PK-guided FVIII-based dosing of this particular concentrate in VWD patients, which potentially results in improvement of quality and cost-effectiveness of care.

OC 19.1 | Comparison of Outcome Between Intrauterine Balloon Tamponade and Uterine Artery Embolisation in the Management of Persistent Postpartum Haemorrhage: A Propensity Score Matched Cohort Study

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Background: Intrauterine balloon tamponade is often used during postpartum haemorrhage. However, efficacy of balloon tamponade to prevent hysterectomy or avert maternal death has never been compared to another invasive management strategy.

Aims: To compare severe maternal outcome in women who received intrauterine balloon tamponade with women who underwent uterine artery embolisation as initial management for persistent postpartum haemorrhage in which an immediate intervention was deemed necessary.

Methods: Propensity score matched cohort of women who experienced persistent postpartum haemorrhage and were initially managed by intrauterine balloon tamponade or uterine artery embolisation. Main outcome measure was a composite of peripartum hysterectomy and/or maternal death.

Results: Our 1:1 propensity score matched cohort comprised of 50 women who had intrauterine balloon tamponade and 50 women who underwent uterine artery embolisation at a blood loss between 1000-7000mL after childbirth. There was no significant difference in hysterectomy rate between both groups (N=6 in each group, OR 1.00, 95% CI: 0.30-3.34), nor in total volume of blood loss (4500mL [IQR: 3600-5400mL] for balloon vs 4000mL [IQR: 3250-5000] for embolisation, P=0.382), nor in median total units of packed red blood cells transfused (7 [IQR: 5-10] for balloon vs 6 [IQR: 4-9] for embolisation, P=0.319). Fifteen women (30%) who were initially managed by intrauterine balloon tamponade still underwent uterine artery embolisation, of whom one had an embolisation-related thrombo-embolic event. Maternal mortality occurred in neither of the intervention groups.

Conclusions: No difference in the rate of peripartum hysterectomy and/or maternal death was observed between women who had

intrauterine balloon tamponade and women who underwent uterine artery embolisation as an initial treatment for persistent postpartum haemorrhage. Although this study was underpowered to demonstrate equivalence, our study design provides a framework for future research in which intrauterine balloon tamponade may prove to be an effective intervention of first choice in the management of persistent postpartum haemorrhage.

TABLE 1 Characteristics before and after propensity score matching

	Before PS matching			After PS matching		
	IUBT N=373	UAE N=82	SMD (%)	IUBT N=50	UAE N=50	SMD (%)
Maternal age, years*	31 (28-35)	32 (29-36)	15.6	32 (29-37)	31 (29-36)	5.7
Gestational age, weeks*	39 (38-40)	38 (37-40)	28.9	39 (37-40)	38 (37-40)	6.9
Multiparity, n (%)	170 (46)	43 (52)	16.8	28 (56)	27 (54)	4.0
Preeclampsia, n (%)	36 (10)	10 (12)	7.5	6 (12)	6 (12)	0.0
Multiple pregnancy, n (%)	23 (6)	6 (7)	7.1	4 (8)	4 (8)	0.0
Prior caesarean birth, n (%)	44 (12)	16 (20)	22.4	9 (18)	10 (20)	5.0
Mode of birth, n (%)	-	-	54.0	-	-	0.0
Vaginal delivery	300 (80)	49 (60)	-	28 (56)	28 (56)	-
Caesarean section	73 (20)	33 (40)	-	22 (44)	22 (44)	-
Cause of haemorrhage, n (%)	-	-	-	-	-	-
Uterine atony	293 (79)	53 (64)	Ref	32 (64)	33 (66)	Ref
Retained placenta	45 (12)	7 (9)	6.6	5 (10)	5 (10)	0.0
Abnormally invasive placenta	24 (6)	7 (9)	19.2	6 (12)	6 (12)	0.0
Other causes	11 (3)	15 (18)	34.7	7 (14)	6 (12)	5.9
Placental abruption	*2*	*1*	-	*2*	*0*	-
Placenta praevia	*2*	*3*	-	*2*	*1*	-
Uterine rupture	*7*	*11*	-	*3*	*5*	-
Estimated blood loss at the time of the intervention, mL*	2500 (2000-3000)	3500 (3000-4500)	62.5	3250 (2500-4000)	3250 (2500-4000)	0.0
Coagulopathy, n (%)	42 (11)	35 (43)	60.4	18 (36)	18 (36)	0.0
Shock symptoms, n (%)	304 (82)	68 (83)	1.3	40 (80)	41 (82)	5.0
Number of uterotonics given*	2 (2-3)	2 (1-3)	28.0	2 (1-2)	2 (1-3)	5.2
Non-uterotonic agents, n (%)	-	-	-	-	-	-
Tranexamic acid	132 (35)	42 (51)	13.5	23 (46)	21 (42)	8.0
Fibrinogen concentrate	6 (2)	13 (16)	30.0	4 (8)	4 (8)	0.0
Recombinant factor VIIa	3 (1)	3 (4)	10.1	1 (2)	1 (2)	0.0
Blood components*	-	-	-	-	-	-
Packed red blood cells	1 (0-2)	4 (3-7)	88.7	4 (2-6)	4 (2-5)	9.2
Fresh frozen plasma	0 (0-1)	2 (1-4)	80.1	2 (0-2)	2 (0-2)	5.5
Platelets transfusion	0 (0-0)	0 (0-1)	48.7	0 (0-0)	0 (0-0)	6.3
Surgical interventions, n (%)	3 (1)	2 (2)	20.8	2 (4)	2 (4)	0.0
B-Lynch sutures	*3*	*2*	-	*2*	*2*	-
Uterine artery ligation	*0*	*1*	-	*0*	*1*	-

PS: Propensity Score, IUBT: Intrauterine Balloon Tamponade, UAE: Uterine artery embolisation, SMD: Standardized Mean Difference, Ref: Reference. *Reported as median with (interquartile ranges).

TABLE 2 Severe maternal outcome in both intervention groups

Table 2. Outcome for women with persistent postpartum haemorrhage who initially had intrauterine balloon tamponade versus women who primarily had uterine artery embolisation between an estimated blood loss of 1000–7000mL.

	Unadjusted analysis			Propensity score matched adjusted analysis		
	IUBT (N=373)	UAE (N=82)	OR (95% CI)	IUBT (N=50)	UAE (N=50)	OR (95% CI)
Composite primary outcome, n (%)	21 (5.5)	10 (12)	2.33 (1.05–5.15)	6 (12)	6 (12)	1.00 (0.30–3.34)
Peripartum hysterectomy	19 (5.0)	10 (12)	-	6 (12)	6 (12)	-
Maternal mortality	2 (0.5)	0 (0)	-	0 (0)	0 (0)	-
Secondary outcome measures*			P-value			P-value
Total number of packed cells, units	4 (3–7)	7 (5–11)	<.001	7 (5–10)	6 (4–9)	0.319
Total volume of blood loss, mL	3500 (3000–4500)	4500 (3350–6000)	<.001	4500 (3600-5400)	4000 (3250-5000)	0.382

IUBT: Intrauterine Balloon Tamponade, UAE: Uterine Artery Embolisation.
*Reported as median with (interquartile ranges).

OC 22.1 | Nonclinical Pharmacology of TAK-748/SHP648 a Novel Factor IX (FIX) Gene Therapy Vector in Mice and Rhesus Monkeys

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Background: Adeno-associated virus (AAV)-based FIX gene therapy has the potential to provide clinical benefit in patients with hemophilia B. TAK-748/SHP648 is a next generation vector exhibiting an improved vector design that includes three cis-regulatory elements (CRM8) to increase the strength of the liver-specific transthyretin (TTR) promoter driving expression of a human FIX transgene.

Aims: The presented studies examine TAK-748/SHP648 dose-response relationships in FIX knockout mice and in rhesus monkeys.

Methods: Male FIX knockout mice (n=12/group) were administered single intravenous doses of $3.9x10^{10}$, $7.7x10^{10}$, $3.9x10^{11}$, or $7.7x10^{11}$ vector genomes (vg)/kg TAK-748/SHP648 or buffer. FIX plasma activity levels were determined at days 7, 14, 28, 42, and 56. At the end of the observation period, bleeding phenotype was assessed in a tail-tip bleeding assay. Three male rhesus monkeys per group received a single intravenous bolus injection of $5.0x10^{11}$ or $1.0x10^{12}$ vg/kg TAK-748/SHP648. Blood samples were taken weekly up to week 18. The primary endpoints were FIX activity and human FIX antigen in plasma.

Results: TAK-748/SHP648 mediated FIX activity was detectable at all dose levels from day 7 to day 56 in plasma of FIX knockout mice. Treatments with TAK-748/SHP648 resulted in dose-dependent supraphysiologic mean FIX levels up to 41.0 IU/mL ($7.7x10^{11}$ vg/kg cohort). TAK-748/SHP648 doses at or above $3.9x10^{10}$ vg/kg reduced blood loss significantly compared with buffer-treated mice (p-value: < 0.05) in the tail-tip bleeding assay. TAK-748/SHP648 administered to rhesus monkeys produced a dose-dependent increase in mean plasma human FIX activity and antigen, resulting in individual peak values that ranged from 1.4 IU/mL ($5x10^{11}$ vg/kg cohort) to 3.7 IU/mL ($1.0x10^{12}$ vg/kg cohort). There were no clinical signs or post-dosing observations considered to be related to TAK-748/SHP648.

Conclusions: Treatment with TAK-748/SHP648 resulted in dose-dependent plasma FIX activity and was well tolerated and efficacious in FIX knockout mice and rhesus monkeys.

OC 22.2 | Therapeutic Levels of Factor VIII Gene Expression Were Achieved by Transcutaneous Ultrasound Mediated Gene Delivery into Canine Liver

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Background: Ultrasound mediated gene delivery (UMGD) combined with microbubbles (MBs) has been shown to be an effective method for non-viral gene delivery, with potential for treating genetic diseases like hemophilia A. Previous studies in our lab demonstrated

successful transfection of swine liver using luciferase reporter plasmids and a transcutaneous UMGD procedure.

Aims: In this study, we investigated using the established transcutaneous UMGD procedure to transfer human factor VIII (hFVIII) plasmid in a canine model.

Methods: A high-expressing liver-specific hFVIII plasmid was injected in combination with RN18 MBs into canine liver lobes through a balloon catheter inserted into the hepatic vein via jugular vein access. Simultaneously, transcutaneous therapeutic ultrasound was applied to enhance gene transfer using an eight-minute pulsed US treatment (4.8 MPa, 1.05 MHz, 50 Hz PRF). Blood was collected at multiple time points for four weeks. A Western blot was performed to examine the presence of hFVIII in the treated animals. ELISA was also carried out to evaluate the levels of hFVIII expression and antibody formation to hFVIII, and transaminase (AST/ALT) assays were performed to assess potential liver damage following treatment.

Results: The Western blot confirmed the presence of hFVIII protein in the plasma of treated dogs. hFVIII expression was detected by hFVIII-specific ELISA with levels between 9 and 35% of normal levels at day three and persisted between 1 and 10% at day seven. Minor elevation in the AST/ALT levels was observed at day one, but these levels decreased to normal rapidly and no other parameters from CBC and whole blood chemistry analysis showed any significant changes.

Conclusions: Transcutaneous UMGD of hFVIII plasmids achieved successful transfection of liver tissue and produced therapeutic levels of hFVIII expression in a canine model. These results show significant promise for a minimally invasive UMGD as a clinically feasible therapy for hemophilia.

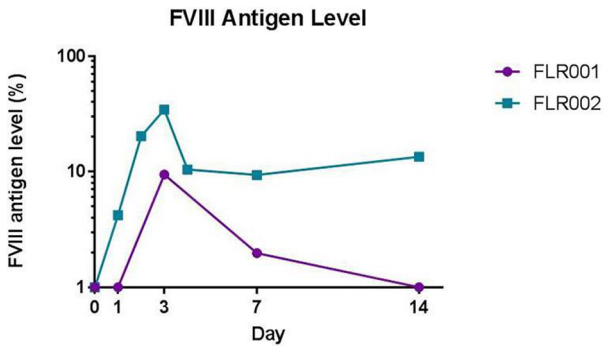

FIGURE 1 Human Factor VIII Antigen Level

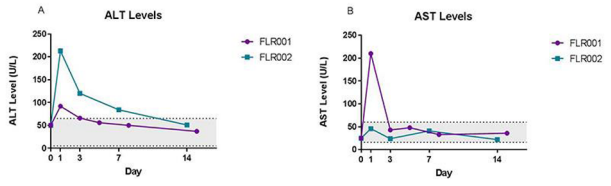

FIGURE 2 Evaluation of Transaminase Levels in Treated Dogs

OC 22.3 | AAV5-mediated Gene Therapy for Hemophilia A with a Factor IX Variant that Functions Independently of FVIII

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Background: Expression of AAV-encoded factor VIII by hepatocytes may not be stable in time and up to approximately 30% of severe hemophilia A patients develop inhibitors that prevent efficacy of replacement therapies with infused blood coagulation factor VIII (FVIII). These patients are dependent on on-demand therapies with bypassing agents e.g. activated prothrombin complex, activated recombinant factor VII, or a bispecific antibody linking FIX(a) to FX(a). Hence there is a need for FVIII-independent approaches in hemophilia A. Previously, factor IX variants have been developed that can promote cofactor-independent coagulation in the absence of FVIII, *in vitro* and in hemophilic mice upon injection of DNA minicircles (Quade-Lyssy et al. 2014). However, hydrodynamic DNA injections are not suitable for human application due to the harsh procedure and transient expression.

Aims: To investigate the feasibility of delivering the FIX variant transgenes using AAV5 vectors.

Methods: C57Bl6 mice, FVIII-KO mice and Cynomolgus Macaques were injected with AAV vectors encoding FIX variants at $5x10^{13}$ or $9x10^{13}$ gc/kg. FIX protein and activity levels were determined by ELISA and chromogenic assay. FVIII-independent activity in the FVIII-KO mice was determined using the one stage clotting assay. Thrombotic and liver function markers were measured as well as vector distribution and mRNA expression in different organs.

Results: Two FIX variants were selected with four amino acid substitutions leading to their FVIII-independent function. AAV5 vector administration to C57Bl6, FVIII-KO mice and non-human primates was well-tolerated and did not show possible induction of thrombogenicity. FVIII-independent restoration of coagulation was confirmed in the FVIII-KO mice upon AAV delivery of the factor IX mutants. Importantly, the obtained transgene expression levels in the non-human primates will translate to therapeutic meaningful FVIII-independent activity.

Conclusions: Our data indicate that development of AAV5 gene therapy based on a FIX variant is feasible and may translate in therapeutic relevant coagulation activity in hemophilia A patients.

OC 22.4 | Oral Nanoparticles with FVIII and IL-10 DNA: Effect on the Immune Response to FVIII in Hemophilia A Mice

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Background: Neutralizing immunoglobulins (inhibitory antibodies) specific to factor VIII (FVIII) constitutes a major challenge in the clinical management of hemophilia A. We have previously reported that oral administration of chitosan nanoparticles containing plasmid DNA coding for FVIII led to detectable transient circulating levels of FVIII protein but no anti-FVIII antibodies in hemophilia A mice. Here, we further investigated the antibody response to FVIII, hypothesizing that adding DNA coding for the anti-inflammatory cytokine IL-10 would modulate the humoral immune response against FVIII.

Aims: To assess the potential immune modulatory effect of nanoparticles formulated with DNA coding for IL-10 and FVIII, and its effect on the development of antibody responses against FVIII in hemophilia A mice.

Methods: Groups of hemophilia A mice (C57BL/6$^{FVIII-/-}$) were treated weekly for 8 consecutive weeks with a single oral administration of chitosan nanoparticles containing DNA coding for either FVIII alone, or for FVIII and IL-10. For the last 4 weeks of the experiment mice were injected weekly with rhFVIII protein to mimic the exposure to FVIII preparations of hemophilic patients. Furthermore, mice were bled regularly and plasmas assayed for the presence of anti-FVIII antibodies by means of quantitative ELISA.

Results: All Hemophilia A mice that did not have earlier exposure to nanoparticles showed high titer of anti-FVIII antibodies in response to weekly rhFVIII protein injections. In contrast, the group of mice treated with FVIII DNA nanoparticles developed lower antibody titer against FVIII following rhFVIII injections. Intriguingly, 66% of mice treated with nanoparticles containing DNA coding for FVIII and IL-10 demonstrated negligible antibody formation by the end of the experiment.

Conclusions: Mice previously treated with FVIII nanoparticles showed reduced antibodies when challenged with rhFVIII, whereas the addition of IL-10 DNA further reduced the antibody response, suggesting the immune modulatory effect of IL-10 with potential application in the treatment of hemophilia A.

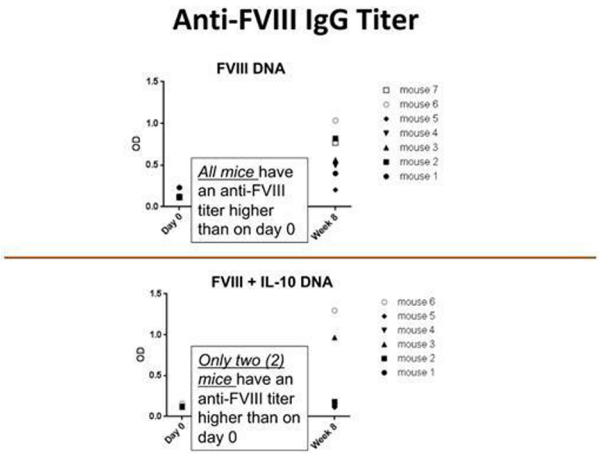

FIGURE 1 Anti-FVIII Titer in Hemophilia Mice

OC 22.5 | Long Term Safety and Efficacy of Adeno-associated Viral Gene Therapy in a Canine Hemophilia A Model

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Background: Recent studies have presented data on efficacy of adeno-associated viral (AAV) gene therapy in patients with hemophilia, however the long-term safety and efficacy of this approach is unknown.

Aims: To evaluate long-term safety, efficacy and fate of hepatic directed AAV-FVIII gene therapy in a canine hemophilia A model.

Methods: A B-domain-deleted canine-FVIII (BDD-cFVIII) AAV vector was administered by portal vein infusion. Chromogenic FVIII activity (FVIII:C) was measured using pooled canine plasma as a standard. AAV-BDD-cFVIII vector genome (VG) quantification was performed by real-time qPCR.

Results: Eight severe hemophilia A dogs were treated with AAV-FVIII (AAV2, 6 or 8; Table 1) at a median dose of 1.25x10^{13} vg/kg (6.0x10^{12}-2.7x10^{13}) at 9.5 months (6-15 months) with follow-up of 10.7 years (8.2-12.0 years). FVIII:C elevation to ≥1% occurred in 6/8 dogs, with stable FVIII expression seen throughout the study and a median terminal FVIII:C 7.2% (1.8-8.6%). Transient (≤4 weeks duration) inhibitor formation occurred in 2 dogs. Reduction in treated annualized bleed rate occurred in all animals (pre=4.0, 0.0-12.0; post=0.4, 0.1-1.7; p=0.017). BDD-cFVIII genomes were detected in all post-mortem liver samples (median=574.7 dsDNA-vg/reaction), with lower levels in spleens of 4 dogs (median=19.4 dsDNA-vg/reaction, 5.8-135.6). BDD-cFVIII genomes were not detected within reproductive organs, kidney, lung, heart, skeletal muscle or a sarcoma (n=1) diagnosed at 9.3 years. Elevation (≥2-fold ULN) in alkaline phosphatase (n=7) and/or alanine aminotransferase (n=4) occurred at a median of 9.1 and 8.6 years, respectively. Post-mortem liver examination (n=6) demonstrated no significant parenchymal inflammation, hepatitis, fibrosis or evidence of hepatocellular carcinoma. Multifocal parenchymal nodularity was seen with variably sized areas of hepatocyte proliferation admixed with foci of liver cells containing cytoplasmic glycogen vacuolation characteristic for nodular hyperplasia and canine vacuolar hepatopathy.

TABLE 1 Summary of AAV-FVIII treated severe hemophilia A dogs. ABR=Annualized bleed rate (treated). *No sample available. # 20ng gDNA/qPCR reaction

ID (Sex)	AAV Serotype	Dose vg/kg	Years post AAV	ABR Pre	ABR Post	Early FVIII:C (years)	Mid FVIII:C (years)	Final FVIII:C (years)	Liver BDD-cFVIII VG#
EL (F)	2	6.0e12	8.18	5.2	0.5	*	4.0% (3.9)	*	181.4
VC (M)	2	1.5e13	10.99	8.7	1.1	3.2% (1.8)	4.0% (7.2)	2.9% (11.0)	2098.3
ANG (M)	2	1.5e13	11.50	5.2	1.7	*	0.4% (7.9)	0.5% (11.4)	200.0
JU (M)	2	2.7e13	11.96	0.0	0.3	6.9% (3.2)	6.1% (8.2)	7.2% (11.9)	985.8
ALX (F)	6	1.0e13	10.50	2.8	0.1	9.4% (1.9)	4.9% (6.7)	8.6% (10.5)	688.1
MZ (M)	6	1.0e13	10.07	1.6	0.3	5.8% (1.3)	4.3% (6.2)	7.9% (10.1)	684.0
MG (F)	6	1.7e13	11.46	12.0	1.3	*	0.8% (8.0)	0.3% (11.3)	52.4
FLO (F)	8	1.0e13	10.47	2.1	0.1	2.8% (1.7)	7.6% (6.5)	1.8% (9.8)	465.3

Conclusions: AAV-FVIII gene therapy in a canine hemophilia model demonstrated durable long-term hepatically derived FVIII expression with correction of the bleeding phenotype.

OC 31.1 | Whole Exome Sequencing of Patients Treated with Adeno-associated Virus Serotype 8-Factor IX (AAV8-FIX) Gene Therapy Reveals Potential Determinants of Persistent Transgene Expression

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Background: The clinical phase I/II study investigated BAX335, an AAV8-based gene therapy for FIX replacement in patients with hemophilia B. BAX335 was well-tolerated in all patients with no clinical thrombosis and no evidence of inhibitors or other FIX-directed immunity. Persistent therapeutic FIX activity of 20% without bleeding or replacement therapy was achieved in only one patient for >4 years (Index patient). In all other patients, FIX activity declined after 5-11 weeks and could not be rescued by corticosteroid treatment.
Aims: Identification of genetic predispositions as molecular determinants for long-term transgene expression in the Index patient using whole exome sequencing (WES).
Methods: High-coverage WES of genomic DNA from patients treated with BAX335 was combined with state-of-the-art variant identification and prioritization. Index patient was compared to the remaining seven patients to identify variants potentially mediating persistent transgene expression. Prioritized variants were evaluated according to the predicted deleteriousness using CADD, PolyPhen-2 and SIFT algorithms and to the phenotypic manifestations of their target genes.
Results: Two of the identified variants found in the index patient could impact transgene expression. The first are compound heterozygous variants of the nuclear receptor co-repressor complex *SMRT/NCOR2*, which mediates transcriptional silencing by tethering general chromatin-modifying enzymes to target genes. Since chromatinized episomal AAV-transgene is regulated by histone modifications[1,2], the identified *SMRT* variants highlight protection from heterochromatinization as one of the mechanisms for ensuring persistent transgene expression.
The most indicative variant was a heterozygous missense mutation in the interleukin-6 receptor gene (*IL-6R*). IL-6R haploinsufficiency is well documented[3-5], thus the identified variant might decrease sensitivity to IL-6-mediated inflammatory stress caused by BAX335 (AAV8-capsid and/or transgene) and protect targeted hepatocytes.
Conclusions: Two important parameters required for successful liver-targeting gene therapy are implicated: (i) design of robust expression cassette resistant to gene silencing by heterochromatinization and (ii) protection of targeted hepatocytes from inflammatory stress induced by viral load.

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Ref. Nr.	Reference
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OC 31.2 | CTLA-4 IgGs - A Powerful Tool to Enable Re-administration in AAV8 Gene Therapy and to Suppress anti-AAV8 T Cell Responses

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Background: Correction of suboptimal expression levels by redosing in adeno associated virus serotype 8 (AAV8) gene therapy is prevented by the formation of anti-AAV8 immunity after the first dose. We developed a strategy using the T cell specific immunosuppressant CTLA4-IgG to enable re-administration of AAV8-human-FIX (AAV8-huFIX) gene therapy. CTLA4-IgG is advantageous because it is also considered to suppress memory T cell responses that could kill transduced hepatocytes.

Aims: Show boosting of human FIX (huFIX) plasma levels by repeated application with an AAV8-huFIX vector in the presence of CTLA4-IgG.

Methods: C57BL/6 mice were treated with AAV8-huFIX gene therapy vector in the presence or absence of abatacept (CTLA-4-IgG). Subsequently, a second dose of AAV8-huFIX gene therapy vector was applied. Anti-AAV8 immunity after AAV8 gene therapy was assessed by analyzing neutralizing antibodies (NAbs) and binding antibodies (BAbs) to AAV8, and IFNg ELISPOT. HuFIX plasma levels were determined by FIX antigen ELISA.

Results: Co-treatment of mice with abatacept, blocking the CD80/CD86-CD28 interaction, resulted in efficient suppression of anti-AAV8 antibodies and AAV8-specific T cell responses, thus, enabling redosing: A boost of huFIX plasma levels from about 2 to 20 µg/ml was achieved by first applying a low dose of AAV8-huFIX followed by a second higher dose of AAV8-huFIX in the presence of abatacept. Furthermore, transient abatacept application was capable to suppress even the function of AAV8-specific memory T cells after re-challenge with AAV8-huFIX in a mouse model bearing pre-existing AAV8-specific memory responses. However, suppression of AAV8-specific T cell responses was not as efficacious as in naïve mice.

Conclusions: Abatacept might enable re-administration of AAV8-huFIX in patients and could be used to specifically dampen AAV8-specific memory T cell function. However, to fully suppress strong memory T-cell responses, more robust immune suppressive regimens might be required.

OC 31.3 | Neddylation and SUMOylation Site Modified Adeno-associated Virus Vectors Demonstrate Enhanced Hepatic Factor IX Gene Delivery *In Vivo*

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Background: The roleof Ubiquitin-likemodifiers (UBLs) like Neddylation and SUMOylation on AAV2 capsid during hepatic gene transfer is unknown.

Aims: To study the role of UBLs during AAV transduction and create novel AAV vectors modified for their Neddylation and SUMOylation status for improved hepatic gene therapy.

Methods: Effect of Neddylation and SUMOylation pathway upon AAV2 infection was measured by transcript analysis. Capsid targets for Neddylation and SUMOylation mutations were predicted and generated by site-directed mutagenesis. *In vitro*screening was performed in Huh7 and HeLa cells and GFP expression was quantified by flow-cytometry. Vectors were evaluated for their therapeutic efficiency in hemophilia B mice(*n=5*) for human factor(F)-IX gene delivery. The levels of F-IX were assessed by ELISA and Immunohistochemistry. Immunogenicity of the novel vectors was enumerated by flow cytometry analysis for T-helper cells(CD3+CD4+), T-cytotoxic cells(CD3+CD8+), T-reg cells(CD4+CD25+FoxP3+), B-cells(CD19+) in recipient mice. Further, the CD8+T-cell response was confirmed by ELISPOT assay in murine splenocytes.

Results: Neddylation and SUMOylation(5.4-6.3-fold) pathway-specificgenes were significantly modulatedin response to AAV2 infection. Upon *in vitro*screening of ten mutant vectors, a Neddylation (AAV2-K665Q) and a SUMOylation (AAV2-K105Q) mutant vector showed superior transduction in HeLa (>97%-150%) and Huh7 (>44%-46%) cells as compared to wild-type AAV2 vector. These vectorswere further testedfor their potential to deliver F-IX gene having liver specific (LP-1) promoter in Huh7 cells, which resulted in 3.9 to 4.5-fold increase in F-IX transgene levels. Mutant vectors injected at low dose (5x10e10 vector genomes) in hemophilia B mice showed a 2-fold increase in F-IX antigen levels, over the period of 4 to 8 weeks. The immunogenicity of the novel vectors in hemophilia B mice were comparable to wildtype AAV2 injected animals.

Conclusions: Our data showsthat Neddylation and SUMOylation modified AAV2 vectors had a significantly improved therapeutic outcome during hepatic gene therapy in hemophilia B mice.

OC 31.4 | Preexisting Immunity against AAV8 and other AAV Serotypes in Healthy Individuals and Patients with Hemophilia B

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Background: Preexisting immunity against vectors is a major challenge for hemophilia gene therapy using adeno-associated virus 8 (AAV8) and current clinical trials exclude patients with preexisting neutralizing antibodies (NAbs). Therefore, it is crucial to carefully determine and characterize anti-AAV8 immunity. Due to the use of different assays in different studies the reported prevalence of anti-AAV8 immunity is not well understood.

Aims: Characterization of the preexisting immunity against AAV8 using validated assays, by assessing anti-AAV8 NAbs, binding antibodies (BAbs), T cell responses and the cross-reactivity to AAV2, AAV1 and AAV5.

Methods: Preexisting anti-AAV8 immunity was assessed in large, international cohorts (Europe, USA): NAbs were assessed using a validated, clinical NAb assay with verified biological relevance. BAbs were assessed by ELISA and T cell responses by IFN-g and IL-2 ELISpot. T cell responses and BAbs against AAV2 and AAV5, and NAbs against AAV1, AAV2 and AAV5 were analyzed to assess co-prevalence.

Results: A high anti-AAV8 NAb prevalence with some regional variation (32%-63%) was found. No differences in NAb prevalence between healthy donors and patients with hemophilia B were observed. NAbs showed high correlation with anti-AAV8 IgG1, but not with anti-AAV8 T cell responses detectable in peripheral blood. A high co-prevalence of NAbs and BAbs against AAV1, AAV2 and AAV5 was detected. Anti-AAV8 NAbs showed long-term stability in healthy donors and in hemophilia B patients treated with AAV8 gene therapy.

Conclusions: Based on our data, approximately 50% of patients cannot be treated with AAV8 gene therapy due to preexisting NAbs. The role of anti-AAV8-specific-T cells in the development of anti-AAV8 NAbs is not clear. For most of the patients with anti-AAV8 NAbs, switching to other AAV serotypes is prevented by co-prevalent NAbs against other serotypes. These data support the development of new strategies for the treatment of patients with preexisting NAbs.

OC 31.5 | Global Seroprevalence of Pre-existing Immunity against Various AAV Serotypes in the Hemophilia A Population

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Background: Adeno-associated virus (AAV)-mediated gene therapy offers great promise to provide a durable reduction in bleeding frequency and treatment burden in the hemophilia population. However, pre-existing immunity against AAV may limit efficacy and therefore patient eligibility for such treatments.

Aims: BioMarin has demonstrated via non-human primate studies that an electrochemiluminescent (ECL)-based method to detect antibodies against AAV5 capsid is suitable to identify those subjects most likely to respond to treatment. A comprehensive global study to evaluate the geographic and temporal distribution of AAV capsid seropositivity in hemophilia A patients has not been reported.

Methods: BioMarin employed an identical ECL-based format to develop and validate assays that detect antibodies against AAV2, 5, 6, 8, and rh10 capsids with comparable sensitivity in a central laboratory. To enable direct comparison of the prevalence of pre-existing immunity to each capsid a global seroprevalence study (BMN 270-901) enrolling up to 100 hemophilia A patients (75 adults and 25 adolescents) has been conducted in each of 8 countries (Russia, Germany, France, UK, USA, Italy, Japan, and South Africa). Plasma samples were collected from all patients following ethics approval and informed consent to determine antibody titers to each capsid. Seroprevalence was evaluated longitudinally in 20% of subjects by collecting additional samples at months 3 and/or 6 to assess variation in pre-existing immunity and antibody titer over time.

Results: Data thus far demonstrate varying prevalence of pre-existing antibodies specific for capsid serotype across countries. While AAV5 consistently presents the lowest seroprevalence among the regions studied, the rate of AAV5 antibody positive patients in the US is 20% compared to 47% in Russia. Consistency was observed in both serostatus and antibody titer (for AAV-positive individuals) over 6 months of time.

Conclusions: These data indicate considerable geographic variability in the percentage of hemophilia A patients with pre-existing antibodies to specific serotypes used in gene therapy.

OC 32.1 | rFVIIIFc for First-time Immune Tolerance Induction (ITI) Therapy: Interim Results from the Global, Prospective verITI-8 Study

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Background: ITI is standard of care for inhibitor eradication and restoration of factor VIII (FVIII) responsiveness in subjects with severe hemophilia who develop high titer inhibitors (HTI). Retrospective data support use of recombinant FVIII Fc fusion protein (rFVIIIFc) in ITI (Carcao *Haemophilia* 2018) but have yet to be confirmed in prospective studies.

Aims: Provide preplanned interim results of verITI-8 (NCT03093480).

Methods: Single-arm, nonrandomized, open-label, ethics-approved study of rFVIIIFc for first-time ITI. Eligible subjects had history of HTI (historical peak ≥5 BU/mL) and provided informed consent. Primary endpoint is time to tolerization, defined by negative inhibitor titer (< 0.6 BU/mL) at 2 consecutive visits; incremental recovery (IR) ≥66% of expected at 2 consecutive visits; and rFVIIIFc half-life ≥7 hours. ITI failure is defined as not meeting above criteria by Week 48. This interim analysis was planned when ≥10 subjects had received ≥6 months of rFVIIIFc ITI.

Results: Results are as of Dec 5, 2018 cutoff. 15 subjects were screened; 14 enrolled and received ≥1 dose of rFVIIIFc for ITI. Median (range) age at start of ITI was 2.6 (0.8-16.0) years and historical peak inhibitor titer was 29.6 (6.2-256.0) BU/mL. Six subjects have been successfully tolerized (Table 1), with median (range) time to first negative titer, normal IR, and tolerization of 2.3 (1.7-15.6), 6.0 (4.3-28.1), and 11.7 (8.1-32.0) weeks, respectively. Seven subjects continue to receive rFVIIIFc ITI (median time on ITI [range]: 16.0 [0.1-35.6] weeks) and one has failed (Table 2). No adverse events related to rFVIIIFc have been reported.

Conclusions: Early results from this prospective/ongoing study of first-time ITI indicate that rFVIIIFc may offer rapid time to tolerization in some subjects (5/14 subjects were tolerized in ≤12.5 weeks) with severe hemophilia A and HTI. Achieving tolerance faster can improve quality of life and reduce costs.

TABLE 1 ITI outcomes for subjects who were tolerized (sorted by time to tolerization)

Age at screening, years	Historical peak inhibitor titer, BU/mL	Inhibitor titer pre-rFVIIIFc ITI, BU/mL	Time from inhibitor diagnosis to start of ITI, wks	ITI outcome	Time to			Time on study, wks
					Negative inhibitor titer,[a] wks	Normal IR,[b] wks	Tolerization,[c] wks	
1	14.1	9	12.14	ITI success	2.29	4.29	8.14	10.86
3	8.11	0.64	18.86	ITI success	2.29	6.86	9.86	31.86
1.7	6.2	0.8	28.43	ITI success	1.71	5.43	11.43	32.14
16	40	2	747.43	ITI success	1.71	6	12	26.57
2.9	9	3.25	71.86	ITI success	2.29	6	12.43	36
1.3	7.1	20	3.86	ITI success	15.57	28.14	32	37.29

BU, Bethesda unit; IR, incremental recovery; ITI, immune tolerance induction; rFVIIIFc, recombinant factor VIII Fc fusion protein.
[a]Time to negative inhibitor titer is defined as the time interval (weeks) from the start date of rFVIIIFc ITI treatment to the date of first negative inhibitor titer which is subsequently confirmed by a sample from a consecutive visit 2 weeks later.
[b]Time to normal IR is defined as the time interval (weeks) from the start date of rFVIIIFc ITI treatment to the date of the first normal IR (≥66%) which is subsequently confirmed by a sample from a consecutive visit 2 weeks later.
[c]Time to tolerization is defined as the time interval (weeks) from the start date of rFVIIIFc ITI treatment to the time meeting all 3 criteria of negative inhibitor titer (<0.06 BU/mL at 2 consecutive visits, normal IR at 2 consecutive visits, and half-life ≥7 hours).

TABLE 2 ITI outcomes for subjects still ongoing ITI or failed (sorted by time on study)

Age at screening, years	Historical peak inhibitor titer, BU/mL	Inhibitor titer pre-rFVIIIFc ITI, BU/mL	Time from inhibitor diagnosis to start of ITI, wks	ITI outcome	Time on study, wks
2	19.2	11	20.43	Ongoing	0.14
10	150	4.2	>156	Ongoing	2.14
0.8	11.3	5.1	23.14	Ongoing	7.57
1.3	132.2	94	4.14	Ongoing	16
5	48	40	71.14	Ongoing	21
5	256	25	55.71	Ongoing	21.43

OC 32.2 | Risk Factors for Inhibitor Development after 50 Exposure Days in Non-severe Hemophilia A - Preliminary Data of a Case-control Study

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Background: The development of factor VIII (FVIII) inhibitors is a major complication in non-severe hemophilia A (HA). Non-severe HA patients have a lifelong risk to develop inhibitors, also beyond 50 FVIII exposure days (EDs). To install preventive measures, we need to identify patients that are at risk for inhibitor development. However, our knowledge on risk factors is only limited to inhibitor development before the 50-75th ED.

Aims: We aimed to identify patient and treatment related factors associated with inhibitor development in non-severe HA patients with more than 50 EDs.

Methods: We performed a case-control study in the INSIGHT cohort (FVIII baseline level 2-40 International Units (IU)/dL), by selecting cases who developed inhibitors after 50 EDs. Each case was matched to 1-4 inhibitor-negative controls for date of birth, cumulative number of EDs and center/country. Data was available for the first ED and the last 30 EDs prior to inhibitor development in cases and up to the same number of cumulative EDs in controls. We performed conditional logistic regression (adjusted for predefined confounders) to identify associations between inhibitor development and patient and treatment related factors (i.e. *F8* genotype, family history of inhibitors, treatment indication, FVIII dose).

Results: Out of 2709 non-severe HA patients, we included 31 cases and 84 controls. *p.Arg2150His* was the most prevalent hemophilia genotype (cases 19%; controls 7%). At the first

treatment, cases were older (35 years, IQR 12-49) than controls (18 years, IQR 8.5-37). During the last 10 EDs, cases received a higher mean FVIII dose (35 IU/kg, IQR 20-45) over a shorter time period (30 days, IQR 9-384) than controls (25 IU/kg, IQR 18-35; 202 days, IQR 23-620). The multivariable analyses will be presented at the conference.

Conclusions: Our preliminary findings suggest that both patient and treatment related factors may be associated with inhibitor development after 50 EDs in non-severe HA.

OC 32.3 | People with Haemophilia and Female Carriers in Sweden have a Higher Risk of Developing Anxiety, Depression and Pain Based on Treatment Patterns as Compared to Matched Controls: Data from a Registry Study over a Period of 11 Years

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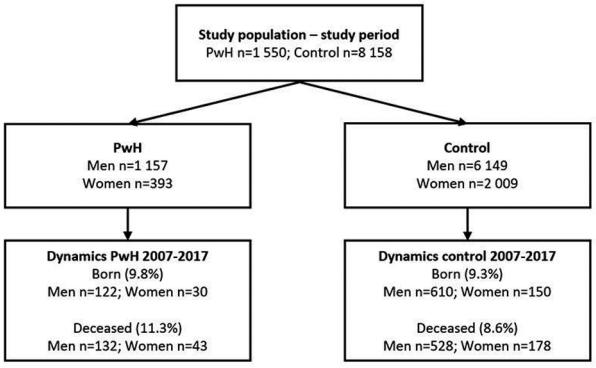
Background: People with haemophilia (PwH) have increased risk of bleeds associated with acute and chronic pain, and long-term disability. Anxiety and depression are other determinants negatively affecting quality of life in PwH. Despite the severity of these comorbidities, they have not been extensively investigated and associations between them even less.

Aims: The MIND study (NCT03276130) aims to identify patterns of prescribed pain, anti-depressive and anti-anxiety medication and management of pain, depression and anxiety in PwH in four Nordic countries, based on patient registry data (A) and surveys (B). Here we describe current and retrospective treatment patterns of prescribed medication (part A) in PwH in Sweden, explore associations with the PwH background and complication characteristics, and compare prescribed medication use between PwH and the general population.

Methods: This 11-year (2007-2017) retrospective population-based registry study, uses international classification systems for diagnoses, surgeries and medications. The study population includes all ages and was identified by diagnosis of haemophilia A or B, or at least one prescription of any factor VIII, IX, or bypassing agent. For each study subject, five age and gender-matched controls were included.

Results: Data were extracted for 1550 PwH. The study population is shown in figure 1. The probability of being prescribed with analgesics, neuroleptics and anti-depressants in PwH compared to controls are shown in Table 1.

Conclusions: A substantially higher prescription of analgesics in Swedish PwH as compared to controls suggests a need for increased focus on pain management including improved bleed protection and



The picture shows study population divided between PwH and controls, men and women and population dynamics (births and deaths) in respective group during the study period 2007-2017

FIGURE 1

TABLE 1

	High factor consumption* (n=431) vs Control (n=2162)		Low factor consumption (n=1003) vs Control (n=4903)		Carrier (n=385) vs Controls (n=1900)	
	OR** (95% CI***)	% diff (p-value) if use>0[#]	OR (95% CI)	% diff (p-value) if use>0	OR (95% CI)	% diff (p-value) if use>0
Opioids	**4.03 (3.17–5.12)**	32.5 (0.074)	**2.54 (2.20–2.94)**	21.1 (0.065)	**2.39 (1.92–2.97)**	0.4 (0.980)
Non-opioids[##]	**4.74 (3.76–5.99)**	**13.7 (0.013)**	**2.21 (1.91–2.57)**	**8.2 (0.032)**	**1.99 (1.62–2.45)**	**15.2 (0.014)**
NSAID	**2.74 (2.20–3.40)**	**34.0 (0.012)**	**1.29 (1.12–1.49)**	**19.6 (0.028)**	1.17 (0.96–1.42)	**50.3 (<0.001)**
Steroids	**2.95 (2.24–3.88)**	**-44.3 (0.019)**	**1.71 (1.40–2.09)**	27.1 (0.076)	**2.36 (1.79–3.11)**	**64.1 (<0.001)**
Neuroleptics	**1.95 (1.45–2.63)**	25.9 (0.076)	**1.65 (1.38–1.96)**	-14.9 (0.085)	**1.50 (1.19–1.90)**	0.2 (0.986)
Anti-depressant	**1.49 (1.01–2.19)**	3.6 (0.884)	**1.35 (1.08–1.69)**	-23.1 (0.099)	**1.43 (1.10–1.86)**	10.5 (0.475)
Anti-epileptics	**2.78 (1.60–4.83)**	**84.6 (0.007)**	**1.80 (1.28–2.53)**	5.6 (0.747)	1.60 (0.97–2.64)	-5.4 (0.816)

Numbers marked in bold shows significant values (<5%). Results by population averaged panel data models for binary and continuous data.
*Definition of high factor consumption: Total filled prescriptions during one year corresponding to FVIII ≥40 IU/kg/ week and FIX ≥10 IU/kg/week, respectively. Not included if factor use >350 IU/kg/week. **OR = Odds Ratio - ≥1 filled prescription of medication. ***CI = Confidence Interval. [#] % diff in volume measured by DDD for individuals ≥1 filled prescription. [##]Non-opioids mainly include (99,97%) acetylsalicylic acid and paracetamol.

prevention of, e.g. arthropathy. The increased prescription of neuroleptics and anti-depressants demonstrates that anxiety and depression are overrepresented in PwH and underscores the importance of identifying and managing the broad PwH population including non-frequent health care users. A similar prescription pattern was seen in female carriers suggesting a need for high medical attention and further research to address knowledge gaps.

OC 32.4 | Health Status of People Living with Non-severe Hemophilia - Insights from the Patient Reported Outcomes, Burdens and Experiences (PROBE) Study

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Background: People living with non-severe hemophilia (PwNSH), FVIII/IX 2-40 IU/dL, generally receive factor concentrate episodically. In recent studies, life expectancy of PwNSH is comparable to the non-hemophilia male population. However, their health status has not been systematically investigated.

Aims: To evaluate the health status of PwNSH.

Methods: PROBE data for PwNSH and participants without a bleeding disorder (controls) were analyzed. A match-paired analysis, using 1:1 ratio of PwNSH and controls was performed. Health status of the two groups was compared using parametric or non-parametric methods.

Results: 366 participants were included in this analysis. Mean age was 44.7 (SD 17.4) years in PwNSH and 45.8 (SD 14.4) in controls. Table 1 demonstrates health status of participants. Acute pain and chronic pain were more commonly reported among PwNSH (72.4% and 68.1%) compared to controls (37.6% and 30.0%), P< 0.001. Pain medications were more commonly used among PwNSH as compared to controls (86.2% vs 68.0%, P< 0.001). Working full-time was more frequent among controls. PwNSH more frequently reported being on long-term sick or disability leave compared to controls. Mean sick days per year were significantly higher in PwNSH (3.7 days vs 44.9 days, P< 0.001). Self-evaluated health status using the visual analog scale (VAS) shows that PwNSH had a significantly lower VAS utility score compared to controls (72.8 vs 83.7, P< 0.001). Overall health status, which was evaluated by mean PROBE score, indicated that PwNSH had worse health status compared with controls (0.71 vs 0.89, P< 0.001).

Conclusions: PwNSH had more health-related problems, worse work/school life, and lower health-related quality of life when compared to controls. PROBE findings demonstrate non-severe hemophilia is not a benign disease. Traditional clinical outcomes, e.g., annualized bleeding rate or mortality, are less sensitive to detect health status in PwNSH. We encourage investigators and clinicians to evaluate PwNSH using a patient-reported outcome assessment tool.

TABLE 1 Health status of people living with non-severe hemophilia compared with Controls

Health related problems				**Work and school life**			
	Non-severe hemophilia (n=183)	**No bleeding disorders (n=183)**	**p**		**Non-severe hemophilia (n=183)**	**No bleeding disorders (n=183)**	***p***
Experiencing problems related to health	142 (67.3%)	69 (32.7%)	<0.001	Working full-time	76 (57.1%)	108 (77.7%)	<0.001
Using mobility aids	97 (53.9%)	27 (15.3%)	<0.001	Student full-time	12 (12.8%)	7 (10.3%)	0.23
Using pain medications	157 (86.3%)	117 (68.2%)	<0.001	On long-term sick or disability	11 (10.5%)	1 (1.5%)	0.02
Acute pain	131 (72.4%)	65 (37.6%)	<0.001	Mean sick days per year (SD), days	3.7 (1.5)	44.9 (8.7)	<0.001
Chronic pain	124 (68.3%)	51 (30.0%)	<0.001				
Difficulty with activities of daily living	97 (53.3%)	18 (10.7%)	<0.001	**Health-related quality of life**			
					Non-severe hemophilia (n=183)	No bleeding disorders (n=183)	***p***
				Mean VAS utility score	72.8 (18.6)	83.7 (13.2)	<0.001

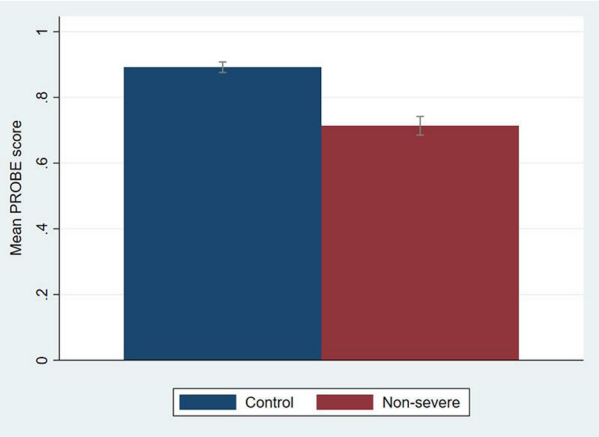

FIGURE 1 PROBE score for non-Severe PWH and Controls (range 0 worst to 1 best health status).

OC 32.5 | Elective Surgery in Patients with Inherited Bleeding Disorders: A Retrospective Analysis of Outcomes and Adherence to Guidelines

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Background: Guidelines are available to aid the management of patients with inherited bleeding disorders undergoing elective surgery. In New South Wales and Australian Capital Territory the Haemophilia Advisory Clinical Committee (HACC) oversees the provision of perioperative factor replacement.

Aims:

1. To review the outcomes of patients with inherited bleeding disorders who received factor replacement approved by HACC for elective surgery.
2. To assess local adherence to published guidelines.

Methods: A retrospective analysis of the Australian Red Cross Blood Service's Supply Tracking Analysis Reporting System (STARS) was performed between 2000 and August 2018 to describe patient characteristics, surgical details and outcomes and the provision of factor replacement.

Results: There were 1065 surgeries performed on 571 patients with inherited bleeding disorders. Diagnoses included Haemophilia A (43.5%), Haemophilia B (9.7%), von Willebrand Disease (45.3%) and rare bleeding disorders (Factor X, XI deficiencies and dysfibrinogenaemia, 1.6%). Surgeries were categorised based on surgical specialty, bleeding risk and whether they were performed in Haemophilia Treatment Centre (HTC). Bleeding complications were reported in 16 surgeries (50:50, HTC: non-HTC) with a further 20 patients receiving factor replacement beyond standard duration of post surgical prophylaxis.

Approximately 50% of all surgeries were performed in a HTC.

Median Biostate doses for minor bleeding risk surgery was 2.0 (0.5-17.3) major 3.7 (0.3-22.2) obstetrics 3.24 (0.3-23.3) and dental surgery 2.0 (0.6-10.6). Approximately 50% of patients with Haemophila received a cumulative factor dose within the seven day range and a further 20% within the 14 day range. 20% received doses lower than recommended and 12% higher.

Conclusions: Elective surgery can be safely performed in patients with inherited bleeding disorders however HTC appear to be underutilised. Factor replacement appears to be appropriately dosed in the majority of cases with the exception of von Willebrand patients undergoing surgery associated with a major bleeding risk, who received doses well below recommended.

OC 39.1 | An Engineered Activated Factor V (^{Super}FVa) for Prevention and Treatment of Acute Traumatic Coagulopathy and Bleeding

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Background: Trauma-associated bleeding and the ensuing acute traumatic coagulopathy (ATC) are often fatal. There is a lack of targeted interventions beyond blood component support. Recent evidence suggests that hyperfibrinolysis and excessive generation of activated protein C (APC) cause rapid depletion of fibrinogen, Factors (F)V and VIII promoting ATC.

Aims: To investigate if targeted intervention with an engineered activated FV variant (^{super}FVa) that is resistant to inactivation by APC reduces traumatic bleeding and ATC.

Methods: Two trauma models in C57BL/6J mice were used to investigate benefits of intervention with ^{super}FVa: a trauma/shock model ("Chesebro") and a "bleed" model. The "Chesebro" model was performed with and without trauma by laparotomy, and with and without blood withdrawal (~500ul) to induce shock (decrease of mean arterial pressure [MAP] from 70-80 to 35±5 mmHg). ^{Super}FVa (0.8mg/kg) was administered by continuous infusion 30 minutes after trauma/shock induction. Activated Partial Thromboplastin Time (aPTT) was determined at the beginning and end (1hour) of experimentation as surrogate for ATC. A liver laceration model was used to determine effects of ^{super}FVa on bleeding and onset of ATC, with ^{super}FVa administered as a bolus at the time of injury. Bleeding (blood absorption with sponges in the abdominal cavity) and aPTT were assessed every 15 min over 1 hour.

Results: In the "Chesebro" model, only mice subjected to both trauma and shock developed ATC, evident from a significant aPTT prolongation (25.4±0.2 to 36.1±0.2 seconds, $p < 0.0001$). ^{Super}FVa normalized the aPTT (27.1±0.2 seconds, $p < 0.0016$). After liver laceration ("bleed" model), most bleeding occurred within the first 15 minutes (0.4±0.01mL) with a decrease of MAP to 35±5 (shock). ATC became evident at 30 min. ^{Super}FVa (0.8mg/kg) reduced bleeding

significantly (0.54±0.01 to 0.34±0.01mL, p< 0.0001) and corrected ATC (26.9±0.3 versus 34.2±0.6 seconds).

Conclusions: ^{Super}FVa rescues traumatic bleeding and ATC, and warrants consideration for clinical development.

OC 39.2 | A New State-of-the-Art Human Fibrinogen Concentrate versus Cryoprecipitate in Pseudomyxoma Peritonei Surgery: Efficacy and Safety Results from a Prospective, Randomized, Controlled Phase 2 Study

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Background: Maintaining adequate plasma fibrinogen levels during cytoreductive surgery for pseudomyxoma peritonei (PMP) may help control hemostasis.

Aims: FORMA-05 compared the efficacy and safety of a new highly purified human fibrinogen concentrate (HFC; Octapharma) with cryoprecipitate in patients with acquired fibrinogen deficiency undergoing PMP surgery.

Methods: FORMA-05 was an off-label single-center, prospective, randomized, controlled Phase 2 study. Patients undergoing PMP surgery with predicted intraoperative blood loss ≥2 L pre-emptively received HFC (4 g) or cryoprecipitate (2 pools of 5 units). The primary endpoint was intraoperative (assessed by surgeon/anesthesiologist) and postoperative efficacy (assessed by hematologist), graded using objective 4-point scales and adjudicated by an Independent Data Monitoring and Endpoint Adjudication Committee (IDMEAC). This Octapharma-sponsored study was approved by the local ethics committee. Informed patient consent was obtained.

Results: The per-protocol set included 43 patients (HFC, n=21; cryoprecipitate, n=22). The mean total intraoperative dose of HFC was 6.5 g, versus 4.1 pools of cryoprecipitate (containing approx. 8.8 g fibrinogen). Median surgery duration was 7.7 h. Overall hemostatic efficacy of HFC was non-inferior to cryoprecipitate and rated excellent or good for 100% of patients in both groups, with similar perioperative blood loss. Intraoperative and postoperative efficacies are shown in Table 1. Due to faster product availability, HFC infusions were initiated a mean of 0.4 h earlier than cryoprecipitate. HFC led to greater mean increases versus cryoprecipitate in FIBTEM A20 and plasma fibrinogen (Table 2).

TABLE 2 Difference in FIBTEM A20 (thromboelastometry) and plasma fibrinogen concentration prior and post pre-emptive dose of HFC/Cryo

	HFC, mean±SD	Cryoprecipitate, mean±SD	Mean difference HFC vs Cryoprecipitate, mean±SD
FIBTEM A20, mm	3.43±2.27 p<0.0001	1.05±3.29 p=0.1507	2.38±2.84 p=0.0088
Plasma fibrinogen, g/L	0.73±0.41 p<0.0001	0.36±0.37 p=0.0003	0.37±0.39 p=0.0038

There were 6 serious adverse events (SAEs) in the HFC group and 17 in the cryoprecipitate group, including 7 thromboembolic events (TEEs; 2 deep vein thromboses, 5 pulmonary embolisms). There were no treatment-related AEs.

Conclusions: These data indicate that HFC is as efficacious as cryoprecipitate in patients undergoing surgery for PMP, with faster availability. No related AEs and no TEEs occurred in patients treated with HFC.

OC 39.3 | Shear-dependent Shedding of Platelet Receptor GPIbα: A Role for Thrombocytopenia during ECMO

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Background: Different mechanisms including platelet consumption or sepsis may account for thrombocytopenia during Extracorporeal

TABLE 1 Intraoperative and postoperative hemostatic efficacy ratings (N=43)

	Intraoperative efficacy				Postoperative efficacy			
	Assessed by surgeon and anesthesiologist		Assessed by IDMEAC		Assessed by hematologist		Assessed by IDMEAC	
4-point efficacy scale	HFC, N (%)	Cryo, N (%)	HFC, N (%)	Cryo, N (%)	HFC, N (%)	Cryo, N (%)	HFC, N (%)	Cryo, N (%)
Excellent	13 (61.9)	12 (54.6)	13 (61.9)	11 (50.0)	21 (100.0)	22 (100.0)	21 (100.0)	22 (100.0)
Good	7 (33.3)	6 (27.3)	7 (33.3)	5 (22.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Moderate	1 (4.8)	4 (18.2)	1 (4.8)	6 (27.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
None	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

Membrane Oxygenation (ECMO). Shedding of glycoprotein (GP) Ibα has been recently identified as a novel mechanism of platelet clearance. There is also evidence that GPIbα-shedding is sensitive to high shear stress, a rheological condition provided by ECMO. We hypothesized that ECMO continuous-flow devices could directly induce thrombocytopenia through shear-induced GPIbα-shedding.

Aims: To assess if ECMO promotes GPIbα-shedding *in vitro* and and *in vivo* and determinate the kinetic evolution of platelet-count and GPIbα-shedding after patient´s implantation.

Methods: Platelet GPIbα-shedding was first investigated *in vitro* using a continuous-flow device (Impella-CP®, Abiomed) loop model. Plasma with normal platelet count (plasma-NPC) was obtained by dilution of platelet-rich plasma obtained from healthy donors in fresh-frozen-plasma. Samples were collected before and after (5, 30, 60 and 180min) perfusion at 37°C of plasma-NPC at intermediate and high-speed (2.6 and 3.6 $L.min^{-1}$ respectively, n=4 each). Platelet-count and GPIbα-shedding were next investigated in 20 ECMO patients before/after implantation of the device (WITECMO-h trial) and in 20 healthy volunteers. The geometric mean-fluorescence-intensity (gMFI) of platelet-GPIbα (PE-staining) and -GPIX (FITC-staining) was measured with a Navios flow cytometer (Beckman Coulter, Miami, FL). Results are expressed as GPIbα/GPIX gMFI-ratio.

Results: *In vitro*, a significant time-dependent loss of GPIbα/GPIX gMFI-ratio was already apparent after 30min (Fig 1) and was more pronounced at high-speed compared to intermediate-speed (pANOVA< 0.001 and p< 0.01 at 180min respectively). The GPIbα/GPIX gMFI-ratio was significantly increased in ECMO-patients compared to healthy subjects 1- and 24-hour after implantation (p< 0.001). A lower platelet-count was observed 1 hour after ECMO implantation (-23% vs baseline, p< 0.01) with a further significant decrease at 24-hours (-53% vs baseline, p< 0.0001) (Fig 2).

Conclusions: ECMO induces an early shear-dependent platelet GPIbα-shedding that may directly account for the early-onset thrombocytopenia observed in ECMO-patients.

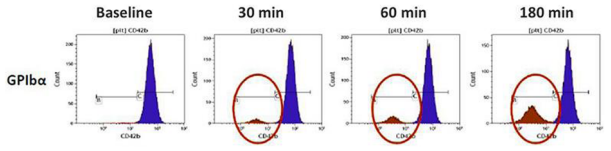

FIGURE 1 loss of GPIb expression in the high-shear continuous-flow loop model

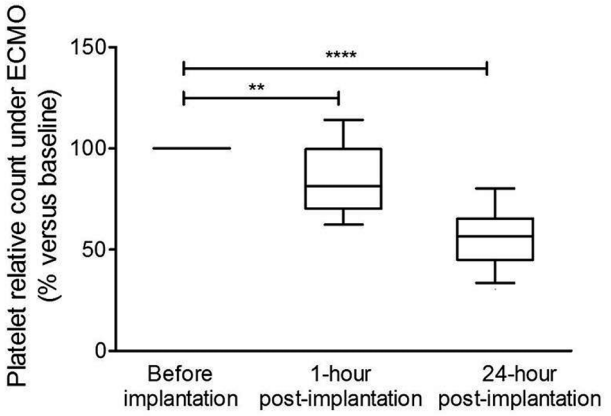

FIGURE 2 Early-onset decrease of platelet-count at initiation of ECMO support

OC 39.4 | Effect of a Recombinant Human Soluble Thrombomodulin (ART-123) on Mortality and Levels of Coagulation Biomarkers in Patients with Sepsis Associated Coagulopathy (SAC)

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Background: ART-123 exerts anti-coagulation and anti-inflammatory effects by binding and modifying thrombin activity to amplify formation of activated protein C. The Phase 3 study was conducted as a randomized, double-blind, placebo-controlled, multinational study to assess efficacy and safety of ART-123in patients with SAC (NCT01598831). We hypothesized that the ART-123 treatment effect would have a measurable improvement in coagulation biomarker levels over the study period.

Aims: To investigate in the SCARLET Study whether ART-123 treatment of patients with SAC can reduce mortality, especially in patients with higher baseline coagulation biomarker levels, and positively impact coagulation biomarker levels over the study.

Methods: 800 subjects with cardiovascular or respiratory dysfunction, and coagulopathy were enrolled and received at least one dose of either ART-123 or placebo. The primary endpoint of the study was reduction of 28 day all-cause mortality, and coagulation biomarkers were evaluated at baseline and multiple time points.

Results: Treatment with ART-123 showed a trend towards reduced mortality, but did not meet the primary efficacy endpoint, with a 2.55% (p=0.318) absolute risk reduction (ARR). ART-123 showed a greater treatment effect in subjects with higher baseline coagulation activation, and improved levels of coagulation biomarkers over time. Post-hoc analysis showed the improvement in mortality was impaired by 21% of subjects not maintaining coagulopathy at baseline (after randomization and prior to first dose). Removal of these subjects from the analysis showed a 5.4% ARR, and the enriched subgroup showed a further increased treatment effect for both measures.

Conclusions: The results of the SCARLET study show that ART-123 treated SAC patients had a trend towards reduced mortality especially with higher baseline biomarker levels, and over time. This effect increased further in subjects with confirmed coagulopathy at baseline. The study results support the potential for ART-123 to reduce mortality in SAC patients.

OC 39.5 | Inhibition of ADAMTS13 Prevents the Loss of High Molecular Weight von Willebrand Factor Multimers in an in vitro Left Ventricular Assist Device

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Background: The bleeding diathesis observed in patients implanted with a left ventricular assist device (LVAD) is linked to the acquired von Willebrand syndrome as all patients have a loss of high molecular weight (HMW) VWF multimers. The loss of HMW VWF multimers might be explained by an increased shear-induced proteolysis of VWF by ADAMTS13. Hence, specifically blocking ADAMTS13 might be an efficient way to rescue the loss of HMW VWF multimers in LVAD patients.

Aims: To investigate if blocking ADAMTS13 using mAb 17C7 can prevent the loss of HMW VWF multimers in an in vitro LVAD circuit using human, ovine and bovine blood.

Methods: Human, ovine and bovine blood (750 mL) was circulated through an in vitro Impella CP® circuit with inhibiting anti-ADAMTS13 mAb 17C7 (n=4) or non-inhibiting mAb 5C11 (n=4) or PBS (n=4). Plasma samples were analysed for VWF multimers, VWF antigen (VWF:Ag) and VWF collagen binding activity (VWF:CB).

Results: Blocking ADAMTS13 using 17C7 prevented the loss of HMW VWF multimers and hence a decrease in VWF:CB/VWF:Ag in the in vitro Impella system with human blood (Figure 1A and 1B) showing the therapeutic potential of 17C7 to rescue the loss of HMW VWF multimers in LVAD patients. However, blocking ovine ADAMTS13 with 17C7 did not prevent the loss of HMW VWF multimers in vitro (Figure 1C). In contrast, when using bovine blood in an in vitro Impella system, the decrease in HMW VWF multimers could be prevented by adding 17C7 (Figure 1D), similar to what was observed with human blood.

Conclusions: Blocking ADAMTS13 is a promising therapeutic strategy to prevent the loss of HMW VWF multimers in LVAD patients. The loss of HMW VWF multimers is also ADAMTS13 dependent in calves, but not in sheep, making the calf the ideal preclinical animal model to study the in vivo effect of this novel therapy.

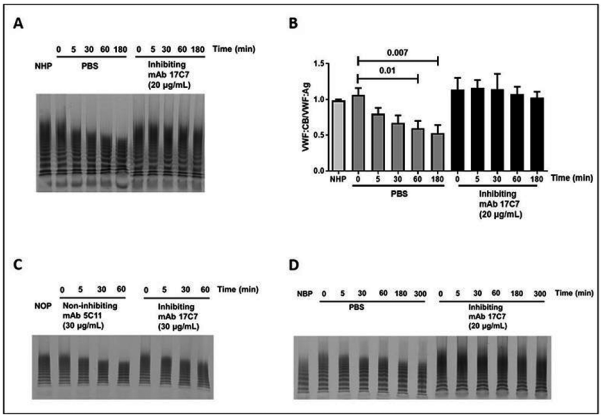

FIGURE 1 Inhibition of ADAMTS13 in an in vitro LVAD system using human, ovine and bovine blood

OC 40.1 | Targeted Exon Skipping as a Potential Gene Correction Therapy for Haemophilia A

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Background: Haemophilia A (HA) is a complex bleeding disorder resultant from mutations in the coagulation protein factor VIII. Factor VIII is encoded by the *F8* gene, and mutations within *exon14* contributes to the aetiology of HA in a subset of patients (~10%); resulting in an incorrectly spliced mRNA transcript and mutant protein. There exists a small degree of functional redundancy in the *exon14* B-domain which may be potential targets for nucleic acid-based therapies.

Aims: To target deletion of selected F8 exon14 sequences from pre-mRNA splicing using 2'Omethyl and/or phosphorodiamidate antisense oligoribonucleotides (AOs) to induce a truncated but functional factor VIII protein.

Methods: AOs targeting predicted F8 exon14 spliceosome elements on the pre-mRNA were introduced in HuH7 cells. Following 24h transfection, total RNA was extracted, converted into cDNA and PCR-assessed using primers spanning exon13&14 and14&15 boundaries. Positive PCR products were sequenced for identity. Factor VIII protein was analysed by immunoblotting with anti-factor VIII antibodies.

Results: We have successfully induced exon skipping as determined by RT-PCR amplification of F8 fragments from AO treated cells. Different AOs induced distinct F8 pre-mRNA splicing in cells. A wild-type product (3.5Kb) was present in all treatments, and excision events were observed in AO1-4, and AO6, but not AO5. Two main *F8* products, 150bp and 800bp, were observed. Sanger sequencing confirmed 2 distinct truncated F8 gene transcripts with different disruptions to the reading frame. One had the intended exon14 sequence completely ablated. An alternative transcript partially excised exon14 sequences, but retained sequences towards the 3'end, including the furin-cleavage site.

Conclusions: Gene skipping of mutated regions of the factor VIII B domain at the pre-mRNA stage is a potential novel treatment strategy for Haemophilia A. This study successfully excised exon14 of the F8 gene to generate a complete B domain deletion, as well as a partially deleted B domain factor VIII.

OC 40.2 | Therapeutic Levels of FVIII Generated by CRISPR/Cas9-mediated *in vivo* Genome Editing in Hemophilia A Mice

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Background: Expression of Factor VIII (FVIII) from a FVIII cDNA that has been integrated into the genome of hepatocytes has the potential to provide a life-long cure for Hemophilia A (HemA).

Aims: Determine if the CRISPR/Cas9 nuclease system can promote non-homologous end joining (NHEJ) mediated insertion of a Factor VIII (FVIII) cDNA into intron 1 of the albumin gene of mice and thereby generate therapeutic levels of FVIII.

Methods: A human FVIII cDNA lacking the signal peptide and flanked by a splice acceptor and polyadenylation signal was packaged in AAV8. Streptococcus pyogenes Cas9 (spCas9) mRNA and a single guide RNA (sgRNA) targeting mouse albumin intron 1 were encapsulated in a lipid nanoparticle (LNP). Cohorts of 5 adult HemA mice or adult NOD *scid* gamma (NSG) mice were injected with 2e12 or 2e13 vg/kg respectively of this AAV8-FVIII donor and 2mg RNA/kg of the LNP. FVIII levels in the blood of HemA and NSG mice were measured with the Coatest® activity assay or a human FVIII specific capture-Coatest® assay, respectively. Droplet Digital PCR was used to quantify the frequency of integration of the FVIII gene in the forward orientation into albumin intron 1 in the liver.

Results: Mice injected with the AAV8-FVIII donor alone had no detectable FVIII in their blood. In HemA mice injected with both the AAV8-FVIII donor and the LNP, 30% of normal human FVIII levels were measured at 2 weeks. NSG mice injected with the AAV8-FVIII donor and LNP had 70% of normal human FVIII levels that were stable through the longest time point measured at 4 months. The frequency of FVIII cassette integration in albumin intron 1 was between 0.5% to 3% of the murine albumin alleles.

Conclusions: CRISPR/Cas9 mediated integration of a FVIII cDNA into albumin intron 1 at low frequency generated therapeutic levels of FVIII in mice.

OC 40.3 | Exon-Specific U1snRNA-Mediated Rescue of Splicing and Missense Changes in Hemophilia A

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Background: Splicing mutations account for 8-10% of Hemophilia A (HA)-causing defects, a highly underestimated proportion since even exonic variants, besides acting on protein biology, can affect splicing regulatory elements. In this context, splicing-rescuing approaches might represent innovative personalized therapies. Over years we demonstrated that engineered variants of the spliceosomal U1snRNA, named exon-specific U1snRNA (ExSpeU1), can correct multiple splicing mutations for therapeutic purposes.

Aims: To elucidate the molecular mechanisms underlying all HA-causing mutations on exon 19 and to test ExSpeU1s as a correction strategy.

Methods: *In vitro* expression of *F8* exon 19 minigenes to assess splicing pattern. Expression via lentiviral vectors (LV) of FVIII missense variants and evaluation of FVIII antigen (ELISA) and activity (chromogenic assays) levels.

Results: Highly variable degree of aberrant splicing was observed, ranging from complete exon 19 skipping for all changes at the 5' splice site (5'ss) to different proportions of exon 19 inclusion for exonic changes (p.Gly2000Ala, p.Arg2016Gly and p.Tyr2036Tyr). FVIII protein expression studies demonstrated that the p.Arg2016Gly change leads to reduced antigen and activity levels (8.3±1.6% and 10.7±1.0% of wild-type, respectively). Differently, the p.Gly2000Ala change did not affect FVIII antigen nor activity, indicating a major effect on splicing for this variant. Co-transfection experiments led to the identification of a single ExSpeU1, designed to minimize potential off-target effects, able to properly restore splicing. In particular, the ExSpeU1 was able to completely rescue (>90%) splicing variants (c.6115+3G>T, c.6115+4A>G and c.6115+6T>A) as well as exonic changes (p.Gly2000Ala and p.Tyr2036Tyr).

Conclusions: Overall, we provided insights into the molecular mechanisms underlying HA caused by all splicing and exonic changes in exon 19, strengthening the notion that also exonic mutations can impair splicing process by affecting splicing regulatory elements. Moreover, we provided evidence of the ability of a single ExSpeU1 in rescuing multiple HA-causing mutations, thus expanding the therapeutic potential of this approach.

OC 40.4 | CRISPR Activation on Coagulation F7 or F8 Promoters Potentiate Trascriptional Activity in the Normal and Mutated Gene Context

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Background: Engineered transcription factors (eTF) have been successfully exploited to modulate gene expression and represent potential therapeutic tools for human disorders. In this perspective, the emerging CRISPR activation (CRISPRa) technology gives great advantages compared to the first eTF, mostly based on Transcription-Activator-like Effectors (TALE). Coagulation factor disorders, in

which even modest protein level increase have a therapeutic impact, represent ideal models to test the CRISPRa.

Aims: To tailor the CRISPRa system on the promoters of coagulation F7/F8 genes and enhance transcription, and thus expression, in the normal and mutated conditions.

Methods: Creation of reporter gene (luciferase) constructs for F7 and F8 promoter. Transient transfection in hepatoma cells and evaluation of the luciferase activity. Evaluation of the FVIII expression by functional assays (FXa generation).

Results: Using reporter gene assays we identified a sgRNA able to trigger the activity of F7 promoter up to ~35-fold, either wild-type, or defective due to the disease-causing c.-61T>G mutation. The effect was higher than that of an engineered TALE-based effector targeting the same promoter region (~15-fold). The transcription increase was confirmed on the endogenous F7 gene, the dCas9-VPR/sgRNAF7.5 combination was more efficient (~6.5-fold) in promoting factor VII (FVII) protein secretion/activity than TALE-TF4 (~3.8-fold). The approach was translated on F8 promoter, whose reduced expression translates into Haemophilia A. Reporter gene assays identified sgRNAs that appreciably increased F8 promoter activity (sgRNAF8.1, ~8-fold; sgRNAF8.2, ~19-fold) with a synergistic effect (~38-fold) when combined.

Conclusions: Over this pioneer study we demonstrated that the CRISPR system can be addressed to increase the expression, or rescue disease-causing mutations, of different promoters, with potential intriguing implications for coagulopathies.

OC 40.5 | Adeno-associated Virus Vector Based on Serotype 3 Represents an Alternative Serotype for Hemophilia Gene Therapy

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Background: Hemophilia represents a disease of choice for gene therapy. Recently successful clinical studies have been conducted on hemophiliacs with the use of adeno-associated virus (AAV) vectors. These demonstrated that hemophilia could be cured by means of gene therapy. However, subsequent intravenous administrations of identical AAV vector serotypes is not possible. This is due to the fact that high-tier neutralizing antibodies against AAV capsid develop after the first administration.

Aims: We searched for an alternative serotype for the second round of gene therapy. We aimed at establishing a strategy for re-administration.

Methods: Various AAV serotypes were compared to express transgenes in hepatocyte cell lines, mouse, and macaque. The intra-portal injection of the vector in a macaque was used for the second administration.

Results: When AAV vectors were intravenously injected in mice, we obtained efficient and specific transgene expression by AAV8 vectors in the liver. AAV8 more efficiently transduced the murine hepatocyte cell line TLR3. While, AAV3 exhibited the strongest expression in the human hepatocellular carcinoma cell line HepG2. Intravenous injection of AAV8 and AAV3 vectors, but not AAV5, yielded FIX's therapeutic levels at a same vector dose in macaques. In addition, in a macaque AAV8 vector administration dramatically induced neutralizing antibodies against AAV8 (1:1,000). Subsequently, the levels of neutralizing antibody for AAV8 gradually diminished. However, it maintained a high-titer (1:100) in the course of a long term follow-up (5 years). Finally, we successfully re-administered the AAV3 vector in the macaque previously treated by AAV8 in combination with intra-portal injection.

Conclusions: AAV3-based vectors represent an alternative serotype for hemophilia gene therapy. We believe that the combination of different serotypes with intra-portal injection may become an attractive approach to re-administer AAV vectors.

OC 42.1 | PK-Guided Rurioctocog Alfa Pegol Prophylaxis in Patients with Severe Hemophilia A Targeting Two FVIII Trough Levels: Results From the Phase 3 PROPEL Study

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Background: Prophylaxis with extended half-life recombinant factor FVIII (FVIII) rurioctocog alfa pegol (BAX855/TAK-660) targeting FVIII troughs ≥1% has been shown to be effective and well-tolerated in patients with severe hemophilia A.

Aims: This prospective, randomized, open-label, multicenter study (NCT02585960) evaluated the safety and efficacy of pharmacokinetic (PK)-guided BAX855/TAK-660 prophylaxis targeting two different FVIII troughs.

Methods: This study enrolled previously treated patients 12-65 years of age, participating in previous BAX855/TAK-660 phase 3 studies (NCT01945593; NCT02210091; NCT01913405); or newly recruited, BAX855/TAK-660-naïve patients. Key inclusion criteria were: FVIII activity <

1%; annualized bleeding rate (ABR) ≥2; and previous plasma-derived/recombinant FVIII treatment (>150 exposure days). Exclusion criteria included history of a confirmed inhibitory antibody to FVIII (≥0.6 Bethesda units) before or at screening; an acquired hemostatic defect other than hemophilia A; and platelet count < 100,000/mL. Ethics committees approved the study and patient informed consent was obtained. After initial PK assessments, eligible patients were randomized into two groups: targeting FVIII trough of 1-3% or 8-12% and evaluated over 12 months (1st 6-months: treatment-adjustment; 2nd 6-months: efficacy evaluation period).

Results: Primary efficacy outcome: proportion of patients with total ABR=0 (all bleeds). Secondary outcomes: total, spontaneous ABR, spontaneous joint ABR. Safety assessment included the occurrence of adverse events (AEs) and serious AEs.

Conclusions: This study will provide relevant data on the question whether higher trough levels and increased total exposure to FVIII lead to better clinical outcomes. These findings will support evidence-based clinical decision making, especially for patients with ambitious treatment goals or active lifestyles, and close a relevant clinical data gap of personalized prophylaxis.

OC 42.2 | The Comparative Pharmacokinetic (PK) Profiles of Two Extended Half-life (EHL) Factor VIII Concentrates [rFVIII-Fc (Eloctate) and rAHF-FL-PEG (Adynovate)] in Adolescent Boys with Hemophilia A: Is there a Difference?

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Background: A head-to-head comparison of the PK of extended half-life (EHL) FVIII concentrates in the same subjects has not yet been reported. Recently in Canada, boys (ages 12-18 years) with hemophilia A were required to switch from rFVIII-Fc to rAHF-FL-PEG.

Aims: Compare the PK profiles of the 2 EHL-FVIIIs in the same boys and correlate PK profiles against various covariates.

Methods: Boys switching from rFVIII-Fc to rAHF-FL-PEG prophylaxis had FVIII levels sampled at 3, 24, 48 and 72 hrs following a regular prophylactic infusion of rFVIII-Fc and then 1-3 months later, of rAHF-FL-PEG. Prophylaxis regimens were unchanged with switching. Testing was done by one-stage (kaolin) and chromogenic methods. PK parameters were determined with the WAPPS-Hemo population PK tool.

Results: 25 boys (mean age 15.3 yr; range: 12.1-18.4; 9 were O blood type) underwent switching. Mean terminal half-lives with the one-stage assay were 16.1 hrs (range: 10.4-23.4; rFVIII-Fc) and 16.7 hrs (range: 11-23.6; rAHF-FL-PEG) (P>0.05). With the chromogenic assay, these were 18.0 hrs (range: 12.0-25.5; rFVIII-Fc) and 16.0 hrs (range: 10.3-22.9; rAHF-FL-PEG) (p=0.001). There were no significant differences in clearance, AUC, Vss or time for FVIII levels to drop to 5%, 3% and 1% between

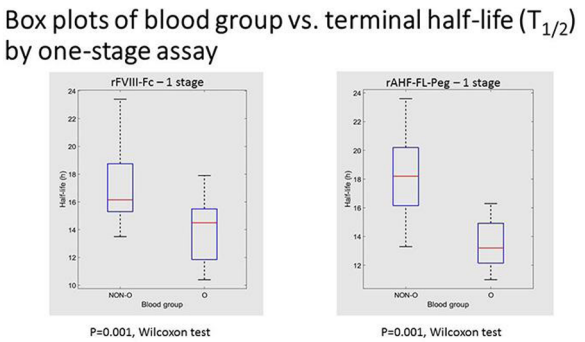

FIGURE 1 Box plots of blood group vs. terminal half-life (T1/2) by one-stage assay

TABLE 1 PK parameters obtained by WAPPS on boys initially with rFVIIIFc and then with rAHF-FL-Peg

	rFVIII-Fc		rAHF-FL-Peg	
Mean (range)	One stage	Chromogenic	One stage	Chromogenic
Vss (L)	3.19 (1.2-4.3)	3.21 (1.6-4.6)	2.99 (1.8-4.9)	2.84 (1.7-4.8)
Clearance (L/h/kg)	0.0023 (0.0012-0.0038)	0.0023 (0.0012-0.004)	0.0022 (0.0013-0.0032)	0.002 (0.0013-0.0034)
AUC (IU/L/h)	18037 (9612-34254)	18472 (10327-32801)	18918 (11262-28003)	19051 (10579-29679)
Terminal Half-life (hr)	16.1 (10.4-23.4)	18.0 (12.0-25.5)	16.7 (11.0-23.6)	16.0 (10.3-22.9)
Time to 5% (hr)	65.4 (39-106)	67.2 (41.8-110.5)	67.7 (42.8-100.5)	70.3 (42.6-105)
Time to 3% (hr)	78.1 (47.2-124)	81.4 (51.7-130.5)	80.9 (51.4-119)	82.9 (50.8-123)
Time to 1% (hr)	108.3 (66.7-166.5)	115.2 (75.6-178.5)	112.2 (72-163.5)	112.9 (70.4-166)

the 2 EHL-FVIIIs (Table 1). At the 72 hr time point, mean observed FVIII levels following a mean dose of 39.3 IU/kg; (range: 30.3-52.6) of rFVIII-Fc were 4.4% (one stage) and 4.4% (chromogenic). For rAHF-FL-PEG, these were 5.1% and 5.3%, respectively following a mean dose of 38.3 IU/kg (range: 29.7-50.4). There was considerable interpatient variation in PK, mainly explained by differences in blood type (Fig 1) and VWF levels.

Conclusions: rFVIII-Fc and rAHF-FL-PEG have almost identical PK parameters (clearance, AUC, Vss, half-life and time to critical FVIII levels) by one-stage assay. For rAHF-FL-PEG, the chromogenic assay gave a smaller Vss and lower half-life but identical times to reach critical FVIII levels.

OC 42.3 | Exploring Methods to Predict an Individual´s Pharmacokinetic Profile when Switching between Factor Concentrates in Hemophilia A

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Background: Hemophilia A patients switch factor concentrate brands for various reasons. There is no protocol to guide switching that uses individual pharmacokinetic (PK) information. Population pharmacokinetic (PopPK) models that quantify how an individual deviates from a population, termed an "eta-value" (η), for any PK parameter, may help with predicting PK during the switching process.

Aims: We explored methods to predict individual PK of a new brand by combining information from the individual PK profile before the switch with a PopPK model of the new brand.

Methods: Individual PK of subjects (n=16; age=13-54) switching from octocog alfa (pre-FVIII) to turoctocog alfa (post-FVIII) were obtained from Martinowitz et al. Three methods to estimate individual PK outcomes of post-FVIII were compared, and all used the post-FVIII PopPK model with covariates (e.g. age, weight) from the Web Accessible Population Pharmacokinetic Service - Hemophilia. Method 1 used no additional information; method 2 used individually scaled ηs from the pre-FVIII PopPK model; and method 3 used a regression line to acquire individual ηs (not predictive, comparator only). Relative errors, regression slopes and 95% CI were calculated between the predicted and observed PK outcomes where a 95% CI that includes a slope of 1 indicates a valid prediction.

Results: Both method 2 and method 3 had lower mean relative errors and ranges than using method 1 across most PK outcomes (Table 1). Regression slopes for methods 2 and 3 were closer to a slope of 1 than method 1 across all PK outcomes (Figure 1). Similar results were observed when predicting pre-FVIII from post-FVIII.

Conclusions: Taking into account population and individual knowledge of pre-FVIII PK when predicting post-FVIII PK (and vice versa) (method 2) was the most accurate means of predicting either factor concentrate's PK. Predicting the PK of the new brand may shorten the time for dose optimization.

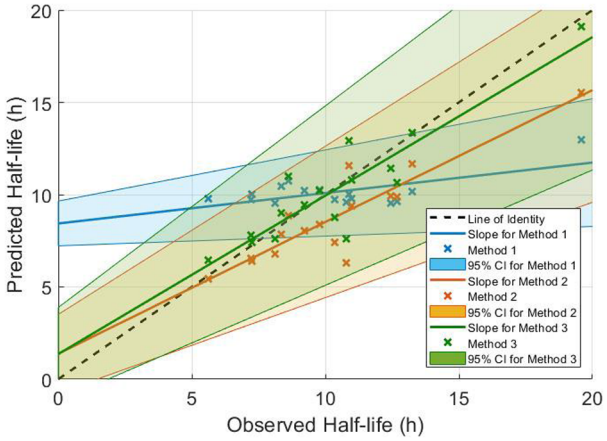

FIGURE 1 Comparison between regression lines using each method to estimate individual half-life on post-FVIII

TABLE 1 Absolute mean relative errors when comparing predictions with observed data across different PK outcomes

PK outcomes (units)	Absolute mean [range] relative error (%) using population value with covariates (method 1)	Absolute mean [range] relative error (%) using population value with covariates + scaled η-values (method 2)	Absolute mean [range] relative error (%) using population value with covariates + η-regression line (method 3)
Clearance (L/h)	26.17 [1-56]	25.53 [1-84]	16.79 [0-48]
Central volume (L)	13.78 [1-41]	16.11 [1-38]	13.74 [0-31]
Half-life (h)	23.12 [5-74]	15.20 [3-42]	10.43 [1-29]
Time at 5% (h)	27.58 [6-85]	16.76 [4-45]	12.04 [1-31]
Time at 2% (h)	26.28 [6-82]	16.34 [4-44]	11.22 [1-31]
Time at 1% (h)	25.42 [6-81]	16.07 [3-43]	10.90 [0-31]
Concentration at 24h (IU/mL)	58.88 [6-260]	26.53 [3-68]	22.96 [0-52]
Concentration at 48h (IU/mL)	76.33 [15-260]	32.75 [7-75]	29.01 [1-99]
Concentration at 72h (IU/mL)	41.90 [14-65]	23.99 [1-51]	21.21 [2-72]

OC 42.5 | First Report of an Extended Half-life FIX in Previously Untreated Patients with Haemophilia B

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Background: Nonacog beta pegol (N9-GP) is an extended half-life recombinant glycoPEGylated factor IX (FIX) developed to treat haemophilia B.

Aims: To report results from ≥20 patients who completed 50 exposure days (EDs) in the ongoing paradigm 6 trial that is assessing immunogenicity, efficacy, and safety of prophylactic N9-GP in previously untreated patients (PUPs) with haemophilia B.

Methods: paradigm 6 is a multicentre, open-label, single-arm, phase 3 trial, which included males aged < 6 years with FIX ≤2%, previously untreated or with ≤3 EDs to FIX-containing products. Patients received N9-GP 40 IU/kg once weekly (prophylaxis) or individualised N9-GP dosing (pre-prophylaxis). Bleeds were treated with N9-GP 40 IU/kg (80 IU/kg if severe). Primary endpoint: incidence of anti-FIX inhibitory antibodies. Secondary endpoints included annualised bleeding rate (ABR) and safety outcomes. The study was approved by relevant ethics committees and patients' guardians gave informed consent.

Results: 37 patients (mean age 0.9 [range: 0-4] years) have been enrolled. 13 patients started directly on prophylaxis. Of 24 patients who started pre-prophylaxis, 15 moved to prophylaxis (N=28). Total in-trial EDs were 2833, representing ~65 patient-exposure years. Two (6.1%) of 33 at-risk patients (those with ≥10 EDs + patients who developed inhibitors) developed high-titre anti-FIX inhibitory antibodies and were withdrawn. In the prophylaxis group, mean N9-GP trough level was 0.150 IU/mL (95% confidence interval [CI]: 0.141-0.160), median ABRs for overall, spontaneous, and traumatic bleeds were all 0.0, and estimated mean overall ABR was 0.31 (95% CI: 0.16-0.57). All 15 bleeds reported in the prophylaxis group were treated with 1 N9-GP injection (see Table 1). No unexpected safety signals or thrombotic events were identified (see Table 2).

Conclusions: We report an inhibitor incidence of 6.1%, which is within the expected range of 5-14% (Francini *et al.* 2016). Moreover, N9-GP prophylaxis was efficacious and well tolerated in PUPs with haemophilia B.

TABLE 1 Treatment outcomes

Study endpoints	Prophylaxis (n=28)	Pre-prophylaxis (n=24)
Exposure		
Number of N9-GP doses, mean (SD)		
Overall	93.4 (65.6)	9.3 (6.9)
Prophylactic doses	92.2 (64.9)	–
Efficacy outcomes		
Bleed-free patients, n (%)	19 (67.9)	13 (54.2)
Number of bleeds/number of patients with bleeds	15/9	33/11
Haemostatic control of bleeding		
Haemostatic response*, N (%)		
Excellent	9 (60.0)	23 (69.7)
Good	5 (33.3)	8 (24.2)
Moderate	1 (6.7)	2 (6.1)
Poor	–	–
Haemostatic success rate**, N (%)	14 (93.3)	31 (93.9)
One injection required per bleeding episode, N (%)	15 (100.0)	27 (81.8)
Median ABR		
Overall ABR	0.0	0.95
$ABR_{spontaneous}$	0.0	0.0
$ABR_{traumatic}$	0.0	0.0
Negative binomial estimate of ABR***		
Overall ABR (95% CI)	0.31 (0.16–0.57)	–
$ABR_{spontaneous}$ (95% CI)	0.08 (0.03–0.21)	–
$ABR_{traumatic}$ (95% CI)	0.23 (0.11–0.46)	–
N9-GP consumption		
Consumption of N9-GP per bleeding episode, IU/kg/bleed, mean (SD)	48.8 (16.9)	55.0 (28.7)

*Assessed on a four-point haemostatic response scale. **Determined as being either an excellent or a good response. ***Estimated from a negative binomial regression model with number of bleeding episodes as the outcome variable and adjusting for exposure time.

%, percentage bleeds/patients; ABR, annualised bleeding rate; $ABR_{spontaneous}$, annualised spontaneous bleeding rate; $ABR_{traumatic}$, annualised traumatic bleeding rate; CI, confidence interval; n, number of patients; N, number of bleeds; N9-GP, nonacog beta pegol; SD, standard deviation.

TABLE 2 Safety outcomes

	Number of patients, N (%) (n=37)*	TEAEs, E (number of AEs per patient-years of exposure)
All AEs	36 (97.3)	377 (5.80)
Serious AEs**	14 (37.8)	23 (0.35)
AEs considered by Investigator to be related to treatment	7 (18.9)	13 (0.20)
Medical events of special interest	9 (24.3)***	14 (0.22)
AEs leading to withdrawal	3 (8.1)†	5 (0.08)

*Includes all treated patients (pre-prophylaxis and prophylaxis).

**Three of the 23 serious AEs were considered to be related to treatment (FIX inhibitors, n=2; anaphylactic reaction in patient with anti-FIX inhibitory antibodies, n=1).

***Rash (n=1), papular rash (n=1), urticarial rash and generalised erythema (n=1), drug hypersensitivity (n=2), hypersensitivity (n=1), anti-FIX inhibitory antibodies (n=2), accidental underdose (n=1), and suspected autism (n=1).

†Includes the two patients withdrawn due to development of anti-FIX inhibitory antibodies and one patient with an urticarial rash and generalised erythema.

AE, adverse event; E, number of AEs; N, number of patients with AEs; TEAE, treatment-emergent AE.

OC 51.1 | An Engineered Protein Analogous to IL-2, Mutein 24, Decreases the Formation of Factor VIII Inhibitors in Hemophilia Mice Receiving Factor VIII Treatment

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Background: Hemophilia A (HemA) patients lack functional factor VIII protein (FVIII), resulting in the inability of their blood to clot. Currently, HemA patients receive infusions of FVIII protein to treat their disease, of which approximately 30% develop inhibitors to FVIII. Previously, mice receiving IL-2 complexed with α-IL-2 mAbs showed a lack of FVIII inhibitor formation after FVIII treatment associated with proliferation and activation of regulatory T cells (Tregs). We evaluated the capability of a computationally designed analogue of IL-2, named Mutein 24 (Mut. 24), to provide similar effects.

Aims: This study aims to increase and activate the Treg population in mice using Mut. 24 dosing prior to FVIII treatment in hopes of preventing the formation of anti-FVIII antibodies in hemophilia mice.

Methods: Mice received intraperitoneal injections of 6μg Mut. 24 in 200μL PBS day 0, followed by another injection of 3μg Mut. 24 in

200µL PBS on day 7. On day 4, mice received hydrodynamic injection of 50µg FVIII plasmid. Flow cytometry was used to characterize peripheral blood cell populations, while ELISA was used to assess inhibitor concentrations in plasma. At week 20, mice received a secondary hydrodynamic injection of 100µg FVIII plasmid.

Results: Mice receiving Mut. 24 showed a dramatic increase in the population of activated Tregs (Table 1). FVIII gene therapy via hydrodynamic injection resulted in high anti-FVIII inhibitor concentration in control mice, while mice treated with Mut. 24 produced low or non-existent inhibitor levels (Figure 1). This difference persisted after the second hydrodynamic injection.

Conclusions: Mut. 24 significantly increased and activated the Treg population in mice, which prevented formation of high-titer anti-FVIII antibodies when administered in coordination with FVIII gene therapy. This tolerance persisted over 6 months, even after a second administration of FVIII gene therapy, implying a potential method for induction of long term immune tolerance of FVIII.

TABLE 1 Characterization of lymphocytes 7 days after Mut. 24 (n=5) or PBS (n=4) injection

Treatment Group	% CD4+/ Lymphocytes	% CD25+Foxp 3+/ CD4+	% CTLA-4+Helios+/ CD4+CD25+Foxp3+
PBS	24.8	8.3	66.4
Mut. 24	25.5	18.9	78.4

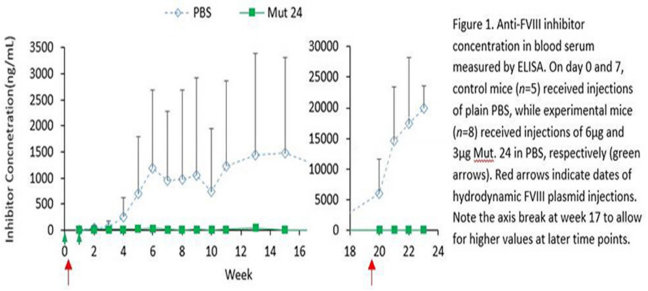

Figure 1. Anti-FVIII inhibitor concentration in blood serum measured by ELISA. On day 0 and 7, control mice (*n*=5) received injections of plain PBS, while experimental mice (*n*=8) received injections of 6µg and 3µg Mut. 24 in PBS, respectively (green arrows). Red arrows indicate dates of hydrodynamic FVIII plasmid injections. Note the axis break at week 17 to allow for higher values at later time points.

FIGURE 1 Anti-FVIII inhibitor concentration in blood serum measured by ELISA

OC 51.2 | High-throughput Immunoprofile Mimotope Variation Analysis in Previously Untreated Patients with Severe Haemophilia A before and after FVIII Exposure

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Background: In recent years, quantitative immunomics has developed rapidly, offering immune response analysis at high-throughput. Mimotope Variation Analysis (MVA) is a powerful tool to characterize the immune response profiles in a sample against millions of synthetic peptide antigens simultaneously.

Aims: To delineate immunoprofiles of patients with haemophilia A and to predict inhibitor occurrence upon response to therapy.

Methods: MVA was used to analyse plasma samples of 80 haemophilia A patients (37 with inhibitor and 43 without) from the SIPPET cohort. Plasma samples before and after treatment of each patient were analysed.

Results: The analysis of Top2500 peptide repertoire across individuals revealed a high dissimilarity in immunoprofiles derived from the pre- and post-treatment samples of the same individual. This dissimilarity suggested that immunoprofile differences might reflect upon the effects of the specific treatment product, environment or maturation of the immune system.

In the pre-treatment stage, clustering analysis of group-specific peptides selected from the entire dataset resulted in 59 motifs exclusive to patients who developed inhibitor and 747 motifs exclusive to patients who did not develop inhibitors. Logistic regression analysis resulted in the selection of 20 most relevant epitope motifs able to predict an inhibitor with 82.9% accuracy.

FVIII-specific immunoprofiles revealed immunogenicity/immunoreactivity "hotspots" in A1, A2 and C2 domains of FVIII protein regardless of the used FVIII product, confirming the dominant epitopes of FVIII from previous studies. Five FVIII epitope motifs were found to correctly classify all inhibitor cases with 85.4% accuracy in the post-treatment stage.

Inhibitor-positive patients treated with rFVIII displayed a much broader immune response targeting FVIII than those treated with pd-FVIII, whereas the situation was quite reverse concerning VWF.

Conclusions: MVA analysis exposed novel, unknown features of immune response in patients with haemophilia, that could provide predictive and prognostic parameters for inhibitor development at early stage of treatment.

OC 51.3 | Exploring Spontaneous Readthrough over Recurrent *F8* Nonsense Mutations: Potential Correlation with Inhibitor Risk?

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Background: Nonsense mutations, relatively frequent (>10%) in Hemophilia A (HA), are considered as "null conditions". However, they have been found also in moderate/mild HA patients and, as compared with large gene alterations, seem to be associated with lower inhibitor risk. This observation suggests residual factor VIII (FVIII) expression

levels that could arise from ribosome readthrough that would produce full-length proteins through mis-recognition of "leaky" nonsense triplets.

Aims: To investigate the occurrence of spontaneous readthrough over the most recurrent *F8* nonsense mutations.

Methods: Transient expression studies in HEK293 cells with the chimeric FVIII-GL protein, where FVIII is fused to the naturally-secreted Gaussia (GL) luciferase, and evaluation of luciferase activity in media and cell lysates.

Results: The FVIII-GL chimeric construct, bearing the selected nonsense mutations, was designed to release the GL moiety alone, to magnify the output arising from readthrough due to the luciferase high sensitivity. Appreciable luciferase activity was detectable in cell lysates from cells expressing all FVIII variants. Noticeably, analysis of media revealed three groups of mutations differing in the secreted luciferase activity, namely i) low (0.15-0.34% of wild-type FVIII-GL) such as the p.R15X (patients' number 14), p.R355X (27), p.R446X (22), p.R1715X (13) and p.R1985X (31), ii) medium (0.58-1.48%) such as the p.R602X (20),v p.2166X (34) and p.R814X (32) and iii) high (3.9-4.9%) such as the p.R1960X (20), p.R2135X (27), p.R2228X (41), and p.R2326X (34).

Conclusions: These preliminary data provide a pioneer indication that spontaneous readthrough occurs over all the investigated *F8* nonsense mutations and identify three classes of mutations. In turn, this would result in different levels of full-length FVIII in HA patients, which could influence the risk for immune response after therapy and help diagnosis and treatment. The relationship of these data with the presence of inhibitors is in progress.

OC 51.4 | A Dysbiotic Gut Microbiota Diminishes Short-Chain Fatty Acid Production and Increases Factor VIII Immunogenicity in Haemophilia A Mice

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Background: Anti-factor VIII antibodies develop in ~30% of haemophilia A (HA) patients however the basis of this adverse event is incompletely understood. The gut microbiota significantly influences the immune system but its mechanistic action on the anti-FVIII immune response remains unresolved.

Aims: To determine the mechanistic basis of changes to the anti-FVIII immune response documented in association with the gut microbiota.

Methods: A microbiologically-confirmed model of gut dysbiosis has been established in HA mice through oral administration of ampicillin. Ampicillin-administered and control mice were given 50 ng of recombinant FVIII biweekly for two weeks and sacrificed two weeks later. Anti-FVIII antibodies were quantified by ELISA and Bethesda assay. Caecal contents harvested before FVIII challenging were sequenced for 16s rRNA or analyzed via nuclear magnetic resonance for short-chain fatty acid (SCFA) quantification. Splenic lysates were analyzed for cytokine profiles and spleens and mesenteric lymph-nodes were evaluated for immune cell frequencies by flow cytometry.

Results: There was no difference in the incidence of anti-FVIII antibodies between ampicillin and control mice, but ampicillin-treated mice produced significantly higher titres of total anti-FVIII IgG (P=0.0023). Elucidation of anti-FVIII IgG antibody subclasses revealed elevated titres of IgG1 (P=0.002), IgG2a (P=0.0003), and IgG2b (P< 0.0001) but not IgG3. Anti-FVIII inhibitory antibodies were also elevated in this group (P< 0.0001). SCFA investigation revealed that prior to FVIII challenging, ampicillin-treated mice had a 1.6-, 1.3-, and 8.1-fold reduction in butyrate, acetate, and propionate (P=0.0035, P=0.0010, and P< 0.0001, respectively) in the caecum. Between both study groups, there were no significant differences in either splenic cytokine profiles nor frequencies of $CD4^+CD25^+Foxp3^+$ and $CD4^+CD25^-LAP^+$ T lymphocytes or $B220^+$ B lymphocytes before FVIII challenging.

Conclusions: Significant reductions in immunomodulatory SCFAs are seen in association with the dysbiotic gut microbiota of HA mice. Diminished SCFA levels precede FVIII exposure and are associated with increased FVIII immunogenicity.

OC 51.5 | Recombinant Canine and Human FXa-I16LProvide Safe and Efficacious Hemostasis during Major Bleeding Events in Hemophilia A Dogs with High-titer Inhibitory Anti-FVIII Antibodies

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Background: Recombinant FXa-I16L (Ile16→Leu) is a zymogen-like variant of activated factor X (FXa) that shows enhanced resistance to inactivation by endogenous inhibitors as compared with wild-type FXa. It corrected the hemophilic coagulopathy in animal models and was safe in normal subjects (Haemophilia 2012;18:87; JTH 2017;15:931) but has not been tested in the presence of high titer, circulating anti-FVIII inhibitory antibodies.

Aims: To determine the safety and hemostatic efficacy of FXa-I16L in hemophilia A dogs with high titer Bethesda Inhibitors during major bleeds.

Methods: Recombinant canine and human FXa-I16L were produced in HEK293 cells and purified by column chromatography. Hemophilia A dogs with an intron 22 inversion (n=5) or a multi-exon deletion (n=1, exons 22-25 deleted) with Bethesda Inhibitors ranging from 6.5 to > 200 BU received canine and/or human FXa-I16L on demand to treat spontaneous muscle, throat, nose, and central

nervous system bleeding events and for emergency major abdominal surgery (8 separate events). The dogs received 1 to 43 doses (10 - 146 µg/kg) given IV every 2 to 4 hours during the day with an ~ 8 hour overnight interval.

Results: No major adverse events were detected. Both canine and human FXa-I16L corrected Thromboelastogram (TEG) parameters (R values decreased from > 60 min to a range of ~10 to 20 mins). Clinical hemostasis was achieved in all cases with cessation of visible bleeding, improved muscle mobility, stabilization of hematocrit without need for further red blood cell transfusion, reduction in hematoma size, improved neurological status, and/or stabilization and healing of laparotomy and gastrotomy incisions. Repeated exposure to human FXa-I16L appeared to produce canine anti-human FXa xenoantibodies (ELISA). Notably, no canine anti-canine FXa antibodies have been detected to date.

Conclusions: Recombinant canine and human FXa-I16L are safe and efficacious and provide life-saving hemostasis in dogs with severe hemophilia A complicated by high titer Bethesda inhibitors.

OC 56.1 | Increased *in vivo* Angiogenesis in von Willebrand Factor (VWF)-deficient Mice

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Background: Patients with inherited and acquired von Willebrand factor (VWF) abnormalities can develop gastrointestinal angiodysplasia and the resulting bleeding can be challenging to treat. Studies have suggested a negative regulatory role for VWF in angiogenesis.

Aims: This study uses a mouse model of VWF-deficiency to examine the time-course of vessel formation and to evaluate *in vivo* angiogenesis.

Methods: To quantify vessel growth over time, ears were excised from C57BL/6 wild type (WT) and VWF-knockout (KO) mice aged one, two, three, four, five, and six months (N = 3 mice/time point) and mounted onto glass slides. Blood vessels were defined as red identifiable structures in the ear and total vessel length was quantified. For the Matrigel plug assay, eight to 12-week old C57Bl/6 WT and VWF-KO mice were injected subcutaneously into the back flank with Matrigel and fibroblast growth factor (FGF) or phosphate buffered saline (PBS; N = 2-5 mice/treatment). One week later, mice were sacrificed and the plugs were excised and stained for hematoxylin and eosin (H&E), DAPI, VWF, and CD31.

Results: One-month old VWF-KO mice had significantly more blood vessels per area than WT mice (VWF KO = 0.0028 ± 0.007 mm^{-1} and WT = 0.0017 ± 0.0005 mm^{-1}; P < 0.05; Figure 1). However, by six months, VWF KO mice had fewer vessels than WT mice (VWF KO = 0.0022 ± 0.0009 mm^{-1} and WT = 0.0033 ± 0.0010 mm^{-1}, P = 0.052; Figure 1) perhaps due to vessel destabilization and pruning.

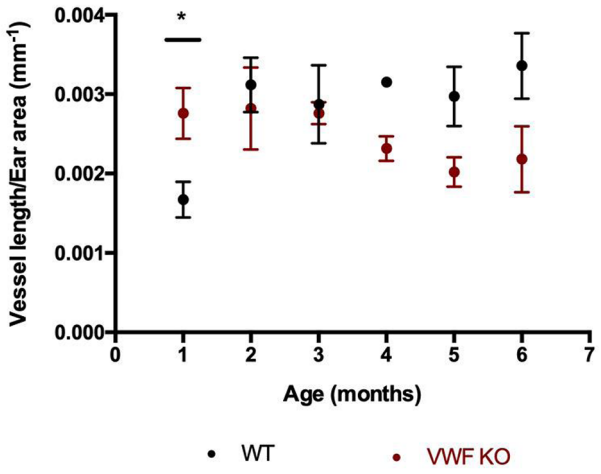

FIGURE 1 Vessel length in WT and VWF-KO mice aged one to six months

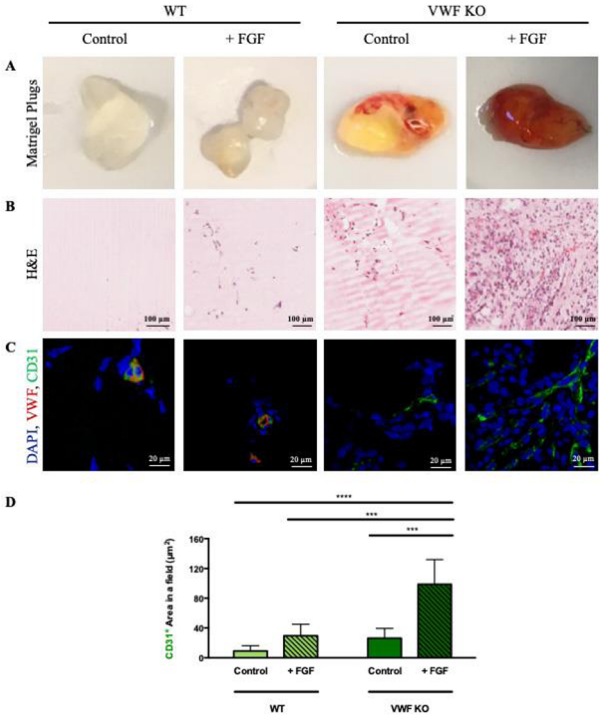

FIGURE 2 Matrigel plugs from WT and VWF-KO mice were imaged and stained for vessel formation

Additionally, when Matrigel was supplemented with FGF, there was increased vascularization in Matrigel plugs excised from VWF KO mice (98 ± 33 $µm^2$) compared to WT type mice (30 ± 15 $µm^2$; P < 0.001; Figure 2D).

Conclusions: Increased vessel formation in the ears and Matrigel plugs of VWF-deficient mice provide further evidence that VWF is a negative regulator of angiogenesis.

OC 56.2 | Allele-specific Inhibition of VWF p.Cys1190Tyr in Patient-Derived Endothelial Colony Forming Cells Corrects the von Willebrand Disease Type 2A Phenotype

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Background: von Willebrand disease (VWD) is mostly caused by dominant-negative mutations in von Willebrand factor (VWF). We hypothesized that inhibition of mutant *VWF* by siRNAs will improve VWD phenotypes. Instead of targeting the dominant-negative mutation itself, targeting heterozygous SNPs located on the same allele as the mutation allows for the design of a small set of siRNAs that will be applicable to a large proportion of the patient population. This hypothesis was recently proved in transfected HEK293 cells, which is an efficient model to select effective siRNAs but do not endogenously produce VWF.

Aims: Test the efficacy of selected siRNAs in healthy control endothelial colony forming cells (ECFCs) and ameliorate the phenotype of patient-derived ECFCs.

Methods: ECFCs were isolated from a healthy control heterozygous for all selected SNPs (c.1451A|G, c.2365A|G, c.2385C|T and c.2880A|G) and a VWD type 2A patient heterozygous for VWF mutation p.Cys1190Tyr and SNP c.1451A|G. siRNA efficacy was determined by allele-specific qPCR. VWF collagen binding (VWF:CB) was used to assess the VWD type 2A phenotype. Informed consent was obtained from the study subjects according to the declaration of Helsinki.

Results: siRNAs targeting c.1451A|G, c.2385C|T and c.2880A|G proved to be effective in ECFCs. Highest efficacy was observed for siRNAs targeting c.1451A and c.1451G with specific *VWF* inhibition of 87.5% and 94.5%, and aspecific inhibition of 56.5% and 54%, respectively. Type 2A ECFCs had a VWF:CB/VWF:Ag of 0.61, compared to 0.94 in control ECFCs. Inhibition of the mutant allele by si1451A improved VWF:CB/VWF:Ag to 0.81; inhibition of the wildtype allele by si1451G worsened VWF:CB/VWF:Ag to 0.41.

Conclusions: Allele-specific siRNAs can distinguish *VWF* SNP-alleles in ECFCs. The phenotype of VWD type 2A ECFCs improved using an siRNA that targets the mutant allele, but deteriorated when inhibiting the wildtype allele. These results show that allele-specific siRNAs are a potential therapeutic approach for dominant-negative VWD.

OC 56.3 | Investigations of the von Willebrand Factor (*VWF*) p.Arg273Trp Variant Using Endothelial Colony Forming Cells (ECFCs)

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Background: The von Willebrand factor (*VWF*) variant, p.Arg273Trp, has previously been described in Types 1 and 3 von Willebrand disease (VWD), resulting in defective multimerization and secretion. *In vitro* studies in Cos-7 cells showed ER retention. To date no investigations have been carried out in patient-derived endothelial cells.

Aims: To investigate the *VWF* p.Arg273Trp variant using patient-derived endothelial colony forming cells (ECFCs).

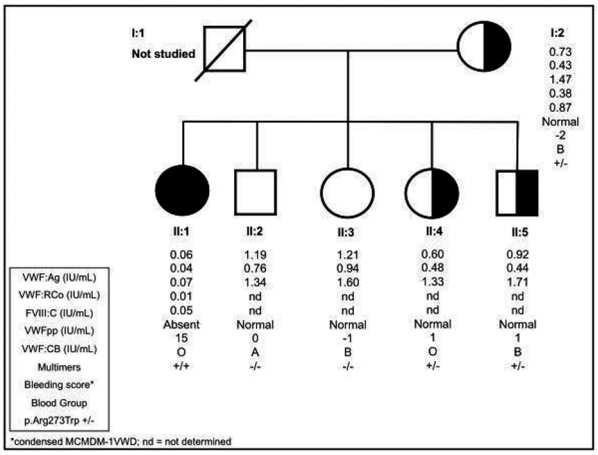

FIGURE 1 Pedigree with laboratory data for family with VWF p.Arg273Trp variant

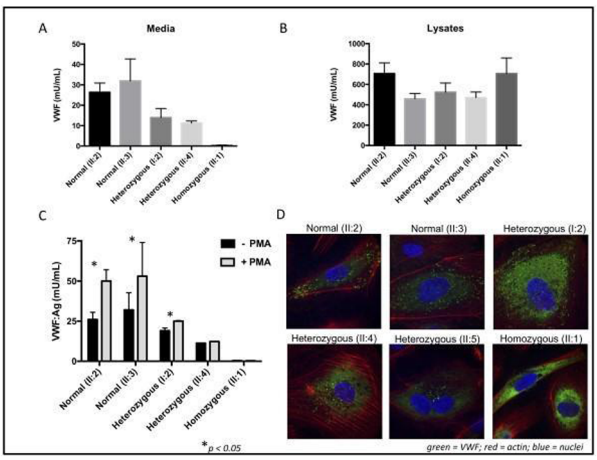

FIGURE 2 VWF expression in ECFCs with VWF p.Arg273Trp variant

Methods: Queen's University Research Ethics Board approval was granted, and all participants gave informed consent. Bleeding scores were obtained and plasma collected for VWF measurements.Peripheral blood was collected to isolate ECFCs. VWF secretion, multimerization, stimulated release, and storage using confocal immunofluorescence (IF) microscopy were examined in ECFCs.

Results: In this family, the variant presents in a classic autosomal recessive fashion and is associated with Type 2A VWD.The pedigree and laboratory results are shown in Figure 1. The bleeding phenotype is severe in the homozygous index case and mild/absent in heterozygous individuals. Plasma VWF multimers were normal for all heterozygous individuals and absent in the homozygous patient. We observed discrepancies in the multimer content between patient plasma and VWF secreted from the patient's ECFCs; heterozygous individuals showed a loss of high molecular weight multimers which did not mirror that in plasma (full range of normal multimers; consistent with normal collagen binding). Figure 2 shows data from ECFCs. VWF release upon stimulation with PMA resulted in a significant increase in one heterozygous ECFCs and non-significant in another. A complete absence of VWF stored in Weibel-Palade bodies (WPBs) and only diffuse VWF in the homozygous cells was observed. Heterozygous cells exhibited a combination of VWF in WPBs and diffuse staining.

Conclusions: The family studied here highlights the variable expressivity of this *VWF* variant as phenotypic differences were observed compared to previous families studied. This study also highlights the value of patient-derived cells in VWD studies.

OC 56.4 | Large Gene Deletion in a VWD Patient Impairs VWF Storage in WPBs and Affects Phenotypic Features of Blood Outgrowth Endothelial Cells

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Background: A heterozygous large deletion of exons 4-34 of von Willebrand factor (VWF) gene was identified in an index patient (IP) with type 3 von Willebrand disease (VWD), as the only mutation. The IP exhibited severe bleeding manifestations despite prophylaxis treatment, with a short VWF half-life after infusion of VWF/FVIII concentrate.

Aims: This study intended to further explore the pathomolecular mechanisms by which this large deletion intensely interrupts VWF biosynthesis and life-cycle.

Methods: Blood outgrowth endothelial cells (BOECs) were isolated from IP and healthy controls. Transcript analysis was done by extracting RNA from the BOECs. The production of VWF in BOECs ($2.5\text{-}3 \times 10^6$ cells/75 cm^2 flak) was evaluated by measuring VWF antigen (VWF:Ag) and multimer analysis of BOECs supernatant. Confocal immunostaining of BOECs was performed. BOECs features such as cell adherence junction and leukocyte-BOECs adhesion were characterized. Screening for alloantibodies to VWF was performed using the enzyme-linked immunosorbent assay.

Results: RNA analysis assured transcription of normal VWF mRNA besides an aberrant deleted transcript. The amount of secreted VWF:Ag from IP- BOECs was not significantly different from that of controls (63% ± 4.9% vs. 72% ± 36.6%, P>0.05). However, IP-BOECs exhibited deficiency in assembly of VWF multimers and formation of Weibel-Palade bodies (WPBs). Confocal imaging of IP-BOECs demonstrated subcellular mislocalization of angiopoietin-2 (Ang-2), with a lesser extent of VWF/Ang-2 colocalization as compared with healthy donors. The IP-BOECs exhibited increased adhesiveness to leukocytes which contained VWF after adhesion. No alloantibody against VWF was detected in IP.

Conclusions: Deleted VWF incorporates to VWF multimers and has a dominant-negative impact on elongation of multimers and WPBs. Aberrant WPBs hinders recruitment of cargos, such as Ang2, resulting in dysregulated release of Ang-2 and subsequent increased BOECs adhesiveness to leukocytes. We presumed the adhered leukocytes phagocytosing VWF may contribute to rapid elimination of VWF from circulation.

OC 56.5 | *In vitro* and *in vivo* Amelioration of the von Willebrand Disease Type 2B Phenotype Using an Allele-specific siRNA

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[1]*Leiden University Medical Center, Internal Medicine, Division of Thrombosis and Hemostasis, Einthoven Laboratory for Vascular and Regenerative Medicine, Leiden, the Netherlands,* [2]*HITh, UMR_S 1176, INSERM, Univ. Paris-Sud, Université Paris-Saclay, Le Kremlin-Bicetre, France*

Background: Von Willebrand disease (VWD) type 2B is associated with increased binding of von Willebrand factor (VWF) to platelets, low platelet count and presence of active VWF. Co-expression of wild-type mouse Vwf (mVwf) and mVwf-p.Val1316Met in $Vwf^{-/-}$ mice recapitulates the heterozygous state of VWD type 2B. Since siRNAs can distinguish two *VWF* alleles by only one nucleotide, we hypothesize that an siRNA that inhibits production of mVwf-p.Val1316Met, but not wild-type Vwf, could correct the VWD type 2B phenotype.

Aims: Correction of the VWD type 2B phenotype in a murine VWD type 2B model via siRNA-mediated allele-specific inhibition.

Methods: siRNAs were screened using mVwf overexpressing HEK293 cells. Heterozygous mice were generated by hydrodynamic co-injection of m*Vwf*/Myc and m*Vwf*-p.Val1316Met/HA cDNAs. siRNA was administered five days later. Wild-type and mutant protein levels were measured by ELISA to assess siRNA efficiency and specificity. Platelet count and an ELISA that captures the open

conformation of the Vwf A1 domain (active Vwf) were used to assess *in vitro* and *in vivo* phenotypes.

Results: We identified an siRNA capable of *in vitro* inhibition of mutant mVwf by 63% with minor inhibition of the wild-type allele. This coincides with a 2.5-fold decrease in active mVwf. In our heterozygous mouse model we observed a severe drop in platelet count and an increase in active mVwf. In a pilot experiment using an siRNA concentration of 1 mg/kg we observed a strong inhibition of total mVwf levels but with a clear specificity of the siRNA towards the mutant allele. This resulted in recovery of platelet count and a 1.6-fold decrease in active mVwf.

Conclusions: We selected an siRNA with the *in vitro* and *in vivo* ability to inhibit mVwf-p.Val1316Met, resulting in decrease of active mVwf. Optimization of conditions to further improve siRNA specificity and correction of the murine VWD type 2B phenotype are ongoing.

OC 60.1 | Surgical Experience from Four Phase III Studies (HAVEN 1-4) of Emicizumab in Persons with Haemophilia A (PwHA) with or without FVIII Inhibitors

E. Santagostino[1]; J. Oldenburg[2]; T. Chang[3]; J. Xu[3]; S. Chebon[4]; M. Doral[3]; V. Jiménez-Yuste[5]; R. Liesner[6]; S. Croteau[7]; T. Lambert[8]; C. Kempton[9]; S. Pipe[10]; C. Dhalluin[4]; N. Selak Bienz[4]; C. Vignal[4]; M. Lehle[4]; G. Young[11]; R. Kruse-Jarres[12]

[1]*Fondazione IRCCS Ca'Granda, Ospedale Maggiore Policlinico, Milan, Italy,* [2]*University Clinic Bonn, Bonn, Germany,* [3]*Genentech Inc., South San Francisco, United States,* [4]*F. Hoffmann-La Roche Ltd, Basel, Switzerland,* [5]*Hospital Universitario La Paz, Autonoma University, Madrid, Spain,* [6]*Great Ormond Street Hospital, London, United Kingdom,* [7]*Boston Hemophilia Centre, Boston Children's Hospital, Boston, United States,* [8]*Haemophilia Care Centre, Bicêtre AP-HP Hospital and Faculté de Médecine Paris XI, Paris, France,* [9]*Emory University School of Medicine, Department of Hematology and Medical Oncology,, Atlanta, United States,* [10]*University of Michigan, Ann Arbor, United States,* [11]*Children's Hospital Los Angeles, University of Southern California Keck School of Medicine, Los Angeles, United States,* [12]*Bloodworks Northwest, Seattle, United States*

Background: Emicizumab is a subcutaneously administered, bispecific, humanized monoclonal antibody that bridges FIXa and FX to restore the function of missing FVIIIa in PwHA.

Aims: To present our experience of surgical procedures in pooled data from four phase III studies: HAVEN 1, 2, 3 and 4 (NCT02622321, NCT02795767, NCT02847637 and NCT03020160, respectively).

Methods: The studies enrolled PwHA of all ages, with or without FVIII inhibitors. Participants requiring minor or unplanned major surgery were managed per the investigator's discretion. We analysed perioperative use of prophylactic coagulation factor (FVIII or bypassing agents), as well as the frequency and management of post-operative bleeds.

Results: Across the HAVEN studies, 214 minor and 19 major surgeries were performed in 113 and 19 participants, respectively. The majority of minor surgeries were dental and central venous access device (CVAD) procedures (Figure 1). The majority of minor surgeries (n=141; 65.9%) were managed without use of prophylactic coagulation factor; of these, 128 (90.8%) did not result in treated post-operative bleeds. Of the 73 (34.1%) procedures managed with prophylactic coagulation factor, 64 (87.7%) did not result in treated post-operative bleeds. Treated post-operative bleeds occurred most commonly following dental procedures, managed either with (5/22) or without (9/41) prophylactic coagulation factor.

Of the 19 major surgeries, 16 (84.2%) were managed with prophylactic coagulation factor; only one of which resulted in a treated post-operative bleed. Of the three major surgeries managed without prophylactic coagulation factor, no post-operative bleeding occurred. A summary of prophylactic coagulation factors used is provided in Table 1. Importantly, no procedure resulted in death, thrombosis, FVIII inhibition, or unexpected bleed.

Conclusions: Emicizumab alone provides good haemostatic coverage for patients undergoing minor surgeries. The majority of minor procedures were performed without prophylactic coagulation factor, and of these, >90% did not result in a treated post-operative bleed.

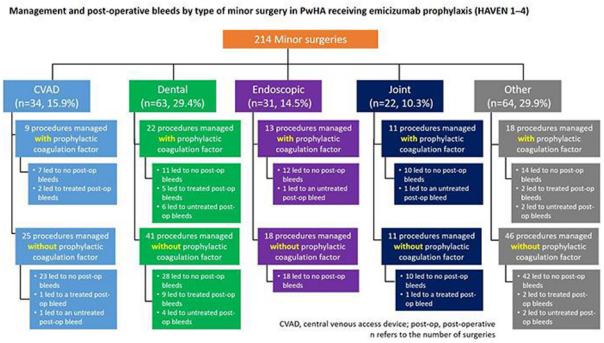


FIGURE 1

TABLE 1

Summary of prophylactic coagulation factor used in PwHA receiving emicizumab who underwent surgery (HAVEN 1–4)

	Minor surgeries		Major surgeries	
	In PwHA with inhibitors	In PwHA without inhibitors	In PwHA with inhibitors	In PwHA without inhibitors
Number of surgeries	106	108	11	8
Prophylactic agents used, n (%)				
rFVIIa	19 (17.9)	0	8 (72.7)	0
aPCC	1 (0.9)	0	1 (9.1)	0
FVIII short-acting	1 (0.9)	47 (43.5)	1 (9.1)	5 (62.5)
FVIII long-acting	0	5 (4.6)	0	2 (25.0)

n refers to the number of surgeries.

In one major surgery (Santagostino et al., 2017), a patient received rFVIIa and FVIII.

PwHA, persons with haemophilia A; rFVIIa, recombinant activated FVII; aPCC, activated prothrombin complex concentrate

OC 60.2 | Emicizumab Treatment is Efficacious and Well Tolerated Long Term in Persons with Haemophilia A (PwHA) with or without FVIII Inhibitors: Pooled Data from Four HAVEN Studies

M. Callaghan[1]; C. Negrier[2]; I. Paz-Priel[3]; S. Chebon[4]; M. Lehle[4]; J. Mahlangu[5]; G. Young[6]; T. Chang[3]; J. Xu[3]; R. Kruse-Jarres[7]; M.E. Mancuso[8]; M. Niggli[4]; P. Kuebler[3]; M. Shima[9]; V. Jimenez-Yuste[10]; E. Asikanius[4]; G. Levy[3]; S. Pipe[11]; J. Oldenburg[12]

[1]Children's Hospital of Michigan, Detroit, United States, [2]Edouard Herriot University Hospital, Lyon, France, [3]Genentech, Inc., South San Francisco, United States, [4]F. Hoffmann-La Roche Ltd, Basel, Switzerland, [5]University of the Witwatersrand and NHLS, Johannesburg, South Africa, [6]Children's Hospital Los Angeles, University of Southern California Keck School of Medicine, Los Angeles, United States, [7]Bloodworks Northwest, Seattle, United States, [8]Angelo Bianchi Bonomi Haemophilia and Thrombosis Center, Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico, Milan, Italy, [9]Nara Medical University Hospital, Kashihara, Japan, [10]Hospital Universitario La Paz, Autonoma University, Madrid, Spain, [11]University of Michigan, Ann Arbor, United States, [12]University of Bonn, Bonn, Germany

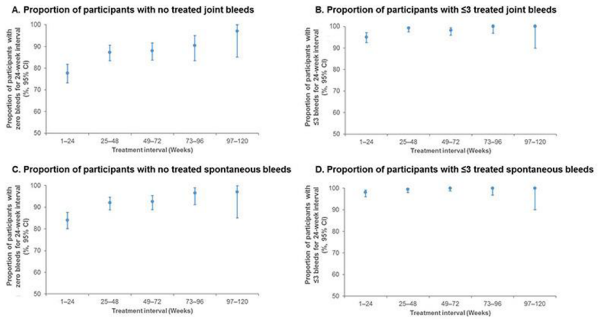
Background: Emicizumab is a subcutaneously administered, bispecific monoclonal antibody that restores haemostasis in PwHA.

Aims: To present the long-term efficacy and safety of emicizumab in PwHA across the four phase III studies: HAVEN 1 (NCT02622321), 2 (NCT02795767), 3 (NCT02847637), and 4 (NCT03020160).

Methods: The studies enrolled paediatric (< 12 years old) and adolescent/adult (≥12 years old) PwHA, with or without FVIII inhibitors. Emicizumab prophylaxis was administered 1.5 mg/kg weekly, 3 mg/kg every 2 weeks, or 6 mg/kg every 4 weeks. All participants assigned to receive emicizumab were included, and data analysed by study or pooled across studies.

Results: Overall, 400 participants in HAVEN 1, 2, 3, and 4 (n=113, 88, 151, and 48, respectively) were included in the efficacy analysis, with a median efficacy period of 82.4 weeks (with 77% of participants treated for ≥74 weeks). The model-based annualized treated bleed rate (ABR; derived via negative binomial regression) was 1.5 (95% CI 1.20-1.84). The mean ABR decreased across consecutive 24-week treatment intervals (Table 1). Across studies, over 87% of participants had no treated joint bleeds in each interval from Week 25 and over 92% had no spontaneous bleeds (Figure 1). No death, thrombotic, or thrombotic microangiopathy events occurred in these trials beyond those reported in HAVEN 1 at the respective primary analysis (Oldenburg et al. NEJM, 2017). Emicizumab continued to be well tolerated, with no participants discontinuing due to adverse events beyond the five previously described (Oldenburg et al. NEJM, 2017; Young et al. ASH, 2018; Mahlangu et al. NEJM, 2018; Pipe et al. WFH 2018).

Conclusions: Emicizumab maintained low bleed rates and favourable safety and tolerability in PwHA of all ages, with or without FVIII inhibitors. Mean ABR decreased and the proportion of participants with zero joint or spontaneous bleeds increased across each treatment interval from Week 25.



Only data for time intervals with ≥10 participants are reported.
CI, confidence interval.

FIGURE 1

TABLE 1

	1–24 weeks	25–48 weeks	49–72 weeks	73–96 weeks
HAVEN 1 (n=113)				
n	109	101	97	73
Mean ABR (95% CI)*	3.1 (0.6–8.8)	0.6 (0.0–4.9)	0.4 (0.0–4.6)	0.4 (0.0–4.5)
% with 0 bleeds (95% CI)	71.6 (62.1–79.8)	84.2 (75.6–90.7)	87.6 (79.4–93.4)	86.3 (76.3–93.2)
% with 0–3 bleeds (95% CI)	89.0 (81.6–94.2)	99.0 (94.6–100)	97.9 (92.8–100)	100.0 (95.1–100)
HAVEN 2 (n=88)				
n	86	64	49	18
Mean ABR (95% CI)*	0.4 (0.0–4.5)	0.3 (0.0–4.4)	0.3 (0.0–4.2)	0.1 (0.0–3.9)
% with 0 bleeds (95% CI)	87.2 (78.3–93.4)	87.5 (76.9–94.5)	87.8 (75.2–95.4)	94.4 (72.7–99.9)
% with 0–3 bleeds (95% CI)	100 (95.8–100)	100 (94.4–100)	100 (92.8–100)	100 (81.5–100)
HAVEN 3 (n=151)				
n	148	144	129	23
Mean ABR (95% CI)*	1.8 (0.2–7.0)	0.9 (0.0–5.5)	0.9 (0.0–5.5)	0.2 (0.0–4.1)
% with 0 bleeds (95% CI)	62.8 (54.5–70.6)	72.9 (64.9–80.0)	77.5 (69.3–84.4)	91.3 (72.0–98.9)
% with 0–3 bleeds (95% CI)	93.2 (87.9–96.7)	97.2 (93.0–99.2)	96.9 (92.3–99.2)	100 (85.2–100)
HAVEN 4 (n=48)				
n	48	45	9	0
Mean ABR (95% CI)*	2.1 (0.3–7.4)	1.5 (0.1–6.4)	NE†	NE†
% with 0 bleeds (95% CI)	64.6 (49.5–77.8)	77.8 (62.5–88.8)	NE†	NE†
% with 0–3 bleeds (95% CI)	91.7 (80.0–97.7)	95.6 (84.9–99.5)	NE†	NE†
Total (n=400)				
n	391	354	284	114
Mean ABR (95% CI)*	1.9 (0.2–7.1)	0.8 (0.0–5.2)	0.8 (0.0–5.2)	0.3 (0.0–4.4)
% with 0 bleeds (95% CI)	70.8 (66.1–75.3)	79.4 (74.8–83.5)	82.7 (77.8–87.0)	88.6 (81.3–93.8)
% with 0–3 bleeds (95% CI)	93.4 (90.4–95.6)	98.0 (96.0–99.2)	97.2 (94.5–98.8)	100 (96.8–100)

*Based on the calculated annualized bleed rate.
†Only data for time intervals with ≥10 participants are reported.

OC 60.3 | A Single-arm, Multicentre, Open-Label, Phase III Clinical Trial to Evaluate the Safety and Tolerability of Prophylactic Emicizumab in Persons with Haemophilia A (PwHA) with FVIII Inhibitors (STASEY): Interim Analysis Results

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Background: Emicizumab is a bispecific monoclonal antibody administered subcutaneously, which bridges FIXa and FX, thereby replacing the function of missing FVIIIa and restoring haemostasis in PwHA.

Aims: Provide interim safety and preliminary efficacy results from the STASEY study of prophylactic emicizumab in PwHA with FVIII inhibitors.

Methods: The primary objective is to evaluate safety (adverse events, including thrombotic events and hypersensitivity). The secondary objective is to evaluate efficacy (number of bleeds over time and quality of life [QoL]). PwHA aged ≥12 years with FVIII inhibitors

receive prophylactic emicizumab 3 mg/kg/week for 4 weeks, followed by 1.5 mg/kg/week for the remainder of the 2-year treatment period (NCT03191799).

Results: At cut-off (15 October 2018), 88 patients had completed 24 weeks on study or discontinued study participation, whichever occurred first; median age, 28.0 (range 12-80) years; median duration of exposure, 39.2 (range 4.4-57.1) weeks. Emicizumab was well tolerated, with no thrombotic events reported (Table 1). Eighteen (20.5%) patients reported an emicizumab-related adverse event, of which injection-site conditions were the most common (13.6%).

TABLE 1 Safety summary (safety-evaluable population)

Adverse events, n (%)	Emicizumab 1.5 mg/kg/week N=88
Total number of patients with ≥1 AE	66 (75.0%)
Total number of AEs	234
Number of patients with ≥1 event	
Fatal AE	1 (1.1%)*
Serious AE	10 (11.4%)
AE leading to treatment withdrawal	0
AE leading to dose modification or interruption	3 (3.4%)
AE leading to study discontinuation	0
Grade ≥3 AE	14 (15.9%)
Study treatment-related AE	18 (20.5%)†
Local ISR	13 (14.8%)
AEs of interest	
Systemic hypersensitivity/anaphylactic/anaphylactoid reaction	0
TE	0
TMA	0
Cases of potential drug-induced liver injury	0
Suspected transmission of an infectious agent by the study drug	0
Most common AEs (≥10% of patients)	
Arthralgia	12 (13.6%)
Headache	10 (11.4%)
Nasopharyngitis	10 (11.4%)

Coded using Medical Dictionary for Regulatory Activities (MedDRA) version 21.1.
*Head injury, unrelated to emicizumab.
†Includes one serious adverse event of catheter site abscess.
Multiple occurrences of the same AE in one individual are counted only once except for "Total number of AEs" row, in which multiple occurrences of the same AE are counted separately.
AE, adverse event; ISR, injection-site reaction; TE, thromboembolic event; TMA, thrombotic microangiopathy.

TABLE 2 Efficacy summary (intention-to-treat population)

Annualized bleed rate[a,b]	Emicizumab 1.5 mg/kg/week N=88
Treated bleeds[c]	
Mean ABR, model based (95% CI)	0.5 (0.29–1.00)
Median ABR, calculated (IQR)	0.0 (0.00–0.00)
Patients with zero bleeds, n (%)	71 (80.7%)
All bleeds	
Mean ABR, model based (95% CI)	1.4 (0.91–2.24)
Median ABR, calculated (IQR)	0.0 (0.00–1.31)
Patients with zero bleeds, n (%)	56 (63.6%)
Treated spontaneous bleeds	
Mean ABR, model based (95% CI)	0.2 (0.08–0.34)
Median ABR, calculated (IQR)	0.0 (0.00–0.00)
Patients with zero bleeds, n (%)	79 (89.8%)
Treated joint bleeds[d]	
Mean ABR, model based (95% CI)	0.3 (0.10–0.84)
Median ABR, calculated (IQR)	0.0 (0.00–0.00)
Patients with zero bleeds, n (%)	81 (92.0%)
Treated target joint bleeds	
Mean ABR, model based (95% CI)	0.1 (0.03–0.18)
Median ABR, calculated (IQR)	0.0 (0.00–0.00)
Patients with zero bleeds, n (%)	83 (94.3%)

[a]A Bleed and Medication Questionnaire was completed by participants/caregivers via an electronic handheld device. Bleed definitions were based on International Society on Thrombosis and Haemostasis (ISTH) criteria (Blanchette, *J Thromb Haemost* 2014;12:1935–39).
[b]Calculated using the negative binomial regression method.
[c]Treated bleeds are defined as a bleed directly followed by a haemophilia medication reported as a treatment for bleed, without an intervening bleed and irrespective of the time between the treatment and the preceding bleed. If multiple bleeds occurred on the same calendar day, the subsequent treatment was considered to apply to each of these multiple bleeds. Bleeds due to surgery/procedure are excluded.
[d]Joint bleeds are defined as bleeds with type reported at 'joint' in combination with at least one of the following symptoms: increased swelling or warmth of the skin over the joint; increasing pain; decreased range of motion or difficulty in using the joint compared with baseline.
ABR, annualized bleed rate; CI, confidence interval; IQR, interquartile range.

The rates of treated, all, spontaneous, joint, and target joint bleeds were low (Table 2). Seventy-one patients had zero treated bleeds (80.7%). Of 17 patients who received treatment for a spontaneous or traumatic bleed, 16 received rFVIIa, and one received FVIII; no thrombotic events were seen with concomitant bypassing agents or FVIII. Clinically meaningful improvements (Wyrwich, *Haemophilia* 2015; Pickard, *Health Qual Life Outcomes* 2007) from baseline in QoL and health status across multiple domains were observed. Of the 80 patients who completed the EmiPref survey, 76 (95.0%) preferred emicizumab to their prior therapy.

Conclusions: No new safety signals were identified. Bleeding rates in PwHA with FVIII inhibitors receiving emicizumab in the STASEY study were in line with previously reported observations from HAVEN 1 (Oldenburg, *NEJM* 2017).

OC 60.4 | Correcting Bleeding Disorders Using Blood Clotting Factors Produced *in vivo* by Shielded Engineered Allogeneic Cells

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Background: Current hemophilia therapies require frequent protein infusions yet are unable to address long-term complications due to suboptimal therapy adherence, non-ideal factor kinetics and generation of inhibitors. To overcome these drawbacks, alternative modalities such as gene and cell therapies are being investigated. Spheres made with Afibromer™ biomaterials shield engineered human cells from immune rejection while also preventing the fibrotic foreign body response around the spheres, thus enabling a sustained therapeutic effect.

Aims: To evaluate whether sustained delivery of blood clotting factors by implantation of engineered human cells producing hFVIII, hFIX or hFVII is dose adjustable, durable, and potentially able to result in better clinical outcomes versus bolus dosing.

Methods: Various doses of engineered cell containing spheres were administered intraperitoneally (IP) to murine wild-type and knockout disease models. Factor production was evaluated via a combination of ELISAs, activity assays and bleeding assays.

Results: Engineered human cells optimized for hFVIII, hFIX and hFVII protein production were placed in spheres and administered IP to wild-type mice. This resulted in therapeutic levels of blood clotting factors. Additional studies in hemophilia A knockout-mice resulted in dose-dependent levels of functional hFVIII in plasma, with a corresponding correction of bleeding time and blood loss in a tail bleeding model. Spheres producing hFVIII demonstrated long-term in vivo viability and protein production (≥6 months).

Conclusions: Taken together, these data demonstrate that administration of Afibromer™ spheres shielding engineered human cells can result in sustained factor production and efficacious correction of the bleeding phenotype in murine preclinical models. The sustained

secretion achieved after a single IP implantation creates a viable alternative to traditional factor delivery or gene therapy with several important advantages. We aim to pursue first clinical studies in patients with Hemophilia A, and to develop therapeutics using Shielded Living Therapeutics™ for other serious bleeding disorders.

OC 60.5 | Development of a Formulation for Oral Delivery of FVIII via a Robotic Pill

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Background: Hemophilia A, a bleeding disorder caused by a deficiency of functional coagulation factor VIII (FVIII), is commonly treated via intravenous replacement therapy 2-3 times weekly with plasma-derived or recombinant FVIII. Recently, a FVIII mimetic bispecific antibody for subcutaneous administration was approved in some geographies. Oral FVIII would substantially improve patients′ life by reducing the burden from intravenous or subcutaneous injections.

Aims: To develop a dry powder formulation suitable for oral administration for FVIII via a robotic pill. The key injectable element of the robotic pill is its drug payload (FVIII) formed as a solid microtablet of precise dose sealed inside a hollow, dissolvable microneedle.

Methods: The recombinant FVIII drug substance was formulated into a dry powder using GRAS injectable excipients suitable for making microtablets. Microtablets were made and then sealed inside microneedles. A qualified, sensitive and selective ELISA was used to measure FVIII activity during process steps.

Results: A dry powder formulation was successfully developed. Using a custom machine and an optimized process, the dry powder was used to create multiple batches of microtablets. In multiple, consecutive batches of microtablets and microneedles, FVIII activity was consistently retained (>90%) with little or minimal loss.

Conclusions: Results confirmed that FVIII could be successfully formulated into microtablets with no impact on activity, providing evidence for the concept of the FVIII as the drug payload in robotic pill. In-life proof-of-concept studies in appropriate animal models are in progress. Successful proof-of-concept studies will lead to an innovative concept for oral delivery of FVIII by using a robotic pill.

OC 62.1 | A Test of the GPIbα β-switch Hypothesis for Platelet-type von Willebrand Disease

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Background: Von Willebrand Factor multimers mediate platelet adhesion through direct interactions between shear-flow exposed A1 domains and platelet GPIbα receptors. Mutations in the β-switch of GPIbα cause gain-of-function in the platelet-type pseudo-Von Willebrand Disease.

Aims: Crystals of free and A1 bound GPIbα suggest that the β-switch undergoes a conformational change from a random coil to a β-hairpin. Platelet-type VWD mutations have been proposed to stabilize the β-switch by shifting the equilibrium in favor of the β-hairpin conformation, a hypothesis predicated on the assumption that the co-crystal complex between A1 and GPIbα is the high-affinity state.

Methods: Hydrogen-deuterium exchange mass spectrometry is employed to test this hypothesis using common G233V, M239V and G233V/M239V disease variants of GPIbα. If true, the expected observation is that hydrogen-exchange in the β-switch would be quenched as result of hydrogen bonds newly formed between the β strands of the β-hairpin conformation.

Results: These experiments reveal that hydrogen exchange in the β-switch is enhanced rather than quenched indicating that the β-switch becomes more dynamic favoring the disordered loop conformation. Hydrogen-exchange is corroborated by differential scanning calorimetry which confirms that these mutations destabilize GPIbα resulting in a decreased unfolding enthalpy which indicates a dissociation of the β-switch from the Leucine Rich Repeat (LRR) domain in the native state. Furthermore, the stability of GPIbα and its A1 binding affinity, determined by surface plasmon resonance, are directly correlated to the extent of local disorder in the β-switch.

Conclusions: These studies demonstrate that GPIbα with a disordered loop is binding competent and support a mechanism by which disorder in the β-switch can dynamically expose the LRR domain of GPIbα. Taken in the context of the recently discovered local disorder of the A1 domain in Type 2B VWD, these results emphasize the role of protein chain disorder in establishing high-affinity interactions between platelets and VWF.

OC 62.2 | Evaluation of Different Therapeutic Strategies to Manage the Rare Coagulation Defect Induced by Thrombomodulin c.1611C>Ap.Cys537Stop Mutation

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Background: A family with unexplained bleeding was identified as having a rare bleeding disorder, caused by elevated soluble thrombomodulin (sTM). A nonsense mutation in the TM gene, *THBD*, (c.1611C>A p.Cys537Stop) results in elevated levels of sTM in plasma and subsequent inhibition of thrombin generation.

Aims: To characterise the effect of different therapeutic strategies on the defect caused by the TM c.1611C>A, p.Cys537Stop mutation.

Methods: Platelet poor plasma (PPP) and platelet-rich plasma (PRP) samples were obtained from a female patient undergoing lithotripsy. Samples were taken prior and after administration of 2 units of platelets. Thrombin generation (TG) was triggered using 1 pM tissue factor and measured using the Calibrated Automated Thrombogram (CAT). In some cases, samples were spiked with 0.5 U/ml activated prothrombin complex concentrate (APCC), 1 µg/ml activated Factor VII (FVIIa) or 50 x 10^9 platelets/litre from a healthy donor. Thrombomodulin antigen was quantified using a commercial kit (Abcam).

Results: The patient had sTM levels approximately 130 x higher than normal controls, 372.6 *vs.* 2.89 ng/ml respectively. The endogenous thrombin potential (ETP) in PPP was decreased 3.8-fold compared to the healthy control. Infusion of platelets abrogated the difference in TG. A similar 1.72-fold increase in ETP was observed with FVIIa spiking in both patient and control plasma. APCC enhanced ETP in patient and control plasma, 5.2- and 1.6-fold respectively. In PRP, TG was 1.3-fold lower compared to control plasma, and spiking with additional control platelets abolished the difference.

Conclusions: We hypothesise that the increased sTM results in enhanced activated protein C generation which reduces TG by degradation of activated Factor V and VIII. Our results indicate that treatment with platelet and FVIIa concentrates may be beneficial for management of this rare bleeding disorder, whilst the effect of APCC may increase risk of thrombosis. The beneficial effect of platelets requires further investigation.

OC 62.3 | Acquired Haemophilia A: 25 Years of Experience in The Netherlands

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Background: Acquired haemophilia A (AHA) is a rare auto-immune bleeding disorder, which can cause severe bleeding. The hemostatic and immunosuppressive treatment of AHA is burdensome and associated with frequent adverse events. Optimal management of AHA is still unresolved.

Aims: Evaluation of Dutch clinical practice of AHA treatment to improve knowledge regarding clinical presentation and efficacy and safety of treatment.

Methods: Dutch multi-center retrospective cohort study (1994-2018) based on the haemophilia complication registry and patient charts.

Results: 140 patients (mean age 73; 52.9% male) were included with a median follow-up of 16.5 months (table 1).

Malignancies (21.5%) and immune related disorders (12.3%) represented the most important underlying conditions, whereas no cause could be identified in 57.7%. All patients presented with bleeding (63.8% severe; based on ISTH criteria). 72.1% of bleeding episodes (155/215) were treated with hemostatic therapy, of which 44.5% (69/155) with recombinant activated factor VII (rFVIIa) and 36.1% (56/155) with activated prothrombin complex concentrate (aPCC). Efficacy of these products was 80.9% and 92.7% respectively, compared with 62.5% for FVIII concentrates and 66.7% for DDAVP.

Initial therapy was mostly steroid monotherapy (67.6%), steroids/rituximab (12.5%) or steroids/cyclophosphamide (11.8%) (table 2). At final follow-up 97 patients (73.5%) were in complete remission; 58 (45.3%) achieved this after first line therapy. A relapse occurred in 26.7% (23/86) after end of therapy. First line remission was significantly lower for steroid monotherapy (34.1%) than for steroids/rituximab (66.7%) and steroids/cyclophosphamide (78.6%), p=0.001. Adverse events occurred in 97/133 patients (73.7%), mostly infections (49/128; 38.3%) and steroid-induced diabetes (44/129; 34.1%). Overall mortality was 57/132 (43.2%); 7.0% due to fatal bleeding and 16.6% due to infection.

Conclusions: AHA is a severe bleeding disorder with an identified underlying disease in nearly half of the patients. Combination therapy of steroids with rituximab or cyclophosphamide is more effective than steroid monotherapy; however, at the cost of higher infection rates.

TABLE 1 Clinical presentation and outcome of AHA patients in the Netherlands, 1994-2018

Time until diagnosis in days (median (range))	22 (0 - 7409)
FVIII level (%) at presentation (median (range))	1.6 (0 - 38)
Anti-FVIII titer (BU) at presentation (median (range))	20.0 (0.7 - 2176.0)
Complete remission (CR) after first line therapy (number/total (%))	58/128 (45.3%)
Time to first CR in weeks (median (range))	11 (1 - 200)
CR at final follow-up (number/total (%))	97/132 (73.5%)
Time to final CR in weeks (median (range))	20 (3 - 710)
Total duration of therapy in weeks (median (range))	25 (0 - 358)
Relapse during steroid withdrawal (number/total (%))	13/66 (19.7%)
Relapse after end of immunosuppressive therapy	23/86 (26.7%)

TABLE 2 Comparison of the most commonly used first line immunosuppressive regimens for AHA

	Steroid mono-therapy N: 92/136 (67.7%)	Steroids/rituxi-mab N: 17/136 (12.5%)	Steroids/cyclo-phosphamide N: 16/136 (11.8%)
CR (number/total (%))	30/88 (34.1%)	10/15 (66.7%)	11/14 (78.6%)
Time to CR in weeks (median (range))	8 (1 - 91)	6 (3 - 24)	11 (3 - 72)
Relapse after end of immunosuppressive therapy (number/total (%))	16/56 (28.6%)	3/12 (25.0%)	1/10 (10.0%)
Adverse events (any) (number/total (%))	65/89 (73.0%)	12/15 (80.0%)	11/15 (73.3%)
Adverse events: Infection (number/total (%))	6/85 (7.1%)	6/15 (40.0%)	6/15 (40.0%)
Adverse events: Steroid-induced diabetes mellitus (number/total (%))	26/87 (32.2%)	7/15 (46.7%)	4/15 (26.7%)
Mortality after end of first line therapy (number/total (%))	14/85 (16.5%)	2/16 (12.5%)	4/16 (25.0%)
Mortality due to fatal bleeding (number/total (%))	4/85 (4.7%)	0/16 (0.0%)	0/16 (0.0%)
Mortality due to infection (number/total (%))	3/85 (3.5%)	1/16 (6.3%)	2/16 (12.5%)

OC 62.4 | Rare Bleeding Disorders in the Netherlands - Update

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Background: Rare Bleeding Disorders (RBD), a disease population consisting of patients with deficiencies of fibrinogen, factor II, V, V&VIII, VII, X, XI, XIII, α2-antiplasmin or plasminogen activator inhibitor 1, have a diverse clinical presentation, varying bleeding scores, bleeding episodes, health-related quality of life and laboratory phenotype. Before the present study, the prevalence and characteristics of this patient population were unknown in the Netherlands.

Aims: Describe epidemiology, bleeding tendency, laboratory parameters, quality of life parameters and molecular genetic spectrum of all known patients in the Netherlands with a RBD. In addition, this study aims to associate both clinical and laboratory phenotype with genotype.

Methods: Cross-sectional multicentre study of patients registered in all six Dutch Haemophilia Treatment Centers with a known RBD, aged 1-99 years. After informed consent, patients are asked to fill out questionnaires on bleeding frequency, socio-demographic characteristics, clinical characteristics (a.o. bleeding tendency, treatment, antibody development), medical history (e.g. hospital admissions and emergency department visits, medication use), needle phobia, sports and physical activity, quality of life and functional limitations. ISTH-BAT and other bleeding assessment tools are performed and validated for RBD. Blood samples are collected for laboratory testing (pro- and anticoagulation factors), including the Nijmegen Hemostasis Assay (NHA). Whole exome sequencing (WES) is performed to unravel the underlying genotypic profile for which a thrombosis and hemostasis specific panel of 145 OMIM-proved genes is used.

Results: Inclusion of patients for the Rare Bleeding Disorders in the Netherlands (RBIN) study was initiated in 2017, and inclusion will be

TABLE 1 Demographics for the group of patients included thus far

Characteristics	Number of patients (%)
Included patients	240
Mean age	41 (range 2-84)
Male sex	86 (38%)
Adults (≥18 y/o)	128 (81%)
Positive family anamnesis	88 (70%)
Patients on prophylaxis	13 (9%)

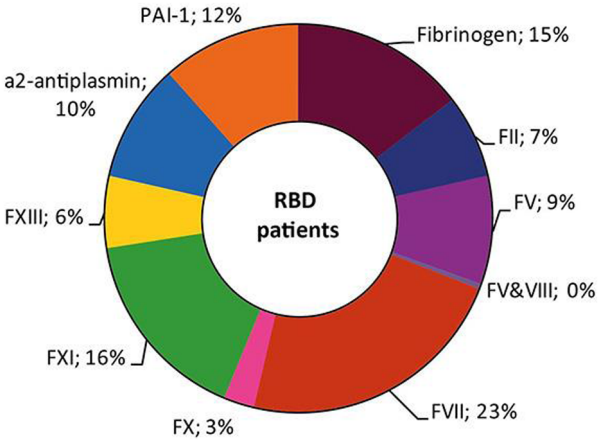

FIGURE 1 Distribution of RBDs in the RBiN study

closed in 2019. So far, 240 patients have been included. The subdivision of these patients is shown in figure 1, demographics in table 1.

Conclusions: The RBiN study will improve insight in associations between both clinical and laboratory phenotype and genotype in rare bleeding disorders.

OC 62.5 | Emicizumab for the Treatment of Acquired Hemophilia A

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Background: Acquired hemophilia A (AHA) is a severe bleeding disorder caused by inhibiting autoantibodies to FVIII. For hemostatic treatment bypassing agents, human or porcine FVIII are standard of care. Although highly effective, these need frequent iv. infusions, have high costs and some risk of thromboembolism. Emicizumab, a bispecific, FVIII-mimetic therapeutic antibody that has considerably reduced the annualized bleeding rates in congenital hemophiliacs with and without inhibitors.

Aims: Preclinical data and pathophysiology suggest efficacy also in AHA.

Methods: We report on 4 patients with AHA, 2 male, 2 female, mean age 62.3yrs (range 50-78), treated with emicizumab. All patients gave informed consent.

Results: Initial FVIII activity was < 1%, max. inhibitor 54.3BU/mL (3.1-81.6). 3 patients had severe bleeding (2 after major surgery), requiring RBC transfusions and therapy with rhFVIIa prior to emicizumab; one patient had skin hematomas, but did not require bypassing therapy.

Emicizumab dose was 3mg/kg sc. weekly/4weeks, then 1.5mg/kg. A chromogenic FVIII assay (FVIII:chr) was used.

APTT normalized 1-3days after the first dose, FVIII:chr exceeded 10% after 13 (7-18)days, bypassing therapy was stopped 4 (2-9)days. FVIII:chr exceeded 50%, after 24 and 42days (n=2), and emicizumab could be stopped after 3-6 injections. One female patient (80yrs, severe comorbidities, major abdominal surgery) experienced minor stroke after the third emicizumab dose (FVIII:chr 10%) in association with rhFVIIa. No other adverse events were observed. All patients received immunosuppression with steroids and rituximab. Patients were discharged after 2weeks (5-30 days) after the first emicizumab injection.

Conclusions: Emicizumab is an effective hemostatic therapy for AHA, with several advantages: weekly sc.administration, good hemostatic efficacy, option to reduce the immunosuppressive therapy. Strategies for patients with comorbidities, severe over- or underweight, after, or in need for major surgery, concomitant risk of thromboembolism need to be developed, and a clinical trial comparing emicizumab with the current standards of care is warranted.

OC 70.1 | Joint Bleeds in Paediatric Patients with Haemophilia A: A CHESS Study Analysis

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Background: There is limited information on real-world outcomes of paediatric patients with haemophilia A (PwHA).

Aims: To describe joint outcomes (including number of joint bleeds, target joints [TJ] and chronically damaged joints [CDJ]) in paediatric PwHA in 5EU countries (France, Germany, Italy, Spain, UK).

TABLE 1 History of joint bleeds* in the pediatric hemophilia population in 5 EU countries

	Current FVIII Inhibitors	No Current FVIII Inhibitors	
	Overall (Moderate and Severe Disease)	Moderate	Severe
N	82	287	416
Number of annual joint bleeds per patient, mean (sd); median [range]**	14.1 (17.7) 4 [0, 76]	4.1 (9.7) 1 [0, 81]	3.2 (8.2) 2 [0, 140]
Number of patients with joint bleeds N (%)			
0 bleeds	2 (2.4)	86 (30.0)	109 (26.2)
1 bleed	9 (11.0)	59 (20.6)	75 (18.0)
More than 1 bleed	71 (86.6)	142 (49.5)	232 (55.8)
Patients with minor joint bleeds N (%)			
0 bleeds	5 (6.1)	93 (32.4)	132 (31.7)
1 bleed	13 (15.9)	68 (23.7)	102 (24.5)
More than 1 bleed	64 (78.0)	126 (43.9)	182 (43.8)
Patients with major joint bleeds N (%)			
0 bleeds	23 (28.0)	170 (59.2)	226 (54.3)
1 bleed	21 (25.6)	51 (17.8)	105 (25.2)
More than 1 bleed	38 (46.3)	66 (23.0)	85 (20.4)

*Bleeds the patient experienced in the last 12 months reported by their physician (Minor joint bleeds: mild pain; minimal swelling; minimal restrictions of motion; resolution within 24 hours of treatment/ Major joint bleeds: pain; effusion; limitation of motion; failure to respond within 24 hours); sd, standard deviation; ** Median of counts are integer values

TABLE 2 Joint disease in the pediatric hemophilia population in 5 EU countries

	Inhibitor patients (Current FVIII Inhibitors)	No Current FVIII Inhibitors	
	Moderate and Severe Disease	Moderate	Severe
N*	82	287	416
Patients with target joints N (%)			
No	53 (64.6)	251 (87.5)	325 (78.1)
Yes	27 (32.9)	31 (10.8)	84 (20.2)
Patients with chronically damaged joints N (%)			
No	53 (64.6)	249 (86.8)	343 (82.5)
Yes	27 (32.9)	33 (11.5)	66 (15.9)
Patients who received prophylaxis before diagnosis of target joint or chronically damaged joint N (%)			
No	7 (8.5)	11 (3.8)	19 (4.6)
Yes	19 (23.2)	19 (6.6)	41 (9.9)
Patients with target joint who had joint surgeries**	16 (59.3)	8 (25.8)	16 (19.0)

* The column sums may be less than the total N due to some patients with missing information

**Target joint surgery includes: arthrocentesis (joint aspiration), arthrodesis (joint fusion), arthroplasty (joint reconstruction/replacement), arthroscopy (joint examination/repair via endoscope), and synovectomy (removal of synovium)

Methods: Cost of Haemophilia across Europe - Socioeconomic Survey in a Paediatric Population (CHESS PAEDs) study was conducted from December 2017 to March 2018. 110 haemophilia specialists provided information on 785 paediatric PwHA they treated, including demographics, treatment and clinical outcomes data. The patient sample was stratified by age group (1-5years/6-11years/12-17years = 1:1:1) and disease severity (severe:moderate = 2:1).

Results: Population included 82 (10%) inhibitor and 703 (90%) non-inhibitor patients (**Table 1**). Among non-inhibitors there were 287 moderate and 416 severe patients. Of the 697 patients with known treatment regimen, 61% were on continuous prophylaxis, 15% on intermittent prophylaxis and 24% were treated on demand. There were 54 patients aged < 2 years, 376 aged 2-11 years, and 355 aged 12-17 years.

Patients with current FVIII inhibitors had an average bleed rate of 14.1 (standard deviation [sd]=17.7) joint bleeds per year, with only 2 (2.4%) of the patients having zero joint bleeds. In comparison, patients without inhibitors had an average of 3.6 (sd=8.9) bleeds per year, with 195 (27.7%) non-inhibitor patients being joint bleed-free.

Overall, TJ were reported in 142 (18%) patients: 33% of inhibitors, 20% of non-inhibitors with severe disease and 11% of non-inhibitors with moderate disease (p-value < 0.01). Similar patterns were observed for CDJ (**Table 2**). The majority of TJ were located in the knees (38%), ankles (22%) and elbows (16%), with 28% of patients with TJ having experienced joint surgery.

Conclusions: Joint bleeding and joint disease are common among paediatric PwHA with moderate or severe disease, signaling remaining unmet need and opportunities for improvement in treatment management.

OC 70.2 | Efficacy and Safety of BAY 94-9027 is Sustained for 5 Years: Outcomes in 36 Patients in the PROTECT VIII Extension Study

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Background: BAY 94-9027 is an extended-half-life, B-domain-deleted recombinant factor VIII (FVIII) that is site-specifically PEGylated with a 60-kDa (2×30-kDa) polyethylene glycol. The efficacy and safety of BAY 94-9027 as prophylactic and on-demand therapy for patients aged 12-65 years with severe hemophilia A were demonstrated in the phase II/III PROTECT VIII trial.

Aims: To explore efficacy and safety outcomes in patients from PROTECT VIII who had completed ≥5 years of prophylaxis as of 31 August 2018.

Methods: In PROTECT VIII, 114 prophylaxis patients received BAY 94-9027 25 IU/kg twice weekly (2×W) for a 10-week run-in period. Patients with ≤1 breakthrough bleed during this period were randomized to 45-60 IU/kg every 5 days or 60 IU/kg every 7 days, or assigned to 30-40 IU/kg 2×W for the 26-week study period. 107 prophylaxis patients entered an optional extension, continuing BAY 94-9027 on any regimen used in the main study. Prophylaxis patients who switched regimen after the first 7 days in the extension were analyzed in a combined variable frequency group. Annualized bleeding rate (ABR), joint ABR and safety outcomes were analyzed.

Results: At the cut-off date, 36 patients had completed 5 years of prophylactic treatment with BAY 94-9027 (median exposure days: 341). Median (quartile [Q1; Q3]) ABR was 1.2 (0.4; 2.1) (**Figure 1**), and joint ABR was 0.8 (0.3; 1.8). There were no study drug-related serious adverse events (**Table 1**). No patients had confirmed FVIII inhibitors (titer ≥0.6 Bethesda units).

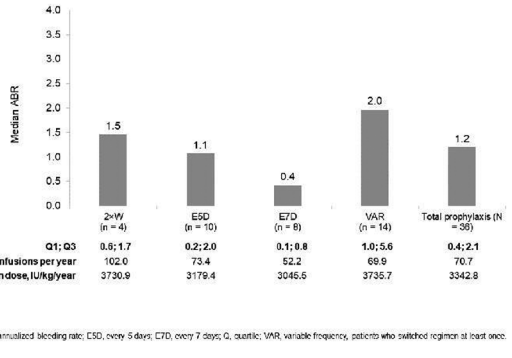

FIGURE 1 ABR in prophylaxis patients with ≥5 years of treatment with BAY 94-9027

TABLE 1 Summary of adverse events (AEs) in prophylaxis patients with ≥5 years of treatment with BAY 94-9027

Patients, n	2×W (n = 4)	E5D (n =10)	E7D (n = 8)	VAR (n = 14)	Total prophylaxis (N = 36)
Any AE	4	10	7	14	35
Any study drug-related AE*	0	0	2	3	5
Any serious AE (SAE)	2	4	3	6	15
Any study drug-related SAE	0	0	0	0	0
Any AE leading to discontinuation	0	0	0	0	0

*Identified as drug-related by investigator assessment. 2×W, twice weekly; E5D, every 5 days; E7D, every 7 days; VAR, variable frequency.

Conclusions: The efficacy of BAY 94-9027 was maintained during the extension using infusion schedules best fitting the needs of individual patients. This long-term evidence supports the favorable risk-benefit profile of this PEGylated product. These data support the use of BAY 94-9027 as a long-term treatment option for patients with hemophilia A.

OC 70.3 | Intracranial Hemorrhage in Hemophilia - A Systematic Review

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Background: Intracranial hemorrhage (ICH) is a life-threatening event in hemophilia. While the focus of most studies in hemophilia patients is joint bleeding, various studies have assessed ICH in hemophilia, reporting a wide range of incidence and mortality rates. More precise estimates of ICH numbers in hemophilia are important to aid in identification of patients at risk and to guide future prevention.

Aims: We conduct a systematic review to assess incidence and mortality of ICH in patients with hemophilia.

Methods: MEDLINE and EMBASE were searched by use of key terms related to hemophilia, ICH and mortality. Studies reporting on a representative congenital hemophilia population were included. Screening, data collection and critical appraisal was performed by two independent reviewers. Random-effects pooled rates are calculated for the whole population and for specified subgroups according to disease severity, age, treatment regimen, historical time period and geographic location. The full protocol can be found at PROSPERO (record ID 100278).

Results: The search yielded 2058 de-duplicated hits on April 12, 2018. We analyzed 56 relevant studies that described 82527 hemophilia patients and 1508 ICH events over 6 decades (mid-year range 1960 to 2014). ICH incidence rate ranged between 21 and 2752 per 100.000 person-years, with a pooled rate of 400 per 100.000 person-years. Over the past decades, the pooled ICH incidence in hemophilia patients declined from around 8% in the period 1960-1979 to 3% in 2000-2018.

Conclusions: In the general population, the incidence of hemorrhagic stroke is estimated at 25 per 100.000 person-years. This is lower than the pooled incidence of 400 per 100.000 person-years that was found for hemophilia patients. At ISTH 2019, we will present our results on ICH in hemophilia in relation to specific subgroups at high risk.

OC 70.5 | Diagnostic and Score Value of Ultrasound and Magnetic Resonance Imaging in Hemophilia Arthropathy

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[1]*Nanfang Hospital, Southern Medical University, Department of Medicine Ultrasonics, Guangzhou, China*, [2]*Nanfang Hospital, Southern Medical University, Department of Hematology, Guangzhou, China*, [3]*The Second People's Hospital of Shenzhen, Department of Ultrasound, Shenzhen, China*

Background: To find a convenient evaluation tool for hemophilia arthritis.

Aims: Referring to MRI on the diagnosis of hemophiliac arthropathy, to explore the diagnostic value of ultrasound on hemophiliac arthropathy.

Methods: The ultrasound and MRI examinations were performed on 42 joints of 42 hemophilia patients (14 knees, 14 ankles and 14 elbows). The consistency of ultrasound and magnetic resonance imaging in the detection and score of joint diseases was compared. Finally, inter-and intra-observer agreements of ultrasound scoring system were tested.

Results: The consistency of ultrasound and magnetic resonance imaging was excellent (κ=0.763-0.896, P< 0.001) in the detection of early soft tissue lesions (effusion or hemarthrosis, synovial hypertrophy, hemosiderin), excellent (κ=0.793, P< 0.001) in the detection of cartilage loss, poor (κ=0.133, P =0.132) in the detection of erosions and poor (κ=0.100, P=0.137) in the detection of subchondral cysts. The consistency of ultrasound and magnetic resonance imaging was

good to excellent (κ=0.684-0.833, P< 0.001) in the score of early soft tissue lesions (effusion or hemarthrosis, synovial hypertrophy and hemosiderin) and poor to good (κ=0.145 -0.635, P< 0.001) in the score of advanced osteochondral lesions (cartilage loss and bone erosions). The inter-observer agreement was good to excellent (κ=0.676-0.870, P< 0.001) for early soft tissue lesions and moderate to excellent (κ=0.421- 0.751, P< 0.001) for advanced osteochondral lesions. The intra-observer agreement was good to excellent (κ=0.705-0.885, P< 0.001) for early soft tissue lesions and moderate to good (κ=0.532-0.732, P< 0.001) for advanced osteochondral lesions.

Conclusions: Ultrasound plays an important role in detecting early soft tissue changes (effusion or hemarthrosis, synovial hypertrophy, hemosiderin) and cartilage loss, which helps follow-up and guide clinical treatment.

OC 75.1 | Extended Stability of Activated TAFI Normalizes Vascular Dysfunction in Hemophilic Joint Disease in Mice

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Background: Excessive vascular remodeling is characteristic to hemophilic joint disease and promotes vessel fragility and re-bleeding. Low thrombin generation in hemophilia leads to impaired activation of Thrombin Activatable Fibrinolysis Inhibitor (TAFI). TAFI deficiency contributes to hemophilic joint bleeding and drives the development of vascular dysfunction in the joints after bleeding in mice.

Aims: To determine the efficacy of induced TAFI expression in preventing joint bleeding and vascular abnormalities in hemophilic ($FVIII^{-/-}$) mice.

Methods: Human wild type (WT-)TAFI or a stabilized variant, TAFI-CIIYQ, was expressed at normal human plasma levels in $FVIII^{-/-}$ mice by hydrodynamic gene delivery. Bleeding was determined after tail clip or joint injury 2 days after DNA injection. Joint vascularity was evaluated 2 weeks after joint injury by Evans-blue leakage, histology and 2-photon microscopy.

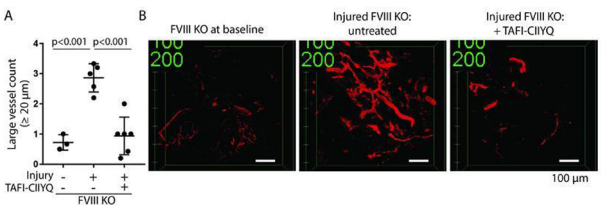

FIGURE 1 (A) Large vessel count and (B) 3D imaging of the vasculature in FVIII KO mouse joints

Results: TAFI-CIIYQ, but not WT-TAFI, corrected acute blood loss (within 20 minutes) after tail clip in $FVIII^{-/-}$ mice. However, TAFI-CIIYQ did not correct delayed bleeding after tail clip or joint injury, resulting in low hematocrits similar to untreated, injured $FVIII^{-/-}$ mice. After joint bleeding, TAFI-CIIYQ, but not WT-TAFI, normalized hemophilic vascular dysfunction. Specifically, TAFI-CIIYQ reduced vascular leakage in injured $FVIII^{-/-}$ mice from 1.8 to 1.3-fold (n=8; p< 0.005). Additionally, TAFI-CIIYQ prevented the formation of abnormally enlarged CD31+ vessels (≥ 20µm diameter), typical for vascular dysfunction in hemophilia after joint bleeding (p< 0.001; Figure A). Finally, 3D imaging of isolectin B4-AF568-perfused synovial vessels confirmed normalization of the vasculature by TAFI-CIIYQ with a 30% reduction in vascular volume compared to untreated, injured $FVIII^{-/-}$ mice (Figure B).

Conclusions: Although plasma TAFI-CIIYQ did not prevent hemophilic joint bleeding, it did normalize bleeding-induced vascular defects in hemophilic joints by stabilizing the vascular barrier and preventing aberrant vessel enlargement. These data support further evaluation of TAFI variants with extended enzymatic stability as a potential therapy to correct vascular dysfunction in hemophilic joint disease.

OC 75.2 | Targeting Protein S Protects Mice with Hemophilia A from Acute and Chronic Hemartrosis

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Background: Hemarthrosis is a major hemophilia-related complication, responsible for a debilitating chronic arthropathy. Recently, we showed that targeting the anticoagulant protein S (PS) in hemophilic mice protects them from acute hemarthrosis (AH).

Aims: To test the efficacy of targeting PS in hemophilic mice and to evaluate the effect of PS targeting on inflammation using different hemarthrosis models.

Methods: AH and chronic hemarthrosis (CH) models were applied to $F8^{-/-}Pros1^{-/-}$and $F8^{-/-}Pros1^{+/+}$mice. For the AH model, mice were subjected to 1 single needle-induced joint injury and for the CH model, to 1 to 3 needle-induced joint injuries/week over 1 to 3 weeks. Inflammatory profile was studied in blood and knee lavage. Immunohistochemistry (IHC) analysis of injured joint was performed.

Results: F8-/-Pros1-/- mice did not develop CH, even after the third injury; in contrast, F8-/- Pros1+/+ mice developed CH (0.17±0.04 vs 0.87±0.6mm, p< 0.001, n=7) (Fig.). IHC analysis of joints after CH showed relevant intra-articular bleeding and insoluble fibrin in F8-/-Pros1+/+ but not in F8-/-Pros1-/- mice. However, preliminary data of safraning staining on injured joints showed comparable cartilage

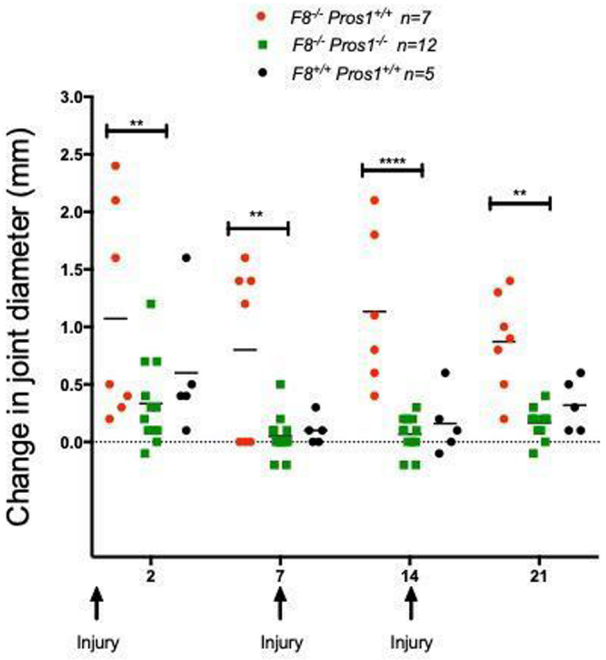

FIGURE 1 Joint swelling according to different genotypes all over the study period

degradation in the two genotypes.After AH, F8-/-Pros1-/- and F8-/-Pros1+/+ mice had comparable circulating M1 (inflammatory) and M2 (patrolling) monocytes. In the knee lavage, the number of M1 and M2 monocytes was comparable in both genotypes after AH. The amount of monocyte chemotactic protein 1 (MCP-1) and IL-6, cytokines secreted by the inflamed synovium, was less abundant in knee lavage of F8-/-Pros1-/- mice than of F8-/-Pros1+/+ mice after AH (P< 0.005).

Conclusions: Targeting PS protects from AH and CH. Inflammatory profile after AH is consistant with the fact that F8-/-Pros1-/- mice, in contrary to F8-/-Pros1+/+ mice, did not develop AH. Further analysis to understand if the lack of PS affects also the inflammatory process that supports the chronic arthropathy is ongoing.

OC 75.3 | Targeting Protease Nexin-1, a Natural Anticoagulant, to Control Bleeding in Hemophilia A

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Background: Hemophilia is a disease caused by the lack of Factor VIII (FVIII) or Factor IX (FIX), leading to insufficient thrombin production, and therefore bleeding. Recently, new therapeutic strategies for hemophilia treatment, that do not rely on clotting factor replacement, have emerged,.

Aims: We propose an innovative approach consisting in targeting a natural and potent thrombin inhibitor, expressed by platelets and called Protease Nexin-1 (PN-1).

Methods: We used the calibrated automated thrombogram (CAT) assay to test the effect of a PN-1 neutralizing antibody, on thrombin generation in platelet-rich plasma (PRP) of hemophiliac patients. CAT assay was also performed on PRP from haemophilia A (HA mice), wild-type, and double knockout mice for PN-1 and FVIII (dKO mice). Thromboelastometry study (ROTEM®) was carried out on murine whole blood to evaluate clot fibrinolysis. Different *in vivo* studies : i) arterial and venous bleeding time, ii) monitoring of mesenteric vessel occlusion after ferric chloride-induced thrombosis, were performed in mice.

Results: The PN-1 neutralizing antibody increases thrombin generation in response to tissue factor, in PRP from patients displaying circulating FVIII greater than 1%. In contrast, PN-1 neutralization improves thrombin generation neither in PRP from severe hemophilia patients nor in PRP from HA mice. However, PN-1-deficiency can improve significantly thrombin generation in PRP from HA mice, after collagen-induced platelet activation. ROTEM® results show increased clot stability and clot lysis time in blood from DKO mice compared with HA mice.

In both bleeding time models, dKO mice display significant reduced blood loss and bleeding time compared to HA mice. Following ferric-chloride-induced injury of mesenteric vessels, platelet recruitment and fibrin(ogen) accumulation are significantly higher in DKO mice than in HA mice.

Conclusions: Our study provides proof-of-concept that PN-1 neutralizing can be a a novel approach for future clinical care in hemophilia.

OC 75.4 | Hemarthrosis in FVIII-deficient Mice Causes Altered Expression of Local and Systemic Iron Regulators and Defective Iron Clearance from the Joint

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[1]University of California San Diego, Department of Medicine, La Jolla, United States, [2]The Scripps Research Institute, Department of Molecular Medicine, La Jolla, United States, [3]University of California San Diego, Center for Computational Biology & Bioinformatics, La Jolla, United States

Background: Hemarthrosis in hemophilia causes toxic iron accumulation in the joint, which contributes to synovitis.

Aims: To explore mechanisms of iron clearance after hemarthrosis, and whether these are defective in hemophilia.

Methods: Hemarthrosis was induced by sub-patellar needle puncture in FVIII-deficient mice and hypocoagulable BALB/c (^{Hypo}BALB/c) mice treated with 10µg/ml warfarin for 7 days and 0.25mg/kg anti-FVIII 2 hours before injury. Warfarin was reversed with 100IU/kg 4-factor prothrombinase complex concentrate on day 2 post-injury +/- twice weekly anti-FVIII treatment. Ferric iron was quantified in joint tissue at 2-4 weeks post-injury by Prussian Blue staining.

Splenic and synovial tissue were harvested from FVIII-deficient mice on day 3 and 2 weeks post-injury for gene expression studies by RNA-sequencing.

Results: Knee injury caused substantial hemarthrosis in all mice. A 5.3-fold increase (p=0.003) in ferric iron, as found in the iron-storage complex, hemosiderin, was detected in FVIII-deficient mice at week 4 only. No ferric iron was detected in ^{Hypo}BALB/c mice despite comparable bleeding and anti-FVIII treatment. 10/56 genes relating to iron transport were differentially expressed (DE) in synovium from FVIII-deficient mice on day 3, persisting to some extent at 2 weeks. These included up-regulation of heme oxygenase-1 (31-fold; $p=3x10^{-6}$) and lipocalin-2 (10-fold; p=0.001), and down-regulation of ceruloplasmin (17-fold; $p=5x10^{-5}$), CD163 (hemoglobin scavenging receptor) (5-fold; p=0.03), and hephaestin (5-fold; p=0.002). In the spleen, there were 12 DE genes on day 3 only, including up-regulation of Steap-3 (4-fold; p=0.0008) and transferrin receptor-1 (3-fold; p=0.005), and down-regulation of lipocalin-2 (4-fold; p=0.01). These changes may influence iron scavenging/retention and transport in synovium, and cellular uptake of iron in the spleen in response to anemia.

Conclusions: Iron accumulation after hemarthrosis appears unique to hemophilia, suggesting impaired clearance. RNA-sequencing created insights into mechanisms of local and systemic iron transport, prompting further investigations to elucidate detrimental synovial iron accumulation.

OC 75.5 | Recombinant Factor VIII Fc Fusion Protein Inhibits Inflammatory Osteoclast Formation *in vitro*

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Background: Decrease in bone mineral density observed in severe hemophilia A (HemA) patients suggests that the absence of FVIII activity and related bleeding episodes may contribute to perturbed bone homeostasis by creating a pro-inflammatory milieu with enhanced monocyte/macrophage-derived osteoclast formation and bone erosion. Previously we found that recombinant FVIII Fc fusion protein (rFVIIIFc)-treated human macrophages exhibit Mox/M2 regulatory phenotype with tolerogenic potential and antioxidant properties driven by NRF2 pathway-associated molecular events.

Aims: Our aim was to investigate whether rFVIIIFc treatment could modify monocyte-derived osteoclastogenesis *in vitro*.

Methods: Monocytes from PBMC of healthy human donors were isolated and cultured with rhM-CSF and rhRANKL to achieve osteoclast formation in the presence or absence of hIgG1, rFVIII or rFVIIIFc. Osteoclast-specific and NRF2 pathway-related genes were measured by qPCR. Osteoclast phenotype was followed by tartrate-resistant acid phosphatase (TRAP) staining and multinucleation. Function of the treated osteoclasts was examined using bone resorption assay.

Results: Monocyte-derived osteoclast development was significantly impaired in the presence of rFVIIIFc observed by TRAP staining and the absence of multinucleation. Gene and protein expression of rFVIIIFc-treated cells showed upregulation of NRF2 pathway-related molecules (NQO1, GCLC, HO-1) and subsequent downregulation of molecules involved in osteoclast formation and function (NFATC1, CATK, RANK). The bone resorption capabilities of rFVIIIFc-treated monocyte-derived osteoclasts were compromised compared to untreated, hIgG1- or rFVIII-treated cells.

Conclusions: Monocyte-derived osteoclast formation was effectively inhibited by rFVIIIFc treatment while hIgG1 or rFVIII treatment had no effect on this process *in vitro*. The presented data suggest that replacement therapy with the extended half-life rFVIIIFc may regulate the immune milieu in people with hemophilia toward an antioxidant and less osteoporotic state. This activity of rFVIIIFc is distinct from its role in blood coagulation and in agreement with the previously described Fc-dependent Mox/M2 macrophage polarization.

IMMUNOTHROMBOSIS AND VASCULAR BIOLOGY

OC 15.4 | DVT-on-Chip: In-vitro Model of Deep Vein Thrombosis Including Valves, Endothelium & Blood Flow

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Background: Deep vein thrombosis (DVT) is caused by endothelial disruption, fluid stasis and hypercoagulable blood (Virchow's triad). Mice models used to study DVT are not predictive as they lack venous valves and exact flow conditions critical to the onset of DVT. We present a microfluidic DVT-on-chip, a vein (1A) containing venous valves, endothelial culture and blood flow.

Aims: NA

Results: Numerical simulations of non-Newtonian blood flow were conducted using ANSYS v19.2 software. The device is made from polydimethylsiloxane (1C). HUVECs were cultured on the channel walls coated with collagen I and fibronectin. Re-calcified citrated

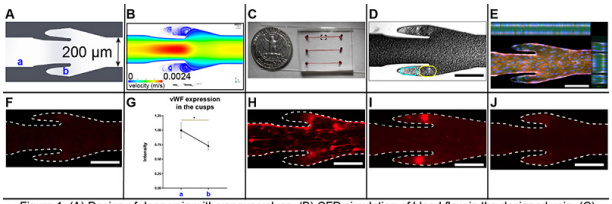

Figure 1: (A) Design of deep vein with venous valves. (B) CFD simulation of blood flow in the designed vein. (C) Fabricated deep-vein-on-a-chip. (D) Perfusion of whole blood throught the fabricated deep vein (yellow - primary vortex, blue - secondary vortex). (E) HUVECs formed confluent lumen in the channels (green - VE-cadherin, red - actin, blue - nucleus). (F) Perfusion of re-calcified citrated whole blood through the lumen resulted in unobservable thrombi. (G) Von Willebrand factor expression at the valves is less than the straight portions (H) Perfusion of re-calcified citrated whole blood through TNF-α treated lumen resulted in thrombus formation. (I) Treatment of blood with lower dosage of heparin resulted in thrombi formation only at the vlaves of TNF-α treated lumen. (J) Further increase in heparin dosage resulted in unobservabel thrombi in TNF-α treated lumen (red - fibrin/fibrinogen).

FIGURE 1 DVT-on-a-chip

blood was perfused through the channel at a shear stress of 2 dynes/cm^2 and fluorescently labelled fibrin and platelets were visualized and quantitated. The lumen and blood were treated with different dosages of TNF-α (0, 5, 100 ng/ml) and heparin (0, 0.25, 0.5 IU/ml) respectively.
Results: Numerical simulations predicted vortices in the valves as observed in defective human veins (1B). Perfusion of whole blood through the fabricated channels also showed flow patterns as observed in vivo (1D). The confluent lumen formed was intact (1E) and blood perfusion resulted in unobservable clots, meaning that the underlying matrix was protected from blood (1F). Further, expression of von Willebrand factor at cusps was less (1G) compared to the straight channels as observed in human veins. But upon stimulation with TNF-α, clots were formed due to overexpression of vascular adhesion molecules (1H). Interestingly, we observed increased fibrin at the venous valve cusps and very limited platelets, which is typical for venous clots in vivo. Finally, this model predicts that high dose of anticoagulation is needed to prevent thrombus formation at the vein cusps, relative to straight sections (1I & 1J).
Conclusions: Our DVT-on-chip can therefore model critical biophysical processes of DVT and we expect it to be applied for investigating mechanisms, drugs and anticoagulation control.

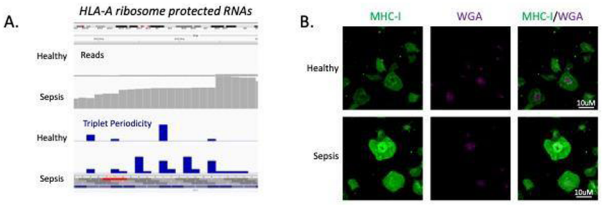

FIGURE 1 Increased MHC-I expression on platelets during sepsis. (A) RNAseq, (B) IF of platelets

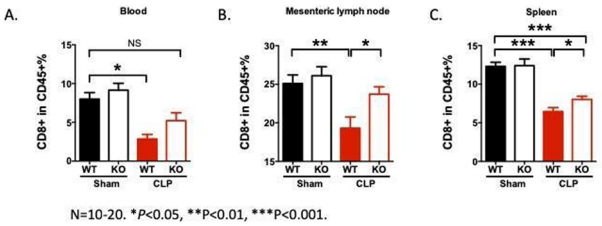

FIGURE 2 Platelet MHC-I dependent loss of CD8+ T cells (CTLs) in a mouse model of sepsis (CLP)

OC 29.1 | Platelets Suppress Antigen Specific CD8$^+$ T Cells in Sepsis through MHC Class I

L. Guo[1]; S. Shen[2]; J. Rowley[1]; W. Jia[3]; B.K. Manne[1]; N. Tolley[1]; K. McComas[1]; J. Su[4]; S. Jacob[1]; R. Campbell[1]; E. Middleton[1]; C. Morrell[5]; M. Rondina[1]; A. Weyrich[1]

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Background: Acute and long-term complications from sepsis cause significant morbidity and mortality worldwide. Survivors of sepsis are left with prolonged impairment of immune functional responses, decreased number of CD8$^+$ T cells (CTLs), and increased risk of secondary infections. Platelets are key effectors in the acute pathogenesis of sepsis, but their role in sepsis immune responses and the long-term sequela of infection is not clear. Recent evidence suggests activated platelets directly engage T cells through MHC class I (MHC-I).
Aims: We aim to study whether MHC-I on platelets regulates CTL responses in sepsis.
Methods: We first examined the expression of MHC-I in platelets from septic individuals. Next, to study the function of MHC-I *in vivo*, we induced sepsis by cecal ligation and puncture (CLP) in mice with platelet-specific MHC-I deficiency (B2Mfl/fl-PF4Cre, or KO) and littermate controls (WT), measured CTL frequency and number on Day4. Third, *in vitro* we co-cultured platelets with antigen specific CTLs (OT-I) and measured CTL proliferation.
Results: MHC-I expression in platelets is increased in sepsis in human and mice as seen from RNAseq, qRT-PCR, immunofluorescent imaging, and flow cytometry data (Fig 1). In mice with sepsis, CTL numbers decreased in the peripheral blood, draining lymph nodes and spleen from WT mice, but were maintained in platelet MHC-I KO mice (Fig 2). To determine whether this *in vivo* suppression of CTLs is through direct platelet-CTL interactions, we co-cultured SIINFEKL pulsed platelets with OT-I CTLs *in vitro*. In our preliminary data, WT platelets from septic mice suppressed OT-I CTL proliferation in an antigen dependent manner, whereas KO platelets from septic mice did not.
Conclusions: Our study provides new evidence that sepsis increases MHC-I in human and mouse platelets, and that MHC-I on platelets is necessary for sepsis mediated CTL suppression. Future studies will focus on how platelets impact chronic adaptive immune suppression in survivors of sepsis.

OC 29.2 | Blocking Activated Factor XII with a Monoclonal Antibody Prevents Organ Failure and Saves Baboons Challenged with Heat-inactivated S. aureus

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Background: Sepsis causes systemic inflammation and robust activation of the coagulation and complement systems, leading to

vasodilation, vascular leakage and coagulopathy, all contributing to organ failure and death.

Aims: We hypothesize that activation of the contact system via factor XII (FXII) plays a pathogenic role in sepsis and serves as cornerstone between inflammation and coagulation.

Methods: We used 5C12, a new high-affinity monoclonal antibody that blocks FXIIa to elucidate the mechanistic role of FXII activation during challenge with a lethal dose of heat-inactivated *S. aureus*.

Results: Treatment with 5C12 (10 mg/kg) efficiently blocked the activation of contact pathway as shown by decreased levels of inhibitory complexes of FXIIa (Fig. 1A) and kallikrein with antithrombin (AT). The treatment attenuated *S. aureus* induced coagulopathy, reducing FXIa-AT and thrombin-AT complexes (Fig. 1B). Similarly, fibrin deposition and degradation was reduced as shown by fibrinogen consumption, immunostaining for fibrin and quantitation of plasma D-dimer (Fig. 1C). Inhibition of FXIIa protected against sepsis-induced capillary leakage and prevented septic shock. Likewise, *S. aureus*-induced inflammation was substantially blunted as shown by significantly reduced plasma TNF, IL1-beta, IL6, IL8, IL10 and GM-CSF. Robust inhibition of plasma nucleosomes and myeloperoxidase support decreased cell death and lowered neutrophil infiltration. Treated animals had considerable better organ function as reflected by kidney and liver tests as well as histopathology analysis at the end of the experiment. Importantly, all treated animals reached the 7-days survival end-point while non treated controls suffered irreversible organ failure and were euthanized before 30 hours post-challenge.

Conclusions: This study provides proof-of principle that contact activation plays an important role in host response to lethal challenge with heat-killed S. aureus, suggesting that pharmacological FXIIa inhibition may have therapeutic potential in the prevention or treatment of S. aureus sepsis.

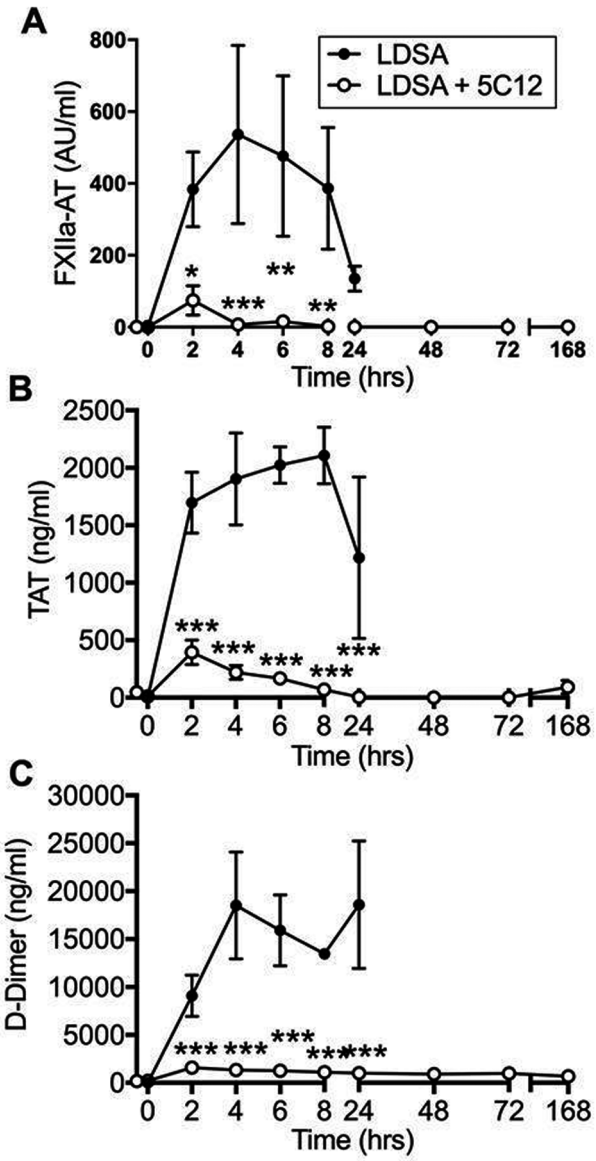

FIGURE 1 Time-course changes of coagulation biomarkers

OC 29.3 | Inflammasome Activation Triggers Blood Clotting and Host Death through Pyroptosis

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Background: Disseminated intravascular coagulation (DIC) is a frequent and fatal complication of sepsis. The presence of bacterial virulence factors can induce blood coagulation, yet the molecular events linking bacteria sensing to initiation of the coagulation cascade remain largely unknown. Inflammasome is the large sensory protein complex in the cytosol that alerts host to bacterial infection. The role of inflammasome in sepsis remains unclear.

Aims: To investigate the role of inflammasome in coagulation and lethality induced by Gram-negative products.

Methods: We used *E. coli* type III secretion system (T3SS) inner rod protein EprJ and LPS to investigate the role of canonical and noncanonical inflammasome activation in sepsis, respectively. For efficient cytosolic delivery, EprJ was fused to the cytosolic translocation domain of anthrax lethal factor (LFn). Binding with LFn, anthrax protein protective agent (PA) delivers the LFn-EprJ fusion protein into the cytoplasma through receptor-mediated endocytosis. We measured prothrombin time, plasma TAT, plasma microvesicle tissue factor activity, total platelet count, and lethality. With caspase 1, caspase 11, gasdermin D (GSDMD), and inducible tissue factor deficient mice, we examined the relationship of inflammasome signaling and tissue factor pathway in Disseminated intravascular coagulation (DIC).

Results: We found that EprJ, the inner rod protein, activated caspase-1 and induced systemic coagulation, while lipopolysaccharide (LPS) produced similar effects via caspase-11 activation. We also

identified gasdermin D (GSDMD) driven macrophage pyroptosis as a mechanism for the release of tissue factor in the form of microvesicles, an essential initiator of coagulation cascades. Genetic or pharmacological inhibition of TF abolishes inflammasome-mediated blood clotting and protects against death.

Conclusions: Blood clotting is the major cause of host death following inflammasome activation and demonstrate that inflammasome bridges inflammation with thrombosis.

OC 29.4 | The Mammalian Target of Rapamycin in Megakaryocytes and Platelets Regulates Mortality and Disease Severity in Sepsis

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Background: Evolving evidence indicates that platelets have unexpected activities during infection. Translational control pathways, including the mammalian target of rapamycin (mTOR), are active in platelets, but how inflammatory diseases modulate mTOR in platelets is unknown.

Aims: To determine if activation of the mTOR pathway in platelets regulate outcomes in sepsis.

Methods: We assessed mTOR activation in septic patients and healthy controls. We generated mice lacking mTOR in platelets (Pf4-cre;mTOR$^{fl/fl}$ or KO) and performed a cecal ligation and puncture (CLP) model of sepsis. Platelet and leukocyte counts and plasma cytokine levels were measured, and mortality was monitored. Ribosomal footprinting of platelets was performed to assess changes in protein translation.

Results: Platelets isolated from septic patients and wild-type (WT) mice both demonstrated significant activation of the mTOR pathway compared to controls (3.2-fold higher, $p< 0.05$). Mice deficient in platelet mTOR had significantly higher mortality than WT mice (n=11, $\beta p=0.005$). KO mice had significantly lower platelet counts compared to WT mice 24-hours after CLP (121 ±20 K/mL vs. 284±94 K/mL, n=7; p=0.008) and significantly lower hematocrits (28.8±8.8% vs. 46.5±3.6%, n=6; p=0.001). IL-1β and TNF-α levels were significantly elevated in KO mice compared to WT mice ($p< 0.001$). Leukocyte counts and circulating bacterial load were not different. Ribosomal footprinting confirmed mTOR deletion significantly reduced protein translation in platelets including integrin αIIb and filamin A, two important regulators of platelet function. WT platelet transfusion into KO mice after CLP rescued mortality, decreased cytokine production, and increased hematocrit while KO platelet transfusion into KO mice had no effect.

Conclusions: These data demonstrate for the first time, to our knowledge, that mTOR pathway in platelets plays a significant role in regulating mortality and outcomes in sepsis. Therapeutic targeting of mTOR in platelets may provide clinical benefit in the setting of sepsis.

OC 29.5 | Sepsis Can Modify mRNA Levels by Dicer-dependent microRNAs in Megakaryocytes and Platelets to Generate Adverse Platelet Reactivity

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Background: In sepsis, platelet activation by LPS via TLR4 can result in microvascular thrombosis. Megakaryocytes (MKs) also express TLRs, thus severe infection can modulate thrombopoesis. Both MKs and platelets are rich in microRNAs (miRNAs) to regulate mRNA function and different cellular events. Murine MKs were previously described to produce platelets with altered mRNA profile in septic mice.

Aims: We characterized sepsis-induced expression of mRNAs with their regulatory miRNAs in *ex vivo* and *in vitro* stimulated platelets as well as in MK cell cultures. The contribution of Dicer function to abnormal miRNA levels was also investigated.

Methods: Leukocyte-depleted platelets of 22 septic and 26 healthy individuals were analyzed for P2RY12 and SELP (P-selectin) mRNAs. TaqMan Open Array was performed for miRNAs, and RT-qPCR were used for verification. The effects of sepsis were also studied in isolated platelets and MEG-01 cells *in vitro* after treatment with TNF-α or LPS with LBP and soluble CD14 for 1-24h. Dicer level was observed by western blotting and fluorescence microscope, while its function was tested by calpeptin in septic MKs and silencing by Dicer siRNA in MEG-01 for P2RY12 and SELP expression.

Results: There was augmented platelet activation as surface and soluble P-selectin were increased in septic patients ($P< 0.001$). Platelets and MKs upregulated P2RY12 and SELP gene expression ($P< 0.01$) accompanied with elevated inflammation-specific miR-155 and decreased miR-223/26b. Additionally, 64 other platelet miRNAs indicated ≥2-fold decrease, while 37 (e.g. miR-191) were increased at the same degree in sepsis vs. controls. After a transient induction of Dicer and miRNAs at 1h, reduced Dicer mRNA and protein levels were seen in LPS/TNF-α treated platelets/MKs after 4-24h causing attenuated miR-223/26b. Calpain inhibition restored miRNA levels, while downregulation of Dicer generated higher P2RY12 and SELP levels in MEG-01.

Conclusions: Modulation of mRNAs by Dicer-dependent miRNAs may occur in MKs and reactive platelets during sepsis.

OC 68.1 | FXa Inhibitors Target Tumor-associated Macrophage FXa-PAR2 Signaling

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Background: The coagulation system is an innate defense pathway that limits infections and restores tissue integrity after injury. These protective properties are exploited by tumor cells to shape the tumor microenvironment (TME). Coagulation protease signaling via protease activated receptors (PARs) converge with other innate immune signaling pathways to control dendritic cell and macrophage phenotypes. Targeting cell-intrinsic macrophage signaling by anticoagulants may therefore alter anti-tumor immunity. We hypothesized that anticoagulants with limited tissue penetrance, e.g. low-molecular weight heparins (LMWH), may differ from small molecule FXa inhibitors in targeting coagulation protease signaling in the intra-tumoral, extravascular space.

Aims: The goals of this study were:

1. to define tumor-promoting coagulation signaling in the TME, and
2. to compare therapeutic efficacy of clinically used anticoagulants in targeting these pathways.

Methods: We analyzed tumor growth and metastasis in spontaneous and in aggressive transplantable preclinical tumor models in immune competent mice. Immune cell composition of the TME and lymph nodes was determined by flow cytometry, mRNA expression profiling and ELIspot assays.

Results: We show that PAR2-activation by specifically macrophage-synthesized coagulation FX controls the immune-evasive phenotype of tumor-associated macrophages at different stages of tumor progression. Whereas LMWH and FXa inhibitor both prevent metastasis, only rivaroxaban attenuates tumor growth in spontaneously developing and transplanted tumor models. As seen with genetic abrogation of FXa-PAR2 signaling, rivaroxaban effectively reprograms macrophage polarization in the TME, activates cross-presenting innate immune cells in tumor-draining lymph nodes, and fosters expansion of tumor-reactive cytotoxic T cells in the TME.

Conclusions: These experiments demonstrate that coagulation innate immune signaling promotes immune-evasion and that tissue-penetrating FXa inhibitors are effective in reprogramming anti-tumor immunity. Oral FXa inhibitors may have clinical anti-cancer benefits beyond thrombosis prophylaxis and may synergize with immunotherapy regimens in cancer patients.

OC 68.2 | Protease Activated Receptor 4 Activation Requires a Coordinated Rearrangement of Extracellular Loop 3 and Thr153 in the Ligand Binding Site Formed by TM3 and TM7

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Background: Protease activated receptors (PARs) are the primary mediators of the protease-initiated cellular signaling. A newly-exposed N-terminus generated by proteolysis serves as a tethered ligand to activate PARs by interacting with its corresponding binding site on the receptor. Despite numerous functional studies on PARs, the structural basis for their activation and the location of their endogenous ligand binding sites (LBS) are unknown. Obtaining the structural information of PARs will further facilitate drug discovery targeting receptors in this family.

Aims: Our goal was to determine molecular basis for tethered ligand activation of the PAR family using PAR4 as a model.

Methods: PAR4 was analyzed by hydrogen deuterium exchange mass spectrometry (HDXMS) and computational modeling to map its structural rearrangement following activation. The structural studies were validated using a panel of PAR4 mutants in cell signaling assays.

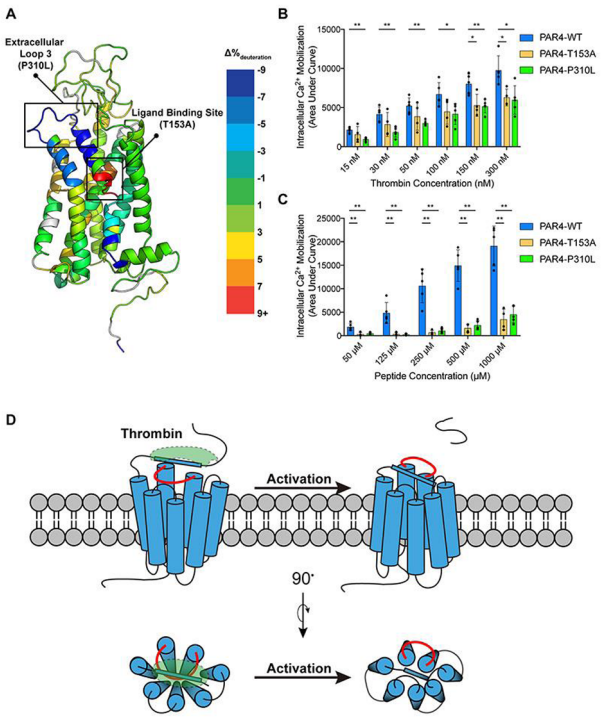

FIGURE 1 Both T153 at LBS and the rigidity of ECL3 are critical for thrombin mediated PAR4 activation

Results: HDXMS identified that the LBS is located in a pocket formed by transmembrane domain 3 (TM3) and TM7 and showed that extracellular loop 3 (ECL3) is a gatekeeper for the interaction between the tethered ligand and this site (Figure-A).Unbiased computational modeling confirmed the HDXMS data and identified a potential interaction between Gly48 from the tethered ligand and Thr153 from the LBS. Mutating Thr153 (PAR4-T135A) abolished PAR4-mediated intracellular calcium mobilization by PAR4-activation peptide (PAR4-AP) and reduced thrombin response by 35.5%. To test if the rigidity of ECL3 is essential for its role as a gatekeeper, we mutated Pro310 to Leu (PAR4-P310L) to increase the flexibility of ECL3. This resulted in a 76.3% and 38.8% reduction in calcium mobilization induced by PAR4-AP and thrombin, respectively (**Figure-B and-C**).

Conclusions: We have uncovered the LBS for PAR4 and identified an unexpected role for ECL3 in the tethered ligand activation mechanism as a gatekeeper (**Figure-D**). This lays the foundation for future work to determine if this mechanism is common for all members of the PAR family.

OC 68.3 | Protease-activated Receptor 4 Protects Mice against H1N1 Influenza A Virus Infection

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Background: PAR4 is the major thrombin-receptor on mouse platelets. In addition, it is expressed within the lung and on immune cells. Previous studies have found crosstalk between PARs and TLRs, including TLR3.

Aims: To investigate the effect of protease-activated receptor 4 (PAR4) deficiency on lung TLR3 responses and H1N1 influenza A virus (IAV) infection in mice.

Methods: PAR4$^{-/-}$ and wild-type (WT) mice were exposed to the TLR3 agonist poly(IC) via intranasal application (100µg in 50µL PBS) up to 3x every 24 hours and the innate immune response was analyzed at day 1 (cytokine/chemokine) and 3 (cell infiltration) in bronchoalveolar lavage fluid (BALF). In addition, progression of IAV infection of the lung was compared between infected PAR4$^{-/-}$ and WT mice.

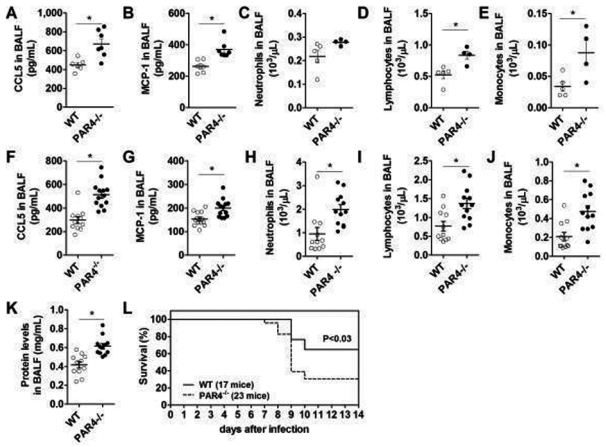

FIGURE 1 Role of PAR4 in lung antiviral immune responses

Results: Poly(IC) administration significantly increased levels of CCL5 and MCP-1 (Figure A,B) and immune cell infiltration (Figure C-D) in the airspace of PAR4$^{-/-}$ mice compared to WT mice at day 1 and 3, respectively. Moreover, PAR4 deficiency was associated with a more pronounced H1N1 IAV infection 5 days after infection with increased cytokine/chemokine levels (Figure F, G) and increased immune cell infiltration in the lung airspace (Figure H-J) compared to WT mice. The more severe H1N1 IAV infection in PAR4$^{-/-}$ mice was associated with more pronounced lung injury (Figure K) and increased death (Figure L) compare to infected WT mice. * P< 0.05.

Conclusions: Our study showed that PAR4 protects from H1N1 IAV infection by inhibiting proinflammatory TLR3-dependent immune responses. PAR4 antagonists are being developed as potential antithrombotic drugs and our data suggest that these drugs may increase the risk and severity of virus infections. We are currently investigating the cellular source of protective PAR4 using conditional PAR4 *knock-out* mice.

OC 68.4 | A Thrombin-PAR1/2 Positive Feedback Loop Amplifies TLR3-mediated Endothelial Procoagulant and Proinflammatory Responses

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Background: Pharmacologic inhibition of PAR1 has been shown to ameliorate TLR3-dependent procoagulant and inflammatory responses in mouse models of viral infection via thrombin-PAR1 signaling on innate immune cells. However, procoagulant responses to TLR3 stimulation have been shown in human endothelium but failed in human myeloid cells which argue the species- and cell type-specific TLR3-mediated responses.

Aims: To characterize the role of thrombin-PAR signaling for TLR3-mediated procoagulant and inflammatory responses in vascular endothelial cells.

Methods: Endothelial in vivo responses to the TLR3 agonist poly(I:C) and PAR1/2 inhibitors were analyzed by endothelial ribosomal RNA sequencing in (*RPL22.Vch5-cre*^{ERT2}) RiboTag mice. In vitro PAR1/2 effects on endothelial procoagulant and cytokine responses to poly(I:C) were studied in human EA.Hy926 and HUVEC, and mouse b.END3 cells. The genome-wide effect of PAR1/2 modulation of

the poly(I:C) response was analyzed by transcriptional profiling of EA.hy926 cells.

Results: Pharmacological inhibitors of PAR1/2 altered the in vivo systemic procoagulant activity (TAT-complex), and the endothelial translatome, indicating that PAR1/2 modify the overall response to poly(I:C). Transcriptional profiling in human ECs revealed that a subset of the poly(I:C) response, including TF, IL8, and chemokines, was augmented by PAR1/2 co-stimulation, indicating an overlapping target spectrum for both receptors. Thrombin, but not the ternary (TF/VIIa/Xa) complex, replicated the potentiating effect of Par1/2 agonist peptides on the poly(I:C)-response. Thrombin-mediated potentiation of the poly(I:C) response was inhibited by PAR1and PAR2 inhibitors, indicating PAR1-PAR2 crosstalk. Thrombin had no effect on procoagulant TF activity induced by LPS. The thrombin-mediated modulation of poly(I:C) responses was reproduced in primary human endothelial cells (HUVEC).

Conclusions: A thrombin-PAR1/2 positive feedback loop amplifies TLR3-mediated endothelial cell procoagulant and proinflammatory responses to poly(I:C). Pharmacologic targeting of PAR1 and/or PAR2 may have therapeutic potential for mitigating endothelial-mediated thrombotic and inflammatory pathologies associated with viral infection, such as DIC.

OC 68.5 | Suppression of Human Macrophage NLRP3 Inflammasome Activity by Activated Protein C or by PAR1-derived and PAR3-derived Peptides

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Background: Inflammasomes are cytosolic multiprotein complexes induced by a range of danger signals, and they promote NLRP3-dependent activation of caspase-1 (casp-1) enzyme activity which results in maturation and release of proinflammatory cytokines, including interleukin (IL)-1β. IL-1β is implicated in immunothrombosis; however, inflammasome regulatory mechanisms are ill-defined. Activated protein C (APC), a natural anticoagulant and anti-inflammatory blood enzyme, suppresses murine NLRP3 inflammasome activity in a mouse model of ischemia-reperfusion injury, in part due to APC acting on protease activated receptor (PAR)1. Whether APC or non-canonical PAR peptide agonists, e.g., PAR1-derived TR47 peptide and/or PAR3-derived P3R peptide that functionally mimic APC-initiated signaling, are anti-inflammatory on human cells has not been defined.

Aims: To determine the anti-inflammatory effects of APC, TR47 and/or P3R on phorbol myristate acetate (PMA)-differentiated monocytes when stimulated to induce casp-1 activity, a marker of inflammasome activity.

Methods: Monocytes (THP-1 cells) were differentiated into macrophages using PMA, then pretreated with APC or peptides prior to stimulation with lipopolysaccharide (LPS) and adenosine triphosphate (ATP) to induce casp-1 whose activity was measured using a luminescent casp-1 substrate.

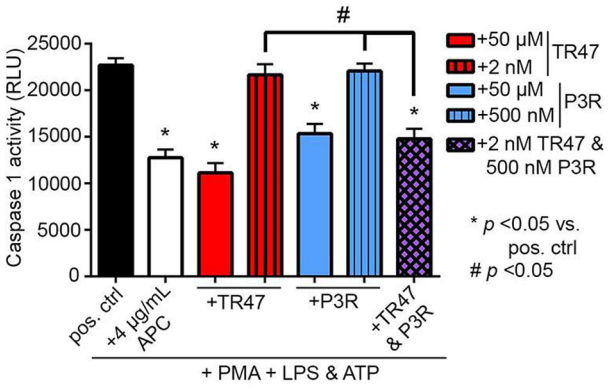

FIGURE 1 APC, TR47 and P3R restrict caspase-1 activity in THP1 cells

Results: APC treatment reduced NLRP3-dependent casp-1 activity in differentiated, stimulated THP-1 cells (Figure). Function blocking antibodies to EPCR, PAR1 or PAR3 each diminished APC's reduction of casp-1 activity, implicating each one as contributory for APC's actions. The TR47 and P3R peptides each individually reduced significantly casp-1 activity in a concentration-dependent manner; remarkably, these two peptides acted synergistically to inhibit activity of casp-1 at low concentrations where either peptide alone had little inhibitory activity (Figure).

Conclusions: APC and the non-canonical PAR1 and PAR3 agonists, TR47 and P3R, respectively, are anti-inflammatory by reducing NLRP3-driven casp-1 activity in human THP-1 cells. APC's casp-1 reduction requires EPCR, PAR1 and PAR3. This new knowledge about the anti-inflammatory activities of APC and of PAR1 and PAR3 agonism has significant translational implications.

OC 77.1 | Blood Endothelial Progenitor Cell-on-a-Chip: A Disease and Patient-specific Cell Source for in vitro Vascular Tissue Engineered Platforms

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Background: Vascular disorders account for some of the highest patient deaths. While animal models are too complex and non-human in many respects, contemporary *in vitro* techniques, such as, organ-on-chip, lack inclusion of an easily obtainable and phenotypically-similar human endothelial cell source. This limits our knowledge of disease mechanisms and hinders drug discovery.

Aims: In this study, we provide a proof-of-feasibility of utilizing Blood Outgrowth Endothelial Cells (BOECs) as a disease-specific primary cell source to analyze vascular inflammation and thrombosis in microfluidic conduits or vessel-on-a-chip.

Methods: The vessel-on-a-chip microchannels (**Fig.1A**) made of PDMS soft lithography are lined with autologous Blood Outgrowth Endothelial Cells (BOEC) derived from whole blood samples. Perfusion of whole human blood containing fluorescently labelled platelets and fibrin at arterial shear rates allows visualization of endothelial dysfunction. Results show mean±SEM. Statistical analysis is performed using Student's t-test in GraphPad Prism®.

Results: After a 24 hour culture under laminar flow, we observe formation of a confluent endothelium with intact junctions on all sides of the channels (**Fig.1B**). These blood-derived BOECs express pro-endothelial factors (**Fig.1C**), confirming their endothelial identity. Culturing SCD-BOECs derived from SCD patient blood in the vessel-on-chip followed by blood perfusion through these endothelialized microchannels yields significantly higher platelet adhesion and "comet" shaped platelet aggregation patterns, only observed during endothelium activation (**Fig.1 D-E**). Contrastingly, commercial HUVECs and control BOECs prevent any platelet adhesion to the surface. This suggests that BOECs in SCD patients exhibit vascular activation as *in vivo*, allowing this device to model thromboinflammatory conditions in SCD. We further find that BOECs of diabetic pigs show increased ROS (**Fig.1F)** and lower proliferation in hyperglycemic conditions compared to normal endothelial cells (**Fig.1G**).

Conclusions: These results demonstrate the potential of autologous endothelial cells easily obtained from blood for creating disease and patient-specific vascular disease models, which may facilitate drug development.

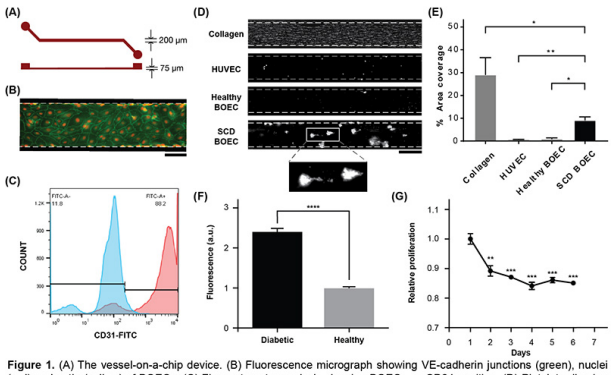

Figure 1. (A) The vessel-on-a-chip device. (B) Fluorescence micrograph showing VE-cadherin junctions (green), nuclei (red) and actin (yellow) of BOECs. (C) Flow cytometry analysis showing BOECs as CD31 positive. (D) Platelet adhesion on collagen, HUVEC, Healthy BOEC and SCD BOEC coated channels after whole blood perfusion. (E) % Area coverage by platelets [*p<0.05, **p<0.005]. (F) ROS generation in BOECs [****p<0.0001]. (G) Relative proliferation of diabetic BOECs compared to healthy cells [**p<0.005, ***p<0.0005].

FIGURE 1 Readouts from the vessel-on-a-chip device

OC 77.2 | Ceramide 1 Phosphate Is a Potent Chemoattractant Factor of Endothelial Colony Forming Cells and Improve Post Ischemia Tissue Regeneration

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Background: Ceramide 1 phosphate (C1P) is a well-known chemotaxis inductor in macrophages and murine progenitor cells. In endothelial colony forming cells (ECFC), we have shown that C1P improved proliferation and tubule formation, although C1P chemotactic effects remain unknown.

Aims: Considering that, C1P levels are elevated in ischemic tissues and that ECFC migration is a key step post-ischemia tissue revascularization, we here aimed to study whether C1P is an ECFC chemoattractant factor and improve their revascularization abilities *in vivo*.

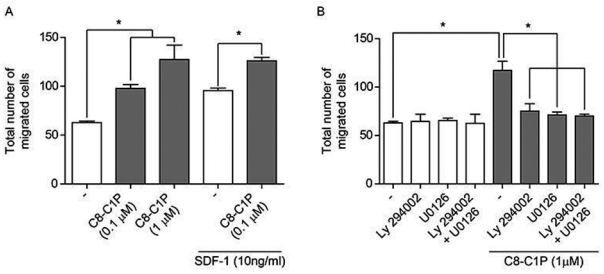

FIGURE 1 C1P driven chemotaxis was abrogated by AKT (Ly294002) and ERK1/2 (U0126) pathway inhibitors

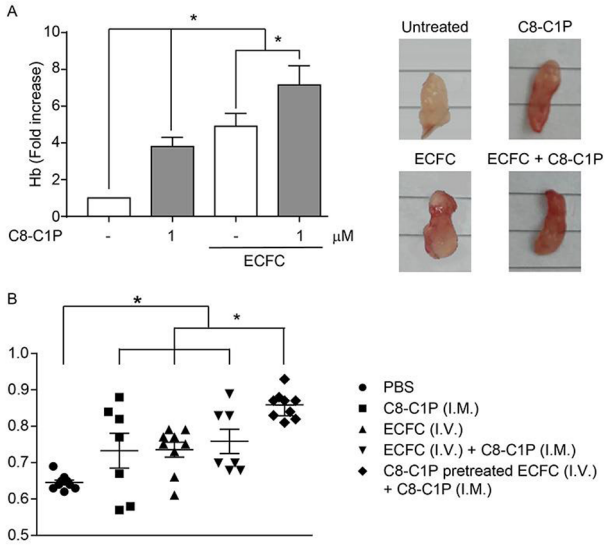

FIGURE 2 C1P attract ECFC to the ischemic muscle and augmented ECFC revascularization abilities in vivo

Methods: Human cord blood-derived CD34$^+$ cells were cultured in EGM2 and, after 14-18 days, ECFC colonies were obtained. ECFC were treated with C1P short chain analog C8-C1P. N=3-6, *p< 0.05, one-way ANOVA.
Results: We found that C8-C1P is a potent chemoattractant factor for ECFC not only *per se*, but also combined with SDF-1 (transwells, Figure 1A). C8-C1P-induced chemotaxis was completely suppressed by pharmacological inhibitors of ERK1/2 and AKT pathways (Figure 1B). *In vivo*, we observed that C8-C1P not only has a potent vasculogenic effect by itself, but also potentiated plug vascularization mediated by ECFC (Geltrex plug implants, Figure 2A). Moreover, in a murine model of hind limb ischemia, intramuscular injection of C8-C1P enhanced blood perfusion in the ischemic limb and slightly increased the revascularization mediated by untreated ECFC transplantation. Furthermore, administration of C1P-pretreated ECFC together with intramuscular C1P resulted in a significant improvement of leg reperfusion compared to each condition alone (Figure 2B).
Conclusions: In conclusion, C8-C1P induce ECFC chemotaxis *in vitro*, through AKT and ERK1/2 activation, and *in vivo* in a hind limb ischemia model, where C8-C1P not only attract ECFC to the ischemic muscle, but also augmented ECFC revascularization abilities. Our results highlight the therapeutic potential of C1P to improve post ischemia tissue regeneration.

OC 77.3 | Neutrophils Prothrombotic Characteristics during Myeloproliferative Neoplasms

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Background: Thrombosis is the most frequent complication during evolution of myeloproliferative neoplasms (MPN) but the events causing these clotting abnormalities remain unclear. Neutrophils are now recognized as important actors of thrombosis, especially by their capacity to emit neutrophil extracellular traps (NETs).
Aims: To assess if *JAK2*V617F neutrophils are more prone to form NETs, thus promoting thrombosis.
Methods: We first studied MPN patients and quantified:

1) NETosis markers,
2) NET formation,
3) reactive oxygen species (ROS) formation.

In a second part, we studied two different mouse models: *PF4-iCre;JAK2$^{V617/WT}$* mice with expression of *JAK2*V617F in hematopoietic cells and MRP8-iCre;*JAK2$^{V617/WT}$* mice with expression of JAK2V617F in neutrophils only. In these models, we quantified: 1) NET formation, 2) plasma levels of circulating DNA, 3) pulmonary thrombus formation. In *PF4-iCre;JAK2$^{V617/WT}$* mice, we analysed thrombus formation after DNAse administration. Finally, we quantified NET formation from JAK2WT or JAK2V617F neutrophils in presence or absence of JAK2WT or JAK2V617F platelets.
Results: In MPN patients, we found:

1) increased plasma levels of free DNA in all patients and increased plasma levels of MPO-DNA complex in patients with history of thrombosis,
2) increased ex-vivo NETs formation,
3) increased neutrophils ROS production.

In mouse models, we observed an increased NETs formation in the two mouse models analysed. We observed increased plasma levels of free DNA and increased thrombus formation in *PF4-iCre;JAK2$^{V617F/WT}$* mice only. Thrombus formation was abrogated in *PF4-iCre;JAK2$^{V617F/WT}$* mice after DNAse administration. We observed an increased NET formation in presence of JAK2V617F neutrophils and JAK2V617F platelets, with a decreased NETs formation after platelets inhibition.
Conclusions: Our results show that neutrophils are prone to form NETs during MPN, in a patient cohort, and in two different mouse models. More, our study demonstrates that JAK2V617F neutrophils alone are not sufficient to promote thrombosis and that the cooperation with platelets is important in NETs formation.

OC 77.4 | Characterization of Blood Outgrowth Endothelial Cells (BOECs) from Hermansky-Pudlak Syndrome (HPS) Type 1

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Background: HPS is an autosomal recessive disorder characterized by abnormal skin or ocular pigmentation and platelet dysfunction with altered biogenesis of lysosome-related organelles (LROs), including melanosomes and platelet dense granules. Nine genes (HPS1, AP3B1, and HPS3 to HPS9) have been identified as causative for HPS in humans. While several studies have explored the function of platelets, melanocytes, fibroblasts and their granules, no studies have explored endothelial cells from HPS.
Aims: To assess the impact of HPS1 mutation on endothelial cells.
Methods: BOECs were isolated from peripheral blood of a HPS1 patient and from healthy controls and characterized by immunofluorescence (IF) and flow-cytometry (FC) using antibodies against endothelial markers (VWF, CD31, CD146, VEGFR-2). Lysosomal-associated proteins LAMP1 and LAMP2 were assessed by IF and FC. Capillary-like structures (CLS)-sprouting was evaluted by angiogenesis-assay in Matrigel-coated plates. BOEC migration was assessed by the scratch assay on collagen-coated plates.
Results: Both HPS1 and control BOECs were positive for the endothelial markers CD31, VWF, CD146 and VEGFR-2 (CD309).

VWF and Weibel-Palade Bodies of HPS1 did not differ from controls, but BOECs from HPS1 showed higher surface expression of LAMP1 and LAMP2 compared to healthy controls (LAMP1: 26.4±2.08% vs 5.4±3.1%; LAMP2: 25.8±2.7% vs 7.4±3.3% p< 0.001), as assessed by both FC and IF.

Moreover, the scratch assay showed a significantly higher residual area in HPS1 (65.1±4.7% vs 33.1±4.3%; p< 0.05), showing defective EC migration. Finally, HPS1 BOECs formed a lower number of CLS (113±2.1 vs 153±2.7, p< 0.001) with shorter lenght (11.2±0.3mm vs 17±0.7mm, p< 0.001) and lesser branches (57.4±2.2 vs 75.2±2.9, p< 0.001) compared to controls, showing defective angiogenesis.

Conclusions: Our results show that HPS1 mutation alters the expression of lysosomal proteins and their trafficking in endothelial cells, resulting in an increased surface expression, but also impairs EC migration and neo-angiogenetic activity.

OC 77.5 | Identification and Functional Characterization of a New Thrombus-resolving Regulatory T-cell Population

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Background: Deep vein thrombosis is associated with long-term complications, including venous thromboembolism, post-thrombotic syndrome and chronic thromboembolic pulmonary hypertension. $CD4^+Foxp3^+$ regulatory T (Treg) have a major direct and non-redundant role in tissue repair and maintenance, distinct from their role in suppression of immune responses. Whether and how Treg participate in thrombus resolution has not been investigated.

Aims: Based on the observation that Treg accumulate in venous thrombi, we aimed to clarify their functional role in venous clot resolution.

Methods: Experimental approaches included the selective manipulation of Treg numbers during thrombus resolution, comprehensive characterization of the histological and cellular changes induced by them, generation of Treg expression profiles and the identification of their clot-dissolving function at the molecular level.

Results: We describe a specialized population of resident 'clot-busting' Treg that develops in venous blood clots, is activated by monocyte-derived cytokines, and regulates thrombus resolution by imposing phenotypic and functional changes in the myeloid compartment.

Conclusions: Our study reveals a crucial role of specialized Treg in ordered blood clot resolution. Increasing Treg activity appears to be an attractive therapeutic approach for improving thrombus resolution and restoring organ function in chronic thromboinflammatory diseases. Treg-modulating reagents, that are already clinically tested, may be used for this purpose.

PEDIATRICS

OC 38.1 | Single-center Clinical Data Analysis of Hereditary Thrombocytopenia in Children with Chronic Thrombocytopenia

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Background: Hereditary thrombocytopenia (HT) is clinically hard to differentiate from immune thrombocytopenia (ITP) in children and thus interferes with treatment.

Aims: Our study aimed to know the detection rate of HT in children chronic thrombocytopenia and its clinical and laboratory characteristics for an early clinical identification and diagnosis.

Methods: Retrospectively collected data from children thrombocytopenia whose present history lasted for ≥1 year and had poor response to immunotherapy. We screened HT by Next Generation Sequencing (NGS) and summarized the clinical and laboratory characteristics.

Results: A total of 161 children chronic thrombocytopenia were included. 43 cases (26.7%) were found with gene mutations. We analyzed the genetic rules of the mutant gene, family verification and the manifestations of the proband. 24 cases (14.9%) was diagnosed as HT and the proportion of males and females was 15:9, the median onset age was 0.58 years, significantly lower than that of non-HT cases (4.36 years), P value < 0.001; proportion of mucosal hemorrhage and visceral hemorrhage (31.8%, 13.7%) of HT was significantly higher than non-HT cases (15.3%, 0.6%) with a P value < 0.001. 12 cases of HT were with positive family history; HT could be divided into small platelet HT, positive platelet HT and large platelet HT according to the platelet morphology in peripheral blood smear. Some cases had well response to immunotherapy but was easy to relapse during the withdrawal period, while the others responded poorly to therapy.

Conclusions: In this study, the detection rate of HT in children chronic thrombocytopenia was 14.9%. The pathogenesis and clinical phenotype of HT was different. Some of them were effective for immunotherapy, which were easily confused with ITP. It is clinically necessary to perform NGS in children thrombocytopenia with early onset, abnormal platelet morphology, prolonged disease course and severe hemorrhage, in order to differentiate HT in time to avoid misdiagnosis.

OC 38.2 | Oxidized Hemoglobin Is Associated with Increased Level of Cell-free microRNAs in Cerebrospinal Fluid of Premature Infants with Intraventricular Hemorrhage: A Pilot Study

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Background: Intraventricular hemorrhage (IVH) can result in brain injury with oxidative hemolysis in premature infants, subsequently causing impaired neurodevelopment. Oxidized forms of hemoglobin can induce inflammation and endothelial cell dysfunction in the intraventricular space. Elevated microRNA (miRNA) levels have recently been linked to cerebral ischemia in adults with subarachnoid bleeding.

Aims: In this study, we analyzed cell-free miRNA levels in cerebrospinal fluid (CSF) samples obtained from preterm infants with IVH, in the presence or absence of oxidized hemoglobin content, in correlation with classic protein biomarkers.

Methods: Infants with high grade IVH (n=19) born at gestational age of 29.3±4.4 weeks were involved in comparison to controls with benign neurologic conditions (n=10). Collected CSF samples were spun down to remove the contaminating blood cells, and supernatants were stored at −80°C until use. Samples were first spiked-in with cel-miR-39, and miRNeasy kit (QIAGEN) was used for RNA extraction, followed by miRNA quantification by RT-qPCR. MiRNA expression was correlated with the concentrations of S100β, E-selectin and ICAM-1 in CSF determined by immunoassays. Oxidized hemoglobin levels were measured by spectrophotometry.

Results: Oxidized forms of hemoglobin were detected in 84% of all the initial CSF samples after grade 3 and 4 IVH. The samples from these patients demonstrated significantly higher miR-223, miR-181b and miR-155 levels (P< 0.01 vs. controls), which were caused not directly by hemolysis. During the follow-up period with total elapsed time of 25.2±13.8 days, decreasing miRNA expression reflected disease regression, and the resolution of IVH could be also monitored. The levels of protein markers were highly elevated in IVH, and the expression of miRNAs was positively correlated with oxidized hemoglobin level (r=0.645, P< 0.001), E-selectin (r=0.410, P< 0.01) and ICAM-1 (r=0.485, P< 0.05) concentrations.

Conclusions: Increased miRNA expression in CSF may indicate the severity of IVH as new biomarkers in preterm infants.

OC 38.3 | Platelet Physiology and Function in Neonates and Children from Different Pathways onto Extracorporeal Membrane Oxygenation (ECMO)

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Background: Extracorporeal Membrane Oxygenation (ECMO) is associated with significant platelet dysfunction and coagulopathy. However, little is known about the impact of prior cardiopulmonary bypass (CPB) on platelet dysfunction and associated bleeding / clotting complications in the setting of paediatric ECMO.

Aims: To determine the effect of prior CPB on platelet physiology and function and associated bleeding or thrombotic events.

Methods: This study was approved by the RCH ethics committee and written informed consent was obtained. Samples were collected from the arterial line of 22 neonates and children placed on veno-arterial ECMO at our institution within the first 24 hours upon ECMO initiation. Using bespoke whole blood flow cytometry panels we measured platelet phenotype, activation, response to stimulation with thrombin receptor-activated peptide 6 (TRAP-6) and formation of platelet-leukocytes aggregates (PLA). The dose-response of platelets to TRAP-6 stimulation was calculated as area under the curve (AUC). Comparisons were made between patients who underwent CPB prior to ECMO vs those who did not. Results were correlated with minor and major bleeding or thrombosis events.

Results: No difference in platelet phenotype, activation, response to stimulation with TRAP-6 or PLA formation was observed between prior CPB and no-CPB patients (Table 1). However, the association between bleeding events and reduced response to TRAP-6 was greater in patients without prior CPB (Table 2). In patients with prior CPB, major bleeding and thrombosis were associated with increased circulating platelet fibrinogen receptor expression and lower PLAs. Conversely, for patients with no prior CPB, major bleeding showed a non-significant trend for increased α-granule exocytosis, yet minor bleeding was associated with a non-significant reduction in platelet responsiveness to stimulation with TRAP-6, as indicated by α-granule release indicator and activated fibrinogen receptor.

Conclusions: This study suggests that bleeding and thrombosis could be associated with different types of platelet dysfunction according to the pathway onto ECMO.

TABLE 1 Platelet-specific or PLA marker expression for patients with or without prior CPB

		Pathways onto ECMO, n (%)		
Platelet marker/receptor (mean % or MFI +/- SD) [MFI, median fluorescence intensity; AUC, area under the curve; SD, standard deviation]		Prior CPB 12 (54.6)	No prior CPB 10 (45.4)	p-value
Phenotype	Fibrinogen receptor (MFI)	27437.25 ± 3577.20	26479.7 ± 1070.84	0.527
Activation	α-granule release indicator (% positive)	2.51 ± 1.48	1.97 ± 0.83	0.293
Response	α-granule release indicator (AUC)	60.15 ± 21.64	52.15 ± 32.65	0.517
Response	Activated fibrinogen receptor (AUC)	98.73 ± 31.99	79.17 ± 42.61	0.248
PLA	Neutrophil-platelet aggregate (%)	9.01 ± 2.76	9.95 ± 5.41	0.671

TABLE 2 Platelet-specific or PLA marker expression according to clinical events

Clinical event	Platelet marker/receptor (mean % or MFI +/- SD) [MFI, median fluorescence intensity; AUC, area under the curve; SD, standard deviation]	Prior CPB			No prior CPB		
		Yes	No	p-value	Yes	No	p-value
Minor bleeding	α-granule release indicator (AUC)	57.98 ± 22.70	63.19 ± 22.26	0.701	32.87 ± 14.53	71.43 ± 35.46	0.071
Minor bleeding	Activated fibrinogen receptor (AUC)	95.95 ± 35.01	102.62 ± 30.70	0.734	55.48 ± 27.05	102.86 ± 44.16	0.082
Major bleeding	Fibrinogen receptor (MFI)	29583 ± 1456.41	25291.50 ± 3870.48	**0.042***	27335.83 ± 1695.76	25195.50 ± 5093.49	0.470
Major bleeding	α-granule release indicator (%)	2.63 ± 1.78	2.40 ± 1.28	0.803	2.33 ± 0.86	1.43 ± 0.44	0.063
Major bleeding	Neutrophil-platelet aggregate (%)	6.85 ± 2.08	11.18 ± 1.01	**0.017***	8.41 ± 3.81	11.50 ± 6.89	0.470
Thrombosis	Fibrinogen receptor (MFI)	29539.33 ± 1249.68	25335.17 ± 3998.16	**0.049***	N/A (2 vs 8 for patient with or without thrombosis)		

OC 38.4 | Predictors of Recurrence and Post Thrombotic Syndrome (PTS) Following Lower Extremity Deep Vein Thrombosis in Children and Adolescents: PTS Risk Reduction Using Thrombolysis As Initial Therapy

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Background: Risk factors for outcomes following pediatric lower extremity (LE) deep vein thrombosis (DVT) are incompletely understood.

Aims: to analyze data on pediatric patients < 21 years with LE DVT in the consented prospective cohort, ThromboPICS.

Methods: High-risk DVT involved the inferior vena cava and/or iliac veins (IVC+/-IV). Outcomes included symptomatic recurrence ≥1 month, post thrombotic syndrome (PTS) using a validated tool ≥6 months or thrombus-related mortality following DVT. Genetic thrombophilia was ranked using an additive scale. Antiphospholipid antibody syndrome (APAS) severity was ranked by antibody number and titer. Factor FVIII, D-dimer and thrombin generation were measured ≥3 months and scored for level. Data were analyzed using paired comparison and univariate logistic regression.

Results: Paired comparisons by age < 13 (35%) vs ≥13 (65%) years included: pulmonary emboli 6 vs. 20%, p = 0.01; obesity 9.5 vs. 16% (NS); genetic thrombophilia 43 vs 53%, NS; APAS 35 vs. 56%, p = 0.006; May-Thurner (MT) and/or atretic IVC 6.4 vs 22%, p=0.007; absence of a trigger 22 vs 53%, p< 0.001; and anticoagulation as initial therapy 81 vs 66%, p=0.035. Estrogen was used by 62% of adolescent females. DVT recurred in 21 vs 23%, NS; PTS occurred in 6.6 vs 19%, p=0.027. There were no deaths related to LE DVT. Univariate analysis for outcomes in adolescents are in Table 1. Age ≥

TABLE 1 Univariable Regression Analysis of Risk Factors for Adverse outcomes in Adolescents ≥13 Years of Age

DVT Group	Outcome	Variable	p
All LE	Recurrence	Age, continuous	0.01
All LE	Recurrence	Ranked APAS	0.02
All LE	Recurrence	D-dimer	0.004
All LE	PTS	IVC/IV	0.033
IVC/IV	PTS	Ranked APAS	0.057
IVC/IV	PTS	D-Dimer	0.011
IVC/IV	PTS	Thrombolysis	0.008

13, high-risk DVT, MT/atretic IVC, APAS and PTS clustered together. FVIII and thrombin generation predicted neither recurrence nor PTS. Sixty-one adolescents ≥ 13 years had high-risk DVT: 28 received anticoagulation alone and 33 anticoagulation following thrombolysis; PTS occurred in 26 vs 13%, p = 0.008.

Conclusions: LE DVT was predominantly in adolescents without a trigger; in these adolescents increasing age, ranked APA and D-dimer correlated with recurrence, while IVC/IV and D-dimer correlated with PTS. Thrombolysis as initial therapy correlated with PTS prevention.

OC 38.5 | Inherited Antithrombin Deficiency and Thrombosis in Childhood: Data from a Large Multicentric Cohort

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Background: Inherited antithrombin (AT) deficiency is a rare autosomal dominant disorder leading to recurrent venous thromboembolism. In contrast to adults, little is known about the presentation in the pediatric population.

Aims: To investigate thrombotic presentation of inherited AT deficiency in children (age ≤18), combining data from two patient cohorts, resulting in the largest pediatric dataset on AT deficiency reported to date.

Methods: This observational, retrospective study was performed on 968 patients of any age with genetically confirmed AT deficiency. When the first thrombotic event occurred before age 19, disease history was evaluated, collecting data concerning site of venous and/or arterial thrombosis, possible transient risk factors or other thrombophilia.

Results: Seventy-three subjects (7.5%) developed thrombosis before age 19. We observed two periods with high prevalence: the neonatal period (< 30 days, n=15) and adolescence (12-18y, n=49). In newborns, thrombosis often presented at unusual sites (upper extremities, renal and cerebral veins) with transient provoking factors in half of them. During adolescence, thrombus localization and risk factors were common to adults.

We noted a high frequency (n=13, 17.8%) of cerebral sinovenous thrombosis, mainly among neonates (8/13).

Five patients suffered from arterial thrombosis (four neonates). : Outcome was severe with fatality in 6 cases (3 neonates) and severe disability in 3 cases, all neonates.

Our subjects predominantly showed type I deficiency (76.7%). From the 14 patients with type II deficiency, 6 carried the p.Leu131Phe mutation, affecting binding to heparin. Four of them were homozygous while the two heterozygous cases also carried Factor V Leiden.

Conclusions: The results of this study show severe thrombotic events in pediatric AT deficient patients. We suggest testing for AT deficiency in children from affected families, particularly in case of type I deficiency. Given the high prevalence of cerebral sinovenous thrombosis among neonates, we recommend avoiding invasive procedures during delivery in women with known AT deficiency.

OC 57.1 | Safety of Dabigatran Etexilate for Secondary Prevention of Venous Thromboembolism in Paediatric Patient

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Background: Dabigatran etexilate (DE) is a direct, oral thrombin inhibitor effective for the treatment of venous thromboembolism (VTE) in adults. In children, current standard of care (SOC) for VTE comprises anticoagulation with heparins or vitamin K antagonists (VKAs). SOC options are currently limited by parenteral administration and frequent monitoring.

Aims: To assess the safety of DE administered for secondary prevention of VTE.

Methods: Open label, single-arm prospective cohort, Phase III clinical trial (NCT02197416) in paediatric patients (stratified by age group: 12-< 18, 2 -< 12, 0-< 2 years of age) with confirmed diagnosis

of VTE treated with SOC for ≥3 months or who completed study treatment in the DIVERSITY study (NCT01895777), and who had an unresolved clinical risk factor requiring further anticoagulation. DE was dosed according to a nomogram derived from the estimated renal function based on a child's age and body weight. Treatment duration was ≤12 months. The primary endpoints were recurrence of VTE, any bleeding events (major, clinically relevant, and minor) and mortality at 6 and 12 months. Main secondary endpoints were occurrence of post-thrombotic syndrome (as per the modified Villalta scale), pharmacokinetic (PK) and pharmacodynamic (PD) parameters, and adverse events. Written informed consent was obtained before participation. The study was approved by all investigational site ethics committees.

Results: Data from ~200 patients from 83 sites in 26 countries will be presented (database lock: March 2019). Interim data showed a low overall frequency of recurrent VTEs and any major bleeding events. The PK-PD relationship was comparable to adults.

Conclusions: This is the first study of a direct oral anticoagulant for secondary VTE prevention in children reporting favourable results on safety with a PK-PD relationship of DE comparable to adults.

OC 57.2 | Investigation of the Age-specific in vitro Effect of Bivalirudin in Children Compared to Adults

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Background: Despite the increased use of bivalirudin in children, there remains a lack of paediatric-specific evidence regarding it's pharmacokinetics, clinical efficacy, adverse event profile and optimal dose schedule in children. Bivalirudin and many other anticoagulation medications are used off label and it is not known whether the response these medications may be age-dependent across infancy and childhood.

Aims: To investigate the *in vitro* effect of bivalirudin in plasma samples from healthy infants and children compared to adults.

Methods: Informed consent was obtained from parents of children and adult participants. This study was approved by the Royal Children's Hospital Ethics in Human Research Committee (#34183). Age-specific plasma pools were created using 20 individual samples specific to the following age groups: neonates, 1 year, 1 to 5 years, 6 to 10 years, 11 to 16 years and adults. Each plasma pool was spiked with 6 different bivalirudin concentrations (0.5 µg/mL, 1 µg/mL, 3 µg/mL, 5 µg/mL, 7 µg/mL and 10 µg/mL).The effect of the drug was quantified using APTT, PT, TCT and dilute TCT assays and thrombin generation.

Results: Results corresponding to the concentrations of Bivalirudin of 10ug/mL are listed in Table 1 as a small part of the overall results. Age-specific differences in Bivalirudin response were observed across most of the tests and across the spectrum of Bivalirudin concentrations tested.

Conclusions: The age-specific differences in the *in vitro* response to Bivalirudin are evidence that weight-based modifications in dose of this drug are not appropriate and that endogenous differences in the haemostatic system need to be considered when considering the use of this drug in children. Further studies need to focus on measuring the effect of Bivalirudin in patients who are receiving this drug.

OC 57.3 | Efficacy and Safety of Dabigatran Etexilate for Treatment of Venous Thromboembolism in Paediatric Patients - Results of the DIVERSITY Trial

M. Albisetti[1]; L. Brandão[2]; L. Bomgaars[3]; E. Chalmers[4]; M. Luciani[5]; L. Mitchell[6]; I. Nurmeev[7]; P. Svirin[8]; T. Kuhn[9]; O. Zapletal[10]; I. Tartakovsky[11]; M. Simetzberger[12]; S. Wiebe[13]; Z. Sun[14]; S. Gropper[11]; M. Brueckmann[11]; J. Halton[15]; on behalf of the 1160. 106 Investigators

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TABLE 1 Coagulation test parameters in response to 10ug/mL Bivalirudin

(mean +/- SD)	Neonates	1 Years	1-5 Years	6-10 Years	11-16 Years	Adults
APTT (sec)	189.2+/-2.43	177.4+/-0.64	172.9+/-0.35	177.9+/-0.31	160.7+/-2.35	153.7+/-0.14
TCT (sec)	999+/-0.00	386.7+/-175.37	458.1+/-6.43	558.5+/-0.00	497.9+/-0.64	433.1+/-8.63
dTCT (sec)	447.9+/-3.75	355.6+/-7.45	352.3+/-2.67	367.7+/-9.48	324.4+/-1.79	258.7+/-2.43
PT (sec)	128.6+/-0.4	76.3+/-1.3	82.3+/-1.3	79.6+/-1.4	76.3+/-1.1	69.2+/-0.5
ETP (nM•min)	1937+/-51.17	2631+/-85.18	3086+/-19.72	2928+/-17.09	2743+/-18.66	3430+/-24.94
Lagtime (min)	12.0+/-0.29	9.8+/-0.24	11.1+/-0.19	11.8+/-0.25	8.8+/-0.24	7.5+/-0
Peak Thrombin (nM)	317.6+/-3.92	488.3+/-19.91	590.8+/-3.84	586.2+/-7.19	569.0+/-6.08	627.75+/-5.58
ttP (min)	15.6+/-0.34	12.0+/-0.41	14.0+/-0	14.4+/-0.34	11.4+/-0.19	10.25+/-0.25

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Background: Dabigatran etexilate (DE) is a direct, oral thrombin inhibitor effective for the treatment of venous thromboembolism (VTE) in adults. In children, current standard of care (SOC) with heparins or vitamin K antagonists is limited by parenteral administration and frequent monitoring.

Aims: To document the efficacy and safety of DE versus SOC, and the appropriateness of a DE dosing algorithm.

Methods: Open-label, randomized, active-controlled, multi-centre, Phase IIb/III trial (NCT01895777) in paediatric patients (stratified by age group: 12-< 18, 2 -< 12, 0-< 2 years of age) with confirmed diagnosis of VTE initially treated with unfractionated heparin or low-molecular-weight heparin. Patients were randomized (2:1) to DE (capsules, pellets, or oral solution) or SOC and treated for 3 months. DE was dosed according to a nomogram derived from the estimated renal function based on a child's age and body weight. The combined efficacy endpoint included recurrence of VTE, VTE-related death, and thrombus resolution. Further endpoints were bleeding events, pharmacokinetic (PK) and pharmacodynamic (PD) parameters, and adverse events. Written informed consent was obtained before participation. The study was approved by all investigational site ethics committees.

Results: Data from ~220 patients from 87 sites in 27 countries will be presented (database lock: March 2019). Interim data indicated DE was comparable versus SOC for the combined efficacy endpoint and safety outcomes (bleeding). Interim data also indicated that PK in paediatric patients followed observations known from adults. PK-PD relationship was also comparable to adults.

Conclusions: The DIVERSITY study is the first study comparing a direct oral anticoagulant to report efficacy, safety and PK-PD relationships in children. The study demonstrates the appropriateness of an age- and body weight-adjusted dosing algorithm for DE in children between 0-< 18 years of age.

OC 57.4 | Comparison of Stroke Incidence in Fontan Patients Receiving Warfarin or Aspirin

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Background: Fontan patients are at an increased risk of stroke due to abnormalities in all three aspects of Virchow's triad. To mitigate this risk, Fontan patients usually receive life-long thromboprophylaxis with either warfarin or aspirin. It is unknown whether the two drugs have different stroke prevention efficacy.

Aims: This study aimed to compare the incidence of cerebral ischemic or haemorrhagic insult in Fontan patients receiving warfarin or aspirin.

Methods: This was a cross-sectional, multicenter study of the ANZ Fontan population. Patients were recruited from the ANZ Fontan registry; informed written consent was obtained for all participants. This study was approved by the Royal children's hospital ethics in human research committee.

Inclusion criteria were: >5 years post Fontan, >10 years of age, receiving aspirin or warfarin exclusively for >5 years prior to study involvement. Cerebral MRI was performed to quantify ischemic and haemorrhagic insult. All scans were reported centrally and classified by a Senior Paediatric Neurologist and Paediatric Neuroradiologist, who were blinded to all clinical information. Pearson's Chi squared test was used to compare outcomes between groups.

TABLE 1 Comparison of Cerebral MRI outcomes of patients receiving warfarin versus aspirin

	Warfarin (N=43)	Aspirin (N=36)	Total (N=79)	Pearson Chi^2	P value
Infarct				0.61	0.44
Absent	25 (58%)	24 (67%)	49 (62%)		
Present	18 (42%)	12 (33%)	30 (38%)		
Micro-Haemorrhage				2.61	0.11
Absent	3 (7%)	0 (0%)	3 (4%)		
Present	40 (93%)	36 (100%)	76 (96%)		
Macro-Haemorrhage				0.85	0.36
Absent	42 (98%)	36 (100%)	78 (99%)		
Present	1 (2%)	0 (0%)	1 (1%)		

Results: There were 79 study participants, all of whom had abnormal brain imaging.

- 96% had micro-haemorrhages
- 8% had stroke
- 87% of strokes were subclinical

There were no statistical differences between warfarin and aspirin groups for stroke (table 1):

- Rate
- Type
- Severity (number of infarcts or micro-haemorrhages)
- Haemorrhage and infarct were highly prevalent and not influenced by thromboprophylaxis type.

Conclusions: This is the first study to demonstrate widespread prevalence of stroke and extensive degree of brain injuries in the Fontan population, regardless of the type of antithrombotic therapy utilised. Whilst the symptomatic stroke rate is similar to those of previous studies, the extensive rate of subclinical stroke highlights the need for further research into the pathogenesis and the implications of these silent events.

OC 57.5 | Antithrombin Activity Levels Below 60% in Children Less than 1 Month of Age Have Implications for Heparin (LMWH) Dosing

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Background: Antithrombin (AT) is a natural anticoagulant inhibiting thrombin and factor Xa and thus accelerating the neutralisation of coagulation proteins. LMWH is responsible for accelerating AT interaction with thrombin and Xa. AT deficiency (acquired or congenital) reduces the ability to achieve the desired anticoagulant effect using LMWH. The anti-Xa test measures the activity of heparin/LMWH against the activity of activated coagulation factor X.
Aims: Determination of the Antithrombin activity levels having an effect on LMWH dosing in a paediatric cohort.
Methods: Prospectively (2 year period) measure AT activity levels in patients < 1 year of age pre-operatively scheduled for LMWH post operatively (n= 335).
Determine the cut-off AT activity affecting Anti-Xa levels by performing *in vitro* heparin recovery.
Evaluate Anti-Xa assay type; AT supplementation (AT+) and without (AT-) on heparin recovery (n=54).
Results: 210/335 patients were found to have AT activity less than 0.60 IU/ml with the majority of patients (n= 209) < 1 month old.
Patients (n=54) receiving LMWH therapy showed that the LMWH assay (AT+) had greater heparin recovery compared with the LMWH (AT-) assay.
In vitro analysis showed that the heparin recovery was dependent on AT levels for both types of anti Xa assays. There was a marked reduction in the amount of heparin recovered when AT activity levels fell below 60%.
Conclusions: The study has shown that anti-Xa requires the presence of AT to carry out its mode of action which may be limiting in determining anti-Xa levels in AT deficient patients. The cut-off concentration at which AT had an effect on Anti-Xa levels was 60% and in this study 210 patients had AT activity < 0.60 IU/ml. This finding has implications for dosing LMWH in neonates under 1 month of age.

PLATELETS AND MEGAKARYOCYTES

OC 08.1 | GFI1B Controls Vesicular Trafficking and Maintains Synchronism during Megakaryocyte Maturation

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Background: Mutations of the hematopoietic transcription factor GFI1B are known to be responsible for inherited bleeding disorders associated with macrothrombocytopenia and abnormal platelet granule content. Although its regulatory effect on cytoskeleton organization has been demonstrated, the wider spectrum of GFI1B's role in megakaryopoiesis is not fully elucidate.
Aims: To investigate GFI1B's role in megakaryocyte physiology and understand the distinctive platelet phenotype observed in affected patient.
Methods: We generated a megakaryocyte lineage specific *Gfi1b*$^{\Delta/\Delta}$ mouse model (PF4-Cre/*Gfi1b*$^{fl/fl}$) and compared the mouse phenotype to affected patients.
Results: PF4-Cre/*Gfi1b*$^{fl/fl}$ mice display a bleeding phenotype associated with severe thrombocytopenia (16±2x10^9/L vs 1011±28x10^9/L, p< 0.0001). Compared to their control littermate, PF4-Cre/*Gfi1b*$^{fl/fl}$ mice exhibit higher TPO plasma levels and megakaryocytic hyperplasia in bone marrow(BM) without sign of fibrosis. Extramedullary haematopoiesis occurs with increased megakaryocytes in the spleen, leading to massive splenomegaly. Both medullary and splenic Gfi1b-deficient megakaryocytes have abnormal morphology: MGG-stained films and TEM revealed a multilobular nucleus at the cell periphery and poorly developed demarcation membrane system(DMS) without defined

platelet territories. These observations suggest a maturation asynchrony between nucleus and cytoplasm, supported by the observation of increased DNA ploidy. In addition, Gfi1b-deficient megakaryocytes display a severe granule deficiency. Although multivesicular bodies are seen in perinuclear areas, no mature cytoplasm granules could be observed. In BM-derived megakaryocytes, *Gfi1b* deletion appears responsible for defective endocytic pathways (including the absence of fibrinogen uptake), incomplete DMS formation and inhibition of proplatelet formation. Murine megakaryocytic defects are consistent with the GFI1B-mutated patient phenotype of thrombocytopenia with a subset of agranular platelets lacking fibrinogen. Moreover, RNA-seq data derived from affected and unaffected individuals revealed dysregulation of more than 20 genes involved in vesicular trafficking linking the human and murine phenotypes.

Conclusions: In this study, we highlight the role of Gfi1b on vesicular trafficking in megakaryocytes, its loss leading to defective platelet production and abnormal granule biogenesis.

OC 08.2 | Megakaryocytes Form Podosome-Like Protrusions *in situ* Leading to the Formation of Transendothelial Pores

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Background: Platelet production requires that megakaryocyte (MK) fragments are released into the circulation after crossing the endothelial cell (EC) layer of bone marrow (BM) sinusoids. The cellular mechanisms involved in this passage remain unclear.

Aims: We characterized the cellular events taking place during MK intravasation.

Methods: Using combined fluorescence, electron and 3D tomography microscopy.

Results: We observed that MK extend protrusions which squeeze the EC leading to the tight apposition of the apical and basal EC plasma membranes. 3D tomography demonstrated that these contacts represent hemifusion sites where local fusion can be initiated and transendothelial pores formed. The MK protrusions were able to deform the EC nucleus indicating that they could develop strong forces. Correlative IF and TEM analyses showed that they contain actomyosin bundles orientated perpendicularly to the endothelial layer. This F-actin organization, the size of the MK protrusions (568±30nm thickness), their finger-like morphology and the protrusive properties suggest a podosome organization. Cortactin and talin, two F-actin-binding proteins classically associated with podosomes, were detected in these structures while beta1-tubulin was not found. Remarkably, in a transgenic mice model, beta1-tubulin deficiency had no impact on the MK/EC interactions. On the other hand, loss of the Arp2/3 complex led to the near disappearance of the podosome-like protrusions and the transendothelial penetration by MK, and loss of myosin IIA led to a smaller protrusion size in MKs indicating that acto-myosin plays a crucial role in MK intravasation.

Conclusions: Thus, MK-derived podosome-like protrusions represent the first events of MK intravasation. This occurs through an actin/myosin-dependent and a beta1-tubulin-independent mechanism. MK protrusions trigger the fusion of opposite EC plasma membranes, leading to the formation of transcellular pores. This allows MK to enter the sinusoids as large fragments which could remodel into proplatelets and detach in the blood stream to ultimately generate circulating platelets.

OC 08.3 | Activation of Toll like Receptors 2 and 4 on CD34$^+$ Cells Increases Human Megakaryo/thrombopoiesis Induced by Thrombopoietin

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Background: Toll-like receptors (TLRs) play a key role in pathogen recognition and regulation of immune responses. Although it is known that activation of platelet's TLR2/TLR4 amplifies the host immune response against infections, its role on human megakaryopoiesis has not yet been investigated.

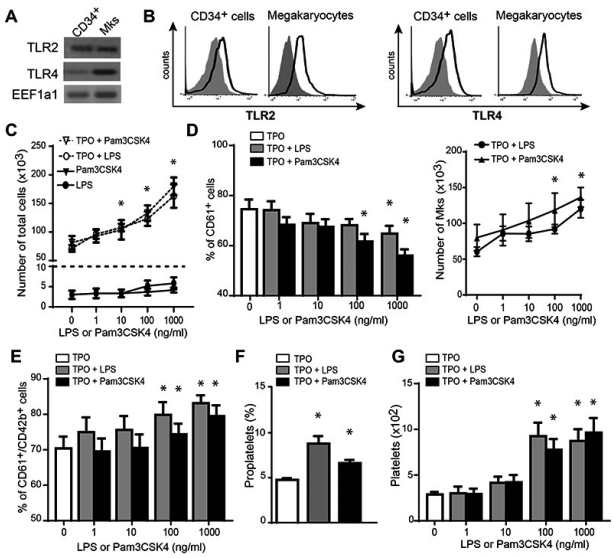
Aims: Since thrombocytosis is usually observed during bacterial infections we evaluated whether Pam_3CSK_4 or lipopolysaccharide (LPS) (components of Gram positive and negative bacteria and TLR2/TLR4 ligands, respectively) modulate human megakaryocyte development and platelet production.

Methods: CD34+ cells derived from human umbilical cords were cultured with TPO; LPS or Pam_3CSK_4; TPO+ LPS or Pam_3CSK_4 during 11-16 days.

Results: CD34+ cells and megakaryocytes express TLR2/TLR4 at both, RNA and protein level. Incubation of CD34+ cells with Pam_3CSK_4 or LPS showed no differences in cell growth vs control. However, both TLR ligands markedly increased TPO-induced CD34+ cell proliferation (Fig.1). Although there was a slight but significant decrease in megakaryocytes differentiation (CD61+), megakaryocyte number, maturation degree (CD42b+), proplatelet and platelet production were higher in cultures treated with TPO + LPS or Pam_3CSK_4 vs TPO alone (Fig.1). Stimulation of CD34+ cells with TPO and TLR2/4 ligands but not with TPO alone triggered IL-6 release (Fig.2). The increased proliferation and IL-6 production induced by TPO + LPS or Pam_3CSK_4 were suppressed by TLR2/4 or IL-6 neutralizing antibodies or PI3K/AKT or NF-kB inhibitors and. The increment in proplatelet and platelet production was associated with

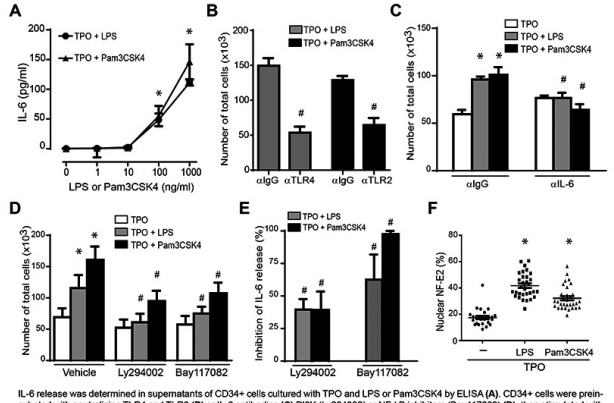
an increased nuclear translocation of NF-E2 (Fig.2). The synergistic effects of TPO-induced megakaryo/thrombopoiesis mediated by TLR2/4 ligands were not observed when Pam_3CSK_4 or LPS were added at day 7 of TPO-treated cultures. Supernatants from CD34+ cells stimulated with TPO + LPS induced CFU-M colonies (cytokine-free methocult).

Conclusions: Our data suggest that recognition of bacteria by CD34+ cell TLR2/4 in the presence of TPO might contribute to thrombocytosis by an autocrine IL-6 loop.



TLR2 and TLR4 expression in CD34+ cells and megakaryocytes (Mks) was determined by RT-PCR **(A)** and flow cytometry **(B)**. CD34+ cells were cultured with LPS or Pam3CSK4 in the presence of TPO (50 ng/ml). Then, at day 11 of culture cell proliferation **(C)**, megakaryocyte differentiation (flow cytometry), total number of megakaryocytes **(D)** and megakaryocyte maturation (flow cytometry) **(E)** were determined. Megakaryocytes were cultured with TLR agonists for 13 days and then seeded over fibrinogen coated glasess. The percentage of proplatelets forming megakaryocytes was analized 16h later **(F)**. After 16 days of culture with TLR agonists and TPO, culture-derived platelets were counted by flow cytometry **(G)**. Representative results or Mean ± SEM, n=4-6, ANOVA).

FIGURE 1



IL-6 release was determined in supernatants of CD34+ cells cultured with TPO and LPS or Pam3CSK4 by ELISA **(A)**. CD34+ cells were preincubated with neutralizing TLR4 and TLR2 **(B)** or IL-6 antibodies **(C)** PI3K (Ly294002) or NF-kB inhibitors (Bay117082) **(D)**, then stimulated with TPO plus TLR agonists and proliferation measured 11 days later. CD34+ cells were preincubated as in D and IL-6 release determined by ELISA 24 hs later **(E)**. NF-E2 activation was determined in megakaryocytes at day 9 of culture with TLR agonists by confocal microscopy **(F)**. * p<0.05 vs. TPO alone, # p<0.05 vs. IgG or vehicle (Mean ± SEM, n=4-6, ANOVA).

FIGURE 2

OC 08.4 | Megakaryocyte Specific Deletion of NMDA Receptor Causes Abnormal Platelet Production and Function, but Normal Megakaryocyte Development

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Background: Calcium signalling regulates megakaryocyte development and platelet activation. We have previously reported that the NMDA receptor (NMDAR), a calcium channel, is functional in megakaryocytes and platelets. NMDAR promotes megakaryocyte maturation *in vitro* and NMDAR inhibitors attenuate platelet function. Here we created a mouse model to determine NMDAR contribution to megakaryocyte development and platelet function *in vivo*.

Aims: To determine the NMDAR role in megakaryocyte differentiation, platelet production and function *in vivo*.

Methods: A Cre-loxP conditional knockout mouse model was used to delete expression of the *Grin1* gene under the Pf4 promoter (Pf4-*Grin1*$^{-/-}$). *Grin1* encodes the obligatory NMDAR subunit. The deletion of *Grin1* was confirmed at the genomic and transcript level using qPCR on CD41-positive megakaryocytes. Platelet function was examined by tail bleeding time, platelet counts, activation, recovery and half-life experiments. Phenotypic changes in megakaryocytes were assessed by histology and ploidy examination by flow cytometry.

Results: Pf4-*Grin1*$^{-/-}$ mice showed a Mendelian ratio of inheritance and no spontaneous deaths. There was mild thrombocytopenia with a 27% decrease in the platelet count (P=0.00014) with no change in platelet size or other counts. Platelets showed a mild impairment in function with a prolonged tail bleeding time (461 vs 251 seconds in control mice; P=0.0016) and decreased activation after stimulation with ADP. Pf4-*Grin1*$^{-/-}$ mice had mildly delayed platelet recovery after acute thrombocytopenia induced by tail-vein injection of anti-platelet serum. Unexpectedly, platelet clearance was also mildly reduced revealed by a mild increase in half-life of X488-labelled platelets. Histological examination showed no gross changes in bone marrow morphology or megakaryocyte numbers, and ploidy analysis showed no change in ploidy distribution.

Conclusions: Our results confirm NMDAR-mediated calcium influx contributes to platelet production and function, but appears redundant for megakaryocyte development *in vivo*. Further studies are under way to characterise the altered platelet release and platelet function in Pf4-*Grin1*$^{-/-}$ mice.

OC 08.5 | Platelet-derived Microvesicles Reprogram Bone Marrow Megakaryocytes in the Setting of Inflammatory Disease

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Background: Inflammatory signaling has multiple and varying effects on platelet production. We have previously shown that platelet-derived chemokine ligand 5 (CCL5, RANTES) increases megakaryocyte maturation and proplatelet production. In addition, platelet and plasma CCL5 increases in inflammatory diseases. However, the mechanism by which this affects megakaryocytes in the bone marrow is unclear.

Aims: We hypothesized that platelet-derived microvesicles, known to contain CCL5, may be acting as sentinels to carry plasma cytokines into the bone marrow, thus contributing to disease pathology.

Methods: Histology, live-cell imaging, and flow cytometry of bone marrow from multiple in vivo models of inflammation were used to examine microvesicle-megakaryocyte interactions.

Results: Immunofluorescence and immunohistochemistry revealed CD41 and P-selectin positive microvesicles in the femoral bone marrow of wild-type mice. To determine that these microvesicles were platelet- and not megakaryocyte-derived, we reconstituted platelet counts in cMpl-/- mice with donor wildtype platelets. After 18 hours, cMpl+ microvesicles were found in the bone marrow of cMpl-/- mice; microvesicles in the marrow increased after LPS challenge. In addition, we found that platelet-derived microvesicles contain CCL5 and could bind to, interact with, and functionally reprogram megakaryocytes via increasing platelet production in vitro. As platelet microvesicles are a product of platelet activation, we moved to an in vivo murine model of inflammation, the NZBWF1 model of spontaneous SLE. We found increased CCL5 in both the platelets (Luminex-based array) and bone marrow (immunohistochemistry) of these mice. Further, platelet-derived microvesicles were enhanced in the plasma and could be found throughout the bone marrow. We hypothesize that platelet-derived microvesicles may be the source of bone marrow CCL5.

Conclusions: Our results show that platelet-derived microvesicles are found throughout the bone marrow and that this increases during inflammatory states. Platelet-derived microvesicles can functionally reprogram megakaryocytes, suggesting they could alter megakaryocytes and the platelets they produce, thus contributing to disease pathology and inflammatory-induced platelet production.

OC 20.1 | Novel Insights into Mouse Models of Ectopic Proplatelet Release

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Background: Bone marrow (BM) megakaryocytes (MKs) produce platelets by extending proplatelets into sinusoidal blood vessels. Defects in proplatelet formation and/or platelet release can lead to thrombocytopenia with increased bleeding risk. We recently found that deficiency of the adhesion and degranulation-promoting adaptor protein (ADAP) affects F-actin organization and β1 integrin activation in MKs and causes microthrombocytopenia in mice partially due to ectopically released (pro)platelets (Spindler *et al*. Blood 2018). Mice lacking the actin-regulatory proteins Wiskott-Aldrich syndrome (WASp), Arp2/3 or Profilin 1 (PFN1) all of which display a thrombocytopenia also show ectopic release of (pro)platelet-like particles into the BM compartment (Fig.1 and published data), pointing to an important axis of actin-mediated directional proplatelet formation. The reason for ectopic release of (pro)platelet-like particles into the BM compartment in those mice is still incompletely understood, but we hypothesize that similar defects account for this observation.

Aims: To investigate the role of actin reorganization and integrin signaling in MKs for ectopic (pro)platelet release in genetically-modified, thrombocytopenic mice.

Methods: We used constitutive (WASp) and conditional knockout mice (ADAP, PFN1) to investigate ectopic (pro)platelet release *in situ* and MK adhesion to the extracellular matrix *in vitro* by confocal microscopy.

Results: ADAP- and PFN1-deficient MKs displayed reduced adhesion to collagen, defective F-actin organization as reflected by reduced F-actin content as well as altered podosome formation, and diminished β1 integrin activation. Interestingly, WASp-deficient

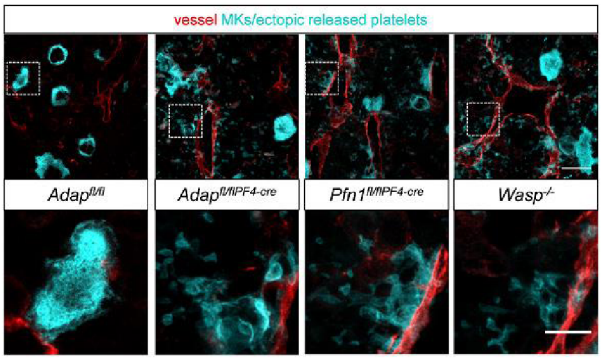

FIGURE 1 In situ analysis of sternal BM from ADAP, PFN1 and WASP-deficient mice

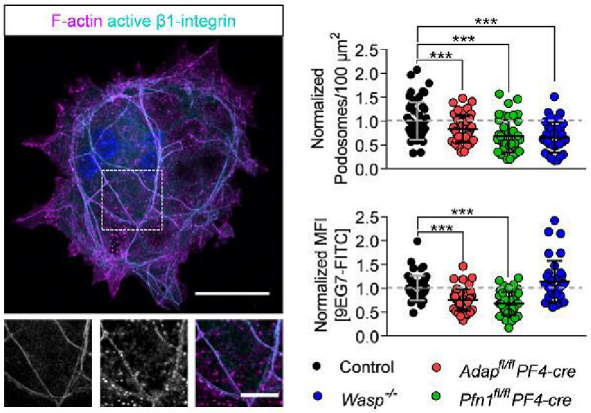

FIGURE 2 F-actin organization and beta1 integrin activation in mutant MKs upon spreading on collagen

MKs showed the strongest reduction in the adhesion assay on collagen, and the mutant MKs, which were able to adhere and spread on collagen, exhibited also altered F-actin organization with reduced podosome formation, but normal β1 integrin activation (Fig.2).

Conclusions: Our results point to a so far unidentified axis including ADAP, PFN1 and WASp in directional platelet biogenesis. Efforts are underway to include Arpc2 mutant mice in our study.

OC 20.2 | Sustained Integrin $\alpha_{IIb}\beta_3$ Activation in Megakaryocytes Impairs Platelet Release in the Bone Marrow

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Background: Megakaryocytes (MK) reside primarily in the bone marrow (BM), where they undergo a series of remarkable morphological changes resulting in proplatelet formation (PPF) and platelet release into the bloodstream.

Rap1, a member of the Ras superfamily of small GTP-binding proteins, is highly expressed in plt and MK. In platelets, Rap1 is critical for the inside-out activation of integrin $\alpha_{IIb}\beta_3$, with Rap1 activity being controlled by the guanine nucleotide exchange factor (GEF), CalDAG-GEFI, and the GTPase activating protein (GAP), Rasa3. Mice with very low Rasa3 expression ($Rasa3^{hlb/hlb}$) exhibit a dramatically reduced plt count and an increased rate of plt clearance. Furthermore, in vitro studies identified an increased interaction of MKs with BM matrix components and a defect in PPF in Rasa3 mutant mice.

Aims: Determine how alterations in the Rap1 signaling pathway affect MK function.

Methods: MK function was analysed in vitro (cultured cells), ex vivo (BM explants), and in vivo (2-photon imaging of the calvarium).

Results: MK lacking both Rap1 isoforms, Rap1a and Rap1b, shows a reduction in the percentage of MK undergoing PPF and an increase in the size of PP extensions. MK from Rasa3 mutant mice ($Rasa3^{hlb/hlb}$) exhibited a marked defect in PPF in vivo. Interestingly, PPF was not impaired in cultured $Rasa3^{hlb/hlb}$ MK, suggesting that the observed in vivo defect resulted from an abnormal interaction of Rasa3 mutant MK with the vasculature. Consistent with this hypothesis, we observed that MK were more tightly associated with sinusoids and spread in bone marrow of $Rasa3^{hlb/hlb}$ mice. Deletion of CalDAG-GEFI or Talin1 partially normalized MK morphology, BM localization, and PPF in $Rasa3^{hlb/hlb}$ mice.

Conclusions: In summary, our studies identify Rap1 as a critical regulator of MK biology. Prolonged Rap1 signaling as seen in Rasa3 mutant mice leads to tighter interaction of MK with BM sinusoids and impaired platelet release into the bloodstream.

OC 20.3 | Unexpected Redundant Function of Twinfilin1 and Cofilin Is Essential for Platelet Biogenesis

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Background: Cytoskeletal rearrangements are pivotal during platelet activation by enabling shape change, which is indispensable for proper platelet function. Hence, defective actin or microtubule reorganization is associated with platelet disorders in humans and mice. Twinfilins are small actin-binding proteins containing two actin-depolymerizing factor homology (ADF-H) domains. While twinfilin 2a (Twf2a) has been identified as a regulator of platelet reactivity, the role of twinfilin1 (Twf1) has remained elusive so far.

Aims: Since recent publications proposed an interaction between Twf1 and another ADF-H-domain-containing protein, namely cofilin (Cof), during actin depolymerization at pointed ends, we generated animals lacking both proteins in megakaryocytes (MKs) and platelets to evaluate a possible redundancy.

Methods: Platelet reactivity and function, especially regarding hemostasis and thrombosis, were assessed *in vitro* and *in vivo*, in particular concerning actin and microtubule dynamics. In addition, MK function and proplatelet formation were assessed *in vitro* as well as *in vivo* by two-photon-microscopy of the bone marrow.

Results: Double-deficient animals presented with a severe macrothrombocytopenia albeit an unaltered platelet lifespan. Strikingly, we found a dramatic increase in MK numbers in bone marrow and spleen. *In vitro*-matured MKs displayed impaired spreading as well as a reduced ability to form proplatelets due to altered microtubule dynamics, which was also observed *in vivo* by two-photon microscopy. Analysis of the platelet cytoskeleton revealed an increased F-actin

content as well as impaired F-actin assembly upon platelet stimulation in the double-deficient platelets. Moreover, platelet spreading on fibrinogen was markedly impaired possibly due to accelerated integrin inactivation. This translated into a slightly increased tail bleeding time as well as a protection from *in vivo* occlusive thrombus formation.

Conclusions: In summary, these findings point to a so far unexpected functional redundancy between Cof and Twf1 during thrombopoiesis and provide direct evidence for the proposed interaction between Twf1 and Cof during actin disassembly.

OC 20.4 | O-glycan Sialylation Regulates Thrombopoiesis through Interferon-secreting Plasmacytoid Dendritic Cells

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Background: Circulating platelet count, an indicator of health and disease, has been linked with platelet glycosylation. Loss of sialic acid during platelet circulation leads to their recognition by the hepatic receptors/lectins, specifically the Ashwell-Morell receptor and the macrophage αMβ2 integrin, prompting their clearance. The role of lectins and glycans in regulation of bone marrow (BM) thrombopoiesis is understudied.

Aims: We investigated the role for BM plasmacytoid dendritic cells, major producers of Type 1 interferon (IFN-I), in regulating thrombopoiesis of aberrantly sialylated megakaryocytes (MKs). The TF-antigen is a cryptic disaccharide on O-glycans usually covered by a sialic acid moiety added by the sialyltransferase ST3Gal1.

Methods: To investigate the role of the TF-antigen in thrombopoiesis, we generated ST3Gal1$^{MK\text{-}KO}$ mice (PF4-Cre) that display increased TF-antigen specifically in MKs.

Results: ST3Gal1$^{MK\text{-}KO}$ mice developed significant thrombocytopenia, but had normal platelet half-life, suggesting that the TF-antigen affected BM thrombopoiesis. *In vitro* MK maturation and proplatelet production from primary ST3Gal1$^{MK\text{-}KO}$ mouse BM cells were normal, pointing to extrinsic factors in the BM environment affecting thrombopoiesis. Immunofluorescence stain of the ST3Gal1$^{MK\text{-}KO}$ BM revealed proplatelet structures positive for GPIbα+ and the TF-antigen, being infiltrated by mononuclear cells resembling lymphocytes.

Platelet counts of ST3Gal1$^{MK\text{-}KO}$ mice were restored to wild-type levels by crossing ST3Gal1$^{MK\text{-}KO}$ mice with Jak3^{KO} mice that have impaired of lymphoid cell development. BM immunostaining showed infiltration of ST3Gal1$^{MK\text{-}KO}$ MKs by immune cells, marked by CD4+ (Fig.1). Bulk RNAseq of CD4+ cells in ST3Gal1$^{MK\text{-}KO}$ BM confirmed a population bias for interferon-releasing plasmacytoid dendritic cells, a cell type regulated by unique sialic acid binding lectins (Siglecs). Inhibition of IFN-I activity, by a blocking receptor antibody, recovered platelet counts in ST3Gal1$^{MK\text{-}KO}$ mice to wild-type levels.

Conclusions: Together, the data shows that recognition of MK O-glycan sialylation by plasmacytoid dendritic cells, likely through inhibitory Siglecs G and H, regulates thrombopoiesis through IFN-I secretion (Fig.2).

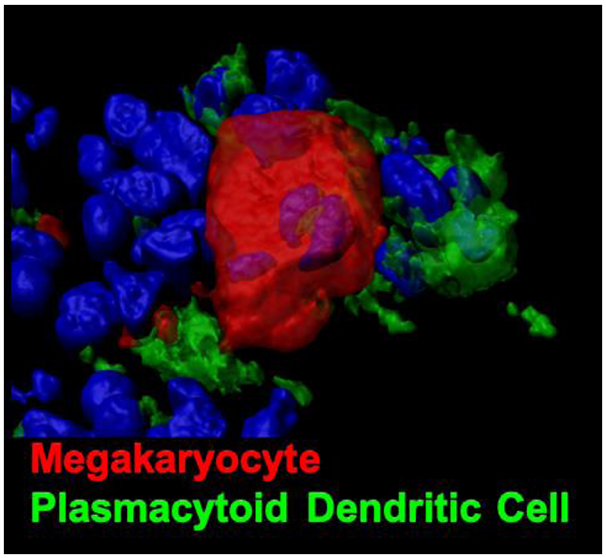

FIGURE 1 Infiltration of plasmacytoid dendritic cells on megakaryocytes in ST3Gal1MK-KO bone marrows

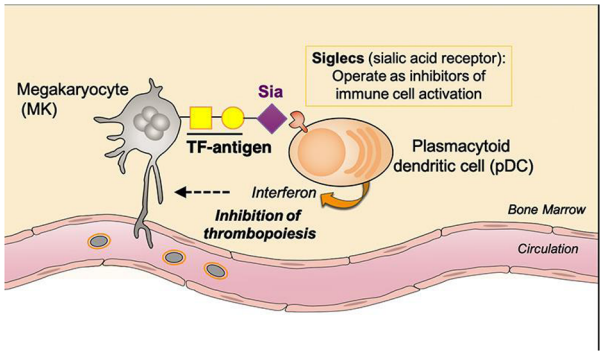

FIGURE 2 Plasmacytoid dendritic cells regulates thrombopoiesis through Siglecs

OC 20.5 | M6PR-specific Targeting of Lysosomal Enzymes Regulates Platelet Production

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Background: Blood platelets are produced in the bone marrow by megakaryocytes (MKs) in unique processes that require

polyploidization, extensive membrane rearrangements, and the formation of secretory granules, including α-granules, dense granules, and lysosomes. Lysosomal enzymes are targeted to the acidic late endosomal/pre-lysosomal compartment from the Golgi apparatus via the mannose 6-phosphate receptor (M6PR) pathway.

Aims: Ours aims were to determine the role of M6PR-specific targeting of lysosomal enzymes in platelet production and function.

Methods: Platelet and MK parameters were investigated in *M6pr*$^{-/-}$ mice lacking the 46-kDa M6PR, the physiological role of which is unclear.

Results: *M6pr*$^{-/-}$ mice had a severe bleeding diathesis, based on the tail bleeding time assay. However, adhesion of *M6pr*$^{-/-}$ platelets in whole blood to collagen was increased under high shear, compared to controls. *M6pr*$^{-/-}$ mice had increased blood platelet count with an abnormally elevated number of circulating proplatelets, appearing as thin and elongated platelet strands, compared to single discoid platelets in controls. *M6pr*$^{-/-}$platelets expressed major glycoproteins on their surface and von Willebrand factor and fibrinogen in their α-granules. Bone marrow hematopoietic stem and progenitor cell and MK counts appeared normal in *M6pr*$^{-/-}$ mice. However, transmission electron microscopy revealed the presence of an abnormal demarcation membrane system (DMS) in *M6pr*$^{-/-}$ bone marrow MKs with poorly defined platelet territories. *M6pr*$^{-/-}$MKs in culture developed abnormal microtubules and showed multilobulated nuclei surrounding a highly disorganized Golgi apparatus.

Conclusions: The data show that M6PR-specific targeting of lysosomal enzymes plays a critical role in the formation of the DMS in MKs, thereby regulating platelet production and function.

OC 27.1 | High Thymic Stromal Lymphopoietin (TSLP) Expression Promotes Platelet Activation and Thrombosis in Kawasaki Disease via Promoting Mitophagy

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Background: Kawasaki disease (KD) is the leading cause of childhood-acquired heart disease in developed countries. Although platelet activation and thrombosis are common in KD patients, the cause of platelet activation has not been previously explored.

Aims: The present study aims to identify the inflammatory cytokines that may play roles in initiating thrombosis following coronary aneurysms in KD patients; and explore whether platelet mitochondrial autophagy contributes to the pathogenesis of KD.

Methods: 37 plasma cytokines were assessed in patients with KD during acute phase and convalescence phase (n=156) as well as from healthy controls (n=50) by protein chip analysis. The expression of phosphatidylserine (PS), P-selectin and platelet mitochondrial membrane potential were examined by flow cytometry. The expression of mitochondrial autophagy protein LC-3B in platelets was measured by immunofluorescence assay, and altered platelet morphology was observed with scanning electron microscopy. The expression of LC-3B and other platelet autophagy-associated proteins as well as apoptosis-related proteins were also determined by western blot analysis.

Results: Among the 37 cytokines analyzed by protein chip analysis, we found that plasma levels of thymic stromal lymphopoietin (TSLP) were significantly higher (2.62 fold) in KD patients with thrombosis than those without thrombosis, which was confirmed by ELISA ($P = 0.0232$). Platelet activation, autophagy, and apoptosis were also increased in patients with thrombosis than those without ($P < 0.05$). Using recombinant TSLP, we found that TSLP induced platelet activation, enhanced thrombus formation and promoted platelet autophagy and apoptosis in vitro. Moreover, an autophagy inhibitor, 3-MA, diminished TSLP-induced thrombus formation.

Conclusions: Our results demonstrate that TSLP can induce platelet activation and apoptosis by the platelet mitochondrial autophagy signaling pathway. To the best of our knowledge, this is the first report to show that elevation of TSLP may play an important role in thrombosis in KD patients.

OC 27.2 | Characterisation of Platelet Microvesicles Containing Mitochondria and their Interaction with Neutrophils

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Background: Platelet activation causes cytoskeletal rearrangement which facilitates the release of microvesicles. Originally described as 'platelet dust', advances in technology have allowed the isolation and characterisation of these cell fragments. Recent research has detailed that microvesicles are a heterogeneous population with varying cargo.

Aims: Here we investigated the existence of a platelet microvesicle subpopulation containing mitochondria and their interaction with neutrophils.

Methods: Washed platelets were prepared by centrifugation (1000g, 10min) followed by resuspension in modified Tyrode's HEPES buffer and diluted to a concentration of 3×10^8/ml with 2mM $CaCl_2$. Neutrophils were isolated by centrifugation over histopaque 1077 (450g, 30min) following dextran separation.

Platelet microvesicles (PMVs) were produced from washed platelets (stained with MitoTracker Orange CMTMRos, 100nm) through incubation with TRAP-6 (37°C, 2hour). Image Stream^{x} MKII analysis was used to characterise PMVs. Agilent Seahorse was used to assess mitochondrial activity of PMVs. Neutrophils (3 x 10^5) were incubated with PMVs (30 x 10^6) for 60min and 120min (37°C, 200rpm). Samples were fixed, stained for LAMP3 and DAPI and imaged with confocal microscopy. Analysis was performed with Image J, Ideas 6.2 and GraphPad Prism.

Results: Image Stream^{X} MKII analysis of the entire PMV population demonstrated that 33±4% were P-selectin positive and 19±1% contained mitochondria (mitoPMVs). Focused characterisation of mitoPMVs indicated almost all expressed P-selectin (96±1%) (n=8). The mitochondria encapsulated within PMVs were functional, exhibiting a basal oxygen consumption rate of 30±3pmoles/min (n=4). Confocal microscopy showed that mitoPMVs interact with neutrophils in a time-dependent manner; individual neutrophils were associated with 11±2 mitoPMV at 60min and 33±6 at 120min (n=3, $p< 0.05$).

Conclusions: Platelet activation causes the production of PMVs that contain functional mitochondria and bear high levels of P-selectin. These mitoPMVs have the ability to interact with neutrophils. Further work is required to understand how this interaction is mediated and its significance in altering neutrophil phenotype.

OC 27.3 | Immune Complexes (ICs) Recruit Platelets in the Synovial Fluid (SF) of Rheumatoid Arthritis (RA) Patients

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Background: In RA ICs bind Fcγ receptors (FcγR) on inflammatory cells inducing their activation and thus eliciting inflammation. Activated platelets are found in SF of RA patients, however, the mechanism by which they are recruited and get activated in the arthritic joint has not been clarified. Human platelets express FcγRIIA and the binding of ICs induces activation. We previously showed that platelets migrate *in vitro* in response to chemotactic stimuli and *in vivo* into inflammed tissue.

Aims: To investigate whether ICs from RA SF can induce *in vitro* platelet migration and activation and to evaluate their role in the pathogenic activity of platelets in RA.

Methods: Platelet count and platelet P-selectin were measured by flow cytometry (FC) in knee joint-SF from 47 patients with RA and 49 with osteoarthritis (OA). ICs were isolated from SF by polyethylene glycol (PEG). In vitro platelet migration (transwell assay) and activation (P-selectin expression) in response to SF or to isolated ICs were assessed.

Results: SF from RA patients contained significantly more activated platelets compared to OA (OA:24.3±10.9 vs RA:160.6±33.7 platelets/μl, OA:9.5±2.5 vs RA:31.7±7.3% P-sel-positive platelets, all $p< 0.01$). No correlation was found between platelet number and levels of the red blood cell marker glycophorin A ($r=0.21$) in SF, excluding passive passage by blood contamination. RA-SF exerted a strong chemotactic (Chemotactic Index C.I.:3.7±0.4 vs OA:1.6±0.2, $p< 0.05$) and activating (Fold increase of P-sel-positive platelets:1.9±0.2 vs OA:1.1±0.1, $p< 0.05$) effect on platelets, both inhibited by FcγRIIA blockade. ICs isolated from RA SF induced strong *in vitro* platelet migration (C.I.:5.5±1.4) and activation (Fold increase of the % of platelet P-sel:1.5±0.1).

Conclusions: ICs present in SF recruit platelets in the joint space and activate them in RA. The blockade of platelet penetration and/or activation in SF by preventing ICs action on platelets may represent a new therapeutic target in RA.

OC 27.4 | R5421 is Not a Selective Inhibitor of Platelet Phosphatidylserine Exposure

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Background: A subset of activated platelets expose phosphatidylserine (PS) on their surface and become pro-coagulant. Platelet PS exposure requires activation of a scramblase protein, TMEM16F, making TMEM16F a potential therapeutic target.

Aims: R5421 has previously been described as a scramblase inhibitor but its molecular interaction with TMEM16F is unknown, as is the specificity of R5421 against other platelet functions.

Methods: Washed human platelets and erythrocytes were pre-incubated with R5421 and stimulated with A23187 or both thrombin and CRP-XL (GPVI agonist) to induce PS exposure which was measured using annexin-V binding by flow cytometry or a plate-based real-time luminescence assay. Platelet integrin activation (PAC1) and granule release (CD62P) were measured in response to TRAP-6 (PAR1 agonist) or CRP-XL. An *in-silico* ligand-based screen was conducted to find compounds which share physiochemical similarity to R5421 and these compounds were screened as above. R5421 and thiodicarb were blind docked *in-silico* into available TMEM16 structures using Autodock 4, Autodock Vina and GOLD software.

Results: R5421 inhibits platelet PS exposure in response to A23187 stimulation in both platelets and erythrocytes, suggesting a direct effect on the TMEM16F scramblase. However, R5421 also inhibits platelet integrin activation and granule release in response to TRAP-6 or CRP-XL, which is not rescued by co-stimulation with ADP or adrenaline. An *in-silico* ligand-based screen revealed the

pesticide, thiodicarb also inhibits platelet PS exposure, integrin activation and granule release, which may contribute to its reported toxicity.

Conclusions: R5421 inhibits platelet integrin activation and granule release in addition to inhibiting scramblase activity. *In-silico* ligand-based screening led us to identify thiodicarb as a novel scramblase inhibitor, though this also has off-target effects. Our *in-silico* docking studies predict residues in TMEM16F that may be key for inhibition by R5421 and thiodicarb and which may be useful for future development of more selective TMEM16F inhibitors.

OC 27.5 | Microthrombocytopenia Accompanied by Cytoskeletal Defects in Platelets Lacking the Small GTPase RhoB

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Background: Cytoskeletal rearrangements are critical for platelet production by megakaryocytes (MKs) as well as for proper platelet function. We have previously shown that mice lacking the small GTPase RhoA in megakaryocytes exhibit a macrothrombocytopenia and reduced platelet activation in response to agonists coupling to $G_{13}/G_{q.}$ In contrast, the role of the Rho subfamily member RhoB in MKs and platelets remained elusive.

Aims: We investigated the functional role of RhoB in platelets *in vitro* and *in vivo*.

Methods: Taking advantage of constitutive RhoB knock-out mice, platelet function and cytoskeletal rearrangements were assessed *in vitro* using biochemical, flow cytometric and microscopy-based assays. Platelet *in vivo* function was studied by assessing tail bleeding time and subjecting mice to models of arterial thrombosis.

Results: We have previously shown that RhoA deficient mice displayed an abnormal localization of MKs within the bone marrow (BM) sinusoids leading to the aforementioned macrothrombocytopenia. Interestingly, RhoB deficient mice displayed a microthrombocytopenia, but a normal MK localization in the BM, indicating a distinct role for RhoB in platelet biogenesis and function. RhoB-deficient platelets displayed a general activation defect for all tested agonists and reduced aggregate formation on collagen under flow. Although F-actin assembly was slightly impaired, platelets were able to fully spread on fibrinogen. Surprisingly, microtubule (MT) coils were reduced and aberrantly localized in spread RhoB-deficient platelets, as observed by superresolution microscopy (dSTORM). Moreover, knockout platelets were unable to reassemble MT coils after cold-induced MT disassembly and displayed decreased amounts of actetylated α-tubulin on protein level, thus indicating a more instable MT network. *In vivo*, RhoB deficiency had no effect on occlusive arterial thrombus formation but caused variable tail bleeding times.

Conclusions: Our results reveal non-redundant roles of RhoA and RhoB in platelet function and an unexpected essential function of RhoB in regulating platelet MT dynamics.

OC 34.1 | Platelet Transcription and Translation Is Altered in Sepsis and Associated with Mortality

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Background: Anucleate platelets are now recognized to mediate critical aspects of sepsis, including thrombo-inflammatory and immune responses. Thrombosis is a well-described consequence of severe sepsis and septic shock and the degree of organ failure may be linked to burden of microvascular thrombosis.

Aims: Whether sepsis can alter the platelet molecular signature and these changes, in turn, contribute to adverse outcomes in sepsis have never been studied.

Methods: We prospectively enrolled 94 septic patients and 37 matched healthy donors. We utilized parallel techniques of RNA-sequencing and ribosome footprint profiling to interrogate the platelet transcriptome and translatome. We further compared sepsis-induced changes in platelet gene expression between septic patients and a murine model of sepsis, cecal ligation and puncture (CLP).

Results: We identified 1,806 significantly differentially expressed (FDR< 0.05) transcripts in platelets from septic patients. Translational events in platelets during sepsis were also upregulated. Platelet transcriptional (rho=0.42, $p=3.2x10^{-285}$) and translational (rho=-0.65, $p=1.09x10^{-56}$) changes were significantly correlated between septic patients and CLP treated mice. Because microvascular thrombosis may play a role in pathophysiology of sepsis we focused on *ITGA2B* (encoding for aIIb), a well-known platelet integrin involved in platelet activation and aggregation. In our cohort of 94 septic patients, *ITGA2B* was significantly upregulated, validated by qT-PCR (~9-fold vs. healthy donors (n=37), p< 0.0001). Transcriptional changes in *ITGA2B* were accompanied by increased *de novo* protein synthesis of aIIb and integrin aIIbb3 activation. Increased *Itga2b* was identified in bone marrow megakaryocytes within 24 hours of sepsis onset, with subsequent

trafficking to platelets at 72 hours post-sepsis. Increased aIIb was associated with mortality in both humans and mice.

Conclusions: These findings provide evidence that human and murine sepsis similarly alters the platelet transcriptional and translational landscape. Moreover, *ITGA2B* is upregulated in sepsis due to trafficking from megakaryocytes, results in increased integrin activation, and correlates with adverse outcomes.

OC 34.2 | Single-cell RNA-seq Transcriptome Analysis of Native Bone Marrow Megakaryocytes Identifies Significant Inflammatory, Metabolic and Mitochondrial Signatures Associated with Platelet Hyperreactivity in Aging and MPN

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Background: Traditional transcriptome analysis of megakaryocytes (MKs) relies upon ex vivo culture and expansion of hematopoietic stem cells and pluripotent precursors under TPO-rich media. This approach has been widely used with significant success. However, it is unclear as to what extent the transcriptome landscape of ex vivo expanded MKs differs from their native state.

Aims: To perform scRNA-seq analysis of freshly isolated bone marrow MKs as a discovery tool to identify potential pathways responsible of aging-associated platelet hyperreactivity.

Methods: Native MKs from young (2 months) and old (>18 months) mice were analyzed using the 10X Genomics platform. Seven discrete clusters of megakaryocytes with significantly high expression of canonical MK transcripts were identified by our bioinformatics analysis. Platelet and mitochondrial studies were performed by FACS, microfluidics, metabolomics and Seahorse analysis.

Results: Bioinformatics analysis revealed significant differences in mitochondrial, metabolic and inflammatory pathways between MKs from young and old mice. Functional characterization of platelets from young and old mice corroborate these findings as platelets from old mice exhibit marked hyperreactivity, different metabolome; increased mitochondrial mass and oxygen consumption (Seahorse analysis) upon activation. Moreover, we found that in vivo exposure to TNF leads to the development of platelet hyperreactivity and mitochondrial changes. This was further corroborated using mice lacking or overexpressing TNF and with a neutralizing antibody in vivo. Finally, preliminary studies on platelets from patients with myeloproliferative neoplasms (MPN), an age-related hematological disorder with significant TNF-driven inflammation and platelet hyperreactivity (as observed in aging), exhibit a significantly similar platelet phenotype as the one found in old mice.

Conclusions: scRNA-seq analysis of freshly isolated megakaryocytes represents a novel approach to study the transcriptome of megakaryocytes. Using this technology we have identified significant age-associated inflammatory, metabolic and mitochondrial modulators of megakaryopoiesis and platelet hyperreactivity.

OC 34.3 | Platelets Regulate CD4+ T effector Memory Cell Responses via Platelet Factor 4-dependent Mitochondrial Biogenesis

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Background: Platelets can regulate immune responses of the total CD4+ T cells. However, it remains elusive how platelets regulate CD4+ T effector cell responses of individual CD4+ T cell subsets.

Aims: To study how platelets regulate effector cell responses of CD4+ T effector memory cells (Tem).

Methods: Human CD4+ Tem cells were stimulated with CD3/CD28 MAbs, and cultured without or with platelets during 7 days. CD4+ T effector cell phenotypes (Th1, Th2, Th17 and Treg) were assessed by flow cytometry.

Results: CD3/CD28 stimulation induced rapid and marked Th1 and Treg cell activation of CD4+ Tem cells. Platelet co-culture enhanced Th1 response transiently, while it persistently enhanced Treg cell activation of Tem cells, with the enhancement plateaued on day 3. Using a panel of neutralising reagents or inhibitors, PF4 was found the most prominent platelet-derived mediator for platelet-enhanced effector cell responses of CD4+ Tem cells, to which platelet-derived TGFβ, RANTES, TxA2 also contributed. PF4 exerted its effects primarily as a chemokine and via its receptor CXCR3, but not via its pro-coagulant activities, as evidenced by ineffectiveness of the thrombin inhibitor hirudin. PF4 elevated mitochondrial transcription factor (TFAM) synthesis, increased mitochondrial biogenesis, and subsequently enhanced Th1 and Treg responses. The observations were supported by the evidence showing that inhibition of mitochondrial function by rotenone counteracted the enhancements by recombinant PF4, and that TFAM overexpression by TFAM-adenovirus infection mimicked PF4 effects. Increased mitochondrial mass and metabolic production were linked to elevated expression of transcription factors,

such as FoxP3, and corresponding $CD4^+$ effector cell responses of Tem cells.

Conclusions: Platelets enhance CD4+ T effector cell responses of Tem cells through the mechanism of PF4-dependent mitochondrial biogenesis. Hence, PF4 may be a promising intervention target of platelet-regulated immune responses.

OC 34.4 | GPIb-vWF Interactions Promote Neutrophil Recruitment Resulting in Aggravated Liver Damage in the Acute Phase of Hepatic Ischemia-reperfusion Injury

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Background: Hepatic ischemia-reperfusion (I/R) injury is a major adverse consequence of liver transplantation, hemorrhagic shock or extensive hepatic surgery and associated with high mortality. The underlying pathomechanisms are incompletely understood and treatment options are limited. The recruitment of leukocytes and subsequent parenchymal injury are hallmarks of liver I/R. In other models of I/R injury, such as ischemic stroke, a contribution of platelets and immune cells has been demonstrated.

Aims: We aimed to assess the contribution of platelets to the acute phase of liver inflammation following I/R injury.

Methods: A mouse model of partial liver ischemia was used, where the blood circulation of 70% of the liver was interrupted for 30 min to trigger sterile inflammation. In the following 3 h of reperfusion the effects of platelet depletion, receptor blockade or deficiencies were assessed with confocal intravital microscopy, in combination with classical histology, flow cytometric analyses and ELISA measurements to determine the immune cell infiltration and liver damage.

Results: Platelet depletion resulted in reduced infiltration of neutrophils and decreased liver damage compared to control mice. GPIIb/IIIa blockade (using JON/A) did not affect leukocyte recruitment or hepatic damage. In contrast, blockade of the GPIbα-von Willebrand factor (vWF) interaction (using pop/B) reduced liver damage following hepatic I/R. This was accompanied by a reduced neutrophil recruitment, while T cell recruitment was not affected. These findings were confirmed in GPIb-IL4R transgenic mice, that lack the extracellular domain of GPIbα, and vWF-deficient animals. Time course analyses revealed a bimodal leukocyte recruitment, with an initial platelet-independent recruitment of neutrophils, and a second phase of neutrophil infiltration that depends on platelet GPIbα-vWF interactions.

Conclusions: Our data suggest, that platelet GPIbα-vWF interactions are a critical factor promoting sustained neutrophil recruitment following I/R, thereby contributing to hepatic damage.

OC 34.5 | Novel Platelet-neutrophil Interaction between Activated αIIbβ3 and SLC44A2 Mediates NETosis under Flow

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Background: Platelet-leukocyte interactions are important in diverse pathophysiological settings from infection to DVT. Previously characterised interactions require full platelet activation (e.g. via P-selectin, CD40L) and/or leukocyte activation (e.g. via Mac-1). However, platelets captured by von Willebrand factor (VWF) under flow also bind leukocytes. We hypothesized that, under flow, GpIbα attachment to VWF A1 domain 'primes' platelets, leading to novel platelet-leukocyte interactions.

Aims: To characterise the interaction between VWF-'primed' platelets and leukocytes under flow.

Methods: VWF, the isolated A1 domain or purified activated $\alpha_{IIb}\beta_3$ were captured onto microchannels. Labelled leukocytes and platelets from whole or plasma-free blood were perfused at defined shear rates ($50s^{-1}$ to $1000s^{-1}$) and recorded in real-time.

Results: Binding of GPIbα to VWF A1 under flow 'primed' platelets causing intracellular Ca^{2+} release and $\alpha_{IIb}\beta_3$ activation. VWF-'primed' platelets captured neutrophils and T cells (but not monocytes and B cells) under low shear. Leukocytes preferentially attached in regions of turbulent flow. Leukocyte binding to 'primed' platelets was independent of P-selectin, but significantly reduced by $\alpha_{IIb}\beta_3$ blockade. Channels coated with activated $\alpha_{IIb}\beta_3$ captured neutrophils under low shear, leading to phenotypic changes, Ca^{2+} release and formation of neutrophil extracellular traps (NETs). NETosis was dependent on shear, intracellular Ca^{2+} release and the NADPH-oxidase pathway. Neutrophil binding to activated $\alpha_{IIb}\beta_3$ was inhibited by blockade of the SLC44A2 receptor. SLC44A2-transfected HEK293T cells bound VWF-'primed' platelets and activated $\alpha_{IIb}\beta_3$, in a manner that could be blocked by SLC44A2 or $\alpha_{IIb}\beta_3$ inhibition.

Conclusions: GPIbα binding to VWF under flow 'primes' (but does not activate) platelets, leading to $\alpha_{IIb}\beta_3$ activation. For the first time, we show that activated $\alpha_{IIb}\beta_3$ directly binds neutrophils via SLC44A2, triggering NETosis under flow. A common SNP in *SLC44A2* recently identified as protective of DVT might be linked to a disruption in this novel interaction. Further work to establish the pathophysiological implications of these findings will also be presented.

OC 43.1 | Fibrin Exposure Increases Platelet ADAM10 Activity in a Calcium-dependent Manner

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Background: Platelet collagen receptor glycoprotein (GP) VI orchestrates platelet signalling, aggregation and A-Disintegrin-And-Metalloproteinase (ADAM) 10-mediated shedding of GPVI. GPVI is shed (sGPVI) in septic patients with minimal collagen exposure. Fibrin/GPVI interactions induce sGPVI release with no signalling requirement. How fibrin enhances platelet activity remains elusive.

Aims: To evaluate fibrin-induced ADAM10 activity and GPVI shedding.

Methods: Platelet aggregate formation and ADAM10 activity using a fluorescence resonance energy transfer assay were evaluated in fibrin-exposed washed platelets; sGPVI release was measured by ELISA. Recombinant tissue-inhibitor-of-metalloproteinases (rTIMPs, 1 µg/mL), 1-10 µM cytochalasin D and 50 µM BAPTA were used to evaluate specific modulatory roles for TIMPs, actin rearrangement and calcium flux, respectively. The effect of charged heparin/chondroitin sulfate (CS) and anti-GPVI monoclonal antibody 1G5-Fab on fibrin-induced ADAM10 activity were also evaluated.

Results: Fibrin-mediated αIIbβ3-independent platelet agglutination required fibrin polymerisation, calcium flux and was reduced by 5 U/mL heparin pre-treatment. Fibrin increased resting ADAM10 activity (p=0.002, n=17), which was inhibited by BAPTA treatment (p=0.0014), or metalloproteinase inhibitors GM6001 (250 µM; p=0.0001, n=8) and GI254023X (2 µM; p=0.0173, n=6) but not by inhibition of actin rearrangement. CS gave a trend to reduce fibrin-mediated activity (19% reduction, n=4), however heparin (5-10 U/mL) reduced fibrin-mediated ADAM10 activity dose dependently and reduced GPVI shedding (p=0.035, n=3). rTIMPs 1-4 did not affect fibrin-induced platelet agglutination, however rTIMP2 reduced fibrin-induced ADAM10 activity (19% reduction), which varied from rTIMP3 reduction of collagen/CRP-mediated ADAM10 activity (67.2% and 54.5% reduction respectively, n=3). 1G5-Fab fragment reduced collagen/CRP-mediated but not fibrin-mediated ADAM10 activity, suggesting fibrin and collagen/CRP binding sites within GPVI differ.

Conclusions: Platelet fibrin exposure increased ADAM10 activity, with blockade only achieved using reagents that directly disrupted fibrin/GPVI interactions or by metalloproteinase inhibitors. These findings highlight differences between collagen- and fibrin-mediated ADAM10 activity and GPVI shedding and help explain elevated sGPVI levels in patients where collagen exposure is limited.

OC 43.2 | GPVI Sustains Platelet Activation to Maintain the Integrity and Stability of a Growing Thrombus

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Background: The rupture of an atherosclerotic plaque forms a mural thrombus which undergoes elevated shear forces. How mural thrombi resist to high mechanical stress and maintain their integrity is unclear.

Aims: We evaluated whether GPVI-induced platelet activation helps the thrombus to resist to mechanical stress during its build-up.

Methods: Computanional fluid-structure interaction analysis was used to finely characterize the mechanical stress exerted on mural thrombi. The importance of GPVI-mediated platelet activation was evaluated in:

1. A numerical model developed for this purpose;
2. A blood flow-assay in which specific anti-GPVI agents were used.

Results: Computational analysis showed that mural thrombi undergo sharp increases in mechanical stress during their growth. A numerical model proposed a key role for GPVI during thrombus build-up, since its blockade results in disaggregation. This was confirmed by real-time video-microscopy, which showed that GPVI blockade with two distinct anti-GPVI Fabs, promotes efficient disaggregation of human thrombi pre-formed on collagen or on human atherosclerotic plaque material. Disaggregation occurring when GPVI is blocked took place under arterial, but not venous flow and increased with shear. This effect is linked to the ability of the anti-GPVI agents to block ligand binding to GPVI, as a third non-blocking anti-GPVI Fab, had no effect. Video-microscopy and nanoscale imaging showed that GPVI blockade reduced thrombus contraction, suggesting an impairment platelet activation. This was confirmed by a marked reduction in disaggregation when thrombi were pre-activated with TRAP or ADP. In agreement, inhibition of 4 key GPVI signalling molecules resulted in a similar kinetic of thrombus disaggregation compared to anti-GPVI Fabs, further indicating that impairing GPVI-mediated platelet activation results in thrombus disaggregation.

Conclusions: This study proposes a key role for GPVI in maintaining the stability of a growing thrombus and suggests that targeting GPVI could induce thrombus disaggregation, underscoring its potential as an anti-thrombotic target.

OC 43.3 | The Interaction of Monomeric and Dimeric GPVI with Variants of Fibrin and Fibrinogen

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Background: Platelet glycoprotein VI (GPVI) initiates thrombus formation via its interaction with collagen. Recent studies have identified fibrin(ogen) as ligand for GPVI, and shown that this plays a role in thrombus growth and stabilization. However, there is controversy as to whether fibrin and fibrinogen bind to monomeric or dimeric GPVI.

Aims: To examine the binding of monomeric/dimeric GPVI to different fibrin(ogen) preparations.

Methods: Recombinant human fibrin(ogen), a novel fibrinogen variant that does not polymerize due to mutations in knobs “a” and “b” (AaR19S/BbR17S), and plasma-purified fibrinogen were used to generate fibrin by the addition of thrombin and calcium. Binding of immobilized fibrins and fibrinogens to GPVI was investigated by ELISA and surface plasmon resonance (SPR). Binding affinity of plasma-purified fibrinogen to GPVI in solution was also measured by microscale thermophoresis (MST). GPVI constructs (D1,D2 monomer and D1,D2-Fc dimer) were expressed in hek293T-cells and secreted protein was purified in-house.

Results: Immobilized plasma-purified and recombinant fibrinogens showed similar binding to GPVI-monomer/dimer by ELISA. Following conversion to fibrin, the binding remained strong and comparable between variants. SPR showed a high-affinity binding profile for the different immobilized fibrinogen forms to GPVI-dimer (K_D 3.4±1.1nM), however, to a much greater than 1:1 stoichiometric response, suggesting multiple GPVI-dimer binding sites acting in a cooperative manner. GPVI-monomer demonstrated low-affinity binding to all forms of fibrinogen (K_D 1.0±0.3μM) with fast on and off rates. Once again, binding of both forms of GPVI remained comparable between all fibrin forms. MST experiments showed that in solution GPVI-dimer bound to plasma-purified fibrinogen (K_D 90.1±14.0nM) at multiple binding sites (Hill cooperativity coefficient >1).

Conclusions: GPVI binds to immobilized fibrinogen and fibrinogen in solution, with higher affinity observed for the dimer vs monomer. Similarities in the binding profiles of different fibrin variants to GPVI, including the non-polymerizing variant, suggest that polymerization is not necessary to elicit GPVI binding.

OC 43.4 | Dimeric form of Platelet Glycoprotein VI Binds to Fibrin Fibers in Fibrin Clots

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Background: The major platelet collagen-receptor GPVI has recently been found to have a separate role as a fibrin receptor. However, whether monomeric or dimeric GPVI functions as the fibrin receptor remains controversial; this may because immobilized fibrin(ogen) has typically been employed to determine GPVI binding.

Aims: Since the physiological large fibers of fibrin only exist in fibrin clots, we analyzed GPVI binding to fibrin clots.

Methods: A mixture of recombinant GPVI-Fc_2 (dimer) or GPVI-His Monomer produced at advanceCOR (Germany), fibrinogen, and control protein was clotted by adding thrombin. Fibrin-fiber--bound GPVI was quantitated by immunoblotting. Clots formed in presence of platelets were analyzed for platelet-derived components bound to fibrin fibers.

Results: GPVI-Fc_2 dose-dependently and saturably bound to fibrin clots (K_d=1.19mM, n=3); crosslinking clots increased its affinity (K_d = 0.508mM, n=4). Binding was inhibited by anti-GPVI-dimer (mFab-F). Control proteins (albumin,Fc fragment) didn't bind significantly to clots. GPVI-His Monomer binding to clots was not saturable and nearly linear. Analyses of clots formed in the presence of platelets show that the main platelet protein bound was integrin aIIbβ3, with small amounts of GPVI. Confocal microscopy verified that platelets bound to fibrin fibers in the clots.

Conclusions: GPVI-Fc_2 binds to fibrin clots showing a typical receptor-ligand binding curve, with K_d similar to those obtained for binding to D-fragment and D-dimer. This suggests that fibrin(ogen)'s GPVI binding site becomes exposed after fibrin formation or cleavage to fragment D. Reported low levels of binding to immobilized fibrin(ogen) would be attributable to partial exposure of the binding site as a consequence of immobilization. However, GPVI-His binding did not fulfill the classical criteria as a receptor. Our results suggest that GPVI has a physiological function in fibrin clots where platelets are trapped inside. The main platelet glycoprotein bound to the fibrin clot is aIIbβ3, with lower levels of GPVI also present.

OC 43.5 | Cancer Cell-derived Immunoglobulin G Activates Platelet via Binding Platelet FcγRIIa

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Background: Tumor-associated thrombosis is the second leading risk factor for cancer patient death, and platelets activity is abnormal in cancer patients. Discovering the mechanism of platelet activation and providing effective targets for therapy are urgently needed. Cancer cell derived IgG has been reported to regulate development of tumors. However, studies on the functions of cancer cell derived IgG are quite limited.

Aims: We investigated the potential role of cancer cell derived IgG in platelet activation.

Methods: We detected the expression of CD62P on platelets by Flow Cytometry and analyzed platelet function by platelets aggregation and ATP release. The content of IgG in cancer cell supernatants was detected by Enzyme-linked immune sorbent assay. The distribution of cancer-derived IgG in cancer cells was analyzed by Immunofluorescence assay. Western blot was performed to quantify the relative expression of FcγRIIa, syk, PLCγ2. The interaction between cancer-derived IgG and platelet FcγRIIa was analyzed by Co-immunoprecipitation.

Results: Higher levels of CD62P were observed in cancer patients' platelets compared with that of healthy volunteers. Cancer cell culture supernatants increased platelet CD62P and PAC-1 expression, sensitive platelet aggregation and ATP release in response to agonists, while blocking FcγRIIa or knocking down IgG reduced the activation of platelets. Coimmunoprecipitation results showed that cancer cell-derived IgG interacted directly with platelet FcγRIIa. In addition, platelet FcγRIIa was highly expressed in liver cancer patients.

Conclusions: Cancer cell-derived IgG interacted directly with FcγRIIa and activated platelets; targeting this interaction may be an approach to prevent and treat tumor-associated thrombosis.

OC 58.1 | Extensive Tyrosine Phosphoproteomic Analysis of the CLEC-2 Signalling Cascade in Human Platelets: New Players on the Team

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Background: C-type lectin-like receptor 2 (CLEC-2) is platelet receptor that plays a crucial role in different physiological and pathological processes. CLEC-2 signals through a tyrosine-kinase-mediated pathway that seems to be highly dependent on the positive feedback exerted by ADP and TXA_2 released upon platelet activation.

Aims: The aim was to analyse the tyrosine phosphoproteome of platelets activated with the CLEC-2 agonist rhodocytin in order to identify relevant p-Tyr sites and proteins involved in platelet activation downstream of this receptor.

Methods: We performed a phosphotyrosine proteomic analysis of platelets stimulated through CLEC-2 receptor in presence and absence of secondary mediators' inhibitors, in comparison with resting state. After protein digestion, phosphotyrosine peptides were enriched by immunoaffinity and identified by mass spectrometry.

Results: We report 363 differentially phosphorylated p-Tyr sites, corresponding to almost all the proteins previously known to participate in CLEC-2 signalling and, interestingly, to relevant signalling proteins that were not previously reported in the CLEC-2 cascade, such as the adaptors Gab1, DAPP1, Dok1, Dok3, CASS4, EFS, Pag1; the kinases FAK1, FES, and FGR, and the membrane proteins G6F, JAM-A, PECAM-1, and TLT-1. Indeed, we evaluated the ADP and TXA_2 relevance in phosphorylating tyrosine sites that are crucial for the enzymatic activity of three key proteins of the CLEC-2 signalling cascade: Syk, Src and PLCγ2. We found that Syk is powerfully phosphorylated at $Tyr^{525+526}$ upon CLEC-2 activation with the positive feedback of secondary mediators, but it also showed a significant up-phosphorylation when those are inhibited. This is not the case for Src phosphorylation at Tyr^{419} and PLCγ2 phosphorylation at Tyr^{759}, for which no significant up-phosphorylation was observed upon CLEC-2 stimulation when secondary mediators are inhibited.

Conclusions: This is the most extensive tyrosine phosphoproteomic analysis of platelets to date, and the obtained data leads to an updated and more complete view of the CLEC-2 signalling pathway in platelets.

OC 58.2 | TMT-based Temporal Quantitative Phosphoproteome of Human Platelets in Response to Collagen

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Background: At sites of injured vessel, exposure of collagen triggers platelet adhesion, activation and aggregation via multiple platelet receptors and subsequent signaling cascades.

Aims: Elucidation of collagen-induced temporal phosphoproteome in human platelets.

Methods: Washed human platelets from 3 independent biological replicates were stimulated with 15 $\mu g.ml^{-1}$ equine type I collagen for 0, 30 and 60 seconds, global proteome and changes of serine/threonine and tyrosine phosphorylation were studied using tandem mass tags (TMT)-based quantitative proteomics analysis. Subsequently, the data were searched by MaxQuant (v.1.5.2.8) against the Swiss-Prot human database combined with

common contaminants concatenated with reverse decoy database. Bioinformatic analysis of the data was further performed to annotate the proteins.

Results: In total we identified 6985 phosphorylation sites from 1952 proteins across three biological replicates, among them 3648 phosphorylation sites from 1015 proteins were quantifiable. Phosphorylation levels of 150 sites/110 proteins were increased (>1.5-fold up) and 4 sites/3 proteins were decreased (>1.5-fold down) after 30 seconds of collagen stimulation, whereas 807 sites/403 proteins and 61 sites/39 proteins were increased and decreased, respectively, after 60 seconds. These covered many platelet functions, including 53 protein kinases (e.g. SYK, DAPK3, STK10, TEC, BTK, CAMK1) with increased and 7 protein kinases (e.g. KALRN, ILK, KSR1, MAP3K5) with reduced levels. Besides, these proteins are involved in many biological signaling pathways. The most relevant 10 signaling pathways are: regulation of cellular component movement, movement of cell or subcellular component, cell adhesion, biological adhesion, enzyme linked receptor protein signaling pathway, regulation of actin filament-based process, transmembrane receptor protein tyrosine kinase signaling, cell-cell adhesion, cell migration and platelet activation.

Conclusions: Our TMT-based quantitative proteomics analysis provides new insights into collagen-induced platelet temporal phosphorylation. A remarkable number of phosphorylation sites and changes have been observed for the first time. The function of these sites require further validation and study.

OC 58.3 | Platelet mRNA Splicing Landscape and its Effect on Platelet Activity: An ACCOuNT Consortium Study

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Background: Cardiovascular disease (CVD) is responsible for >630,000 deaths in the U.S. every year and is the leading cause of death both in the U.S. and worldwide. Aberrant platelet activity has been linked to an increased risk of CVD, including thrombosis, arteriosclerosis, stroke, heart attack and pulmonary embolism. Differences in platelet activity have been linked to polymorphisms in platelet-related genes, but little research has investigated the role of differential alternative splicing on platelet activity.

Aims: This study will characterize the splicing landscape in African American (AA) and European American (EA) platelets, and examine the functional consequences of differential mRNA splicing events occurring in platelets between the two populations.

Methods: Platelet RNA-Seq data from public repositories (i.e. Gene Expression Omnibus, N = 55) and diverse cohort of healthy volunteers (N = 25 of both EA and AA) were analyzed to catalog mRNA splicing events. Analysis of public RNA-Seq data from healthy volunteers identified 7,293 expressed genes with an average of 5 mRNA splice variants per expressed gene.

Results: Clusters of unique splicing patterns could be identified in small subsets of healthy volunteers. For example, isolated mRNA splicing patterns for the ARRB1, GP6, ITGB3 and TBXAS1 genes were identified in $\leq$ 5% of healthy volunteers. These unique splicing events portend protein isoforms leading to aberrant platelet activity. The functional consequences of these isolated splicing events are currently under investigation. In addition, a comparison of the splicing landscape in platelets from EA or AA was initiated to identify population specific splice variants. Preliminary analysis of the RNA-Seq data identified 225 genes exhibiting differential RNA splicing between the two populations.

Conclusions: This study reveals that the mRNA splicing landscape varies between individuals, and these differences may affect platelet function and serve as biomarkers to identify CVD risk.

OC 58.4 | Hepatic Thrombopoietin Gene Silencing Induces Platelet Count Reduction within the Hemostatic Range and Reduces Breast Cancer Progression in MMTV-PyMT Mice

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Background: Activated platelets accumulate in some cancers and may contribute to cancer progression by promoting cell proliferation and metastasis. However, platelet inhibitors cause bleeding and have not been clinically evaluated for efficacy in malignancies. We hypothesize that moderate platelet count reduction should reduce platelet activity without increasing the risk of bleeding, and could be used to evaluate the role of platelets in cancer.

Aims: Reduce platelet count within the hemostatic range by selective targeting of hepatic thrombopoietin gene expression using an antisense oligonucleotide (TPO-ASO) and investigate its effect on the development and progression of spontaneous metastatic breast cancer in mice.

Methods: Murine TPO-ASO was synthesized and evaluated for antiplatelet efficacy in mice. In dose finding studies, wild-type FVB/n female mice were administrated TPO-ASO weekly for 70 days and platelet count and bone marrow megakaryocyte density

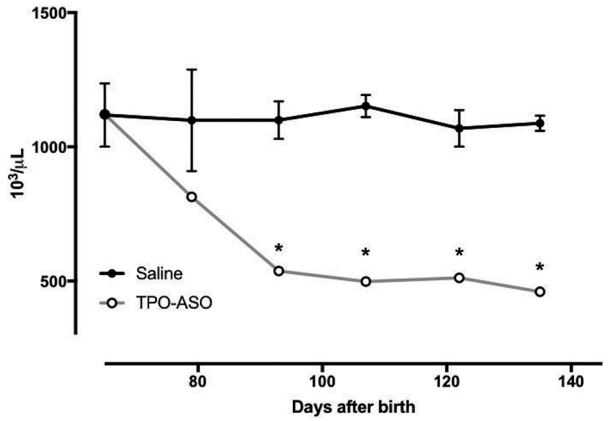

FIGURE 1 Platelet count over time in wild-type FVB/n mice

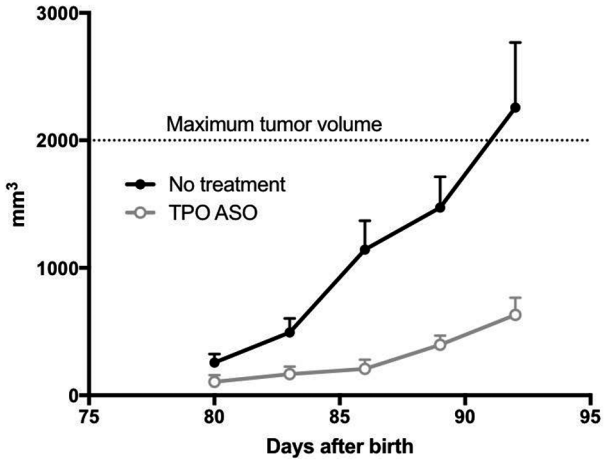

FIGURE 2 Tumor growth in MMTV-PyMT mice

were monitored. Plasma TPO levels were measured using ELISA. Subsequently, transgenic MMTV-PyMT mice were treated with TPO-ASO from 40 days of age, and then monitored for mammary gland tumor development and growth, and time to euthanasia at reaching combined tumor volume of 2 cm^3. Pulmonary metastases, deposition of platelets and macrophages in the tumors, and tumor vessel and Ki-67 positive cell density were analyzed by histology.

Results: Platelet count reduction plateaued at 4 weeks of TPO-ASO treatment and halved platelet count (Fig 1), plasma TPO levels, and megakaryocyte density. TPO-ASO suppressed tumor growth (Fig 2), inhibited spontaneous pulmonary metastasis, extended time to euthanasia, reduced intra-tumoral platelet deposition and vessel density, tumor-infiltrating macrophages, and proliferating (Ki-67 positive) cells in the mammary tumors.

Conclusions: Hepatic TPO gene silencing reduces platelet count within the hemostatic range and inhibits spontaneous breast cancer development and metastases in mice, raising the possibility that using a liver-specific TPO-ASO could represent a reasonably safe approach to evaluation of antiplatelet therapy in cancer

OC 58.5 | Novel Single Cell Mechanobiology Approach Reveals an Intermediate State of Integrin αIIbβ3 that Mediates Platelet Aggregation under Blood Flow Disturbance

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Background: Platelets are extremely sensitive to shear forces in disturbed blood flow induced by atherosclerotic plaques, vessel stenosis or medical device intervention. The resulting occlusive thrombosis leads to cardiovascular disease or medical device failure.

Aims: Understanding how force stimulates platelet aggregation has direct clinical relevance, since the prothrombotic effects of disturbed blood flow are not eliminated by conventional antiplatelet agents like aspirin and clopidogrel. However, whether the αIIbβ3 integrins are activated similarly or differently by biomechanical and biochemical (agonists, e.g., ADP, thrombin, thromboxane) stimuli is still elusive. Hereby, we developed a series of Nano-Mehcanobiology approaches to examine the molecular mechanisms underlying shear-induced thrombosis.

Methods: Combining a stenosed microfluidic device with high-resolution live-platelet imaging, we found that biomechanical thrombosis in blood flow disturbance is mainly supported by platelet integrin αIIbβ3 with an extended-closed headpiece conformation. To characterise this novel integrin biomechanical activation pathway, we developed a dual biomembrane force probe to visualise single integrin dynamics on a living platelet.

Results: We demonstrated that these integrins have an affinity and bond lifetime for ligands that are intermediate between those well-recognized inactive and active states. This intermediate state is inducted by mechano-signaling of GPIb via a pathway distinct from that of chemical signaling of ADP receptors. This inside-out mechano-signaling potentiates the outside-in mechano-signaling of αIIbβ3, promoting its transition from the intermediate to the active state, resulting in mechanical affinity maturation. Our work mapped the landscape of integrin αIIbβ3 activation on single live platelets, and elucidated different requirements of αIIbβ3 upregulation in the development of biomechanical and biochemical thrombi.

Conclusions: Furthermore, our work extends the fundamental knowledge on integrin structure-function relationship with identification of a semi-stable intermediate integrin state and suggests targeting this intermediate integrin activation pathway as a perspective anti-thrombotic strategy. This platelet mechanobiology study has recently been accepted by *Nature Materials*.

OC 59.1 | Shear Rate Gradients Promote a Bi-phasic Thrombus Formation in a Vwf-dependent Manner

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Background: Blood flow profoundly varies throughout the vascular tree due to its pulsatile nature and to the complex vessel geometry. While thrombus formation has been extensively studied *in vitro* under steady flow, and *in vivo* under normal blood flow conditions, the impact of complex hemodynamics such as flow acceleration found in stenosed arteries has gained increased appreciation.

Aims: We investigated the effect of flow acceleration, characterized by shear rate gradients, on the function of platelets adhering to weak adhesive surfaces such as fibrinogen.

Methods: The effect of shear rate gradients on plasma VWF and on platelet function was evaluated:

i) *in silico*, in numerical models;
ii) *in vitro*, by perfusing hirudinated human blood on immobilized fibrinogen in original microfluidic flow devices presenting restrictions to mimic vessel stenosis;
iii) *in vivo*, in experimental thrombosis models in which the injured mouse carotid artery was severely stenosed with a micromanipulator.

Results: While we confirmed that under steady flow, fibrinogen is a weak adhesive protein, we observed that under shear rate gradients, this surface becomes highly thrombogenic, supporting efficient platelet adhesion, activation and aggregation and leading to occlusive thrombus formation. Interestingly, this phenomenon is general as it occurs on other weak adhesive matrices including laminins and thrombospondin-1. This shear rate gradient-driven thrombosis is bi-phasic with an initial step of slow, weak and heterogenous platelet recruitment supported by direct plasma VWF adsorption to immobilized fibrinogen and followed by a second phase of explosive thrombosis initiated by VWF fiber formation on platelet monolayers. *In vivo* experiments confirmed that shear rate gradients accelerate thrombosis in a VWF-dependent manner.

Conclusions: This study reports that shear rate gradients found in pathological settings, such around evolved atherosclerotic plaques, promotes a very rapid and bi-phasic thrombosis, which can be initiated on weak adhesive surfaces, relies on plasma VWF and can result in vessel occlusion.

OC 59.2 | Characterization of a Novel *GPIbα* Knockin Mouse that Lacks the Last 24 Amino Acids of its Intracellular Domain

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Background: The platelet GPIbα-VWF A1 domain interaction is essential for platelet tethering under high shear conditions. Under flow, VWF binding to GPIb-IX-V complex induces intracellular calcium fluxes and activation of tyrosine kinases that culminate in the activation of $\alpha_{IIb}\beta_3$. We recently demonstrated that human platelets are 'primed' (but not activated) following binding to VWF under flow and are able to recruit neutrophils via activated $\alpha_{IIb}\beta_3$.

Aims: To evaluate the (patho)physiological relevance of VWF/flow-dependent signalling through GPIbα.

Methods: *GPIba*$^{\Delta sig/\Delta sig}$ mice, in which the last 24 amino acids of the intracellular tail were deleted, were generated via CRISPR-Cas9 technology. Platelet function was assessed *ex vivo* by flow cytometry, platelet aggregometry, immunostaining, microfluidic assays, and *in vivo* by tail bleeding and thrombosis models.

Results: *GPIba*$^{\Delta sig/\Delta sig}$ mice exhibited mildly reduced platelet count (~80%) and slightly enlarged (~140%) platelets compared to *GPIba*$^{+/+}$ littermates. Activation of *GPIba*$^{\Delta sig/\Delta sig}$ platelets with ADP and thrombin was unaffected; *GPIbα*$^{\Delta sig/\Delta sig}$ platelets stimulated with collagen-related-peptide (CRP) exhibited markedly decreased P-selectin exposure and $\alpha_{IIb}\beta_3$ activation. Consistent with this, reduced platelet spreading was observed on CRP, but not on fibrinogen. *GPIba*$^{\Delta sig/\Delta sig}$ platelet tethering/aggregation was slightly reduced on VWF- and collagen-coated microchannels. Finally, *GPIba*$^{\Delta sig/\Delta sig}$ mice exhibited normal haemostasis and normal platelet and fibrin accumulation in the laser-induced thrombosis model.

Conclusions: We have generated a novel transgenic mouse with truncated GPIbα cytoplasmic tail. Although the primary filamin binding site has been preserved, *GPIba*$^{\Delta sig/\Delta sig}$ mice have slightly lower platelet counts and modestly enlarged platelets. The defect observed in *GPIba*$^{\Delta sig/\Delta sig}$ platelets after CRP stimulation corroborate *in vitro* data suggesting that the intracellular tail of GPIbα (and its associated signalling machinery) is important for GPVI-dependent signalling. Data from flow assays to monitor platelet priming and neutrophil recruitment under flow, and *in vivo* thrombosis models are underway to characterize the physiological and pathophysiological role of the GPIbα-A1 signalling.

OC 59.3 | Role of β-arrestin2 in Regulation of Platelet Activation and Thrombus Formation through GPCR Desensitization

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Background: The arrestins are a class of soluble proteins that function in concert with the GPCR kinases to stop or arrest GPCR-mediated intracellular signaling. Although β-arrestins function in the desensitization of most GPCRs, the functional differences of β-arrestin1 versus β-arrestin2 in the regulation of GPCR signaling in platelets and the mechanisms of desensitization of platelets to GPCR agonists by β-arrestins have not been understood.
Aims: To identify the role of β-arrestin2 in the regulation of platelet function and GPCR signaling in platelets.
Methods: We used mice lacking β-arrestin1 and β-arrestin2 to evaluate the functional role of β-arrestin2 in platelet activation.
Results: Platelet aggregation, dense-granule secretion, and fibrinogen receptor activation induced by 2-MeSADP, U46619, thrombin, and AYPGKF were significantly potentiated in β-arrestin2 -/- platelets compared to WT platelets. In contrast to β-arrestin2 -/- platelets, AYPGKF-, thrombin-, and U46619-induced platelet aggregation and secretion were significantly inhibited in β-arrestin1 -/- platelets compared to WT platelets, while 2-MeSADP-induced platelet aggregation and secretion were minimally affected in β-arrestin1 -/- platelets. However, CRP-induced platelet aggregation and secretion were not affected in both β-arrestin1 -/- and β-arrestin2 -/- platelets. These results suggest that β-arrestin2 functions as the predominant regulator in the termination of GPCR signaling in platelets. In addition, platelet aggregation was restored in response to second challenge of 2-MeSADP and AYPGKF in β-arrestin2 -/- platelets whereas re-stimulation of agonist failed to induce aggregation in WT and β-arrestin1 -/- platelets, indicating β-arrestin2 contributes to $P2Y_1$, $P2Y_{12}$, and PAR receptor desensitization. Furthermore, 2MeSADP- and AYPGKF-induced Akt and ERK phosphorylations were significantly potentiated in β-arrestin2 -/- platelets. Finally, β-arrestin2 -/- mice show an enhanced and stable thrombus formation after $FeCl_3$ injury to the carotid artery, indicating β-arrestin2 is critical for thrombus formation *in vivo*.
Conclusions: β-arrestin2 plays an important role in regulation of platelet function through selective GPCR desensitization.

OC 59.4 | Decoding $G\alpha_q$ Signaling in Platelets

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Background: Most platelet agonists work through G protein-coupled receptors (GPCRs). Of the heterotrimeric G proteins expressed in platelets, G_q has been shown to be especially important, triggering the increase in cytosolic Ca^{++} needed for a full response to most platelet agonists. However, much remains to be learned about the mechanisms that modulate this critical event in order to achieve an optimal platelet response to injury.
Aims: Our aims are to decode G_q signaling in platelets, to investigate G_q effectors and how they can be regulated, and to understand their implications in platelet activation and thrombus formation.
Methods: To decipher the regulatory complex of G_q signaling in platelets , we used CRISPR-Cas9 genome editing to generate mice bearing the G188S substitution in exon 4 of $G_{q,}$
Results: The results show that the G_q(G188S) substitution disrupts interactions between G_q and the Regulators of G protein Signaling (RGS) proteins that are expressed in platelets. However, in contrast to the analogous substitution in G_{i2}, which blocks interactions with RGS and produces a *gain* of function in platelets, platelets from G_q(G188S) mice showed a substantial *loss* of function in vitro and in vivo. Underlying this loss of function is a reduction in the binding of G_q to phospholipase β_3 (PLCβ3) and an increase in binding to the GPCR kinase, GRK2. An analysis of available crystal structures indicates that G_q(G188S) resides in a region important for the binding of both PLCβ and RGS proteins, but not GRK2.
Conclusions: Collectively, these observations show for the first time that 1) a G_q/PLCβ/RGS/GRK2 signaling node is present in platelets, 2) this complex plays a role in modulating G_q-dependent signaling during platelet activation, and 3) the Ga_q(G188S) substitution affects G_q-mediated signaling by disrupting G_q interactions with PLCβ and RGS, but not GRK2.

OC 59.5 | Sodium-calcium Exchanger Reverse Mode is Required for Generation of Procoagulant COAT Platelets

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Background: The combined activation of platelets with collagen-plus-thrombin induces the formation of procoagulant COAT platelets. Dichotomous intracellular signaling generating a subpopulation displaying procoagulant activity instead of traditional aggregating endpoints is still not fully elucidated. However, it has been demonstrated that secondary messengers such as calcium and sodium play a critical role.
Aims: To investigate calcium and sodium ion kinetics and the role of sodium-calcium exchanger (NCX) during platelet activation induced by convulxin-plus-thrombin.
Methods: Flow cytometry was used to characterize distinct populations of human platelets forming after activation with convulxin (collagen receptor GPVI agonist) and thrombin. Free cytosolic calcium

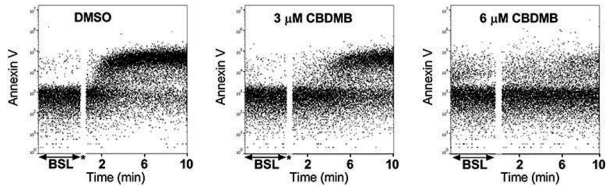

FIGURE 1 Time course of COAT platelet generation in presence of DMSO or CBDMB. (*) activation; baseline, BSL.

and sodium were measured with specific ion fluorescent indicators (Fluo-3 and ION NaTRIUM Green-2), and Annexin-V co-staining allowed discriminating procoagulant COAT platelets. NCX function was modulated with 5-(4-chlorobenzyl)-2'-4'-dimethylbenzamil (CBDMB) inhibitor.

Results: Specific ion fluxes were observed in distinct procoagulant and aggregating platelet subpopulations. High and prolonged intracellular calcium concentration (>1 μM), and transient sodium increase were observed in procoagulant COAT platelets, whereas aggregating non-COAT platelets rapidly decreased their calcium content and maintained higher cytosolic sodium. CBDMB dose-dependently diminished (IC_{50} = 2.85 ±0.3 μM) and delayed (shown in Figure) generation of procoagulant COAT platelets. Modulation of NCX by CBDMB affected ion kinetics: calcium efflux was retarded and reduced in aggregating platelets while sodium efflux was reduced in procoagulant COAT platelets. This suggests that both forward and reverse NCX modes are used in convulxin-plus-thrombin-activated platelets. According to our data, non-COAT aggregating platelets predominantly use forward mode NCX, by pumping calcium out and moving sodium in, while procoagulant COAT platelets mostly rely on reverse NCX function, which exchanges cytosolic sodium with extracellular calcium.

Conclusions: This study demonstrates that NCX is a critical regulator of the convulxin-plus-thrombin platelet response. The present data show that calcium influx from NCX reverse mode is required for achieving an efficient procoagulant signaling.

OC 69.1 | Platelet Deficiency of ERp5 Is Compensated for by Increased Levels of ERp57 and PDI

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Background: Endoplasmic reticulum 5 (ERp5) is a member of the vascular thiol isomerase family, which also includes protein disulphide isomerase (PDI), ERp57 and ERp72. The thiol isomerases are released from activated platelets and associate with platelet integrin αIIbβ3. Platelets deficient in PDI, ERp57 or ERp72 have decreased aggregation and thrombus formation *in vivo*.

Aims: A platelet-specific knockout of ERp5 was made in mice to probe its platelet function.

Methods: Conditional ERp5 knockout mice (PF4Cre+ Pdia6 fl/fl) (CKO) were generated by CRISPR-Cas9 technology. Mice carrying the floxed ERp5 gene without PF4Cre (PF4Cre- Pdia6 fl/fl) were used as controls. Thrombin activation of integrin αIIbβ3 was measured from binding of JON/A antibody. Platelet adhesion to fibrinogen under flow was measured in a microfluidics device. The reductase activity of the platelet surface was measured from dieosin-GSSG cleavage.

Results: ERp5 protein levels in CKO mouse platelets were < 2% of control platelets. Depletion of ERp5 resulted in increased levels of ERp57 (3-fold) and PDI (2-fold) protein. ERp5 CKO mice had normal platelet counts, normal expression of platelet surface integrin αIIbβ3, and similar levels of JON/A binding to αIIbβ3 following thrombin stimulation. Adhesion of the CKO platelets to fibrinogen was also similar at shear rates of 100 s^{-1} and 500 s^{-1}. In contrast, the reductase activity of ERp5 CKO platelets were increased compared to controls.

Conclusions: ERp5 is the first platelet vascular thiol isomerase that, when ablated, is compensated for by increased levels of other platelet isomerases. It has been proposed that the vascular thiol isomerases may function as a redox chain, shuffling electrons among themselves and to substrate disulfides. ERp5 is more reducing than PDI and ERp57, suggesting that electrons flow from ERp5 to PDI to ERp57. Our results demonstrate that ablation of ERp5 leads to upregulation of 'downstream' oxidoreductases, which supports the redox chain hypothesis.

OC 69.2 | Impact of miR-204 on Human Platelet-like Structures

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Background: Several platelet-derived miRNAs are associated with platelet reactivity (PR) and clinical outcome in cardiovascular patients. We previously showed a correlation between miR-204-5p and PR in stable cardiovascular patients but functional validation is lacking.

Aims: To functionally validate miR-204-5p as a regulator of PR in platelet-like structures (PLS) derived from human hematopoietic stem cells and to address mechanistic issues.

Methods: We used a model where human hematopoietic stem cells are differentiated into megakaryocytes, allowing transfection of miR-204-5p and recovery of derived PLS. The functional impact of miR-204-5p was assessed by several methods, including a flow-based assay and a spreading assay on fibrinogen. Transfected megakaryocytes and PLS were morphologically characterized by FACS

and microscopy. Quantitative PCR and western blot were used to assess the impact of miR-204-5p on selected gene targets. Finally, miR-204-5p plasma level was quantified in an independent cohort of cardiovascular patients.

Results: Functional assays showed an increased reactivity in megakaryocytes and PLS after transfection with miR-204-5p compared to control. In megakaryocytes, miR-204-5p transfection induced cytoskeletal changes, involving regulation of Rho family GTPase through downregulation of cdc42 protein, while megakaryocytes released abnormal chapelets bearing enlarged proplatelet tips. In a cohort of cardiovascular patients treated with aspirin (n=187), miR-204-5p level was correlated with PR induced by various agonists.

Conclusions: We functionally validated miR-204-5p as a regulator of PR that occurs through actin dynamics regulation. These data support a potential role of miR-204-5p as a biomarker of PR.

OC 69.3 | Platelet Lamellipodia Formation Is Not Required for Thrombus Formation and Stability

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Background: During platelet spreading, the actin cytoskeleton undergoes rapid rearrangement, forming filopodia and lamellipodia. Controversial data has been published on the role of lamellipodia in thrombus formation and stability.

The WAVE complex, which has been shown in other cells to drive lamellipodia formation by enhancing actin nucleation via the Arp2/3 complex, is activated by Rac1 interaction with the WAVE complex subunit Cyfip1. However, the role of Cyfip1 in platelet function is unknown.

Aims: To investigate the impact of Cyfip1-deficiency on platelet physiology and the role of lamellipodia in thrombus formation and stability.

Methods: *Cyfip1*$^{flox/flox}$ *Pf4-Cre* mice were analyzed *in vitro* and *in vivo*.

Results: *Cyfip1*$^{-/-}$ mice displayed normal platelet counts and unaltered glycoprotein surface expression. Activation of mutant platelets was moderately reduced to all tested agonists, as measured by αIIbβ3 integrin activation and P-Selectin surface exposure. *Cyfip1*$^{-/-}$ platelets adhered to multiple adhesive surfaces including fibrinogen, but strikingly lamellipodia formation was completely abolished (Fig.1). Despite defective lamellipodia formation, *Cyfip1*$^{-/-}$ platelets formed stable thrombi, when perfused *ex vivo* over collagen fibers at different shear rates and *in vivo* in two models of occlusive arterial thrombosis. Similarly, the hemostatic function was unaltered in the mutant mice. Even in the reverse passive Arthus reaction model,

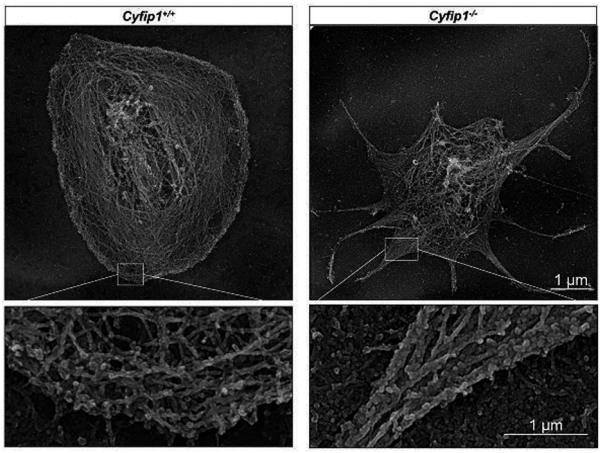

FIGURE 1 Lamellipodia formation is abolished in Cyfip1-deficient platelets

Cyfip1-deficient platelets maintained vascular integrity during inflammation of the skin. Investigation of *Cyfip1*$^{-/-}$ platelet morphology and the cytoskeletal ultrastructure in an induced thrombus under flow by scanning and transmission 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 (Fig.2).

Conclusions: We provide for the first time direct evidence that 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.

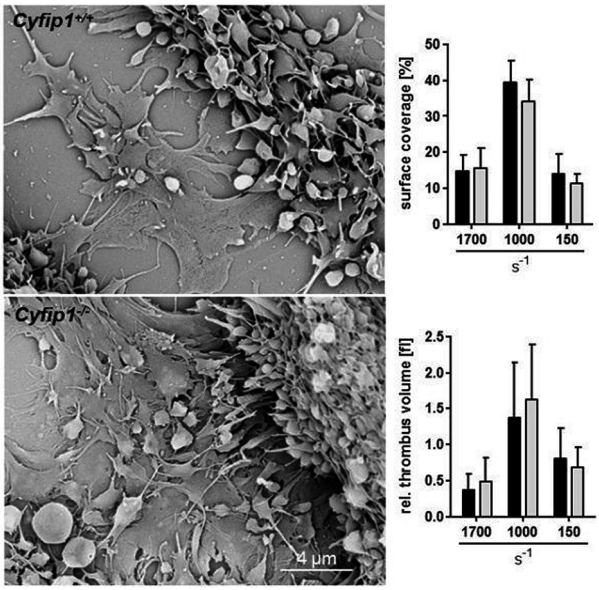

FIGURE 2 Cyfip1-deficient platelets form stable thrombi under flow

OC 69.4 | Platelet Actomyosin Response to Flow or Substrate Stiffness-induced Biomechanical Forces

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Background: Platelets are sensitive to their local biomechanical environment, demonstrated by the lack of spreading on soft substrates like endothelial cells. Identifying mechanisms that drive their force-sensitive response will provide insight into platelet binding and adhesion stability to various substrates in static or flow environments.

Aims: Utilize a clutch model of actomyosin to predict outcomes of integrin ligation in both static and flow environments

Methods: A simple stochastic (discrete event) clutch model was made to investigate the molecular machinery of platelet force-dependent responses. It incorporates force-dependent binding kinetics, actin polymerization, and myosin contraction. Parameters from the model were determined from platelet spreading experiments on fibrinogen with different inhibitors. The model was then validated by investigating platelets under various shear rates in a microfluidic device.

Results: Integrin ligation leads to actin polymerization, resulting in cell spreading, while non-muscle myosin II creates contractile forces that pull actin (and integrin) toward the granulomere thereby pulling the platelet body into the substrate-akin to ratcheting something down. Resistance to these contractile forces caused by a stiff substrate or drag forces in flow can result in 'clutch' (or ratchet) slippage. Using platelet spreading and evaluating fibrinogen-coated bead movement, we were able to quantify these clutches with spreading rates of 14.6 ± 3 nm/s and ratcheting at 7.7 ± 1.7nm/s in a static environment. These parameters were incorporated into the model, and significantly predicted platelet spreading rates with an R^2=0.92. Spreading under flow was predicted with R^2=0.87.

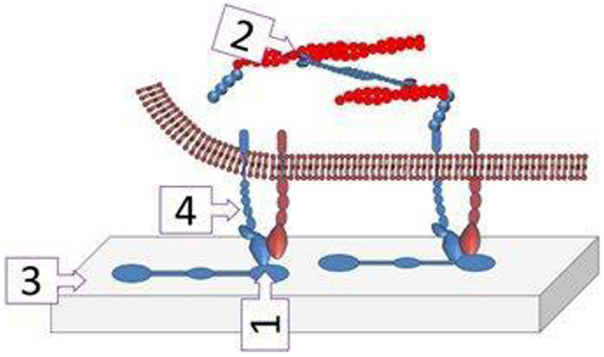

FIGURE 1 Model: 1) integrin ligation 2) myosin motor 3) surface stiffness 4) mechanical properties

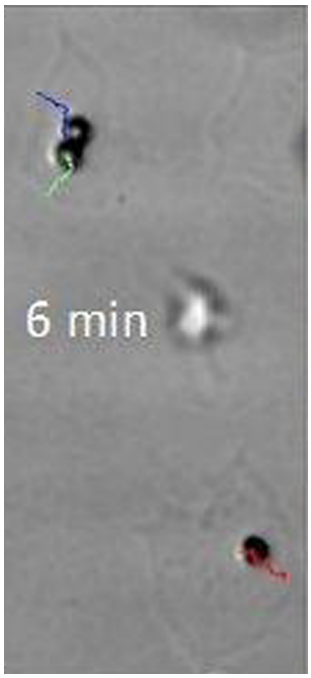

FIGURE 2 Fibrinogen-coated bead path toward granulomere

Conclusions: Here, we present a model for platelet responses to flow or elastic surfaces, with experimental validation. As surface stiffness or drag forces increase, it causes force producing mechanisms within the platelet to slip. Combined, this work provides a first step toward elucidating complex platelet responses under flow, potentially providing new insight into unexplained bleeding disorder or targets for antiplatelet therapies.

OC 69.5 | Using a Mathematical Model of Platelet Aggregation in an Extravascular Injury to Investigate Modifiers of Primary Hemostasis

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Background: Mathematical models of flow-mediated coagulation have been used to predict regulatory mechanisms of thrombosis; results from such models were validated with experiments. The computational efficiency of these models allows for systematic exploration of large sets of parameters that would be impossible to resolve experimentally.

Aims: The objective of this work was to create a mathematical model that captures: (i) the transport of platelets and proteins; (ii) the dynamics of platelet adhesion and aggregation; (iii) aggregate

structure; and to use the model to identify parameters that are most likely to modify primary hemostasis.

Methods: We developed an ordinary differential equation (ODE) model that incorporates platelet aggregation and simplified dynamics of blood flow, where the contribution of increased resistance due to aggregate growth is coupled to flow through an injury. Flow and aggregation calculations were computationally and experimentally validated using analogous partial differential equation (PDE)[1] and microfluidics[2] models. We explored the effects of the agonist ADP on $P2Y_1$- and $P2Y_{12}$-mediated platelet function via aggregate growth.

Results: Computational experiments with a constant supply of ADP concentration produced platelet aggregation with injury occlusion. Titration experiments in which we reduce ADP concentration, a surrogate for the addition of 2MeSAMP, led to prolonged occlusion times. We found that, with the inclusion of ADP-mediated granule release, the transport of ADP downstream reduced the local agonist concentration and resulted in occlusion times of over 20 minutes.

Conclusions: These results agree with experimental identification of the inhibition of $P2Y_1$- and $P2Y_{12}$-dependent platelet function as a modifier of hemostasis. The unexpected result associated with ADP-mediated granule release suggests that flow-mediated dilution of ADP may also be a key modifier of hemostasis.

This work was supported by the NIH (R01HL120728).

1. Leiderman, K. and A.L. Fogelson. Math. Med. Biol. 2011; 28(1):47-84.

2. R.M. Schoeman, et al. Cellular and Molecular Bioengineering, 10(2017): 3-15.

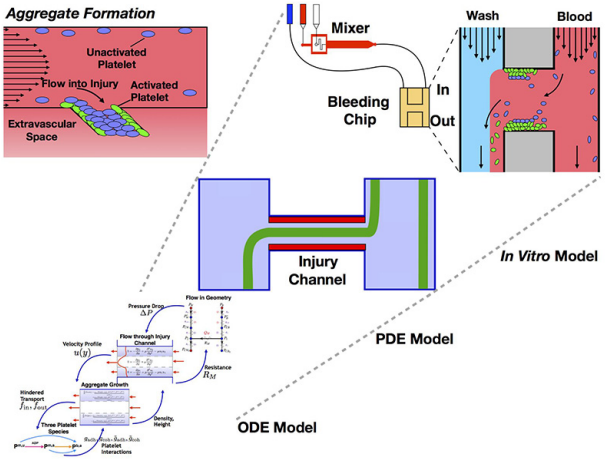

Fig. 1: Three nested models **(i)** 3-D MFA; **(ii)** 2-D partial differential equation (PDE) model; **(iii)** reduced-order ordinary differential equation (ODE) model; contribute to a system's biology approach to investigating platelet function and coagulation. All models incorporate intravascular and extravascular compartments, resulting in the flow of blood in multiple directions. Crosstalk among these experimental and computational models is used to identify and quantify injury occlusion sensitivity.

FIGURE 1

OC 78.1 | Novel NADPH Oxidase 1-selective Inhibitors Impair Collagen-dependent Platelet Activation and Thrombus Formation *in vitro* and Ferric Chloride-induced Carotid Occlusion *in vivo* with Negligible Effects on Thrombin Responses and Bleeding Time

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Background: NADPH oxidases (NOXs) produce reactive oxygen species in platelets and their enzymatic activity is essential for platelet activation.

Aims: Despite its poor pharmacokinetic properties, the NOX1-selective inhibitor 2-acetyl phenothiazine (2-APT) can inhibit collagen-dependent platelet responses *in vitro*. We synthesised a series of novel chemical derivatives with improved physicochemical profile compared to 2-APT and tested their ability to affect platelet responses *in vitro* and *in vivo*.

Methods: We utilised turbidimetry, flow cytometry, thrombus formation under flow or electron paramagnetic resonance to measure platelet activation or oxidative stress *in vitro* and ferric chloride-induced carotid occlusion or tail bleeding assays to assess thrombosis or haemostasis *in vivo*.

Results: Amongst the newly synthesised compounds, 2APT-PD06, showed significantly higher potency as collagen-selective inhibitor than 2-APT *in vitro*. When administered in mice, 2APT-PD06 inhibited collagen-dependent aggregation in platelet rich plasma (PRP) and thrombus formation under flow in whole blood. Carotid occlusion time was also significantly increased, while tail bleeding time was only marginally affected. The effect of 2APT-PD06 reproduced the functional phenotype that we observed for genetically silenced $NOX1^{-/-}$ mice (reduced collagen response *in vitro* and reduced carotid occlusion *in vivo*). The administration of 2APT-PD06 in $NOX1^{-/-}$ mice did not reduce carotid occlusion further, confirming the $NOX1^{-/-}$ selectivity of this compound.

Conclusions: This study describes the generation of novel NOX1-selective inhibitors able to target collagen-dependent platelet responses *in vitro* and carotid occlusion *in vivo*. The limited effect on thrombin-induced responses *in vitro* and tail bleeding time *in vivo*, supports the hypothesis that NOX1 is dispensable for platelet activation in response to thrombin and for the haemostatic response induced by acute vascular injury, while it is essential for collagen-dependent platelet activation and vascular occlusion induced by endothelial dysfunction. Taken together, these data indicate NOX1 as an interesting target for the development of selective antiplatelet drugs with limited bleeding side effects.

OC 78.2 | Safety and Tolerability, Pharmacokinetics and Pharmacodynamics of ACT017, an Anti-platelet Glycoprotein VI Fab: First in Human Healthy Volunteer Trial

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Background: Glycoprotein VI (GPVI) is accepted as being a target to develop effective antiplatelet agents without bleeding risk. ACT017 is an anti-GPVI clinical-grade fragment of monoclonal antibody. Preclinical studies of ACT017 in non-human primates have shown its *ex vivo* biological efficacy, excellent tolerability and established a protocol of administration.

Aims: This first-in-human study aimed to evaluate the safety and tolerability, PK and PD of single ascending intravenous doses of ACT017.

Methods: Protocol, subject information and informed consent form, were approved by the Independent Ethics Committee (IEC) of QPS the competent authority of the Netherlands (CCMO). Six cohorts of eight healthy male and female subjects each, received ascending single doses (62.5 to 2000 mg) of ACT017 as a 6-hour *i.v.* infusion, with ¼ of the total dose administered within 15 minutes and the rest of the dose within the next 5 hours and 45 minutes. Safety and tolerability parameters, including bleeding time platelet counts and platelet GPVI expression, biological efficacy on collagen-induced platelet aggregation, and plasma concentrations of ACT017 were evaluated up to 48 hours post dose.

Results: All doses of ACT017 were well tolerated and no serious adverse events occurred during the study. None of the subjects reported an infusion site reaction. Bleeding time was not affected in a clinically significant manner by any of the ACT017 doses. Plasma concentrations increased linearly with the dose received and PK values were established. There was no modification in the platelet count and platelet GPVI expression. Administration of ACT017 inhibited collagen-induced platelet aggregation and the extent and duration of the effect were dose-dependent.

Conclusions: The novel antiplatelet agent ACT017 has consistent pharmacokinetic/pharmacodynamic properties and favourable safety and tolerability profiles in healthy volunteers. This first in human study paves the way for the subsequent assessment of ACT017 safety and efficacy in patients with thrombosis.

OC 78.3 | First Generation PI3KC2α Inhibitors are Potently Anti-Thrombotic and Improve on the Safety Profile of Current Anti-Platelet Therapies

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Background: The class II PI3K, PI3KC2α, is a lipid kinase expressed in many cells, including platelets. Mice genetically deficient in PI3KC2α are protected against thrombosis but do not bleed. The mechanism behind this effect is incompletely understood but is believed to involve a dilation of the platelet internal membrane, the open canalicular system (OCS). However, due to an absence of PI3KC2α inhibitors, whether or not a similar mechanism exists in human platelets remains unknown.

Aims: To determine the efficacy of first generation PI3KC2α inhibitors and to assess the viability of PI3KC2α as a target for novel antiplatelet therapies.

Methods: A rational drug design approach was used to develop PI3KC2α inhibitors which were tested for their effects on recombinant PI3KC2α, isolated platelets, ex vivo whole blood thrombosis, and mouse models of in vivo thrombosis.

Results: MIPS-19416 emerged as a lead inhibitor, with an IC_{50} of 13 nM against recombinant enzyme. MIPS-19416 completely reproduced the unique changes to the OCS in mouse platelets and induced near-identical effects in human platelets. MIPS-19416 only modestly impaired in vitro human platelet activation (aggregation, P-selectin expression, $\alpha_{IIb}\beta_3$ activation) but was potently anti-thrombotic in whole blood experiments. Mice treated with MIPS-19416 were protected against thrombosis in two distinct in vivo thrombosis models, but did not exhibit impaired haemostasis. Finally, MIPS-19416 markedly impaired human ex vivo thrombosis that was resistant to broad inhibition of platelet activation pathways (thrombin, ADP and thromboxane).

Conclusions: Development of a first generation PI3KC2α inhibitor revealed that PI3KC2α regulates the OCS in both mouse and human platelets. Inhibition of PI3KC2α produced an anti-thrombotic effect not fully explained by the modest effects on platelet activation and may be largely independent of canonical platelet activation. These findings reveal a novel target for the development of anti-thrombotic therapies with an improved safety profile over current approaches.

OC 78.4 | Two Birds, One Stone: Anti-β3 Integrin PSI Domain Antibodies Inhibit Both Platelet Aggregation and Blood Coagulation

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Background: Integrin αIIbβ3 plays key roles in thrombosis and hemostasis through mediating platelet adhesion and aggregation. We recently reported the active site of thiol-isomerases, CXXC, is expressed twice within the plexin-semaphorin-integrin (PSI) domain across all integrins (Blood, 2017). We developed anti-β3 integrin PSI domain monoclonal antibodies (mAbs) that inhibit the thiol-isomerase activity of β3 PSI domain and decrease platelet adhesion, aggregation, and thrombosis without increasing bleeding. Interestingly, the mAbs inhibition of thrombosis *in vivo* was 10-20x greater than on *in vitro* platelet aggregation.

Aims: Mechanistically explore how the PSI domain contributes to blood coagulation.

Methods: Utilize our proprietary anti-β3 PSI domain mAbs in *in vitro / ex vivo* coagulation assays.

Results: Clot retraction assays performed with human and mouse blood showed less clot retraction and a significantly lower dry clot weight in whole blood treated with anti-PSI mAbs compared to controls. Laser scanning confocal microscopy of platelet-rich plasma (PRP) samples with labelled fibrin(ogen) showed that fibrin network formation of anti-PSI mAb treated clots was significantly decreased. To measure blood coagulation, thromboelastography (TEG) was performed. Blinded TEG showed that coagulation decreased in anti-PSI mAb-treated whole blood (human and mouse) and PRP. We further compared our anti-PSI mAbs with other anti-β3 integrin mAbs. TEG analysis uncovered that the anti-PSI mAbs significantly decreased coagulation parameters, much more than M1, JAN D1, and the Abciximab precursor 7E3, which more potently inhibit αIIbβ3-fibrinogen binding and platelet aggregation. This suggests the decreased coagulation may result from the anti-thiol-isomerase activity of the anti-PSI mAbs and its impact on coagulation factors. The mechanistic features of this inhibitory effect are under investigation.

Conclusions: This is the first evidence of an allosteric therapeutic target of β3 integrin and suggests that targeting the PSI domain partially blocks platelet aggregation and coagulation, resulting in less bleeding while maintaining strong anti-thrombotic effects.

OC 78.5 | Olfactory Receptors and Dysregulated Platelet Activity in Abdominal Aortic Aneurysms

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Background: AAA growth and rupture are associated with platelet activation and thrombus in aneurysmal segments. We hypothesized that specific mechanical signals activate platelets and contribute to AAA progression and rupture.

Aims: Using platelets isolated from humans with AAA and relevant murine models of AAA, we investigated whether the platelet phenotype changes.

Methods: Platelets were isolated from human control and AAA patients and mice, and activation was assessed by flow cytometry. A screen for transcripts in AAA patient platelets compared to healthy platelets was assessed by mRNA-seq, and validated by qPCR and Western blotting. Blood flow was simulated *ex vivo* using a cone/flow system. Clonal cell lines of platelet olfactory receptors (OR) were created for a ligand screen. OR activity was assessed by downstream cAMP Response Element [CRE] function by luciferase activity, and cAMP binding Protein (CREB) activity by phospo-antibody. A murine AAA model mimicking the human condition of AAA with luminal thrombus prior to rupture was used.

Results: Human platelets from AAA patients were reactive through thromboxane and PAR receptors. Platelets from patients with AAA expressed increased OR2L13 mRNA and surface protein compared to healthy platelets. Platelets in a murine AAA model were markedly reactive early in AAA progression but became less reactive coincident with increased platelet OR2L13 expression. An *ex vivo* system to recapitulate steady (laminar) or disturbed (turbulent) blood flow (S-flow and D-flow, respectively) showed OR2L13 is redirected to the platelet membrane >10-fold under D-flow conditions. Human and murine platelet activation in response to agonists was attenuated by OR2L13 ligands.

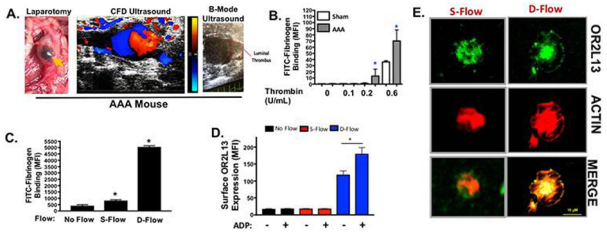

FIGURE 1 AAA and Platelet Olfactory 2L13

Conclusions: D-flow, such as in AAA segments, activates platelets which secrete enzymes capable of remodeling aorta. Platelets from patients with AAA and mice with AAA express increased OR2L13, and platelet activation is reduced by OR2L13 ligands. Platelet OR2L13 expression may be protective in response to D-flow in AAA.

OC 79.1 | Neutrophils Are Responsible for Colitis-induced Anemia and Thrombosis in P2X1-deficient Mice

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Background: Chronic inflammatory bowel diseases are associated with intestinal bleeding and systemic thrombotic events. However, the mechanisms underlying this hemostatic imbalance remain largely unknown. Particularly, the respective contributions of platelets and neutrophils to intestinal bleeding and extra-intestinal thrombosis have never been investigated.

Aims: To study the mechanisms involved in the maintenance of vascular integrity and thrombosis under intestinal inflammatory conditions.

Methods: We used a mouse model of acute colitis induced by oral administration of dextran sodium sulfate for 7 days. Diarrhea and bleeding scores were determined upon depletion of platelets, neutrophils or glycoprotein VI, treatment with aspirin or clopidogrel or in $P2X_1^{-/-}$ mice. Platelet and neutrophil count, red blood cell (RBC) indices and plasma levels of erythropoietin (EPO), IL-6 and G-CSF were analyzed. Extra-intestinal thrombosis was assessed using a laser-induced injury model of thrombosis in cremaster muscle arterioles. Accumulation of platelets, neutrophils and fibrin generation were recorded in real-time.

Results: Thrombocytopenia or $P2X_1$ deficiency led to macrocytic regenerative anemia due to intestinal hemorrhage. Hemoglobin levels and RBC count were severely decreased while mean corpuscular volume (MCV) and red cell distribution width (RDW) were higher as compared to wild-type controls. EPO plasma levels were elevated. Spleens were enlarged and contained higher fraction of immature erythroblasts. Strikingly, $P2X_1^{-/-}$ mice exhibited higher neutrophil count due to increased G-CSF levels, while platelet count was unchanged. Neutrophil depletion limited blood loss and fully restored RBC indices $P2X_1^{-/-}$ mice. Colitic $P2X_1^{-/-}$ mice showed a strong increase of neutrophil accumulation at the site of arteriolar wall injury. Fibrin generation was reinforced despite impaired platelet recruitment in the growing thrombi.

Conclusions: In inflamed intestine, platelets and P2X1 ion channels are mandatory to preserve vascular integrity. Upon P2X1 deficiency, neutrophils are responsible for massive intestinal bleeding associated with extra-intestinal pro-thrombotic tendency due to formation of neutrophil-rich thrombi.

OC 79.2 | Platelet C-type Lectin-like Receptor 2 (CLEC-2) is Required for Optimal Regulation of Erythropoiesis

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Background: CLEC-2 is a platelet activating receptor, which was first identified as a receptor for a snake venom, rhodocytin. CLEC-2 is mainly expressed on platelets and megakaryocytes (Mgk). We also identified podoplanin (PDPN) as an endogenous ligand for CLEC-2. We have previously reported that PDPN-expressing stromal cells positively regulate megakaryocytic expansion and proplatelet formation via CLEC-2 (Tamura et al., Blood, 2016). Although deletion of CLEC-2 in mice exhibits significant anemia and thrombocytopenia, its contribution to erythropoiesis remains unknown.

Aims: The aims of this study were to uncover a role of CLEC-2 in erythropoiesis.

Methods: We used flow cytometry to analyze maturation of erythroblasts, apoptotic cell death, and cell cycle distribution. Conditioned media, prepared from PDPN-expressing stromal cells stimulated with CLEC-2 and Mgk stimulated with PDPN, were analyzed by cytokine array and ELISA, and co-cultured with immature erythroblasts.

Results: We analyzed maturation of erythroblasts in bone marrow from Mgk/platelet-specific CLEC-2 knockout (CLEC-2 cKO) and wild-type mice using CD71/Ter119 flow-cytometric assay (Figure). The number of subpopulation 2 (S2; CD71+ Ter119+), S3

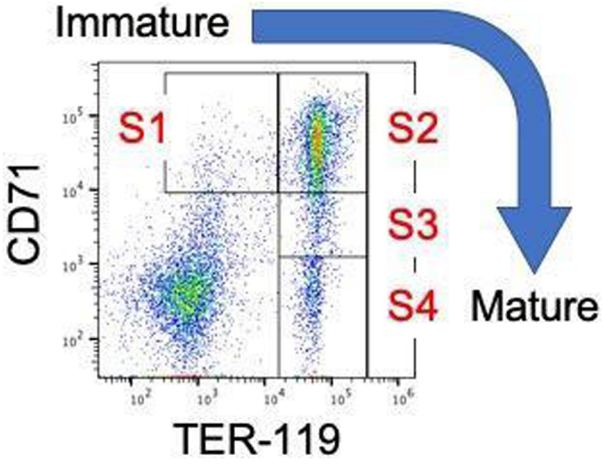

FIGURE 1 Erythroid cells are divided into subpopulations S1 to S4 based on their CD71/TER119 expression

(CD71dim Ter119+) and S4 (CD71- Ter119+), but not S1 (CD71-Ter119-), were decreased in CLEC-2 cKO mice. We further found that apoptotic cell death was increased in S2, without alteration of cell cycle distribution. These data indicated that increased cell death and/or maturation arrest were responsible for the anemia. We next prepared conditioned media from PDPN-expressing stromal cells stimulated with CLEC-2 and Mgk stimulated with PDPN. The former medium contained high concentration of β-chemokines and IL-6. Among them, only IL-6 showed anti-apoptotic effects on immature erythroblasts. The latter one contained high concentration of TGF-β, which exhibited erythroblast maturing effects. These data suggest that insufficient secretion of those cytokines/chemokines was one of the causes for the anemia in CLEC-2 cKO mice.

Conclusions: CLEC-2 on Mgk/platelets not only contribute to megakaryopoiesis but also positively regulate erythropoiesis.

OC 79.3 | Inhibition of Platelet GPVI Induces Intratumor Hemorrhage and Increases Efficacy of Chemotherapy in Mice

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Background: Platelets are small anucleated cells that play a central role in the arrest of bleeding. Previous studies have identified platelets as major regulators of tumor vascular integrity, since their depletion selectively renders tumor vessels highly permeable and causes massive intratumoral hemorrhage. While these results established platelets as potential targets for anti-tumor therapy, their depletion is not a treatment option due to their essential role in hemostasis. Thus, a detailed understanding of how platelets safeguard vascular integrity in tumors is urgently required.

Glycoprotein (GP) VI is a platelet-specific activating receptor, regulating multiple platelet functions including activation and pro-coagulant activity. GPVI has been proposed as a potentially safe anti-thrombotic target, since its blockade reduces arterial thrombosis without impairing hemostasis. GPVI also contributes to the maintenance of vessel integrity under conditions of inflammation. However, its role in the regulation of tumor vessel integrity remains unknown.

Aims: We investigated the role of GPVI in the maintenance of vascular integrity in primary tumors and studied the potential impact of GPVI-blockade on the efficacy of chemotherapy.

Methods: Primary tumors were induced by heterotopic and orthotopic implantation of prostate (TrampC1) and breast (AT-3) cancer cells. Tumor vascularization and growth were analyzed in wild-type and *Gp6*$^{-/-}$ mice and upon treatment with the GPVI-blocking antibody JAQ1 F(ab´)$_2$ alone or combined with chemotherapeutic drugs.

Results: We show that blockade of GPVI induces tumor hemorrhage and reduces tumor growth with similar kinetics and to a comparable extent as platelet depletion. Moreover, GPVI blockade in mice increased intratumoral accumulation of co-administered chemotherapeutic drugs. Consequently, combination of GPVI-blockade with chemotherapy resulted in a profound and prolonged anti-tumor effect.

Conclusions: Our findings identify platelet GPVI as a key regulator of vascular integrity especially in growing tumors and could serve as a novel therapeutic target for the development of anti-tumor strategies that impede platelet function.

OC 79.4 | Transforming Growth Factor β Released by Platelets Primes Macrophage Activation and Extracellular Trap Formation, Thereby Influencing Breast Cancer Growth in Mice

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Background: Leukocytes release their chromatin fibers, referred to neutrophil extracellular traps (NETs), which are composed by DNA, histones and granule content. NET formation facilitates tumor cell aggressiveness in the context of systemic inflammation. Earlier, it has been shown that platelets could promote NET formation during bacterial infections. Recent studies indicated that activated platelets may also induce monocyte/macrophage-dependent extracellular trap (MET) formation, a process termed as METosis, thereby exacerbating organ injury and inflammation. However, the role of activated platelets in METosis during tumor growth has not been investigated.

Aims: We examined the role of platelets in the formation of METs during breast cancer growth.

Methods: Breast tumors were induced by orthotopic injection of two different breast cancer cell lines called AT-3 and E0771. Tumor growth was analysed in wild-type (WT) mice and megakaryocyte/platelet specific TGFß-knockout mice (*TGFß*$^{fl/fl\text{-}PF4\text{-}Cre}$) which were treated with

(i) DNAse I,
(ii) macrophage-depleting chlodronate liposomes,

(iii) platelet- and
(iv) neutrophil-depleting antibodies.

Infiltration of immune cells into the growing tumors was followed by immunofluorescence confocal microscopy and flow cytometry.

Results: We show that macrophage depletion decreased tumor volume in WT mice to a similar extent as DNAse I treatment. In contrast, depletion of neutrophils did not alter tumor growth in WT mice, indicating that neither neutrophils, nor NETs are critical for breast cancer growth. Moreover, we found that platelet depletion or TGFß deficiency in platelets inhibited METosis. Depletion of macrophages and MET formation attenuated tumor growth and this process was strongly dependent on TGFß released by activated platelets.
Conclusions: Platelet-derived TGFß enhances tumor growth by priming macrophage activation and METosis. Our findings reveal that inhibition of TGFß released by activated platelets may represent a new therapeutic strategy to decrease breast cancer growth by interfering with pro-tumorigenic macrophage function.

OC 79.5 | Targeting Activated Platelets in the Tumor Microenvironment for Localized Delivery of Antibody-drug Conjugates to Tumors and Metastases

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Background: Apart from their classical role in hemostasis, platelets are increasingly recognized as important mediators of tumor growth and metastasis. Experimental studies have demonstrated that tumor cells release inflammatory mediators which may provide a prothrombotic environment, promoting platelet activation.
Aims: 1. To confirm if activated platelets are present and abundant in the tumor microenvironment.
2. To determine the utility of a single-chain antibody which binds to the activated integrin GPIIb/IIIa for targeted cancer diagnosis and therapy.
Methods: We developed a theranostic agent based on a single-chain antibody which specifically binds to the high affinity, activated integrin GPIIb/IIIa on activated platelets ($scFv_{GPIIb/IIIa}$). The $scFv_{GPIIb/IIIa}$ was conjugated to 1) A highly potent microtubule inhibitor, monomethyl auristatin E (MMAE), incorporated with a cathepsin B cleavable linker for drug release, for therapy, and 2) Cyanine7 for *in vivo* imaging.
Results: *In vivo* fluorescence imaging demonstrated that $scFv_{GPIIb/IIIa}$-Cy7-MMAE localized to primary tumors and metastases in a mouse xenograft model of triple negative breast cancer, a particularly difficult to treat tumor for which a selective tumor-targeting therapy remains to be clinically established. Next, we demonstrated that the $scFv_{GPIIb/IIIa}$-Cy7-MMAE displays marked efficacy as an anti-cancer agent, reducing tumor growth and preventing metastatic disease, without any discernible toxic effects.
Conclusions: Overall, we confirmed the utility of a novel theranostic based antibody-drug conjugate that targets a potent cytotoxic drug to activated platelets and specifically releases the cytotoxic agent within the confines of the tumor. This unique double targeting mechanism holds major promise as a novel therapeutic approach for the treatment of a broad range of primary tumors and metastatic disease, particularly for tumors that lack a specific molecular surface epitope for drug targeting.

PLATELET DISORDERS

OC 06.1 | Antithrombotic Therapy with an Anti-glycoprotein VI Humanized Fab Does Not Increase the Risk of Inflammation-associated Bleeding

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Background: Glycoprotein VI (GPVI), the main platelet receptor for collagen, has emerged as a new target for anti-thrombotic therapy because its blocking inhibits platelet aggregation and arterial thrombosis without increasing bleeding time after trauma. However, recent studies have indicated that GPVI contributes to prevention of inflammation-induced bleeding in the skin and lungs, thus suggesting that GPVI inhibition might cause an increased risk of bleeding in those organs upon inflammation.
Aims: Here we investigated the risk of inflammation-induced bleeding associated with GPVI inhibition by Act017 (Acticor Biotech), an antithrombotic drug candidate currently in phase 2 clinical trial.
Methods: Mice humanized for GPVI (GPVIh mice) were treated with Act017 at high antithrombotic dose (16 mg/kg) and subjected to either immune complex-mediated skin inflammation or LPS inhalation-induced lung inflammation. The bleeding phenotype of control and Act017-treated GPVIh mice subjected to those models was compared to that of mice treated with a platelet-depleting antibody, or with partial (GPVI+/-) or complete (GPVI-/-) deficiency in GPVI.

Bleeding at the reaction site was quantified by measurement of skin hemoglobin content or hemoglobin concentration in bronchoalveolar lavage fluid.

Results: GPVIh mice treated with Act017 and GPVI+/- mice, which have half the normal platelet surface level of GPVI, did not bleed during immune complex-mediated skin inflammation. This was in contrast to the petechial skin bleeding at the reaction site observed in platelet-depleted and GPVI-/- mice. Absence of skin bleeding in GPVI+/- and Act017-treated GPVIh mice was not due to altered neutrophil recruitment as this was comparable to that in control GPVIh and GPVI+/+ mice. Only mice immunodepleted of platelets bled in the LPS inhalation-induced lung inflammation model.

Conclusions: Our results show that, in contrast to platelet depletion or GPVI deficiency, treatment with the anti-GPVI humanized Fab Act017 used at antithrombotic dose does not increase the risk of inflammation-associated bleeding.

OC 06.2 | The Antiplatelet Drug Ticagrelor Kills Resistant Gram-positive Bacteria

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Background: Ticagrelor is a potent oral antiplatelet drug prescribed annually to millions of patients presenting with acute coronary syndrome worldwide. A post-hoc analysis of the large randomized PLATO trial revealed that patients receiving ticagrelor had a lower risk of infection-related death as compared to clopidogrel. In the recent XANTHIPPE study, ticagrelor administration to patients hospitalized for pneumonia was associated with improved lung function.

Aims: We assessed whether ticagrelor or one of its metabolites could possess antimicrobial properties.

Methods: We synthetized ticagrelor and its four main metabolites. Bactericidal efficacy was tested in time-kill assays against Gram-positive methicillin-resistant *S. epidermidis*, methicillin-sensitive *S. aureus* (MSSA), Glycopeptide Intermediate *S. aureus* (GISA), methicillin-resistant *S. aureus* (MRSA), and vancomycin-resistant *E. faecalis* (VRE). Effect on biofilm formation was assessed *in vitro* with crystal violet staining and in a mouse model of *S. aureus* polyurethane implant infection.

Results: Ticagrelor and its most abundant metabolite AR-C124910 displayed *in vitro* bactericidal efficacy against all Gram-positive bacteria tested, including MRSA and VRE. MRSA bacterial growth was promptly delayed in the presence of 5µg/ml ticagrelor. At minimal bactericidal concentrations (MBC), ticagrelor killed late exponential phase MRSA cultures with similar efficacy as daptomycin. It was superior to conventional vancomycin against MRSA, MRSE and VRE. Ticagrelor dose-dependently inhibited biofilm formation by these strains. Despite the fact that the effective *in vitro* antibacterial concentrations were higher than ticagrelor Cmax values for an antiplatelet effect in humans (1.2µg/ml after one 180mg LD and 0.75µg/ml at 90mg bid steady-state), treatment of mice with doses equivalent to antiplatelet doses (3mg/kg LD, 1.5mg/kg bid), inhibited biofilm growth on *S. aureus* pre-infected implants and bacteria dissemination in surrounding tissues.

Conclusions: We uncovered unprecedented bactericidal effect of ticagrelor, which may provide mechanistic explanation for reduced infection-related death in patients receiving ticagrelor in clinical studies.

OC 06.3 | Novel Assay Demonstrates Increased Procoagulant Platelet Response in Essential Thrombocythemia Segregating With JAK2V617F Mutation Status and Thrombosis

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Background: Essential thrombocythemia (ET) has increased thrombotic risk, however, standard platelet function tests do not predict thrombosis.

Aims: To characterise the procoagulant platelet response in ET in relation to thrombosis risk.

Methods: Patients with ET, polycythaemia vera (PV) and healthy controls were recruited in Concord Hospital, Sydney, Australia (LNR/17/CRGH/251) and Singapore General Hospital (CIRB2016/2058). Procoagulant platelets were measured by combined uptake of GSAO and P-selectin expression by flow cytometry: unstimulated or with increasing doses of thrombin (0.5 - 5U/mL) or thrombin plus collagen (collagen, 10mg/mL) in whole blood or washed platelets. Both standard and FlowSOM FACS analysis were applied (FlowJo™). Light transmission aggregometry (LTA) and platelet-poor-plasma thrombin generation (ETP) with/without thrombomodulin (TM) were performed on a subset.

Results: ET patients (n=70) and PV (n=20) demonstrated markedly higher procoagulant platelet response compared with healthy controls (n=32) at all concentrations of thrombin ($p< 0.001$) and thrombin with collagen ($p< 0.001$), with stratification of response according to JAK2V617F mutation status (Figure 1). No correlation was seen with ETP±TM or LTA. In multivariate analysis including age, gender, history of thrombosis, presence of cardiovascular risk factors, use of hydroxyurea, platelet and leukocyte counts, JAK2V617F mutation was the sole predictor of procoagulant platelet hyperresponsiveness in

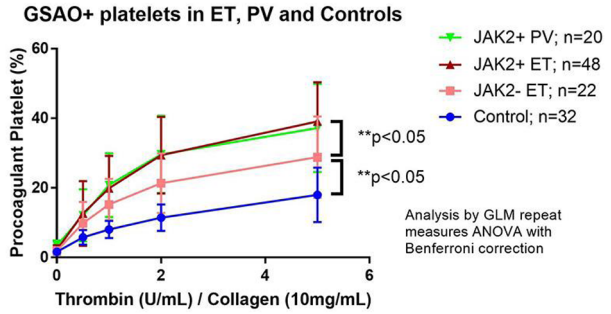

FIGURE 1 Procoagulant platelet response of ET, PV and controls in response to thrombin+collagen

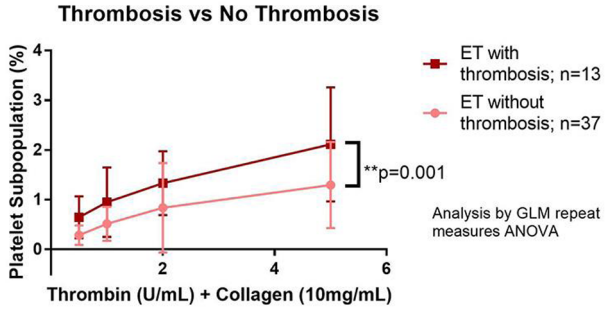

FIGURE 2 Subpopulation of GSAO+ platelets in ET with/without thrombosis in response to thrombin+collagen

ET(p=0.002). Comparison of washed platelets and whole blood within patients (n=10) and controls (n=5) demonstrated both platelet and non-platelet contribution to increased procoagulant platelet response(p< 0.05). Unbiased multidimentional clustering analysis identified a subpopulation of GSAO+ platelets overexpressed in ET patients with thrombosis (Figure 2) in univariate and multivariate analysis.

Conclusions: GSAO assay demonstrates ET patients have a heightened procoagulant platelet response related to JAK2V617F mutation status, independent of ETP or LTA but influenced by factors both intrinsic and extrinsic to the platelet. A procoagulant platelet subpopulation overexpressed in ET patients with thrombosis was identified which may warrant prospective evaluation as a potential thrombosis risk marker.

OC 06.4 | A Large Spectrum of Variants Identified in Moderate Thrombocytopenic French Blood Donors: Results of the ABC Study

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Background: Thrombocytopenia (less than 150G/l) may be a reason for donors deferral for blood donation in France.

Aims: Our project aimed at exploring potential genetic variations responsible for isolated moderate thrombocytopenias and to define their prevalence in a blood donor population from 3 French regions: Alsace, Centre-Atlantique and Bretagne.

Methods: After a medical questionnaire and obtaining an informed consent, we collected a saliva sample from 448 donors with platelet counts < 150 G/l on two consecutive blood donations. Following DNA extraction the coding sequences and intron/exon junctions of 17 candidate genes were amplified by PCR Ampliseq™ and sequenced on a Ion Proton© instrument. The annotation of the variants was performed using the SeqNext© software. Variants of interest were selected when the frequency in the population was less than 1%. The pathogenicity of the variants was assessed using data from the literature and bioinformatics prediction tools.

Results: Most donors (59%) were aware of their low platelet count but only 17% knew of a family history of thrombocytopenia. 84 heterozygous variants of interest spread over 12 genes. 23 variants are deleterious or probably deleterious, the rest of the variants being of unknown significance (VUS) and requiring functional validation. 59 variants were found on gene already studied in patients with inherited thrombocytopenia (8 GPIBA, 2 GPIBB, 3 WF, 9 MYH9, 11 TUBB1, 9 ACTN1, 13 ITGA2B and 5 FLNA) with a surprisingly high frequency of ITGA2B and TUBB1 variants. The remaining 24 variants where identified on genes suspected to be responsible for thrombocytopenia in human or already studied in mouse models (7 TUBA8, 8 ITGB3, 5 ITGA2, 2 RASGRP2 and 2 TUBA4A).

Conclusions: These results obtained in a population of blood donors indicate that at least 5% of healthy donors with moderate thrombocytopenia carry a constitutional anomaly in a gene responsible for this platelet count defect.

OC 06.5 | Assessment of Platelet Thrombus Formation under Flow Condition in Patients with Acute Kawasaki Disease

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Background: Kawasaki disease (KD) is an acute systemic vasculitis of unknown etiology. The acute phase of KD is thought to involve platelet activation, and antiplatelet therapy is included in the treatment protocol for the past decades. The evidence for platelet activation, however, is limited to a few studies without consideration of shear stress. The total thrombus-formation analysis system (T-TAS) was developed for quantitative analysis of platelet thrombus formation (PTF) by the use

of microchips with thrombogenic surfaces. This system can measure the process of PTF mediated by platelet-collagen interaction under selected shear rates in a situation closure to the *in vivo* condition.

Aims: To assess the PTF under flow condition in patients with acute KD.

Methods: Thirty-eight patients with acute KD were investigated. A whole blood was obtained immediately before treatment (Pre), one week (1W) and one month (1M) after starting treatment with antiplatelet drugs. We assessed by the platelet chip (PL-chip) coated with the type I collagen under high share stress of 1000/s (PL12) and 2000/s (PL24). We assessed AUC3 and 10 (area under curve the flow pressure curve for 3 and 10 min) to quantify the onset facility of PTF and the overall stability of thrombus.

Results: In pre, PL12-AUC3 was 3.7U (1.1-11.9) and PL24-AUC3 was 27.5U (1.4-59.5), respectively. They were increased compared with controls (1.0U (0.0-9.3) and 6.1U (0.6-99.5)), respectively ($p<0.001$ and $p=0.026$). PL12-AUC10 was 151.7U (14.0-310.8), and PL24-AUC10 was 360.5U (72.9-481.6), respectively. PL12-AUC10 was decreased compared with controls (234.2U (100.6-364.2), $p<0.001$). In 1W and 1M, both PL12- and PL24-AUC3 got decreased, and there was no statistical difference in aspirin dosage or alternative drugs.

Conclusions: The present study suggests early onset but weak stability of PTF was characteristic in acute KD.

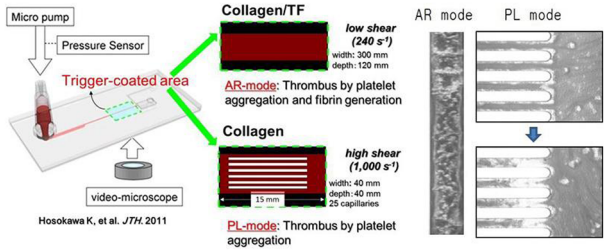

FIGURE 1 The total thrombus-formation analysis system (T-TAS)]

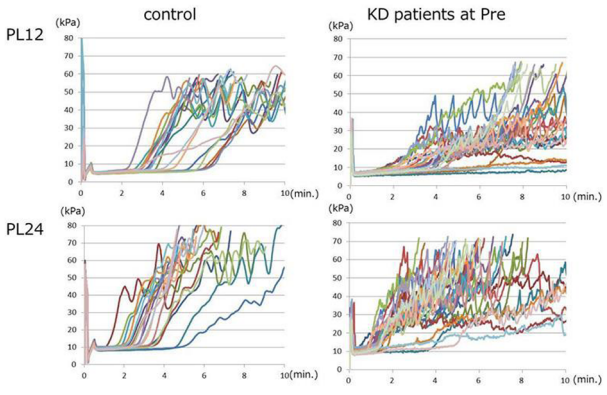

FIGURE 2 The pressure curves of controls and KD patients at Pre

OC 16.1 | Premature Platelet Clearance via Clustering of the Hyposialylated GPIbα Receptor in GNE-related Thrombocytopenia

I. van Asten[1,2,3]; R. Schutgens[1,2]; A. Lakerveld[3]; T. Noordermeer[3]; A. Koekman[3]; K. Hage[3]; S. Sebastian[3]; G. Pasterkamp[3]; A. Huisman[3]; D. van den Heuvel[4]; H. Gerritsen[4]; S. Korporaal[3]; M. Bierings[5]; J. van der Smagt[6]; M. van Gijn[6]; R.T. Urbanus[1,2,3]

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Background: Congenital thrombocytopenia due to hyposialylated platelets has been described and is associated with mutations in the Glucosamine(UDP-N-Acetyl)-2-Epimerase and N-Acetylmannosamine Kinase(GNE) gene, which encodes the rate limiting enzyme in sialic acid synthesis. We identified 2 female siblings with a severe macrothrombocytopenia(< 10 G/L) from birth, extensive bleeding and a homozygous R420Q mutation in the GNE gene. The exact mechanism of GNE-related thrombocytopenia remains unclear.

Aims: To investigate the molecular mechanism behind GNE-related thrombocytopenia.

Methods: Platelet sialylation was measured on the flow cytometer. Pathogenicity of the R420Q GNE variant was confirmed in GNE-deficient HEK293 cells through overexpression of GNE variants. GPIbα clustering due to desialylation was measured with fluorescence life time imaging. Phagocytosis of control platelets, desialylated platelets and patient platelets by THP-1 macrophages and HepG2 hepatocytes was measured on a flow cytometer. Inhibition of GPIbα clustering with GM3 and silencing of CD11b, the counter receptor for clustered GPIbα, was used to study the platelet binding interaction with THP-1 macrophages and HepG2 hepatocytes.

Results: Patient platelets were strongly hyposialylated. Overexpression of GNE-R420Q in GNE-deficient HEK293 cells confirmed the association between the R420Q variant and hyposialylation. Indium-labeled autologous platelet scanning in one patient showed hepatic sequestration of platelets and a decreased platelet half-life. Consistent with these findings, uptake of patient platelets by THP-1 macrophages and HepG2 liver cells was increased up to 6-fold. Uptake of desialylated platelets by THP-1 macrophages depended on GPIbα and the presence of αMβ2 on macrophages. Patient platelets, as well as sialidase treated platelets, showed substantial GPIbα clustering. Preventing GPIbα clustering in desialylated platelet attenuated platelet uptake by THP-1 macrophages. GPIbα was not involved in uptake by HepG2 liver cells. Platelet count in one patient increased to 50G/L when thrombopoiesis was enhanced with romiplostim.

Conclusions: GNE-related thrombocytopenia is characterized by increased hepatic platelet clearance, largely attributable to clustering of the hyposialylated GPIbα receptor

OC 16.2 | Gray Platelet Syndrome: More than an Inherited Bleeding Disorder

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[1]*University of Cambridge, Haematology, Cambridge, United Kingdom*, [2]*University College London, London, United Kingdom*

Background: Gray platelet syndrome (GPS) is an autosomal recessive thrombocytopenia characterised by a deficiency in platelet α-granules caused by biallelic loss of function variants in *NBEAL2*. Fewer than 100 cases have been reported worldwide, which makes defining the clinicopathological manifestations challenging.
Aims: To improve diagnosis and treatment by investigating a large, international cohort of GPS patients.
Methods: An international collaboration through the NIHR BioResource enrolled a cohort of 45 patients from 19 hospitals across 11 countries. Detailed clinical phenotype data, DNA and RNA sequencing, epigenetic profiling, and proteomics were used to characterise GPS on a clinical, cellular and molecular level. Additionally, the *Nbeal2*$^{-/-}$ mouse was used to study the clinical manifestations associated with age, including quantification of emperipolesis and myelofibrosis.
Results: The cohort includes 28 females and 17 males, with a mean age at presentation of 15 years (range from 2 months to 67 years). We identified 37 novel GPS-causing variants in *NBEAL2*. Bleeding symptoms vary in severity, B12 levels are elevated in 90% of cases, 1/3 of patients have myelofibrosis and nearly 25% have an autoimmune disease. Transcriptomic and epigenetic profiling of CD4+ T lymphocytes, monocytes, neutrophils and platelets showed >100 differentially expressed genes per cell type, which were enriched in gene ontology terms related to granular processes and innate immunity. This aligns with previously published studies in *Nbeal2*$^{-/-}$ mice, where neutrophils, mast cells and NK cells showed granule abnormalities and megakaryocytes revealed a pro-inflammatory state. Furthermore, the plasma proteome featured discriminatory proteins involved in canonical pro-inflammatory pathways.
Conclusions: The absence of functional Nbeal2 results in significantly different cellular profiles for lymphocytes, neutrophils and monocytes correlated with a pro-inflammatory plasma proteome and a wide spectrum of autoimmune diseases. The clinicopathological complexity of GPS reaches far beyond bleeding and this cohort provides unique opportunities to define the long-term consequences of this severe rare inherited condition.

OC 16.3 | Whole-genome Sequencing of Rare Disease Patients in a National Healthcare System

E. Turro; NIHR BioResource

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Background: Most patients with hereditary rare diseases do not receive a molecular diagnosis and the aetiological variants and mediating genes for half such disorders remain to be discovered.
Aims: To streamline diagnosis and to discover unknown aetiological variants, in the coding and non-coding regions of the genome.
Methods: In a pilot study for the 100,000 Genomes Project, we generated whole-genome sequencing (WGS) data for 13,037 participants, of whom 9,802 had a rare disease, including 1,169 patients with a bleeding, thrombotic or platelet disorder (BTPD).
Results: We provided a genetic diagnosis to 1,040 of the 7,065 patients with detailed phenotypic data, of whom 148 had a BTPD. Over 60% of the solved BTPD cases had causal variants in only 7 genes (*MYH9*, *ANKRD26*, *ACTN1*, *GP1BB*, *RUNX1*, *RNU4ATAC*, *CYCS*). By examining WGS read alignments for patients with complete absence of a protein encoded by a diagnostic-grade gene but carrying an explanatory variant call on only one haplotype, we identified causal complex variants in intron 9 of *ITGB3*: a tandem repeat and an SVA retrotransposon in two patients with Glanzmann thrombasthenia. We identified 99 Mendelian associations between genes and rare diseases, of which at least 80 are confirmed aetiological and of which 17 are associated with a BTPD. Finally, we reported novel non-coding variants causing disease through the disruption of transcription: a hemizygous deletion of a *GATA1* enhancer and *HDAC6* in a 9-year old boy with a new syndrome of autism and macrothrombocytopenia; and a regulatory SNV that, together with deletion of exon 10 of *MPL*, reduced MPL expression to pathological levels in a 10-year old thrombocytopenic boy with a marrow that was only moderately depleted of megakaryocytes.
Conclusions: Our study demonstrates a synergy by using WGS for diagnosis and aetiological discovery in routine healthcare, and demonstrates the role of non-coding regulatory variants in BTPDs.

OC 16.4 | Rare Variants in IKAROS Family Zinc Finger 5 (IKZF5) Are Responsible for Autosomal Dominant Thrombocytopenia

K. Freson; on behalf of the NIHR BioResource and the UK 100,000 Genomes Project

University of Leuven, Cardiovascular Sciences, Center for Molecular and Vascular Biology, Leuven, Belgium

Background: In the 100,000 Genomes pilot study, we recruited 13,037 participants into 15 rare disease domains, including 1,169

bleeding and platelet disorder (BPD) patients enrolled by the NIHR BioResource.

Aims: The 100,000 Genomes Project aimed to identify DNA variants underlying unresolved Mendelian disorders using whole genome sequencing.

Methods: Using the statistical genetic association method BeviMed, we identified a genetic association between IKZF5 and thrombocytopenia with a posterior probability of 0.748.

Results: The association signal was due to rare missense variants in four probands of European ancestry. We next analyzed a separate internal whole exome sequencing BPD dataset and identified two probands with thrombocytopenia carrying rare missense variants in IKZF5. All variants were unobserved in 125,748 samples from gnomAD. IKZF5 belongs to the Ikaros transcription factor (TF) family containing five zinc fingers (Znf) and is ubiquitously transcribed in haematopoiesis but of unknown function. Five of the six variants which were located in or near a Znf and were evolutionarily conserved. Co-segregation studies showed that two variants in two probands occurred de novo and in the remaining three pedigrees we identified eight relatives with thrombocytopenia who carried the IKZF5 variant. A canonical DNA-Znf binding model predicts that these missense variants alter DNA recognition. Expression studies using myc-tagged IKZF5 showed nuclear entry of wild type TF while mutants remain in the cytoplasm. Platelets from affected cases appeared normal on blood smears, but electron microscopy showed a reduced number of alpha-granules in normal-sized platelets. Proplatelet formation was reduced in megakaryocytes from seven affected cases compared to six controls. RNA sequencing from platelets, monocytes, neutrophils and CD4-T cells from three affected cases and twelve controls showed hundreds of differentially expressed transcripts for platelets in contrast to only subtle changes in the transcriptomes of the other types of blood cells.

Conclusions: IKZF5 is a novel TF regulating transcription during platelet formation. :

OC 16.5 | A Novel F153S ITGB3 Mutation Found in a Glanzmann Thrombasthenia Patient Reveals a Structural Clasp that Modulates Integrin Activation

S.B. Koukouritaki[1,2]; A.M. Myat Thinn[3,4]; K.J. Ashworth[5]; J. Fang[1,2]; H.S. Slater[1,2]; L.M. Du[1,2]; A.T. Nurden[6,7]; J. Di Paola[5]; J. Zhu[3,4]; D.A. Wilcox[1,2,3]

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Background: We report the characterization of a constitutively active form of αIIbβ3 due to a novel F153S *(F179S including the signal peptide)* mutation in the βI-Domain of the integrin β3 subunit discovered in a patient affected with a mild variant form of the inherited platelet bleeding disorder, Glanzmann Thrombasthenia.

Aims: The F153S mutation was characterized as a homozygous T556C nucleotide substitution detected in exon 4 of *ITGB3* of platelet cDNA by Sanger sequencing. Alignment analysis shows that F153 is completely conserved among β3-integrin subunits from several species and among all human β-integrin subunits suggesting that this amino acid may play an essential role in maintaining proper integrin structure.

Methods: To test this hypothesis, mutagenesis expression analysis of F153 was performed with immortalized cell lines co-transfected with αIIb and different forms of β3 (F, W, Y, A, and S)153.

Results: Our results demonstrate that co-expression in HEK293FT cells of αIIb with β3 encoding amino acids with bulky side chains (F, W, Y)153 were expressed normally in the resting state and αIIbβ3 became activated only by co-transfection of the talin-head domain *(that initiates inside-out integrin activation)*. In contrast, β3 mutants with amino acids containing small side-chains (S, A)153 rendered integrin αIIbβ3 constitutively active on the cell surface and capable of binding the activation-dependent IgM monoclonal antibody, PAC1 in the absence of extracellular activation. In silico modeling suggests that substitution of small amino acids at F153 facilitates an unhindered inward movement of the α2- and α1-helixes of the βI-domain leading to the constitutively active αIIbβ3 conformation.

Conclusions: Thus, this work establishes that a single amino acid F153 within the βI-domain of integrin β3 serves a vital structural role as a "Clasp" to keep an integrin complex in a normal resting state and its disruption has pathological consequences for integrin and platelet function.

OC 26.1 | Familial Immune Thrombocytopenia (ITP) and Hereditary Persistence of Fetal Haemoglobin (HPFH) Associated with a Pathogenic Variant of *MYB*

I.M. Morison[1]; B.M. Zhang[2]; S. Cross[3]; R.J. Weeks[1]; J.L. Ludgate[1]; U. Khatoon[4]; D. Fisk[2]; B. Lo[5]; J.L. Zehnder[2]

[1]University of Otago, Dunedin, New Zealand, [2]Stanford University School of Medicine, Stanford, United States, [3]Canterbury District Health Board, Christchurch, New Zealand, [4]University of Auckland, Auckland, New Zealand, [5]Sidra Medical and Research Center, Al Rayyan, Qatar

Background: Familial ITP, which is extremely rare, has the potential to reveal causative mechanisms of disease.

Aims: A four generation family with incompletely penetrant autosomal dominant ITP affecting six individuals was documented (Figure 1). Affected family members presented at the ages of 1 to 47 years (mean 19 years) with severe thrombocytopenia that was responsive to intravenous immunoglobulin or immunosuppressive therapy and usually had relapsing disease. The aim was to identify

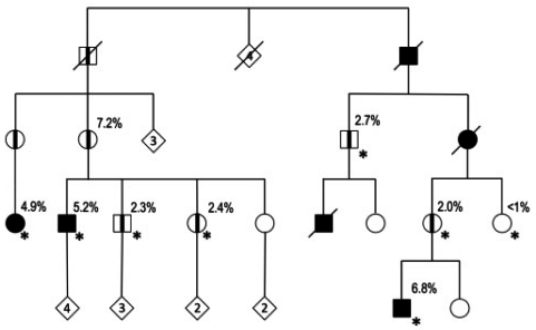

Figure 1. Immune thrombocytopenia pedigree. Filled symbols show affected individuals. Vertical bars show unaffected carriers (obligate or sequenced). Astericks show sequenced individuals. The percentage figures show HbF levels. To enhance anonymity, the pedigree been altered without affecting the quality of the genetic associations.

FIGURE 1

the causative genetic variant and to determine the mechanism by which the variant predisposes to immune destruction of platelets.

Methods: Whole genome sequencing and targeted sequencing were used to identify and confirm the variant and its effect on RNA splicing. The study was approved by the University of Otago Human Research Ethics Committee and all participants gave informed consent.

Results: A pathogenic variant affecting the canonical splice donor site in intron 12 of *MYB* was identified in three distantly related affected individuals and in four unaffected individuals. Affected and unaffected carriers of the *MYB* variant had HbF levels of 2.0-7.2% (mean 4.2%; normal < 1%). The mean platelet count of unaffected carriers was 278 x 10(9)/L (normal 150-400). Amplification of peripheral bood cDNA showed alternative splicing: reference and alternatively spliced transcripts from exons 12 to 13 were present in equal amounts. The alternative transcript potentially codes for a protein in which the final 111 amino acids are replaced with 52 alternative residues. Collation of genome-wide association study results shows a strong association of the *MYB* locus with HbF and other red cell, platelet and granulocyte parameters, but very few, low probability associations with immune-mediated diseases.

Conclusions: Familial ITP, with associated HPFH, was attributable to a pathogenic variant of *MYB*. The absence of association of the *MYB* locus with other autoimmune diseases suggests that the familial predisposition to ITP is driven by platelet factors.

OC 26.2 | Receptor Editing Deficiency Causes B-Cell Central Intolerance and Autoantibodies Accumulation in Primary Immune Thrombocytopenia

S. Wang[1]; Z. Sheng[1]; Y. Gao[1]; M. Hou[1]; S. Li[2]; J. Peng[1]

[1]*Qilu Hospital, Shandong University, Department of Hematology, Jinan, China,*
[2]*Qilu Hospital, Shandong University, Department of Oncology, Jinan, China*

Background: The antibody-mediated platelet destruction is the most important pathogenic mechanism in primary immune

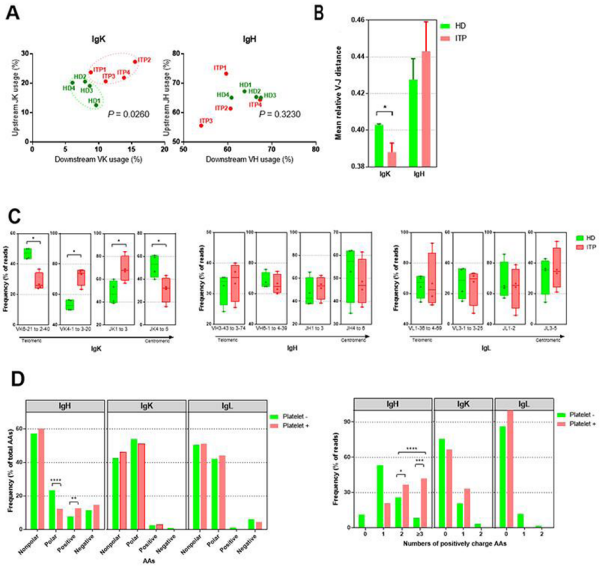

FIGURE 1 Sequence features of the Ig repertoire in ITP peripheral naive B cells

thrombocytopenia (ITP). Receptor editing is a process to change the specificity of immature B cell receptors (BCRs), which is the main part of B-cell central tolerance.

Aims: To investigate the generation of anti-platelet antibodies and the status of B-cell central tolerance in ITP.

Methods: We performed next-generation sequencing on peripheral B cells of ITP patients and healthy controls. We conducted single cell RT-PCR on naïve B cells to clone Ig heavy chain (IgH) and Ig light chain (IgL) genes from the same cells. The paired IgH and IgL were co-transfected and the recombinant monoclonal antibodies were expressed. The reactivity of these antibodies against platelet antigens was determined by ELISA, immunofluorescence, and flow cytometry. Ig gene sequence features were analyzed by IgBLAST and VHRFA-1.

Results: The deep sequencing and the PCR products revealed that the ITP-derived Igκ preferentially used the downstream Vκ and upstream Jκ segments compared with healthy controls, which indicated

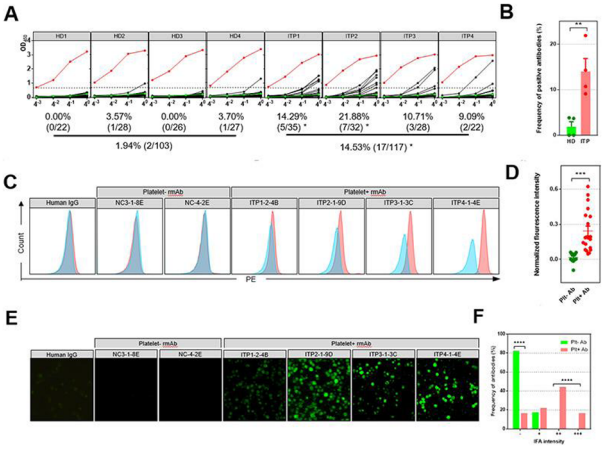

FIGURE 2 Platelet-reactivity of the rmAbs derived from ITP naive B cells

a defective receptor editing of B cells in ITP patients (Fig. 1A-C). Utilizing single cell RT-PCR, 220 recombinant antibodies were cloned derived from naïve B cells in 4 ITP patients and 4 healthy controls. ELISA showed that 14.53% of the ITP-derived naïve B cells were platelet-reactive, in contrast to 1.94% in those of healthy donors, suggesting a defective B-cell tolerance in the early developing stage (Fig. 2). The anti-platelet reactivity was confirmed by immunofluorescence and flow cytometry. Sequence analysis revealed that positively-charged amino acids were highly enriched in the complementarity determining region 3 of the anti-platelet antibodies (Fig. 1D), which possibly interacted with the negatively-charged glycoproteins.

Conclusions: In summary, this study provides evidence of defective early B-cell tolerance and accumulation of anti-platelet naïve B cells in ITP. This defect might be a result of insufficient receptor editing in IgL.

OC 26.3 | Interleukin-37 Reduces Inflammation and Impairs Phagocytosis of Platelets in Immune Thrombocytopenia

G. Li; Y. Zhao; X. Ni; X. Liu; J. Peng; M. Hou

Qilu Hospital, Shandong University, Jinan, China

Background: Immune thrombocytopenia (ITP) is an autoimmune and inflammatory disease characterized by low platelet count with heterogeneous bleeding manifestations. Severe bleeding in ITP is not completely related with low platelet count. Interleukin-37 (IL-37) is an anti-inflammatory cytokine that participates in the process of several inflammatory and autoimmune diseases. However, the role of IL-37 in the pathogenesis of ITP is unknown.

Aims: Our study aimed to evaluate the regulatory role of IL-37 in the process of ITP and its association with disease severity.

Methods: Plasma IL-37 was detected by enzyme-linked immunosorbent assay (ELISA). We cultured the monocytes/macrophages from ITP patients to investigate the regulatory role of IL-37 on monocytes/macrophages. Fcγ receptors (FcγRs) of macrophages were analyzed using flow cytometry and q-PCR. Signaling pathways were determined by western blotting. Phagocytic capacity of macrophages was measured by the engulfment of opsonized platelets.

Results: IL-37 expression was elevated in ITP patients, which was correlated with platelet count and the severity of bleeding in ITP according to the ITP Bleeding Scale (IBLS) system, indicating that IL-37 could be a candidate in evaluating disease severity of ITP. IL-37 initiated an anti-inflammatory effect on monocytes/macrophages from ITP patients by down-regulating the phosphorylation of MAPK, AKT, and NF-κB signaling pathways. Moreover, IL-37 restored the balance of activating and inhibitory FcγRs and decreased antibody-mediated platelet phagocytosis by monocytes/macrophages, suggesting that IL-37 might be a potential therapeutic agent in ITP.

Conclusions: Our study demonstrated that ITP patients exhibited higher IL-37 expression, especially patients with severe hemorrhage or low platelet count. IL-37 decreased antibody-mediated platelet phagocytosis, restored the balance of activating and inhibitory FcγRs, and inhibited inflammatory activity via MAPK, AKT, and NF-κB signaling pathways among monocytes/macrophages. Therefore, IL-37 might be a candidate in evaluating the severity of ITP and a promising therapeutic agent for the management of ITP.

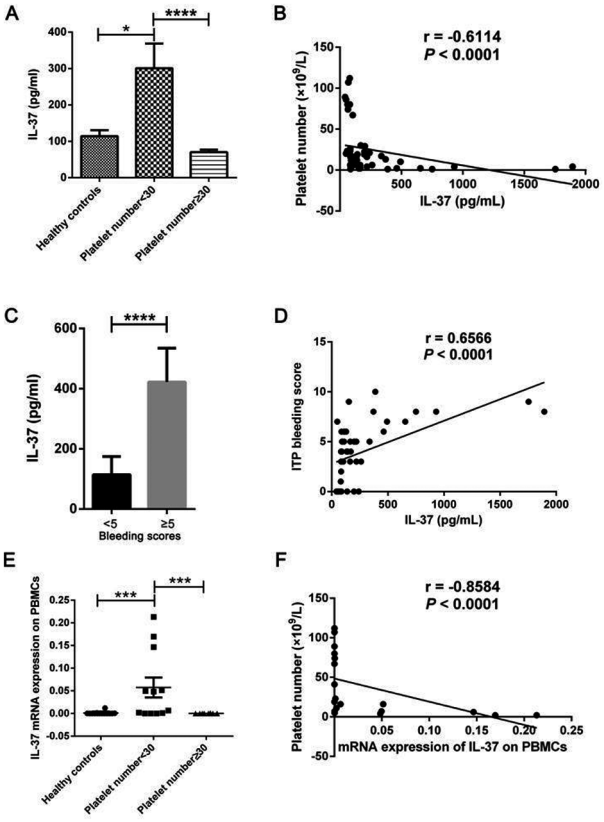

FIGURE 1 IL-37 expression is elevated in active ITP patients

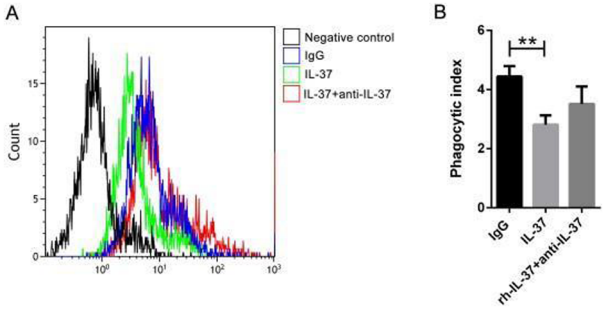

FIGURE 2 IL-37 treatment decreases phagocytic capacity of monocytes/macrophages

OC 26.4 | Autoantibody Mediated Changes in Platelets´ Glycan Pattern: Potential Impact on Platelet Function and Lifespan in Immune Thrombocytopenia

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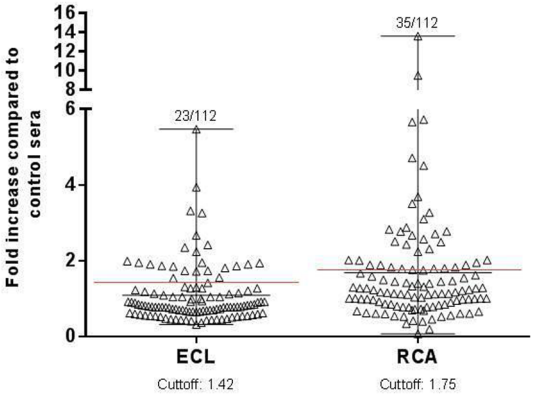
Background: Desialylation, the loss of sialic acid content on platelets (PLTs) glycoproteins (GPs) was recently identified to contribute in immune thrombocytopenia (ITP). However, the potential impact of autoantibodies (AAbs) on megakaryocyte (MK) sialylation remains unclear.

Aims: To investigate the effect of ITP AAbs on PLTs and MKs sialylation and the subsequent impact on PLT survival.

Methods: AAb mediated GP-modifications were tested using a lectin binding assay. After incubation of MKs or PLTs with ITP or control sera, glycan changes were analyzed by flow cytometry (FC). To investigate the impact of desialylation on PLTs life-span, the NOD/SCID mouse model was used.

Results: 112 ITP sera were investigated in this study. 35 (31%) sera induced a significant increase in RCA signal on PLTs surface compared to control sera from healthy donors (RCA-mean fold increase (RCA-FI): 3.23, range: 1.76-13.61, p=0.0001). In addition, 23 (21%) sera caused higher ECL binding to test PLTs (ECL-FI: 2,31, range: 1,54-5,47, p=0.0001). Injection of desialylating AAbs resulted in accelerated clearance of human PLTs from the circulation of the NOD/SCID mice which was significantly reduced by a specific neuraminidase inhibitor that prevents desialylation on the PLT surface (survival of human PLTs after 5h: 29%, range 22-40% vs. 48%, range 41-53%, p=0.014, respectively). Most interestingly, a subgroup of ITP sera induced desialylation on MKs surface. In particular, 8 out of 13 (62%) induced a significant increase in RCA signal on MK surface (mean RCA-FI: 2.19, range: 1.15-3.66, p=0.01); and 10 out of 13 (77%) sera with PLT-desialylating AAbs increase ECL binding (mean ECL-FI: 1.29, range: 1.01-1.7, p=0.005).

Conclusions: Our findings suggest that ITP AAbs of different specificities are able to induce desialylation on PLTs as well as on MKs which seems to result in an impairment of function and contribute to PLT destruction in vivo.



A subset of human ITP AAbs induce significant changes in the glycan pattern of PLTs from healthy donors.

Lectins: ECL, Erythrina cristagalli lectine targeting N-Acetylglucosamine; RCA, Ricinus communis agglutinin targeting β-Galactose

FIGURE 1

OC 26.5 | TNF-α Blockade Corrects the Monocyte/Macrophage Imbalance in Primary Immune Thrombocytopenia (ITP)

Y. Zhao; X. Liu; J. Peng; M. Hou

Shandong University, Department of Hematology, Jinan, China

Background: Primary immune thrombocytopenia (ITP) is an acquired autoimmune bleeding disorder. Deviation of monocyte/macrophage subsets plays an important role in the pathogenesis of ITP. TNF-α blockade is widely used in many autoimmune diseases with satisfactory effects. However, the effect of TNF-α blockade on monocyte/macrophage system in ITP remains unknown.

Aims: Our study aimed to investigate whether TNF-α could restore the disturbed balance of monocyte/macrophage subsets and be a potential therapeutic strategy for ITP.

Methods: Plasma TNF-α was detected by ELISA. The monocyte/macrophage subsets were analyzed using flow cytometry and immune fluorescence. Macrophage polarization was analyzed using western blot, q-PCR, and flow cytometry. Phagocytic capacity of macrophages was measured by the engulfment of opsonized platelets. T-cell priming ability of macrophages was determined through co-culturing with $CD4^+$ T cells and $CD8^+$ T cells. We established a passive model of murine ITP. Platelet count was measured every other day. The retention of platelets in liver and spleen was assessed by the fluorescence intensity detected using the in vivo imaging system.

Results: ITP patients manifested with an increased TNF-α expression which was inversely correlated with platelet count. Moreover, the numbers of TNF-α-expressing macrophages in spleen and $CD16^+$ monocytes were more than that of healthy controls. TNF-α blockade inhibited M1 macrophage polarization by suppressing the NF-κB signaling pathway, and dampened the phagocytic capacity and T-cell priming ability of macrophages. Moreover, anti-TNF-α therapy reduced the number of non-classical monocytes and M1 macrophages, ameliorated the retention of platelets in spleen and liver, and significantly increased the platelet count of ITP mice.

Conclusions: Our study demonstrated that TNF-α blockade could decrease the number and functions of pro-inflammatory subsets of monocytes/macrophages by inhibiting NF-κB signaling pathway, leading to remarkable attenuation of antibody-mediated platelet destruction both *in vitro* and *in vivo*, thus might be a promising therapeutic strategy for the management of ITP.

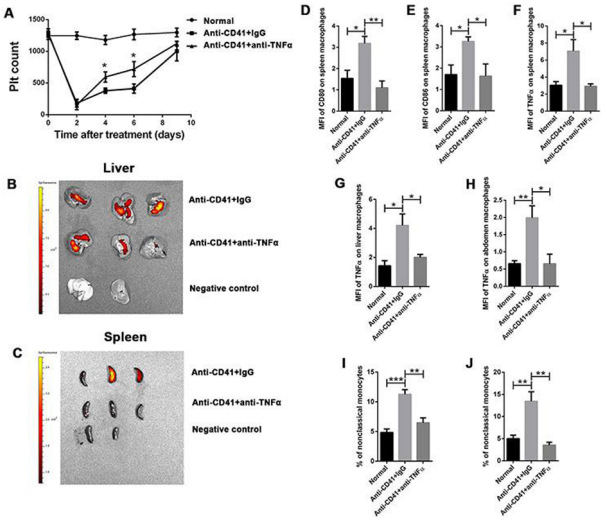


FIGURE 1 TNF-α blockade ameliorated thrombocytopenia in ITP mouse model

OC 36.1 | Prospective Validation of a Rapid and Accurate Diagnostic Algorithm for Heparin-induced Thrombocytopenia

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Background: Heparin-induced thrombocytopenia (HIT) carries a high mortality and morbidity if left untreated, while switching unnecessarily to alternative anticoagulants leads to bleeding complications and increased costs. Clinical suspicion of HIT therefore requires rapid laboratory assessment to guide clinical management.

Aims: To develop a diagnostic algorithm able to accurately confirm or exclude HIT with a short laboratory turn-around-time (TAT).

Methods: Based on our previous evaluation study (05.2014-12.2016, n=526), we developed an in-house diagnostic algorithm that relies on the Bayesian combination of pre-test clinical probability (4T-score) and quantitative results of sequential rapid immunoassays (IAs) for anti-PF4/heparin antibodies (AcuStar HIT-IgG and PaGIA-H/PF4) in order to predict the outcome of the functional gold-standard assay (HIPA). We are prospectively validating this algorithm (01.2017-01.2019, n=430).

Results: 100% negative (NPV) and positive (PPV) predictive cut-off values for a positive HIPA were set at AcuStar HIT-IgG values of < 0.13 U/ml and >3.0 U/ml, respectively. For H/PF4-PaGIA the cut-off titers were < 2 and >8, respectively. Likelihood ratios were determined for intermediate results. During the prospective validation study, AcuStar HIT-IgG was employed as a single IA in 345/430 (80%) of cases (TAT 30 min); H/PF4-PaGIA was performed as a second line IA in 85/430 (20%) of initially unsolved cases (TAT 60 min). Our Bayesian diagnostic approach could exclude HIT in 380/430 (88.3%) and correctly predict it in 31/430 (7.2%) of patients with suspected HIT, leaving thus only 14/430 (3.3%) of unsolved cases after 60 minutes. In 5 cases (1.2%), the positive prediction of our algorithm was not confirmed by HIPA. However, laboratory work-ups and clinical evolutions were very suggestive of HIT.

Conclusions: The combination of the estimated clinical probability of HIT and the sequential application of two rapid IAs for anti-PF4/heparin antibodies enables a rapid and accurate diagnostic work-up within 60 minutes for up to 97% of patients with suspected HIT.

OC 36.2 | Thrombosis in Heparin-induced Thrombocytopenia Is Mediated by Neutrophils

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Background: Heparin therapy may induce an immune reaction, termed heparin-induced thrombocytopenia (HIT), in which platelets are activated via FcϒRIIa receptors leading to thrombocytopenia and severe thrombosis. Neutrophil extracellular traps (NETs) are DNA structures released by neutrophils in response to diverse stimuli. The involvement of NETs in HIT is yet to be fully explored.

Aims: To determine if HIT immune complexes induce NETs formation and to evaluate the role of NETs in the pathogenesis of HIT.

Methods: The study was approved by the institutional Human and Animal Ethics Committees. Sera were collected with informed consent from HIT patients and healthy controls. HIT was reconstituted *ex vivo* in a microfluidics system and *in vivo* in FcϒRIIa^{+}/hPF4^{+} transgenic mice. NETs formation was evaluated by detection of extracellular DNA, citrullinated histone H3 and myeloperoxidase using confocal microscopy, Western blotting and flow cytometry. Thrombi were quantified by MicroCT scanning and immunohistochemistry. HIT in the absence of NETosis was evaluated with NETs inhibitors and in NETs-deficient mice (hFcϒRIIa^{+}/hPF4^{+}/PAD4$^{-/-}$).

Results: NETs markers are present in HIT patients' sera and neutrophils. HIT immune complexes activate neutrophils via FcγRIIa and through platelets/neutrophil interactions mediated by P-selectin/PSGL-1 and induce NETosis. *Ex vivo*, HIT thrombi are rich in platelets, neutrophils, extracellular DNA and citrullinated histone 3. Lack of neutrophils prevents thrombus formation. Conversely neutrophil reconstitution restores thrombus deposition. Purified neutrophils treated with HIT IgG formed thrombi containing NETs. HIT mice demonstrated markers of NETosis in plasma and in thrombi. Treatment of HIT mice with DNase I or the

NETs inhibitor GSK484 led to a substantial decrease in thrombosis. Importantly, no thrombi were detected in hFcϒRIIa^{+}/hPF4^{+}/PAD4$^{-/-}$ mice.

Conclusions: HIT immune complexes induce NETs formation and this is essential for the development of thrombosis. These observations suggest a new concept of the pathogenesis of thrombosis in HIT and as such are clinically relevant.

OC 36.3 | Dissecting the Mechanisms of Thrombocytopenia and Thrombosis in HIT

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Background: Heparin induced thrombocytopenia (HIT) is a life-threatening complication to heparin therapy caused by an antibody that recognizes the heparin-platelet factor 4 complex. It is currently believed that this immune complex binds to and activates platelets, resulting in thrombocytopenia and thrombosis. We propose that thrombocytopenia and thrombosis are distinct events governed by independent pathways.

Aims: To uncover the mechanisms that lead to thrombocytopenia and paradoxical thrombosis in HIT.

Methods: For in vitro and ex vivo experiments, blood from healthy donors was treated with IgG isolated from HIT patients and heparin. For in vivo experiments, FcγRIIa/PF4 mice were injected with heparin and HIT IgG to recapitulate the HIT condition. Cells of interest were isolated and/or depleted from whole blood for various experiments by MACS or cell-specific antibodies. Cell activation was inhibited pharmacologically or by using FcγRIIa/PF4/PAD4 knockout mice. Confocal microscopy, flow cytometry, microfluidics experiments and mouse imaging systems were used to analyse cell activation, clot formation and platelet counts. This study was approved by local Human Research Ethics Committee and UNSW Animal Care and Ethics Committee.

Results: In the presence of the FcγRIIa blocking antibody agIV.3, transgenic mice were protected from thrombocytopenia and thrombosis. When platelets were depleted in vivo, fibrin-rich clots were formed suggesting that platelets are not essential for the thrombotic process. In the absence of platelets, the HIT complex directly binds to and activates neutrophils via the FcγRIIa receptor expressed on neutrophils, resulting in NETosis. Depleting neutrophils and/or blocking NETosis abolished thrombus formation, however, showed no improvement in thrombocytopenia.

Conclusions: In contrast to current understanding, we have shown that thrombocytopenia and thrombosis are due to independent processes. Neutrophils and NETosis are the key drivers of thrombosis in HIT and do not require platelets for clot formation. Targeting neutrophils directly and inhibiting their activation is required for the treatment of thrombosis in HIT patients.

OC 36.4 | Low-dose Argatroban Use in Child-Pugh B and C Chronic Liver Disease with Suspected Heparin-induced Thrombocytopenia at King's College Hospital

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Background: Argatroban is used in the treatment of heparin-induced thrombocytopenia (HIT). It is contra-indicated in severe liver disease, however, a previous case report describes low-dose argatroban use in Child-Pugh C. Since November 2014, King's College Hospital has

TABLE 1 Argatroban Dosing Protocol

Clinical category		Severe hepatic impairment	Critically ill	Other
Definition		Child-Pugh C, bilirubin >25µmol/L, ascites, or elevated PT/INR in the absence of warfarin	Critical care patients, Child-Pugh B, post cardiac surgery	Standard patients with no significant organ impairment
Recommended Starting Dose		0.2 mcg/kg/min	0.5 mcg/kg/min	1 mcg/kg/min
Dose Adjustment Schedule as per APTT(s)/ Baseline APTT	<1.5	Increase by 0.05 mcg/kg/min	Increase by 0.1 mcg/kg/min	Increase by 0.2 mcg/kg/min
	1.5-2.5	Maintain infusion rate	Maintain infusion rate	Maintain infusion rate
	2.5-3.0	Reduce by 0.05 mcg/kg/min	Reduce by 0.1 mcg/kg/min	Reduce by 0.2 mcg/kg/min
	>3 (or APTT >100 s)	Stop infusion Check APTT every 2 h until 1.5-3 x baseline Restart at 50% reduced rate and recheck APTT within 2 h	Stop infusion Check APTT every 2 h until 1.5-3 x baseline Restart at 50% reduced rate and recheck APTT within 2 h	Stop infusion Check APTT every 2 h until 1.5-3 x baseline Restart at 50% reduced rate and recheck APTT within 2 h

TABLE 2 Results of argatroban use in Child-Pugh B and C Liver Disease

Clinical Category of Liver Disease	Child-Pugh B		Child-Pugh C	
Starting argatroban dose prescribed (mcg/kg/min)	0.5 (n =1) 0.65 (n=1) 0.2 (n=3)		0.2 (n=3) 0.02 (n=1) 0.25 (n=1) 0.5 (n=1)	
Mean infusion rate (mcg/kg/min) [SD] required to reach 'steady state'	**0.59 [0.09]**		**0.21 [0.17]**	
Dose adjustments required to reach steady state according to starting dose for each patient	**Starting Dose**	**Achieved steady state with <4 dose adjustments?**	**Starting Dose**	**Achieved steady state with <4 dose adjustments?**
	0.5 0.65 0.2	Yes Yes No	0.02 0.2 (n=2) 0.2 (n=1) 0.25 0.5	Yes Yes No Yes Yes
Safety & efficacy at 30 days	No cases of recurrent/progressive thrombosis 1 minor bleeding complications 2 major bleeding complications 4 deaths likely unrelated to argatroban			

used low-dose argatroban in severe hepatic impairment as per the protocol summarized in *Table 1*.

Aims: To determine the safety and effectiveness of our dosing protocol in patients with Child-Pugh B and C chronic liver disease.

Methods: A retrospective case note review was conducted for patients who received argatroban for suspected HIT between 1st January and 31st December 2017. APTT results, argatroban dosing and clinical progress were reviewed for each patient up to 7 days following commencement of argatroban.

Results: 26 patients received argatroban for suspected HIT. The records of 20 patients were available, of which 5 had Child-Pugh B liver disease and 6 had Child-Pugh C. Of these 11 patients, the mean age was 55 years (range 28 to 77). 8/11 (73%) were male. Further results are summarized in Table 2.

Of the 5 Child-Pugh B patients, the 3 who started at lower doses than protocol needed more >4 dose adjustments to achieve steady state. Of the 6 Child-Pugh C patients, 3 had starting doses as per protocol. One had previously received argatroban and was found to be extremely sensitive; hence commenced at 0.02 mcg/kg/min.

Conclusions: To the best of our knowledge, this is the first case series reporting argatroban use in severe liver disease and suggests that low doses of argatroban can be safely used. Encouragingly, mean steady state infusion rates were very close to the starting rates suggested by our protocol.

OC 36.5 | 5B9, a Useful Tool for Evaluating the Performances of Platelet Function Tests for the Diagnosis of Heparin-induced Thrombocytopenia

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Background: Serotonin Release Assay (SRA) is the "gold standard" to detect pathogenic heparin-induced thrombocytopenia (HIT) antibodies. However, this method is time-consuming, expensive and necessitates the use of ^{14}C-radio-labelled serotonin, explaining that an alternative method is mandatory. Heparin-Induced Multiple Electrode Aggregometry (HIMEA), Light Transmission Aggregometry (LTA) using platelet rich plasma (PRP) or washed platelet (WP), ATP release, and Flow Cytometry (FC) are potential alternative options.

Aims: We aimed to evaluate the sensitivity of these 5 functional methods for the detection of HIT antibodies in comparison with SRA, using 5B9, a monoclonal IgG which fully mimics HIT antibodies.

Methods: Platelet activation induced by 5B9 (50, 20, 10µg/mL) without, with "therapeutic" or high concentrations of unfractionated heparin was assessed by each of the following methods with blood samples from 20 unselected healthy donors: 1- HIMEA (whole blood) 2- LTA (PRP)3- FC based on the assessment of P-selectin expression (PRP) 4- ATP release (WP) 5- LTA (WP) 6- SRA (WP)

TABLE 1 Comparative sensitivity of SRA and 5 other assays for detecting heparin-dependent platelet activation induced by 5B9

Sensitivity (%)	5B9 50 µg/mL	5B9 20 µg/mL	5B9 10 µg/mL
SRA	100	100	95
HIMEA	100	100	65
Flow Cytometry	90	75	40
ATP release	100	70	30
LTA with WP	100	70	30
LTA with PRP	100	65	5

SRA: Serotonin release assay; HIMEA: Heparin induced multiple electrode aggregometry; ATP release: adenosine triphosphate release; LTA: Light transmission aggregometry; WP: washed platelets; PRP: platelet rich plasma

Results: All method exhibited a good sensitivity for detecting the highest concentration of 5B9 (50 µg/mL). Only SRA and HIMEA were positive in 20/20 donors when testing 5B9 20 µg/mL, followed by FC, positive in 3/4 donors. SRA was the only method able to detect platelet activation induced by 10 µg/mL 5B9 with all donors tested except one, and the other methods were less sensitive (table).
Conclusions: These results confirm that 5B9 is a very efficient tool to assess the performances of platelet function tests for the diagnosis of HIT. SRA is likely the more sensitive functional assay to detect low concentrations of HIT antibodies. HIMEA or FC, which are rapid assays, exhibit a better sensitivity than LTA or ATP release, and could be potential alternatives to SRA. 5B9 deserves to be evaluated in a multicenter study as a quality control.

OC 52.1 | Feasibility of a New, Rapid Functional Assay for the Diagnosis of Heparin-induced Thrombocytopenia: A Standardized Flow Cytometry Assay

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Background: Washed platelet assays, which are regarded as gold-standard for the diagnosis of heparin-induced thrombocytopenia (HIT) are rarely available in a timely manner. A simple, standardized platelet flow cytometry assay was developed to overcome this important limitation.
Aims: To explore the diagnostic utility of a new, standardized platelet flow cytometry assay for the diagnosis of HIT in clinical practice.
Methods: Frozen serum samples of 103 consecutive patients, evaluated for suspected HIT at our institution in 2017, and characterized with 4Ts score, IgG-PF4/heparin ELISA (GTI), HemosIL®Acustar (IgG), as well as heparin-induced platelet activation test (HIPA), were further tested using a flow cytometry assay, determining P-selectin release of donor platelets after incubation with patient's serum (Emosis HIT Confirm®). Platelet-rich plasma of one donor was used, and patient's serum was heat-inactivated before determination. Diagnosis of HIT was defined as a positive HIPA result.
Results: HIT was confirmed in 15 out of 103 patients corresponding to a prevalence of 14.6%. HIT Confirm was positive in 11 patients (10.7%), negative in 88 patients (85.4%), and inconclusive in 4 patients (3.9%). Among patients with positive HIT Confirm, median OD of ELISA was 3.1 (range 0.2, 3.8), median Acustar 16.5 CU/ml (0, 128), and median 4Ts score 6 points (range 5, 7.5). Among patients with a negative HIT Confirm, median ELISA was 0.2 (0.1, 2.9), median Acustar 0 (0, 45.6), and median 4Ts score 3 (0, 6). Removing inconclusive results from the analysis, the number of true positives was 9, the number of true negatives 83, the number of false negatives was 5, the number of false positives 2. This corresponds to a sensitivity of 64.3%, and a specificity of 97.6%.
Conclusions: Proposed test can confirm HIT in most patient samples. Further test modifications such as increasing the number of platelet donors might improve sensitivity.

OC 52.2 | FcγRIIa Polymorphisms and Allele-Specific Haplotypes in Heparin-Induced Thrombocytopenia (HIT)

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Background: Polymorphisms of FcγRIIa (Q27W and H131R) may influence receptor function and are linked to susceptibility to infections or autoimmune diseases. FcγRIIa H131R polymorphism has been associated with increased thrombosis risk in HIT patients. Engagement of FcγRIIa or platelet-specific collagen receptor, glycoprotein (GP)VI, induces platelet activation and metalloproteinase-mediated ectodomain shedding of GPVI, generating soluble GPVI (sGPVI) in plasma. Combinations of these polymorphisms may influence platelet reactivity and potentially modulate the risk of HIT.
Aims: Assess haplotype-based association between FcγRIIa polymorphisms Q27W and H131R, and platelet reactivity and HIT susceptibility.
Methods: Genomic DNA was isolated from whole blood of healthy individuals (n=45), non-HIT thrombocytopenic patients (n=31) and patients sent for HIT testing (n=105, 17 confirmed HIT by serotonin-release assay). *FCGR2A* haplotypes were determined in phase by genomic DNA amplicon sequencing (PacBio Sequel platform). Plasma sGPVI was measured by ELISA.
Results: Homozygous 131HH (n=46) was associated with lower sGPVI levels in all cohorts (p=0.0088 compared to 131HR/RR, n=134). 15/17 (88%) of confirmed HIT patients carried at least one R allele at H131R versus 98/133 (74%) in non-HIT individuals. 90% of query-HIT patients who experienced a thrombotic event (n=20) carried an R allele. Phased haplotype analysis of *FCGR2A* revealed that 27W and 131R variants never co-occurred on the same allele (n=181). There was no apparent overrepresentation of haplotype combinations (Q|H, W|H, Q|R) among the confirmed HIT compared to non-HIT individuals (χ^2=2.264, df=2, p=0.3224, n=180).

Conclusions: Here, HIT occurrence was not significantly associated with a particular *FCGR2A* haplotype combination at positions 27 and 131. This haplotype combination could have a small effect size and should there be an associated phenotype, a larger cohort would be required to reach statistical significance. *FCGR2A* 131R is a potential risk allele for thrombosis and together with plasma sGPVI may help evaluate platelet reactivity in the presence of HIT antibody.

OC 52.3 | Anti Platelet Antibodies Predict Platelet Desialylation and Apoptosis in ITP

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Background: Immune thrombocytopenia (ITP) is a bleeding disorder caused by anti-platelet and anti-megakaryocyte antibodies. Certain antibodies induce platelet desialylation and platelet clearance by hepatocyte Ashwell-Morell receptors. In addition, autoantibody mediated platelet apoptosis has been demonstrated. Nevertheless, the association between platelet desialylation or apoptosis is unclear in ITP.

Aims: To 1) establish the proportion of ITP patients with platelet desialylation; 2) examine its association with platelet apoptosis; 3) determine if detectable anti-platelet antibodies predict platelet desialylation/apoptosis;4) study the effect of desialylation inhibition in a murine model of ITP using auto-anti IIbIIIa patient antibodies.

Methods: The study was approved by the institutional Human and Animal Ethics Committees. Sera were collected with informed consent from 60 ITP patients and 20 healthy controls. To examine the presence of detectable anti-platelet antibodies or induction of platelet desialylation and apoptosis, washed platelets were treated with patients' or controls' sera followed by incubation with anti-human IgG, RCA-1, anti-Neu1 antibody and DiOC6, respectively and analysed by flow cytometry. Finally, a nonobese diabetic/severe combined immunodeficient murine model of ITP was established to examine the therapeutic effect of oseltamivir in platelet preservation from auto-anti IIbIIIa antibody mediated platelet clearance.

Results: Sera from ITP patients with detectable antibodies caused significant platelet desialylation and apoptosis ($p< 0.05$, $p< 0.01$, $p< 0.001$ for RCA-1, NEU-1 and DiOC6 respectively; one-way ANOVA with post hoc Tukey's HSD). *In vivo* experiments demonstrated that neuraminidase inhibitor protected human platelets from auto-anti-IIbIIIa antibody induced clearance ($p< 0.01$ Mann Whitney test).

Conclusions: ITP patients with detectable anti-platelet antibodies are more susceptible to platelet desialylation and apoptosis. Neuraminidase inhibitor is likely to protect platelet removal in patients with auto-IIbIIIa antibodies. A collaborative clinical trial is warranted.

OC 52.4 | Clinical Association of Increased Antioxidant Enzymes Levels in Immune Thrombocytopenic Purpura

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Background: Immune thrombocytopenic purpura (ITP) is a disorder characterized by immune-mediated accelerated platelet destruction and suppressed platelet production. Increased Oxidative stress and reduced antioxidants levels may be a cause of this disease. Oxidative stress occurs as a result of increased activity of free radical-producing enzymes, decreased activity of free radical-removing enzymes, and insufficient levels of antioxidants.

Aims: Current study has been done to evaluate antioxidants levels in ITP patients and their possible association with occurrence of ITP.

Methods: This 1:1 case control study was conducted on 111 ITP patients selected at NIBD, Karachi from January 2016 to December 2017. CBC was performed by Sysmex XN-1000. Total antioxidant status (TAS), Superoxide dismutase (SOD) and Glutathione Reductase (GR) enzymes levels were determined using Randox® kits on Selectra Chemistry analyzer. Fisher Exact test was used to evaluate difference on SPSS version 23.

Results: The patients' age ranged from 9months to 83 years. All ITP patients were further divided into newly diagnosed/persistent (n=64) and chronic ITP (n=47) respectively. Mean platelets count was 32.9±35.1 (p=≤0.05), 39±39.2 (p=≤0.05) and 278±58.9 for newly diagnosed/persistent ITP, chronic ITP and controls. There was no significant difference of TAS in patients and controls but significantly increased levels of GR and SOD was observed with mean of 90.1±33.5 and 9818.5±525 in newly diagnosed/persistent ITP, 100.5±32.7 and 8486.9±498 in chronic ITP as compared to controls which were 68.4±11.3 and 757.4±425.5.

Conclusions: Oxidative stress may be one of the cause of ITP. Higher production depicts hyper-activity of anti-oxidant enzymes thereby confirming oxidative stressed status in ITP patients irrespective of their type of disease. Evaluation of oxidative stress and antioxidants levels can be helpful in understanding the pathogenesis of ITP.

OC 52.5 | Immune Phenotyping Predicts Prognosis and Treatment Response in Newly Diagnosed Childhood Immune Thrombocytopenia

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Background: A key clinical question in newly diagnosed childhood immune thrombocytopenia (ITP) is whether a child will have a transient disease course or will develop chronic ITP.

Aims: To investigate whether heterogeneity in leukocyte subpopulations at diagnosis is associated with spontaneous recovery and treatment response in children with newly diagnosed ITP.

Methods: Two hundred children with newly diagnosed ITP were randomized to a single dose intravenous immungobulins (IVIg) or careful observation and followed for one year (TIKI trial; Heitink-Pollé *Blood* 2018). Immune phenotyping was performed by flow cytometry on pre-treatment samples (N=159). Absolute and relative counts for total T, CD4, CD8, B and NK cells, regulatory T cell (Treg; $CD25^{+}/CD127^{low/neg}$) and T memory cell subsets (CD45RO and CD27 expression) were obtained. Complete recovery from ITP was assessed by platelets $\geq 100x10^{9}$/L. Cox proportional-hazard models were used for multivariate association analysis of cell frequencies and complete recovery.

Results: Recovery from ITP was considered at the same time for spontaneous recovery or after IVIg therapy by stratification for treatment effects. After adjustment for age, a lower CD4/CD8 ratio was associated with reduced recovery rates with a hazard ratio (HR) of 0.64 (95% confidence interval [CI], 0.46 - 0.89; P=0.009); this was driven by higher CD8 levels. Furthermore, an increased frequency of $CD45RO^{+}CD27^{-}$ effector CD4 T cells was associated with reduced recovery rates with a HR of 0.87 (95% CI, 0.79 - 0.97; P=0.008). Both variables remained significant in multivariate analysis (P=0.014). Recovery was not associated with Treg levels. CD4/CD8 ratio and effector T levels were not associated with a preceding infection, leukocyte or total lymphocyte counts, or presence of anti-platelet autoantibodies.

Conclusions: Major T cell frequencies were independently associated with spontaneous recovery and treatment response in newly diagnosed childhood ITP. Immune phenotyping may potentially aid clinicians in identifying patients who recover spontaneously or do not benefit from IVIg treatment.

OC 72.1 | A RNA Interference-based Approach to Autosomal Dominant Glanzmann Thrombasthenia: A Step towards Personalized Treatment

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Background: RNA interference (RNAi) is emerging as a promising precision medicine strategy for gene-therapy of dominant negative hereditary disorders acting by suppressing the expression of the dominant mutant gene.

Autosomal dominant Glanzmann Thrombasthenia (GT) is caused by gain-of-function variants of *ITGA2B* or *ITGB3* which generate a constitutively activated $\alpha_{IIb}\beta_3$ leading to platelet dysfunction and defective proplatelet formation.

We previously characterized an autosomal-dominant GT with a heterozygous c.2134+1G>C *ITGB3* variant leading to the in-frame deletion of exon 13 and to the generation of a c.2015-2134del transcript deriving from exon12-exon14 fusion. The resulting mutant $\alpha_{IIb}\beta_3$ exerts a dominant negative effect on normal $\alpha_{IIb}\beta_3$, making this gene variant an ideal candidate for a RNAi-based therapeutic approach.

Aims: Aim of this work was to assess whether an allele-specific siRNA can block selectively the synthesis of the dominant negative β_3 protein thus restoring the expression and function of $\alpha_{IIb}\beta_3$.

Methods: A siRNA molecule (ITGB3bkp12-14 siRNA) targeting the breakpoint region exon12-exon14 was designed. CHO cells expressing wild-type α_{IIb} were transfected using expression vectors with either wild-type or mutant β_3 and treated with the ITGB3bkp12-14 siRNA (1-25 nM). The expression of normal and mutant *ITGB3* transcripts was assessed by real-time PCR, while expression and function of $\alpha_{IIb}\beta_3$ were assessed by flow cytometry.

Results: A ITGB3bkp12-14 siRNA concentration of 7nM efficiently silenced mutant β_3 (by 76±17%) without silencing the wild-type transcript (9±2%). CHO cells expressing both normal and mutant $\alpha_{IIb}\beta_3$ constitutively bound PAC-1 (which recognizes active $\alpha_{IIb}\beta_3$). Treatment with the ITGB3bkp12-14 siRNA abrogated constitutive binding of PAC-1 and restored normal $\alpha_{IIb}\beta_3$ activation triggered by DTT, reestablishing normal phenotype.

Conclusions: Our data show that a RNAi-based strategy for gene therapy of autosomal dominant GT is feasible and represents a first step to personalized treatment for this rare disorder.

OC 72.2 | Impaired Hemostatic Activity of Healthy Transfused Platelets in Select Inherited Platelet Disorders: Mechanisms and Implications

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Background: Platelet transfusions often fail to prevent bleeding in patients with select congenital platelet disorders, including patients with mutations in *RASGRP2*, the gene encoding the critical platelet signaling protein CalDAG-GEFI.

Aims: To investigate whether dysfunctional platelets can interfere with the hemostatic function of healthy platelets in the context of platelet transfusion.

Methods: We performed real-time epifluorescence and spinning disk confocal (SDC) imaging of hemostatic plug formation in the saphenous vein of mice following laser ablation. Wild-type (WT) and mutant platelets were differentially labeled for visualization, and bleeding time and platelet adhesion were quantified.

Results: *Rasgrp2*$^{-/-}$ mice were refractory to WT platelet transfusion, unless the ratio between mutant and WT platelets was below 3 to 1. Thrombocytopenic recipient mice (*IL4R/GPIb-Tg*) then received transfusions of labeled WT and *Rasgrp2*$^{-/-}$ platelets. Surprisingly, both mutant and WT platelets incorporated effectively into the hemostatic plug, but the presence of mutant platelets significantly delayed the adhesion of WT platelets. SDC Z-stacks of hemostatic plugs demonstrated sequestration of WT platelets into distinct regions, while mutant platelets loosely filled the remaining areas. Real-time SDC revealed that plugs containing both mutant and WT platelets were loosely packed, continuously shedding single platelets. Transfusion studies in murine models for Glanzmann's Thrombasthenia (GT) and Bernard-Soulier Syndrome further demonstrated that dysfunctional platelets reduce access of WT platelets to von Willebrand factor exposed at sites of injury. Human GT platelets also interfered with the function of healthy platelets using the Impact-R assay. Finally, plugs containing WT and "dual anti-platelet therapy" platelets (aspirin-treated *P2y12*$^{-/-}$ platelets) formed quickly but destabilized in the center, leading to re-bleeding.

Conclusions: These studies suggest that the post-transfusion ratio of donor versus endogenous platelets can predict the success of platelet transfusions in the treatment of select inherited and acquired platelet function disorders. Our findings may also have clinical implications in patients receiving anti-platelet drugs.

OC 72.3 | New Constitutional GATA1 Variants Study Reveals the Contribution of GATA1 in MYH10 Silencing During Megakaryopoiesis

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Background: Megakaryocyte (MK) polyploidization requires *MYH10* expression silencing through the action of transcription repressors (ie RUNX1 and FLI1). We identified two new constitutional *GATA1* variants c.617A>T (F1) and c.802C>A (F2) in thrombocytopenic patients.

Aims: To study the role of GATA1 in *MYH10* regulation and to characterize the phenotype of the GATA1 variants´ carriers.

Methods: Platelet function, granule content and MYH10 expression were investigated (aggregation and mepacrine fluorescence assays, electron microscopy, western-blot). Patients CD34^{+} cells-derived MKs differentiation was evaluated (flow cytometry). Transfected cells were used to study GATA1 subcellular localizations and partner interactions. Transcriptional regulation was analyzed using luciferase reporter plasmid.

Results: Severe thrombocytopenia (15x10^{9}/l) was noticed at diagnosis in F1-patient, whereas the initially normal platelet counts observed in the two F2-affected siblings progressively dropped during childhood (>50x10^{9}/l). At the time of normal platelet count, F2 patients suffered spontaneous bleedings associated with aggregation defects. We identified platelet granule deficiencies in carriers. Bone marrow smears showed hypolobular MKs. Patients-derived MKs were smaller then controls and showed ploidy defect. MYH10 overexpression found in the *GATA1* variant carriers' platelets suggests a defective silencing during megakaryopoiesis. Genetic variations did not alter GATA1 nuclear localization and disrupted GATA1-FOG1 interaction was noticed in F1. Analysis of normal megakaryocytes ChIP-seq data (*Tijssen et al.*, 2011) showed GATA1, FLI1 and RUNX1 co-occupancy in a promoter and an intronic regions of *MYH10*. The two identified sequences were cloned in luciferase-reporter constructs. Each sequences associated with decreased luciferase activity in the hematopoietic-cell line HEL. In MSR-cells co-transfected with reporter construct and GATA1, only the intronic region associated with altered luciferase activity. Expression of GATA1 variants led to a reduction in regulatory activity.

Conclusions: Thrombocytopenia severity differs with the GATA1 variants. We propose MYH10 as a new GATA1 target involving both a promoter and an intronic regulatory element.

OC 72.4 | Confirmation of ACTN1 Variants as the Cause of Macrothrombocytopenia Using an in Vitrocellular Model

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Background: Genetic variants of alpha-actinin-1 (ACTN1) have been reported to cause autosomal dominant macrothrombocytopenia, however, confirmation of pathogenicity requires proof of altered cellular structure.

Aims: To assess the pathogenicity of *ACTN1* variants of uncertain significance (VUS) in macrothrombocytopenic patients using an *in vitro* cellular model.

Methods: To evaluate the effect of *ACTN1* VUS on the cytoskeleton, CHO cells were transfected with Myc-tagged wild-type (WT) or mutated *ACTN1*. Cytoskeletal and cytosol fractions were prepared for Western blotting. Myc-tagged ACTN1 was semi-quantitated in both fractions. Transfected cells also were spread on fibronectin, and stained with anti-cMyc antibody and phalloidin. Previously reported pathogenic variants Val105Ile and Leu395Gln were used as abnormal controls.

Results: Four heterozygous missense *ACTN1* variants were identified by targeted sequencing of macrothrombocytopenic subjects and confirmed by Sanger sequencing. Ser43Thr (c.127T>A) and Trp128Cys (c.384G>T) are located within the actin-binding domain (ABD) while Leu547Pro (c.1640T>C) and Asn703Ser (c.2108A>G) are in the rod domain.

By Western blotting, cytoskeletal Myc-ACTN1 was significantly increased for ABD variants S43T, W128C and V105I compared to WT, while cytosolic Myc-ACTN1 was reduced suggesting increased actin affinity. Redistribution of Myc-ACTN1 to the cytoskeleton was not seen with L547P, N703S and L395Q . Immunofluorescence on spread cells showed diffuse staining and finely dispersed ACTN1 filaments colocalizing with actin for WT and N703S transfected cells, whereas cells transfected with S43T, W128C, V105I and L395Q showed less diffuse staining and coarse, irregular fibers suggesting a less fluid actin network. Results for L547P were inconclusive with mixed patterns.

Conclusions: Using an *in vitro* cellular model, the two novel ABD variants of ACTN1 may increase affinity for actin, leading to a more rigid cytoskeleton that may explain the macrothrombocytopenia. Our model has proven useful for confirming the pathogenicity of ABD variants, but will need refining to assess the impact of rod domain variants.

OC 72.5 | The Thrombocytopathy in the Netherlands Study: A Horizontal Diagnostic Approach for Suspected Congenital Platelet Defects

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Background: Congenital platelet defects (CPD) are rare disorders of primary hemostasis caused by congenital defects in platelet production or function. Identification of CPDs is challenging and usually requires highly specialized tests and multiple hospital visits.

Aims: This study reports the results of a horizontal diagnostic approach in patients suspected for a CPD.

Methods: Laboratory tests consisted of a complete blood count, light transmission aggregometry, nucleotide measurement, flow cytometry and whole exome sequencing with a selected 88 gene panel. The study was approved by the Medical Ethical Committee of the University Medical Center Utrecht and all participants gave written informed consent in accordance with the declaration of Helsinki.

Results: In total, 200 patients were evaluated: 19 patients with a previously diagnosed CPD that served as positive control and 181 patients suspected for a CPD, of whom 45 had a thrombocytopenia. A CPD was identified in 59% (107/181) of patients. They were classified as dense granule deficiency or defects in the ADP, thromboxane A2 or GPIb-V-IX pathway. The remainder were categorized as isolated thrombocytopenia or complex abnormalities. A (likely) pathogenic mutation was identified in 7% (12/181) of patients and was reported more often in thrombocytopenic patients than in those with normal platelet counts (16% versus 4%). In patients with a normal platelet count and no objective platelet function defect (66/181, 36%), no mutations were found.

Conclusions: A horizontal diagnostic approach leads to the identification of a CPD in more than half of adult patients, but only a small proportion receives a molecular diagnosis.

PROTHROMBOTIC STATES

OC 23.1 | Increased Risk of Thrombosis in Antiphospholipid Syndrome Patients Treated with Direct Oral Anticoagulants. Results from an International Patient-level Data Meta-analysis

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Background: Direct oral anticoagulants (DOACs) are approved for prevent thrombotic recurrence in venous thromboembolism disease. Their use in APS patients is controversial because conflicting data

TABLE 1 Characteristics of APS patients treated with DOACs

	APS without recurrence thrombosis (n= 374)	APS with recurrence thrombosis (n= 73)	p value
aPL profile			
LA	168/248 (68)	44/59 (75)	
aCL	152/245 (62)	53/60 (88)	< 0.05
Antiβ2GP1	109/244 (45)	45/58 (78)	< 0.05
Triple positivity	61/267 (23)	33/59 (56)	< 0.05
Oral anticoagulant			
Rivaroxaban	240/374 (64)	50/73 (68)	
Dabigatran	122/374 (33)	22/73 (30)	
Apixaban	12/374 (4)	1/73 (1)	

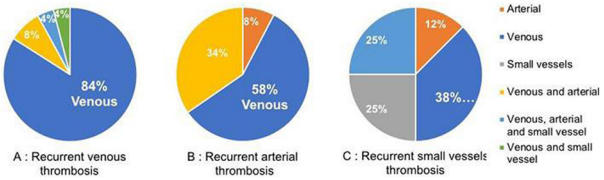

FIGURE 1 Previous thrombotic manifestations according to the localization of the recurrence

have been published from case reports, case series and two controlled randomized trials (RAPS and TRAPS).

Aims: We summarize available literature on DOACs use in APS patients through a systematic review in order identify risk factors predisposing to thrombotic events.

Methods: We conducted a systematic literature search in the MEDLINE, Embase and Cochrane Databases. We included all articles published up until 15/03/2018.

Results: The literature search identified three hundred and nighty three studies. Finally, we included forty seven studies representing 447 APS patients. Among them, there were seventy-three thrombotic events (16%).

The three DOACs used was rivaroxaban (N=290), dabigatran etexilate (N=144) and apixaban (N=13) ; there was no difference between them. Triple positivity was associated with a 4-fold increased risk for recurrent thrombosis (23% versus 56%; p< 0,05). Comparisons of patients' characteristics according to the presence of a thrombotic recurrence while on DOACs are available in Table 1. In patients treated with anti-Xa inhibitors, history of arterial thrombosis was associated with a higher risk of recurrent

thrombosis (32% vs 14%; OR=2.8 [95%CI; 1.4-5.7], p=0.006). However, the most of patients with a recurrent thrombosis had previous venous APS manifestation only (figure1). Moreover, there were 3 catastrophic APS and all of them had previous venous thrombotic manifestation only.

Conclusions: High-risk APS patients with triple positivity have a **4-fold increased risk** of thrombosis recurrence while on DOACs. Previous arterial and small vessel manifestation are associated with thrombotic recurrence but single VTE also. So DOACs should be used with caution in APS patients. Future randomized controlled trials should determine which factors are associated with a poor prognosis while on DOACs and which APS patients could be treated safely with these drugs.

OC 23.2 | Detection of Anti-domain I Antibodies Enables the Identification of High-risk Antiphospholipid Syndrome Patients: Results of a Multicenter Study

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TABLE 1 ORs of aPLs for the indicated clinical manifestation of APS

		Thrombosis*			Pregnancy complications**		
		N	OR	95%CI	N	OR	95%CI
LAC		851	3.4	2.5-4.7	481	4.2	2.8-6.4
Anti-CL IgG		851	4.1	2.6-6.3	481	4.4	2.6-7.4
Anti-β2GPI IgG		851	2.7	1.9-3.9	481	2.7	1.7-4.3
Anti-DI IgG	In total population	851	5.2	3.1-8.5	481	5.6	3.1-9.9
	In anti-β2GPI IgG or IgM positive population	204	3.9	2.0-7.4	112	3.8	1.7-8.3
	In anti-β2GPI IgG positive population	192	3.8	2.0-7.4	101	5.5	2.3-13.1

Abbreviations: aPLs, antiphospholipid antibodies; N, number of patients; ORs, odds ratios; 95% CI, 95% confidence intervals

*Thrombotic APS + non-APS thrombosis versus non-thrombotic population (AID + normal control);**: Obstetric APS + non-APS obstetric versus non-obstetric population (female AID + female normal control)

Background: Classification of the antiphospholipid syndrome (APS) relies predominantly on measuring antiphospholipid antibodies (aPLs), including lupus anticoagulant (LAC), immunoglobulin (Ig)G/IgM anti-cardiolipin (anti-CL) and anti-β2glycoprotein I (anti-β2GPI) antibodies. Especially aPLs against a domain I (DI) epitope of β2GPI are pathogenic in APS.

Aims: Investigate the clinical value of detecting anti-DI IgG in a multicenter study.

Methods: From eight European centers 1005 patients were enrolled: 379 APS patients, 237 non-APS thrombotic/obstetric controls, 196 patients with autoimmune diseases and 193 normal controls. LAC was determined by the local center; anti-CL, anti-β2GPI (HemosIL AcuStar™) and anti-DI IgG (QUANTA Flash β2GPI Domain I) by a single technician. To avoid a classification bias, data were analyzed with outcome variable 'clinically affected versus not-affected'.

Results: Odds ratios (ORs) of anti-DI IgG for clinical manifestations were higher than those of the conventional assays. Upon restriction to patients positive for anti-β2GPI IgG/IgM or anti-β2GPI IgG, anti-DI IgG positivity still resulted in significant ORs for clinical complications **(Table 1)**. When anti-DI IgG was added to the criteria or used as substitute for anti-β2GPI IgG and anti-CL IgG, ORs hardly improved (OR(95%CI) of (2.9(2.2-3.8)/2.7(2.0-3.6) versus 2.8(2.1-3.8) for thrombosis; 5.4(3.6-8.2)/5.0(3.3-7.5) versus 5.2(3.5-7.9) for pregnancy morbidity). Upon removing anti-DI IgG positive patients, LAC remained significantly correlated with clinical complications (OR2.5(1.7-3.5) and 3.9(2.4-6.4) for thrombosis and pregnancy morbidity), in contrast with anti-CL and anti-β2GPI IgG. Most of the anti-DI IgG reactive patients were triple-positive (83%/70% for the thrombotic/pregnancy morbidity subpopulation) and triple-positive patients were characterized by higher levels of anti-DI compared to single/double-positive patients.

Conclusions: Detection of anti-DI resulted in the highest ORs for clinical manifestations in comparison with the current criteria. Although addition of anti-DI to the criteria hardly improved the association with clinical symptoms, detection of anti-DI can be used as a risk stratification tool,identifying high-risk patients.

OC 23.3 | IgM Antiphospholipid Antibodies: Long Term Follow-up and Thrombotic Risk

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Background: Antiphospholipid antibodies (aPL) represent risk factors for clinical events in antiphospholipid syndrome (APS). Although IgM aPL are included in the current APS classification criteria, the clinical relevance of IgM aPL has been debated. Moreover, IgM aPL titres fluctuate and frequently turn negative in the longitudinal follow-up.

Aims: In this study, we retrospectively followed patients with IgM aPL and evaluated whether long-term presence of IgM aPL associated with thrombotic risk.

Methods: Samples remitted to our laboratory for aPL testing during a 15-year period were investigated. aPL testing comprised of IgG/M anticardiolipin antibodies (aCL), IgG/M antib2Glycoprotein I antibodies (ab2GPI), and IgG/M phosphatidylserine-dependent antiprothrombin antibodies (aPS/PT) analyzed by enzyme linked immunosorbent assays, and lupus anticoagulant by clotting assays. Patients with at least one IgM aPL positive and with ≥ 3 samples tested for IgM aPL during the follow-up period were included in the study. Patients' clinical records were retrospectively reviewed.

Results: 143 patients with a total of 1146 samples analyzed. A history of thrombosis was found in 69 patients. Persistent IgM aPL (defined as positive either aCL, ab2GPI or aPS/PT IgM in ≥ 2 occasions >12 weeks apart) was found in 69 (48%) patients while 74 (52%) had transitory IgM aPL (aCL, ab2GPI or aPS/PT IgM positive only once). Thrombotic events were more frequently observed in patients with persistent IgM aPL compared to those with transitory aPL IgM (64% vs 34%, OR: 3.4, 95%CI: 1.7-6.9, p< 0.001). IgM aPL remained positive 6, 12 and 24 months in 94%, 75% and 68% of patients, respectively. Patients with IgM aPL positive >2 year had higher thrombotic frequency compared to those positive < 2 years (7.66/100 person-years vs 5.88/100 person-years).

Conclusions: Persistent IgM aPL represent a risk factor for thrombosis. Testing IgM isotype of aPL contribute to a precise evaluation of thrombotic risk.

OC 23.4 | Predictive Value of Thrombocytopenia in Antiphospholipid Syndrome

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Background: Predicting antiphospholipid related-event is unmet need. Thrombocytopenia is the most frequent non-criteria manifestations in antiphospholipid syndrome (APS) and is thought to be the consequence of platelet activation, leading to the hypothesis

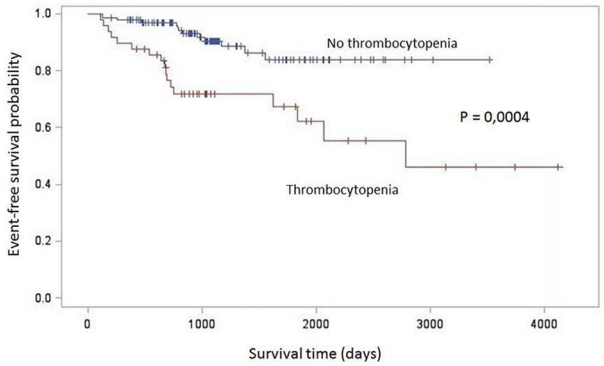

FIGURE 1 Estimates of the risk of thrombosis, obstetrical event or death (Kaplan-Meier method)

that thrombocytopenic patients platelet activation could be more intense, and lead to a more severe prognosis.

Aims: To determine whether history of thrombocytopenia was predictive of APS-related event during follow-up.

Methods: APL patients with or without associated SLE from our tertiary care centers were prospectively enrolled. Thrombocytopenia was defined as platelet count bellow 150 G/L at least once. Main outcome was time to first event during follow-up, with a combined criterion being the occurrence of thrombosis, obstetrical event or death. Secondary outcomes were thrombosis, obstetrical event or death taken individually. Consent was obtained from each patient.

Results: 192 patients were enrolled in our study, including 85 primary APS, 48 APS with underlying autoimmune disease, 52 asymptomatic APL carriers, and 7 patients with autoimmune disease and APL, not fulfilling APS criteria. Underlying autoimmune disease (AID) was SLE in 52, rheumatoid arthritis in 2, others AID in 16, and 118 had no AID. Thrombocytopenia was present in 58 patients (27.1%). After a follow-up of 728.9 patient-years, thrombosis, obstetrical event and death occurred in respectively 21, 4 and 6 patients. Multivariate survival analysis revealed that thrombocytopenia (Figure) and hypertension were independent predictive factor for thrombosis, obstetrical event or death (respectively, HR = 3.11 [95CI, 1.49 to 6.50] and HR = 3.49 [95%CI, 1.68 to 7.23]), while only hypertension was independent predictive factor for either thrombosis (HR = 4.72 [95CI, 1.95 to 11.42], or obstetrical event (HR = 13.23 [95%CI, 1.82 to 96.43]).

Conclusions: Thrombocytopenia in patients with APL with or without associated SLE identifies patients at risk of thrombosis, obstetrical event or death. Thus, thrombocytopenic patients should undergo a tailored monitoring,

OC 23.5 | The Prothrombin/MHC Class II Complexes on Procoagulant Cells as the Novel Immune Target for Pathogenic Antiphospholipid Antibodies

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Background: Antiphospholipid syndrome (APS) is a systemic autoimmune disorder characterized by thrombosis or pregnancy morbidity in the presence of antiphospholipid antibodies (aPL). Phosphatidylserine-dependent antiprothrombin antibodies (aPS/PT) recognize the phosphatidylserine/prothrombin (PS/PT) complex, and are strongly correlated with APS. Recently, a novel antigen presentation mechanism has been proposed that misfolded proteins are transported to the cell surface by major histocompatibility complex class II (MHC II) molecules without protein degradation, thus behaving as 'neo-self-antigens'. These complexes of misfolded proteins with MHC II molecules are considered major targets of autoantibodies in autoimmune diseases.

Aims: To investigate weather misfolded prothrombin (PT) are transported to the cell surface by MHC II in procoagulant cells, consequently being targeted by aPS/PT.

Methods: 1) The interaction between PT and several MHC II alleles overexpressed by HEK293T cells was analyzed using flow cytometry (FCM). 2) Synthesis of PT in monocytes was evaluated in phorbol-12-myristate-13-acetate (PMA) treated THP-1 cells by western blotting and FCM. 3) Cell surface transportation of synthesized PT was investigated by FCM on THP-1 cells or human monocytes treated with PMA and interferon gamma.

Results: 1) Overexpressed PT/MHC II complexes were recognized by a mouse monoclonal aPS/PT on THP-1 cell surface. We confirmed the dependency of MHC II allele differences on aPS/PT binding. 2) PMA treated THP-1 cells synthesized PT, which showed stronger binding with aPS/PT compared with non-pathogenic monoclonal anti-PT antibody.3) aPS/PT binding to PT/MHC II complexes were confirmed in treated THP-1 cells and human primary monocytes. No binding was observed between non-pathogenic monoclonal anti-PT antibody and PT/MHC II complexes on these cells.

Conclusions: PT molecules were syntheszed in procoagulant monocytes and presented on the cell surface via MHC II. The expression of PT/MHC II complexes on treated monocytes were considered as one of the antigenic targets for pathogenic aPS/PT.

OC 30.1 | Haemostatic Biomarkers for Prediction of Therapy Response and Prognosis of Disease in Patients with Metastatic Colorectal Cancer

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Background: Haemostatic biomarkers have a predictive value for cancer-associated venous-thromboembolism (VTE). However, their association with treatment-related oncologic outcomes like progression-free-survival (PFS), disease-control-rate (DCR) and overall-survival (OS) in metastatic colorectal cancer (mCRC) is only partially known.

Aims: To evaluate the role of haemostatic biomarkers for prediction of treatment-response and prognosis in mCRC.

Methods: Within the framework of the Vienna Cancer and Thrombosis Study (n>2000), a prospective observational cohort-study, we investigated the association of six well-known biomarkers of hypercoagulability with oncologic outcomes in 99 patients with mCRC (newly diagnosed or progressive disease after remission) prior to chemotherapy-initiation (median age: 61 years, 37.4% female, most frequent primary-tumour: 40.4% rectum; metastasis: liver 80.8%, lung 35.4%; bevacizumab: 58.2%). Written-informed-consent was provided by all participants and the study was approved by the local ethics-committee.

TABLE1 Association of elevated levels of haemostatic biomarkers with progression free survival (PFS) and overall survival (OS)

Biomarker	Median PFS in months (95%CI)		Median OS in months (95% CI)	
	7.8 (4.8-14.6)*		21.1 (15.1-27.2)*	
	Biomarker ≤ Q3	**Biomarker > Q3**	**Biomarker ≤ Q3**	**Biomarker > Q3**
Factor VIII	8.3 (6.5-10.1)	5.0 (1.7-8.2)	24.4 (13.4-35.4)**	13.9 (7.0-20.9)**
sP-Selectin	8.0 (6.5-9.5)	6.2 (2.9-9.6)	21.1 (9.3-33.0)	16.3 (4.4-28.1)
D-Dimer	8.3 (6.5-10.2)	6.7 (5.8-7.6)	26.0 (10.3-41.6)**	13.8 (7.6-19.9)**
F1+2	8.0 (6.5-9.5)	6.2 (4.2-8.2)	21.5 (12.9-30.2)**	13.9 (7.0-26.4)**
Peak-thrombin-generation	8.3 (6.8-9.9)	6.0 (4.6-7.5)	23.7 (14.5-33.0)	15.3 (10.9-19.6)
Fibrinogen	8.8 (7.0-10.7)	6.0 (5.4-6.7)	28.2 (17.8-38.7)**	15.8 (12.9-18.6)**

*Median OS and PFS in the overall study population (n=99); **: statistically significant differences in Kaplan-Meier-estimates (log-rank); Abbreviations: PFS: progression free survival; OS: overall survival; 95%CI: 95% confidence interval; ≤Q3: patients with biomarker-levels equal to / under the third quartile (≤75th percentile); >Q3: patients with biomarker-levels above the third quartile (>75th percentile); sP-selectin: soluble p-selectin; F1+1: prothrombin fragment F1+2;

TABLE 2 Association between elevated levels of haemostatic biomarkers and disease control rate (DCR)

Biomarker	DCR		OR for DCR	
/	**Biomarker ≤ Q3**	**Biomarker > Q3**	**Univariate OR**	**Adjusted OR*****
Factor VIII	68.9%*	45.8%*	0.28 (0.11-0.70) **	0.23 (0.09-0.62) **
sP-selectin	69.3%*	45.8%*	0.75 (0.38-1.48)	0.77 (0.38-1.54)
D-dimer	64.3%	65.2%	0.83 (0.62-1.12)	0.84 (0.62-1.15)
F1+2	70.4%*	43.5%*	0.35 (0.16-0.75)**	0.36 (0.16-0.82) **
Peak thrombin generation	64.0%	62.5%	0.94 (0.59-1.50)	1.03 (0.63-1.68)
Fibrinogen	64.0%	62.5%	1.68 (0.55 -5.14)	1.68 (0.50-5.66)

*: statistically significant differences in DCR according to dichotomized biomarkers (Chi-square-test); **: statistically significant results in binary logistic-regression analysis of the association between DCR and doubling of biomarker-levels on a continuous scale; ***: adjusted for age, sex, VTE, number of metastatic sites; Abbreviations: DCR: disease-control-rate (composite of stable-disease + complete- + partial-remission according to response-evaluation-criteria-in-solid-tumours 1.1); OR: odds-ratio; ≤Q3: patients with biomarker-levels equal to / under the third quartile (≤75th percentile); >Q3: patients with biomarker-levels above the third quartile (>75th percentile); sP-selectin: soluble p-selectin; F1+2: prothrombin-fragment F1+2;

Results: PFS: Despite shorter PFS for elevated levels (>75th percentile) across all biomarkers in Kaplan-Meier-estimates (Table1), no significant associations were observed in multivariable Cox-regression-analysis (co-variables: age, sex, number of metastatic sites, VTE; adjusted hazard-ratio (aHR) [95%CI] for disease-progression per doubling of levels: factor VIII: 1.42 [0.92-2.19], sP-selectin: 1.14 [0.80-1.61], D-Dimer 1.10 [0.95-1.28], prothrombin-fragment 1+2 (F1+2): 1.24 [0.87-1.78], peak-thrombin-generation: 1.16 [0.90-1.49], fibrinogen: 1.01 [0.53-1.94]).

DCR: We observed significantly smaller DCR in patients with elevated levels in 3/6 biomarkers (Qui^2-test, Table2). In multivariable logistic-regression-analysis (co-variables: age, sex, number of metastatic sites, VTE) two biomarkers were significantly associated with DCR (adjusted odds ratio [95%CI] for DCR per doubling of levels: FVIII: 0.23 [0.09-0.62], F1+2: 0.36 [0.16-0.82]).

OS: We observed shorter median-OS in patients with elevated levels across all biomarkers (Table1). In multivariable analysis 4/6 Biomarkers were significantly associated with risk of mortality (aHR [95%CI] for death: factor VIII: 2.06 [1.28-3.30], sP-selectin: 1.55 [1.07-2.24], D-Dimer: 1.40 [1.18-1.65], F1+2: 1.64 [1.10-2.46]).

Conclusions: Haemostatic biomarkers, reflecting hypercoagulability, predict treatment-related oncologic outcomes and overall survival in patients with mCRC.

OC 30.2 | Hemostatic and Non-hemostatic Functions of Magnesium Transporter MAGT1 in Mice

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Background: Magnesium (Mg^{2+}) infusion or dietary supplementation is a common practice in the treatment of hypertension and cardio-/cerebrovascular pathologies. In addition, Mg^{2+} homeostasis has been investigated in some cases of cancer development and metastasis. However, despite beneficial effects of Mg^{2+} in prevention of thrombo-inflammatory diseases, the pathophysiological role of platelet Mg^{2+} homeostasis remains elusive.

Aims: We investigated the role of Mg^{2+} transport mechanisms for platelet functions in relation to arterial thrombosis and hemostasis as well as cancer metastasis.

Methods: Platelet function was studied in a broad range of *in vitro* assays and *in vivo* models of hemostasis, thrombosis and thrombo-inflammatory brain infarction using Mg^{2+} transporter 1 (MAGT1)-deficient (*Magt1*$^{-/y}$) mice. In addition, *Magt1*$^{-/y}$ and wild type (*WT*) mice were assessed in tumor metastasis models induced by injection of MC38 (colon) and LLC (Lewis Lung Carcinoma) cells.

Results: *Magt1*$^{-/y}$ mice displayed accelerated occlusive arterial thrombus formation *in vivo*, shortened bleeding times, and a dramatically worsened brain damage upon focal cerebral ischemia. Interference with Mg^{2+} transport activity in *Magt1*$^{-/y}$ mice resulted in imbalanced platelet Mg^{2+} homeostasis upon stimulation of glycoprotein VI (GPVI). This resulted in an increased Ca^{2+} influx, enhanced ATP and thromboxane A_2 release, which further reinforced platelet reactivity and aggregation responses. $MgCl_2$ supplementation inhibited this prothrombotic phenotype and normalized the aggregation responses of *Magt1*$^{-/y}$ platelets to control level. Furthermore, we demonstrate that MAGT1 deficiency enhances tumor metastasis in *Magt1*$^{-/y}$ mice. $MgCl_2$ supplementation or knockdown of ATP-gated purinergic receptor P2X7 in tumor cells could attenuate platelet-tumor cell interaction in a MAGT1-dependent manner.

Conclusions: These results suggest that reduced platelet Mg^{2+} uptake through MAGT1 signalling could be a risk factor for arterial thrombosis, stroke and cancer metastasis. Our findings furthermore suggest a possible platelet-dependent mechanism underlying the beneficial effects of Mg^{2+} supplementation not only in the therapy of cardiovascular diseases, but also beyond.

OC 30.3 | Hemostatic Biomarkers and Prediction of Prognosis in Newly Diagnosed Patients with Gastrointestinal Metastatic Cancer: Results from the HYPERCAN Study

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Background: Hypercoagulability and venous thromboembolism (VTE) are frequent in advanced gastrointestinal (GI) cancer patients and, due to the reciprocal interaction between coagulation and cancer, can be involved in the high incidence of treatment failure and disease progression (DP).

Aims: In a cohort of patients from the HYPERCAN study (ClinicalTrials.gov ID#NCT02622815) with newly diagnosed metastatic GI cancer, we prospectically assessed whether the pre-chemotherapy levels of hemostatic biomarkers may predict for DP.

Methods: The study cohort included 160 consecutive cancer patients (100 colorectal/60 gastric; median age 65 yrs). Clinical data were recorded at enrollment, after informed consent, and during scheduled follow-up visits for at least two years. DP and VTE were monitored by imaging every 3 chemotherapy cycles. Fibrinogen, D-Dimer, and thrombin generation (TG) were measured at diagnosis, before starting any curative chemotherapy.

Results: At diagnosis, patients presented with a hypercoagulable state, as shown by significantly ($p< 0.01$) higher plasma levels of fibrinogen, D-Dimer and endogenous TG potential (ETP) compared to healthy controls.

After 6 months from the beginning of chemotherapy, cumulative incidence of DP was 23.7% (CI 95% 17-30.1) and VTE 11.2% (CI 95% 7.5-17.5). Of interest, 50% of patients with VTE in the first 6 months had a DP within the year. Furthermore, patients with DP were characterized by significantly ($p< 0.05$) higher pre-chemotherapy levels of ETP compared to patients with no DP after 6 months [2,050 vs 1,699 nM*min]. By multivariate Cox regression analysis, D-Dimer (HR=1.08) and TG ETP (HR=1.01) plasma levels, together with gastric cancer diagnosis (HR=2.2), were identified as independent risk factors for DP.

Conclusions: Hypercoagulability biomarkers are a promising tool to identify GI patients at high risk of treatment failure. These results provide the basis for creating risk assessment models useful to tailor a risk-adapted treatment.

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OC 30.4 | Role of Neutrophils and Neutrophil Extracellular Traps in Mice Bearing Human Pancreatic Tumors

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Background: Pancreatic cancer is associated with high incidence of venous thromboembolism (VTE). Neutrophils have been shown to contribute to thrombosis in part by releasing neutrophil extracellular traps (NETs). A recent clinical study showed that increased plasma levels of the NET biomarker, citrullinated histone H3 (H3Cit), are associated with VTE in pancreatic and lung cancer but not in other types of cancer, including breast cancer.

Aims: To determine the contribution of neutrophils and NETs to venous thrombosis in mice bearing human pancreatic tumors.

Methods: Nude mice bearing human pancreatic BxPc-3 tumors were used. Thrombosis was induced by inferior vena cava stasis.

Results: We found that tumor-bearing mice had increased circulating neutrophil counts and levels of granulocyte-colony stimulating factor, neutrophil elastase, H3Cit and cell-free DNA compared with controls. In addition, thrombi from tumor-bearing mice contained increased levels of the neutrophil marker Ly6G, as well as higher levels of H3Cit and extracellular DNA. Thrombi from tumor-bearing mice also had thinner fibrin fibers consistent with increased thrombin generation. Importantly, neutrophil depletion and administration of DNase I reduced thrombus size in tumor-bearing but not in control mice.

Conclusions: Our data indicate that neutrophils and NETs contribute to venous thrombosis in a mouse model of pancreatic cancer. Targeting this pathway may prevent VTE in pancreatic cancer patients without increasing bleeding.

OC 30.5 | NETs as Thrombotic Risk Indicators in Biliopancreatic Cancer Patients at Diagnosis

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Background: Venous thromboembolism (VTE) is a common complication of cancer that increases mortality and morbidity in these patients. However, limited tools are available to identify the patients at need for anticoagulation due to a high thrombotic risk. Neutrophil extracellular traps (NETs) are highly prothrombotic networks released by neutrophils upon activation, made up of DNA, myeloperoxidase (MPO), calprotectin and nucleosomes, among other elements.

Aims: To evaluate the ability of NETs to identify biliopancreatic cancer patients at high risk of developing VTE during follow-up.

Methods: 125 biliopancreatic cancer patients were prospectively recruited and followed for two years. Blood was drawn at diagnosis

and every three months. Ethical approval and consent from all participants were obtained. In every plasma sample obtained from patients, we measured NETs markers: DNA (PicoGreen), nucleosomes, MPO and calprotectin (specific ELISAs). We estimated the predictive potential of VTE of NETs using R (v3.5.0).

Results: We conducted a Cox regression survival model with a time-dependent covariate, including the NETs variables measured in each sample of the patients in rank form. We obtained that, for each unit that the logarithm of the calprotectin concentration increases, the VTE risk in biliopancreatic cancer patients increases 6 times (P=0.009). The other markers of NETs showed no predictive capacity of VTE. Next, we adjusted an Elastic Net logistic regression model and obtained a predictive model of VTE with calprotectin as a predictor (AUC=0.77).

Conclusions: The concentration of calprotectin at diagnosis allows us to estimate the risk of VTE in biliopancreatic cancer patients. This information could be very useful to prevent thrombotic events in these patients through closer monitoring and personalized thromboprophylaxis. ISCIII-FEDER (PI12/00027, Red RIC RD12/0042/0029, PI14/00079, PI14/00512, FI14/00269, CPII15/00002, PI17/00495), Generalitat Valenciana (PrometeoII/2015/017, ACIF/2017/138), Sociedad Española de Trombosis y Hemostasia and OBEL Family Foundation (26145).

OC 41.1 | A Validated Profile of 6 miRNAs as Predictor of Early Incidental Post-surgical Pulmonary Embolism in Glioma Patients

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Background: Intracranial tumors increase the risk of thrombosis, and often cause incidental post-surgical pulmonary embolism (PE). New biomarkers are needed to early identify brain cancer patients with high thrombotic risk. microRNAs (miRs) regulate protein expression and seem to regulate cancer progression and thrombosis.

Aims: To generate and validate a predictive model of miRs capable of predicting the risk of early incidental post-surgical PE in glioma patients.

Methods: We recruited and prospectively followed after surgery 50 glioma patients. Ethical approval and consent were obtained. All had negative baseline lung perfusion scans. PE was objectively diagnosed in 17 patients within 7 days post-surgery. In plasma collected before surgery, we measured the expression of 179 miRs with the Serum/plasma Focus microRNA PCR Panel V4 (Exiqon) in 10 selected glioma patients (5 developed PE and 5 did not). Using a multivariable logistic regression model, the predictive ability for post-surgical PE was estimated as the area under the ROC curve (AUC) with R (v3.5.0). Next, we validated the predictive model in an independent subset of 40 glioma patients (12 developed PE and 28 did not). We identified in *miRWalk* and *KEGG* their predicted target proteins, related to the coagulation cascade and complement.

Results: We adjusted an elastic net model for PE risk in glioma patients with 6 miRs before surgery: miR-363-3p, miR-93-3p, miR-22-5p, miR-451a, miR-222-3p and miR-140-3p. We validated this model in an independent cohort of glioma patients obtaining an AUC=0.78 (95%CI:0.63-0.94), which outperforms the predictive ability of the Khorana score in these patients (AUC=0.52).

Conclusions: Before surgery, the expression level of 6 miRs is a good predictor of early incidental post-surgical PE in glioma patients. Our predictive model outperforms the Khorana score, currently used in clinical practice. This predictive model could be useful to tailor post-surgical thromboprophylaxis in these patients. ISCIII-FEDER(PI14/00079-PI14/00512-FI14/00269-CPII15/00002-PI17/00495), Generalitat Valenciana (PrometeoII/2015/017-ACIF/2017/138), SETH and GR-2011-02347854.

OC 41.2 | Utility of the Thrombin Generation Test in Various Clinical Phenotypes to Predict Thrombotic Recurrences in Cancer Patients without Anticoagulant Treatment

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Background: Venous thromboembolism (VTE) is a frequent complication in cancer patients, which causes greater morbidity and mortality in these patients. In addition, patients with cancer and VTE have a high rate of thrombotic recurrences.

Aims: To explore the ability of the thrombin generation test (TGT) in various clinical phenotypes to predict thrombotic recurrence in cancer patients who had already suffered a VTE event, but in whom the anticoagulant treatment was withdrawn.

Methods: We prospectively recruited 167 cancer patients who suffered VTE. Ethical approval and consent from all participants were obtained. We obtained a sample of citrated blood at anticoagulation withdrawal (recruitment), as well as 21 days and 3 months after. Of these, 22 patients (13.2%) suffered a recurrence (median: 3 months,

1-40 months). At each visit we measured FVIII, tissue factor, soluble P-selectin, D-dimer, C-reactive protein, procoagulant phospholipid activity, and the different TGT parameters by automated test (CAT, Thrombinoscope). Differences between the variables studied during the patients' follow-up were analyzed with the Wilcoxon test using R (v3.5.0).

Results: The variables that allowed us to differentiate cancer patients with thrombotic recurrences were: P-selectin at 21-day follow up (P=0.006), D-dimer at recruitment (P=0.034), D-dimer at 21 days (P=0.0003), C-reactive protein at 21 days (P=0.0098) and lag time at 3-months (P=0.039).

Conclusions: The concentration of P-selectin, D-dimer, C-reactive protein and the lag time of TGT are parameters that seem to have a prognostic utility during the follow-up of oncological patients who have suffered VTE, in order to predict a thrombotic recurrence. This information could lead to personalized thromboprophylaxis in these patients to avoid recurrences.

OC 41.3 | A Validation, with New Clinical Applicability, of a Clinical-genetic Risk Model that Predicts Thrombosis with High Sensitivity in Patients with Lymphoma

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Background: The incidence of venous thromboembolic events (VTE) in patients with lymphoma is estimated around 10%, increasing the morbidity and mortality for this group of patients. Khorana scale has been developed to assess VTE risk in cancer patient but it is not useful for lymphoma patients. Recently, a new risk score have been developed to predict VTE in this specific population (Throly). Likewise, the incorporation of genetic variables into a VTE risk model improved VTE risk assessment in solid tumors (TiC-ONCO).

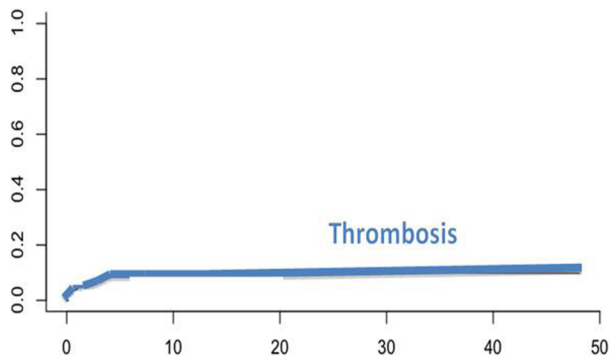

FIGURE 1 Cumulate incidence of Thrombosis

Score	Sensitivity	Specificity	PPV	NPV
Tic-Lympho	93,55	54,49	23,36	97,98
Khorana	35,48	71,79	16,60	84,41
Throly	45,16	64,74	50	86,63

Score	AUC	(95% CI)	p
Tic-Lympho	0,783	(0,721-0,837)	<0,0001
Khorana	0,503	(0,431-0,574)	0,902
ThroLy	0,579	(0,50-0,648)	0,0319

FIGURE 2 Sensitivity, specificity, predictive value and AUC of the Khorana, Throly, and TiC-Lympho

Aims: The aim of our study was to evaluate if the same approach of TiC-ONCO is applicable to patients with lymphoma.

Methods: Between 2014 and 2018, 254 lymphomas were diagnosed in our center, 33 of them presented VTE. The F5 rs6025, F5 rs4524, F13 rs5985, SERPINA10 rs2232698, in addition to clinical variables (immobilization, tumor type, Ann Arbor score, mediastinum extension, and personal thrombotic history) were significant associated to VTE risk and used to build the TiC-ONCO-associated Lymphoma score (TiC-Lympho). The Khorana, ThroLy and TiC-Lympho scores were compared through predictive value and area under the curve (AUC).

Results: We observed a cumulative incidence of VTE of 9% (Fig.1). The results of sensitivity, specificity, predictive value and AUC of the Khorana, Throly, and TiC-Lympho are shown in Table 1 and Table 2 (Fig.2).

Conclusions: Our results demonstrated that the incorporation of genetic-clinical data to assess VTE risk it is not only valid to solid tumors, but it is also relevant in lymphoma. In fact, it is the first time that this approach has been applied to haematological patients. It is noteworthy that the predictive capacity for VTE in lymphoma patients of our score far exceeds that obtained by the Khorana and Throly scores. Therefore, TiC-Lympho will allow us to categorize lymphoma patients adequately at diagnosis and offering an individualized thromboprofilaxis.

OC 41.4 | Subsequent Venous Thromboembolism after Upper Extremity Deep Venous Thrombosis in Patients with Cancer

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Background: Upper extremity deep venous thrombosis (UE-DVT) is a common complication seen in patients with cancer. The incidence of subsequent thrombosis: UE-DVT, pulmonary embolus (PE), or lower extremity (LE-DVT) in patients with cancer and index UE-DVT is not as well described.

Aims: To determine the 12-month cumulative incidence (CI) and location of subsequent venous thromboembolism (VTE) after index UE-DVT among 14 common cancer sites, adjusted for the competing risk of death.

Methods: Using the California Cancer Registry and the California Patient Discharge (PDD) and Emergency Department Utilization (EDU) datasets (2005 - 2014), cancer patients with UE-DVT were found using specific ICD-9-CM codes. Patients with a prior VTE were excluded. Subsequent UE-DVT was defined as a subsequent admission with a specific UE-DVT code in the principal or second position code if the cancer was the primary diagnosis code. Subsequent PE or LE-DVT was defined as an admission with a specific PE or LE-DVT code.

Results: There were 5,210 index UE-DVT amongst 785,444 patients. Overall, the 12-month CI of subsequent VTE varied from 6.8% to 18% depending on the tumor type. The 12-month cumulative incidence by location of subsequent VTE and cancer site is shown in Figure 1. The highest incidence of subsequent VTE were in patients with brain and ovarian and these tumors were also associated with the highest risk of subsequent PE. The median time from index UE-DVT to subsequent VTE was 52 days (quartile range: 12-209 days). The lowest risk of subsequent VTE was in patients with prostate cancer and myeloma.

Conclusions: Although the majority of subsequent VTE after UE-DVT were in the UE, a significant proportion were PE and LE-DVT. Further study is ongoing to determine the risk factors for subsequent VTE after an index UE-DVT, and the association of UE-DVT with mortality.

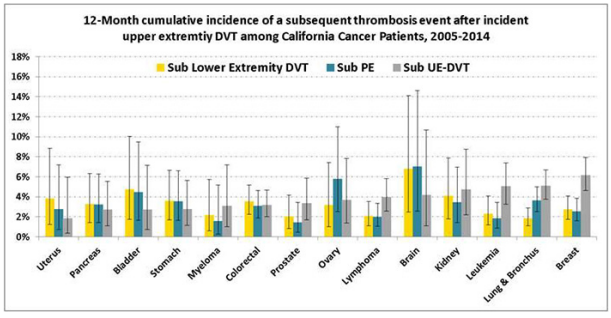

FIGURE 1

OC 45.5 | Tissue Factor and Tissue Factor in Combination with Alpha-1 Antitrypsin or Reg4 Associate with Venous Thromboembolism in Patients with Colorectal Cancer

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Background: New and better biomarkers are urgently needed to predict which cancer patients are at increased risk for venous thromboembolism (VTE) and benefit most from thromboprophylaxis. We recently identified a VTE RNAseq profile that associates with occurrence of VTE in colorectal cancer (CRC) patients, with *REG4*, *SPINK4*, and *SERPINA1* as the top-3 upregulated genes at mRNA level.

Aims: To establish whether protein expression of Reg4, Spink4 and alpha-1 antitrypsin (A1AT, encoded by *SERPINA1*), and Tissue Factor (TF) in the tumor associates with VTE in an independent cohort of CRC patients.

Methods: At the Leiden University Medical Center, 18 patients who developed VTE 1 year before or after cancer diagnosis were age, sex, and cancer stage-matched to 18 CRC patients who did not develop VTE. Protein expression was detected by immunohistochemical staining and scored blindly by assessing the H-score (intensity[0,1,2,3] * % positive tumor cells). Means were compared using paired t-tests and the association with VTE was assessed by means of logistic regression, using patients below the median of the control group as reference group for each protein.

Results: All levels of protein expression are depicted in the Table below. Particularly TF was increased in the tumors from CRC patients who developed VTE vs. patients without VTE which led to a 5.0-fold increased risk (Fig. + Table). In contrast, a trend or no association with development of VTE was observed for A1AT, Reg4 or Spink4 protein expression levels. TF expression levels in combination with A1AT ($TF^+/A1AT^+$) or Reg4 ($TF^+/Reg4^+$) demonstrated increased

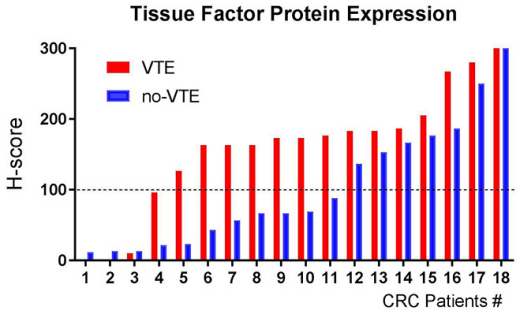

Fig. 1. Tissue Factor protein expression in tumors as detected by immunohistochemical staining of tumors from colorectal cancer (CRC) patients that developed, or did not develop venous thromboembolism (VTE).

FIGURE 1

TABLE 1

	Mean (VTE pts)	Mean (no VTE pts)	Mean difference (95%CI)	OR pos vs neg (95% CI)
TF	158.4	102.5	55.9 (11.5 to 100.1)	5.0 (1.1-23.5)
A1AT	142.3	84.6	57.8 (-4.9 to 120.5)	3.5 (0.8-14.5)
Reg4	71.7	61.2	10.5 (-53.0 to 74.8)	1.6 (0.4-5.9)
Spink4	58.0	56.6	1.3 (-37.3 to 39.8)	2.0 (0.5-7.4)
TF+/A1AT+	N/A	N/A	N/A	6.5 (0.9-49.7)
TF+/Reg4+	N/A	N/A	N/A	9.0 (0.8-100.1)
TF+/SPINK4+	N/A	N/A	N/A	5.0 (0.7-33.2)

ORs of 6.5 (95%CI 0.9-49.7) and 9.0 (0.8-100.1), respectively, for the risk for VTE when compared to tumors negative for these factors.

Conclusions: TF as well as the combination TF with A1AT or Reg4 appear to associate with VTE, and are therefore potential biomarkers to identify CRC patients at high risk for VTE.

OC 46.1 | Crosstalks between ADAMTS13 and Complement in Murine Models of Thrombotic Microangiopathy

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Background: Severe deficiency of plasma ADAMTS13 activity is the primary cause of thrombotic thrombocytopenic purpura (TTP) while an overwhelming activation of the alternative complement pathway may result in an atypical hemolytic uremic syndrome (aHUS), two prototypes of thrombotic microangiopathy (TMA). However, the clinical and pathogenetic distinction between TTP and aHUS can be difficult. There is a sufficient clinical evidence to suggest that there may be a crosstalk between ADAMTS13 and complement, which may play a role in pathogenesis and phenotypical expression of TMA.

Aims: In this study, we determined the synergistic role of severe Adamts13 deficiency and a heterozygous or homozygous mutation in complement factor H *(cfh)*gene in the murine models of thrombotic microangiopathy.

Methods: Knock-in cfh^{W1206R}, knock-out *Adamts13*, and double mutations were generated to recapitulate the clinical presentations of human TMA. Complete blood counts, plasma levels of LDH, BUN, creatine, haptoglobin, tissue histology, and Kaplan-Meier survival curve were determined in these mice.

Results: Our results demonstrate that mice with $Adamts13^{-/-}cfh^{W1206R}$and cfh^{R1206R}or$Adamts13^{-/-}cfh^{R1206R}$manifested with a marked thrombocytopenia, reduced plasma levels of haptoglobin, and an increased number of fragmented red blood cells in peripheral blood, as well as increased plasma levels of LDH, BUN, and creatinine, consistent with the phenotypical expression of thrombotic microangiopathy. Additionally, mice with $Adamts13^{-/-}cfh^{W1206R}$,$Adamts13^{+/+}cfh^{R1206R}$or $Adamts13^{-/-}cfh^{R1206R}$exhibited a significantly increased mortality rate when compared with $Adamts13^{-/-}$or cfh^{W1206R}or *wild-type* mice. Histological analysis revealed the presence of platelet-rich microvascular thromboses in the terminal arterioles and capillaries of major organ tissues in$Adamts13^{-/-}cfh^{W1206R}$,$Adamts13^{+/+}cfh^{R1206R}$or $Adamts13^{-/-}cfh^{R1206R}$mice died or sacrificed.

Conclusions: We therefore conclude that there is a significant crosstalk between ADAMTS13 and complement system and a synergistic role of severe ADAMTS13 deficiency and complement activation in pathogenesis, phenotypical expression, and outcome of the murine models of thrombotic microangiopathy.

OC 46.2 | Anti-ADAMTS-13 Autoantibody-specific Anti-idiotypic Antibodies Restore ADAMTS-13 Activity in Immune-mediated Thrombotic Thrombocytopenic Purpura (iTTP) Patients

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Background: Severe ADAMTS-13 deficiency in patients suffering from iTTP is due to anti-ADAMTS-13 autoantibodies. At least 50% of iTTP patients surviving a first acute episode subsequently experience further episodes. The mechanisms that uphold remission in certain patients but not in others are not yet understood.

Aims: We hypothesize that remission in iTTP patients is partially the result of anti-idiotypic antibodies neutralizing anti-ADAMTS-13 autoantibodies, the former we aim to identify and characterize.

Methods: From splenic mononuclear cells of two frequently relapsing iTTP patients, the anti-idiotypic IgG_1 Fab κ/λ repertoire was amplified by phage display technology. Selecting antibodies consisted of three sets of previously generated monoclonal anti-ADAMTS-13 Fabs (Schaller et al., Blood 2014), corresponding to specific paratopes, CDR3 motifs 1, 2 or 3. Equimolar pools (200nM; termed A or C, according to patient origin) of selected anti-idiotypic Fabs, were

then tested for their ability to neutralize functional ADAMTS-13 inhibitors in plasma samples of 22 (11 with 1-2BU/ml and 11 with >2BU/ml functional inhibitor) unrelated, randomly selected iTTP patients.

Results: We sequenced 24 anti-idiotypic Fab clones, of which eight were further studied. Anti-idiotypic Fab pools A and C partially neutralized functional ADAMTS-13 inhibitors of >2BU/ml in 3/11 (27%) and 5/11 (45%) patient samples, respectively. For functional inhibitors of 1-2BU/ml, full neutralization was seen in 2/11 (18%) and 3/11 (27%) patient samples with pool A and C, respectively.

Conclusions: In the splenic immunoglobulin repertoire of two relapsing iTTP patients, we have identified functionally effective anti-ADAMTS-13 anti-idiotypic antibodies, supporting our hypothesis of an anti-idiotypic immune response in iTTP. Already at a low concentration of 200nM, the anti-ADAMTS-13 anti-idiotypic antibodies partially restored ADAMTS-13 activity in unrelated iTTP patients. Analyses of further iTTP patients, with functional ADAMTS-13 inhibitors titrated to endpoint (beyond 2BU/ml), dose dependent pool incubation, as well as full characterization of the functional properties of the anti-idiotypic Fabs are underway.

OC 46.3 | Thrombotic Thrombocytopenic Purpura: 10 Year Analysis of the United Kingdom TTP Registry

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Background: Thrombotic thrombocytopenic purpura (TTP) is a thrombotic microangiopathy, confirmed by ADAMTS13 activity < 10%).

Aims: Epidemiological analysis of the UK TTP registry.

Methods: UK study of patients presenting with acute TTP between 2009 and 2018.

Results: Out of 1087 acute TTP episodes affecting 663 patients, 475 (73% female, 27% male) were consented to the registry. Median age at presentation was 43 years (range 9-93). 60% were Caucasian, 22% Afro-Caribbean. 84% had immune-mediated TTP (idiopathic 76%; secondary 24%) and 16% had congenital TTP. From 564 episodes, relapse rate was 19%.

Commonest presenting findings were neurological (71%), raised troponin (55%) and bleeding/petechiae (48%). Of the 45% admitted to the intensive care unit, 10% required intubation. There was no difference in presenting features between idiopathic and secondary TTP.

At presentation, 92% had ADAMTS13 activity < 10% and 25% had platelet counts < 10 x10^9/l (Table 1). 81% received steroids and a median of 11 (range 1-75) plasma exchanges (PEXs) (Table 2). 74% received rituximab, at a median of 3 days after admission and median of 4 doses (range 1-10). 44% of patients had platelets < 150 x10^9/l at 7 days, compared to 4% at 1 month. Normalisation of ADAMTS13 occurred in 61% of patients by 1 month, with 79% achieving at least a partial response (ADAMTS13 ≥30%). 13% of cases attained haematological remission but had ADAMTS13 persistently < 10%. Median time to relapse was 26 months (range 1-114) and 61% were successfully re-treated with Rituximab.

89 patients received Rituximab prophylaxis to prevent acute relapse. The true number is likely to be higher, as this data was not initially collected in the registry.

Conclusions: TTP is a heterogeneous condition. Number of PEXs to remission appears to have decreased over the years (Table 2). Although Rituximab usage in the acute setting remains consistent, its use prophylactically has increased.

TABLE 1 Change in platelet count and ADAMTS13 activity after treatment

	Percentage of patients			
	At diagnosis	Day 7	1 month	3 months
plt <10 x10 9/L	27%	2%	0%	0%
plt <50 x10 9/L	86%	15%	0%	1%
plt <150 x10 9/L	98%	44%	4%	5%
plt >150 x10 9/L	2%	56%	96%	95%
ADAMTS13: 0-10 %	92%	58%	13%	14%
ADAMTS13: 0-30 %	97%	67%	20%	21%
ADAMTS13: 30-60 % (PR)	2%	14%	18%	12%
ADAMTS13: ≥60 % (CR)	0%	16%	61%	67%

TABLE 2 Comparison of acute TTP treatment by year-group

	PEX usage: Median (range)	% of acute cases where Rituximab was used
2009-2010	14 (2-75)	72%
2011-2012	12.5 (1-74)	69%
2013-2014	11 (4-51)	76%
2015-2016	10 (3-45)	75%
2017-2018	8 (3-65)	78%
Overall	**11 (1-75)**	

OC 46.4 | Patient Anti-ADAMTS13 Autoantibodies Induce an Open ADAMTS13 Conformation in Immune-mediated Thrombotic Thrombocytopenic Purpura

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Background: Recently we showed that acute immune-mediated thrombotic thrombocytopenic purpura (iTTP) patients have an open ADAMTS13 conformation, while 78% of iTTP patients in remission with ADAMTS13 activity (TS13:act) >50% have a folded ADAMTS13. However, the ADAMTS13 conformation in iTTP patients in remission with a persistent (< 10%) or moderately restored (10-50%) TS13:act and the cause of the open ADAMTS13 conformation is unknown.

Aims: Determine ADAMTS13 conformation in plasma of iTTP patients during acute iTTP and remission with TS13:act < 10%, 10-50% and >50%. Investigate if anti-ADAMTS13 autoantibodies induce conformational changes in ADAMTS13.

Methods: ADAMTS13 conformation and presence of anti-ADAMTS13 autoantibodies was determined by ELISA in 118 iTTP plasmas from acute (n=44) or remission phase (n=74) patients. Additionally, ADAMTS13 conformation was tested in healthy donor (HD) plasma after addition of purified IgG's from 18 acute iTTP patients.

Results: ADAMTS13 was open in 98% (43/44) of the acute phase 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. Next, we could demonstrate that open ADAMTS13 was linked with the presence of anti-ADAMTS13 autoantibodies (chi square, P< 0.0001). Finally, we showed that patient anti-ADAMTS13 autoantibodies can open the ADAMTS13 conformation as purified IgG's from 14 of the 18 (78%) acute iTTP plasmas did induce the open conformation in folded ADAMTS13 from HD plasma, demonstrating that patient anti-ADAMTS13 autoantibodies can indeed induce conformational changes in ADAMTS13.

Conclusions: We show that besides acute iTTP patients, also iTTP patients in remission when TS13:act is < 10% or 10-50% have an open ADAMTS13 conformation, implying that the underlying pathophysiology is still ongoing. In addition, we showed that anti-ADAMTS13 autoantibodies cause conformational changes in ADAMTS13 in iTTP. Hence 'opening' anti-ADAMTS13 autoantibodies are linked to the pathophysiology of iTTP.

OC 46.5 | HLA Variant rs6903608 is Associated with Disease Onset and Relapse in Immune-mediated Thrombotic Thrombocytopenic Purpura

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Background: Immune-mediated thrombotic thrombocytopenic purpura (iTTP) is a rare, life-threatening, autoimmune disease caused by ADAMTS13 severe deficiency, which may recur in up to 50% of patients surviving a first acute episode. Common HLA variant rs6903608 was previously found to increase iTTP risk by 2.5 fold (95%CI 2.05-3.05), but whether this variant is associated with disease relapse is unknown.

Aims: To precisely estimate the impact of rs6903608 on iTTP onset and relapse.

Methods: We performed a case control and cohort study in 161 Italian patients referred for a first episode of iTTP between January 2002 and March 2018, and 456 Italian healthy controls. iTTP was defined by thrombocytopenia, microangiopathic hemolytic anemia (without alternative causes), ADAMTS13 activity < 10% and anti-ADAMTS13 antibodies. rs6903608 was genotyped with TaqMan assay. Logistic, linear, Cox regression, and Kaplan-Meier analysis were used to study the association with a first episode or a relapse of iTTP.

Results: Most patients were women (77%), with a median age at onset of 45 years [interquartile range 33 54]). rs6903608 was strongly associated with iTTP onset (odds ratio [OR] of risk homozygotes 4.68 [95%CI 2.67-8.23]); OR of heterozygotes 1.64 (95%CI 0.95-2.83]) (Table 1), which occurred over three years earlier for each extra risk allele (β -3.34, 95%CI -6.69-0.02). Of 153 survivors (median follow-up 4.8 years [95%CI 3.8-5.9]), 44 (29%) relapsed. Homozygotes for the risk allele had an over 2-fold increased relapse rate compared with both heterozygotes (hazard ratio [HR] 2.44 [95%CI 1.26-4.72])

TABLE 1 Association of the rs6903608 genotype and the occurrence of a first iTTP episode

rs6903608 genotype	Cases (n=161)	Controls (n=456)	OR (95% CI)
TT, n (%)	20 (12)	119 (26)	Reference
CT, n (%)	67 (42)	243 (53)	1.64 (0.95 to 2.83)
CC, n (%)	74 (46)	94 (21)	4.68 (2.67 to 8.23)

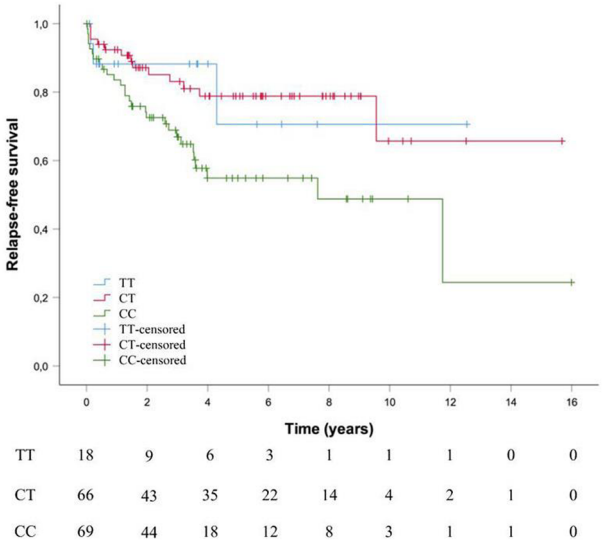

FIGURE 1 Relapse-free survival in iTTP patients according to rs6903608 genotype

and reference allele homozygotes (HR 2.30 [95%CI 0.70-7.57]) (Figure 1).

Conclusions: HLA variant rs6903608 is a risk factor for both iTTP onset and relapse. Given the high severity of acute bouts, which may be fatal despite prompt treatment, this newly identified predictive factor may prove useful to identify patients at high risk of relapse, who may benefit from closer follow-up or more intensive therapy.

OC 61.1 | Efficacy of Recombinant ADAMTS13 (TAK-755/SHP655) in a Humanized Mouse Model for Sickle Cell Disease

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Background: Sickle Cell Disease (SCD) is a hereditary red blood cell (RBC) disorder, caused by a single amino acid substitution of the β chain of hemoglobin (Hb). Sickle Hb polymerizes when deoxygenated and causes rigid, sticky and sickle-shaped RBCs, which disrupt blood flow and contribute to vascular occlusions in the microcirculation. Interestingly plasma concentration of ultra-large von Willebrand Factor (ULVWF) multimers is high in SCD patients, and increasing evidence points to an important role of ULVWF in the pathogenesis of vaso-occlusions.TAK-755/SHP655 is a human recombinant ADAMTS13, the plasma metalloprotease which regulates the size of VWF multimers.

Aims: To provide proof of concept for the disease modifying activity of rADAMTS13 (TAK-755) in a humanized animal model of SCD.

Methods: A humanized mouse strain (Tim Townes mouse) was used as a model for SCD and challenged by hypoxic conditions to generate acute vaso-occlusive crisis (VOC). Mice were intravenously administered vehicle or TAK-755 (3000 U/kg) 1 hour before exposure to hypoxia (8% oxygen for 10 hours), which was followed by 3 hours reoxygenation (H/R). The impact of TAK-755 on mortality was evaluated by exposure to severe hypoxia (7% oxygen). Hematologic parameters were measured and molecular studies carried out on lung and kidney, known target organs for SCD.

Results: In SCD mice exposed to H/R, TAK-755 prevented the H/R-induced increase in circulating neutrophils and anemia. TAK-755 significantly decreased the H/R-induced (i) inflammatory cell infiltrates and local thrombi formation; (ii) inflammatory vasculopathy; and (iii) endothelial dysfunction. Under severe hypoxia, the survival rate of TAK-755 treated mice was higher than vehicle-treated animals.

Conclusions: TAK-755 prevented organ damage and reduced systemic inflammation and hemolysis in a humanized mouse model of SCD exposed to H/R stress. Therefore, administration of TAK-755 may provide therapeutic benefit in clinical management of acute events in SCD.

OC 61.2 | Pregnancy Outcomes in Patients with Atypical Haemolytic Uraemic Syndrome

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Background: Pregnancy outcomes in patients with atypical haemolytic uraemic syndrome (aHUS) are not well reported.

Aims: Here, we present characteristics and outcomes for patients with aHUS who became pregnant while enrolled in the Global aHUS Registry.

Methods: The Global aHUS Registry (NCT01522183), initiated in April 2012, collects demographics, disease history, treatment and outcomes data for patients with aHUS. The current analysis includes patients from the Registry with evaluable pregnancy data supplemented with pharmacovigilance information; numbers of pregnancies, outcomes and treatment status were evaluated.

Results: As of December 2018, 39 pregnancies were recorded in 36 patients. In 3 cases, no outcomes were reported; two pregnancies were ongoing. Outcome data were available for 34 pregnancies: 24 with

eculizumab exposure and 10 without (Table). Of eculizumab-exposed pregnancies, 17 patients were dosed according to recommendations in the prescribing information. There was a higher proportion of live births in non-exposed vs eculizumab-exposed patients (80% vs 58%, respectively) and a more elective terminations in eculizumab-exposed patients (33% vs 10%, respectively; reasons cited in Table footnote). Relapse of aHUS occurred in 9% of patients (1 exposed, 2 non-exposed) with known pregnancy outcomes. Five patients received dialysis during pregnancy, all of whom were eculizumab-exposed (including 1 patient with an aHUS relapse). No malformations or anomalies were reported at or within 3 months of birth. There was a higher proportion of complement mutations in eculizumab-exposed patients with known outcomes (63% vs 33%, respectively).

Conclusions: Real world data from the Global aHUS Registry showed no increase in poor outcomes in pregnant patients exposed to eculizumab with the proportion of miscarriages consistent with the general population (up to 20% of pregnancies[1]). Further evaluation of this aHUS subpopulation is ongoing.

TABLE 1

Table. Pregnancy outcomes in patients with aHUS per eculizumab exposure

Outcome	Pregnancy with exposure to eculizumab (n=24)	Pregnancy without exposure to eculizumab (n=10)
Live birth	14 (58)	8 (80)
Elective termination[a]	8 (33)	1 (10)
Miscarriage	2 (8)	0
Late foetal death	0	1 (10)
aHUS relapse	1 (4)[b]	2 (20)[c]
Genetic mutation identified/tested[d,e]	12/19	6/13
Genetic mutations listed (n)	C3 (1); C3, CFH (1); C3, CFH, MCP, CFB (1); CFH (3); CFH, MCP (3); MCP (2); THBD (1)	CFH (3); MCP (2); THBD (1)

Data presented as n (%) unless otherwise stated. [a]Reasons for elective termination were given for 5 patients citing the following reasons: health concerns of the mother; medical advice due to eculizumab treatment; severe maternal medical condition; high-risk medical reason; maternal pathology and risk of exacerbation affecting prognosis. [b]Relapse occurred during first trimester. [c]Relapse occurred during second trimester. [d]Two patients had one pregnancy each with eculizumab exposure and one without eculizumab exposure; genetic data are included in both groups. [e]Number of patients tested includes those without known outcome. C3, complement component 3; CFB, complement factor B; CFH, complement factor H; CFI, complement factor I; MCP, membrane cofactor protein; THBD, thrombomodulin.

Reference

1. Griebel CP, et al. *American Family Physician* 2005;72(7):1243–50

OC 61.3 | Age at First Disease Manifestation in Relation to Residual ADAMTS13 Activity in Patients with Congenital Thrombotic Thrombocytopenic Purpura

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Background: Congenital thrombotic thrombocytopenic purpura (cTTP) is an autosomal recessive inherited disease with a clinically heterogeneous course and an incompletely understood genotype-phenotype correlation. Some data suggest that the severity of disease in cTTP is related to residual ADAMTS13 activity.

Aims: This study explores the relationship between overt disease onset and ADAMTS13 activity in confirmed cTTP patients from the Hereditary TTP Registry.

Methods: The Hereditary TTP Registry is an international open and ambidirectional cohort study on cTTP. Clinical and laboratory data at enrolment were recorded for each confirmed cTTP patient retrospectively by physicians responsible for patient care. Diagnosis of cTTP was confirmed by severely deficient ADAMTS13 activity (< 10 % of normal) in the absence of a functional inhibitor and the presence of ADAMTS13 mutations on both alleles. Enrolment data until the end of 2017 were analysed. All patients or their legal representatives provided written informed consent before enrolment.

Results: 123 confirmed cTTP patients were enrolled from Europe (55), Asia (52), the Americas (14), and Africa (2). First recognized disease manifestation occurred around birth up to the age of 70 years. Of the 98 different ADAMTS13 mutations detected, c.4143_4144dupA (exon 29; p.Glu1382Argfs*6) was the most frequent mutation, present on 60/246 alleles. We found a larger proportion of compound heterozygous than homozygous carriers of ADAMTS13 c.4143_4144dupA with overt disease onset at < 3 months of age (50% versus 37%), despite the fact that ADAMTS13 activity was < 1% in 18/20 homozygotes compared to only 8/14 compound heterozygotes. Evaluating overt disease onset in all patients with available anamnestic information and ADAMTS13 activity values measured in the TTP centers of Bern (Switzerland) and Nara (Japan) (n=97), we found a weak but significant correlation between age at overt onset and ADAMTS13 activity (r_s=0.25, p< 0.01).

Conclusions:

Residual ADAMTS13 activity only partially explains the age at first disease manifestation. :

OC 61.4 | Successful Treatment of Pregnant Patients with Congenital Thrombotic Thrombocytopenic Purpura Using Prophylactic Fresh Frozen Plasma Infusion

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Background: Congenital thrombotic thrombocytopenic purpura (cTTP), also known as Upshaw-Schulman syndrome, is an extremely rare thrombotic disease caused by congenital *ADAMTS13* gene mutations. Female patients with cTTP often develop hemolytic attack and was diagnosed with cTTP during pregnancy. However, the differences in delivery outcomes between women with or without FFP and the optimal dosage of prophylactic fresh frozen plasma (FFP) infusion are not clear.

Aims: To clarify this issue, we performed a retrospective cohort study using a Japanese cTTP registry.

Methods: Thirty-four pregnancies in 20 patients with cTTP who were included in the Japanese registry by 2017 were enrolled in this study. cTTP was diagnosed mainly based on 1) a deficiency of ADAMTS13 (less than 10% activity) without any inhibitor, and 2) *ADAMTS13* gene mutation. We analyzed delivery outcomes and prophylactic FFP infusion during pregnancy.

Results: The characteristics of these pregnancies are shown in Table 1. Twenty-six pregnancies were identified before the diagnosis of cTTP. Of these, 14 were live births and 12 were stillbirths. The causes of stillbirth were intrauterine fetal death in 11 cases and miscarriage in one case, and the median gestation period at stillbirth was 25 weeks (range 20-31). By contrast, all eight pregnancies from six women who became pregnant following the diagnosis of cTTP lead to live births successfully. Seven received prophylactic FFP infusion, while one did not because maternal plasma ADAMTS13 levels remained higher than 5%. As shown in Figure 1, over 5 mg/kg/week of FFP infusion might be required to maintain pregnancy. Interestingly, one patient with 2.4 mg/kg/week of FFP infusion underwent emergency cesarean section at 30 weeks of gestation due to fetal distress.

Conclusions: Our study indicated that the amount of FFP should be increased from 20-25 weeks of gestation, when pregnancy-associated cTTP exacerbation was seen. Enough FFP infusion could improve the outcome of pregnancy in cTTP patients.

TABLE 1 The characteristics of cTTP pregnancies

	Pregnancy before cTTP diagnosis	Pregnancy after cTTP diagnosis
Number of patients	16	6
Median age of mother at delivery	28.5 (range: 23-33)	28 (range: 22-33)
Number of pregnancies	26	8
Infant outcomes	14: live birth 12: stillbirth	8: live birth
Treatment during pregnancy	3: FFP infusion 23: none	7: FFP infusion 1: close watching
Median weeks of gestation at live birth	36.5 (range: 25-39)	37 (range: 30-39)
Labor Procedure at live birth	10: cesarean section 1: vaginal delivery 3: not available	6: cesarean section 2: vaginal delivery
Median weeks of gestation at stillbirth	25 (range: 20-31)	Nothing

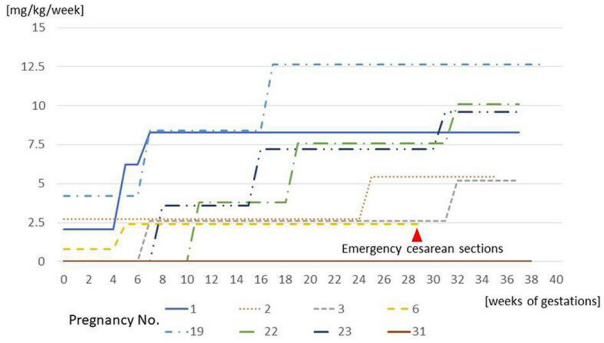

FIGURE 1 Changes of FFP amounts for cTTP patients during pregnancy

OC 61.5 | Genetic Landscape in Korean Adult Patients with Atypical Hemolytic Uremic Syndrome

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Background: Atypical hemolytic uremic syndrome (aHUS) is a rare thrombotic microangiopathy (TMA) with characteristics of microangiopathic hemolytic anemia, thrombocytopenia, and acute kidney injury. It has been reported that genetic defects are related with the disease development and prognosis.

Aims: We investigated the genetic landscape of Korean adult patients with aHUS.

Methods: The study subjects were 793 patients diagnosed with thrombotic microangiopathy (TMA) from 2012 to 2018. The diagnosis of aHUS was clinically made according to the recent guidelines with exclusion of TMA patients having ADAMTS13 activity < 10%, STEC-HUS, and TMA of secondary etiology. Molecular genetic workup included the initial screening of hotspot mutations in CFH and C3 genes by RFLP tests in 768 patients. Among mutation-negative patients by RFLP, 59 patients with a high index of suspicion were selected for whole exome sequencing (WES). Variants were classified using the 2015 ACMG standards and guidelines.

Results: In the cohort, total 55 pathogenic or putative pathogenic variants were found in 36 aHUS patients. Thirty-one patients were identified from WES and ten patients from RFLP. Affected genes were CFH (12/55, 22%), C3 (7/55, 13%), THBD (6/55, 11%), CFHR4 (5/55, 9%), PLG (5/55, 9%), MCP (4/55, 7%), CFHR5 (4/55, 7%), CFB (2/55, 4%), CFHR2 (2/55, 4%), C4BPA (2/55, 4%), CFI (2/55, 4%), and one each case (2%) of C4BPB, CFHR1 and CFHR3. Fourteen patients (14/36, 39%) had more than two pathogenic or putative pathogenic variants. THBD D486Y, classified as variant of uncertain significance, were recurrently identified in aHUS patients with a higher frequency than in general Korean population (Fisher's exact test, P=0.007). A pathogenic variant in C4BPA was firstly found in one patient in our cohort.

Conclusions: This is the largest study to delineate the genetic etiology in Korean adult patients with aHUS. We found novel pathogenic variants and a high frequency of multiple variants in our series.

VENOUS THROMBOEMBOLISM

OC 04.1 | Incidence of Residual Perfusion Defects by Lung Scintigraphy in Patients Treated with Rivaroxaban Compared with Warfarin for Acute Pulmonary Embolism

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Background: Residual perfusion defects as detected by lung scintigraphy occur in over 50% of patients with acute pulmonary embolism (PE) treated with vitamin K antagonists but there is lack of data in patients treated with direct oral anticoagulants.

Aims: To retrospectively estimate the incidence of residual perfusion defects detected by Ventilation Perfusion (VQ) scan at 3-6 months in patients with first acute symptomatic PE treated with Rivaroxaban compared to warfarin.

Methods: Consecutive eligible patients with confirmed PE treated with rivaroxaban as part of a previous study were identified. The Monash Health Radiology database was used to identify a historical cohort of age matched (± 5 years) PE patients treated with warfarin. Follow-up VQ scans were classified as normal (no perfusion defect) or abnormal (matched or unmatched perfusion defects) by 2 independent nuclear medicine physicians blinded to treatment.

Results: One hundred and ninety patients with PE (95 in each cohort) were included (mean age, 56.8 years; 41.1% males; 54.2% unprovoked). Baseline demographics were similar but there was a significant difference in modality of the index scan in warfarin versus rivaroxaban treated patients (Table 1). In the overall cohort, 31.1%

TABLE 1 Characteristics of study population and incidence of residual perfusion defects in rivaroxaban compared to warfarin treated PE (n = 190)

Characteristic	Overall study cohort n = 190	Warfarin n = 95	Rivaroxaban n =95	P value
Mean age, years (SD)	56.8 (18.0)	56.8 (17.4)	56.7 (18.7)	0.97
Male gender, n (%)	78 (41.1)	41 (43.2)	37 (38.9)	0.56
Mean Weight, kg (SD)	87.6 (21.0)	86.9 (21.1)	88.1 (21.1)	0.72
Unprovoked PE	103 (54.2)	51 (53.7)	52 (54.7)	0.12
Mean time to follow-up scan, days (SD)	146.1 (31.4)	154.7 (27.1)	137.6 (33.3)	<0.01
Index scan VQ scan (%)	89 (46.8)	59 (62.1)	30 (31.6)	<0.01
Index Scan CTPA (%)	101 (53.1)	36 (37.9)	65 (68.4)	<0.01
Index scan involving ≥ 3 segments in patients with VQ scan	55 (61.8)	32 (54.2)	23 (76.7)	0.04
Index scan involving main PA or saddle PE in patients with CTPA (%)	32 (31.7)	15 (41.7)	20 (30.8)	0.27

had residual defects. There was a significantly lower incidence of residual perfusion defects in rivaroxaban treated patients 23.2% (95% CI 15.8 to 32.6), compared to warfarin 38.9% (95% CI 29.8 49.0, p = 0.02) despite a similar proportion of patients with central pulmonary artery involvement and a higher proportion of patients with involvement of ≥ 3 segments on index CTPA or VQ scan respectively.

Conclusions: Treatment with rivaroxaban was associated with a significantly lower incidence of residual perfusion defect detected by VQ scan at 3-6 months compared to warfarin. This supports recent evidence that direct Xa inhibitors may enhance fibrinolysis but requires further evaluation in a larger cohort of patients.

OC 04.2 | The Rule-out Criteria for Chronic Thromboembolic Pulmonary Hypertension can Identify Patients without Haemodynamic Abnormalities and Functional Limitation 3 and 12 Months after Acute PE

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Background: Up to one third of patients report persisting hemodynamic abnormalities and functional limitation over long-term follow-up after acute pulmonary embolism (PE).

Aims: We tested whether a validated algorithm designed to rule-out chronic thromboembolic pulmonary hypertension (CTEPH) after acute PE can be used for identifying patients at lower risk of presenting with persisting symptoms and echocardiographic abnormalities.

Methods: The multicentre Follow-up of Acute Pulmonary Embolism (FOCUS) cohort study prospectively enrolled 1,100 consecutive patients diagnosed with acute symptomatic PE; two year follow-up is ongoing. We focused on the scheduled visits for 3- and 12-month follow-up. The rule-out criteria are based on: the absence of ECG signs of right ventricular dysfunction and normal NT-proBNP/BNP values. Echocardiographic abnormalities were defined according to the presence of abnormal parameters indicating an intermediate/high probability of pulmonary hypertension as recommended by the 2015 ESC/ERS Society Guidelines on Pulmonary Hypertension. The presence of functional limitation was defined based on a World Health Organization classification grade ≥3, a Borg dyspnoea index ≥4, or a 6-minute walking distance < 300 m.

Results: We included 323 patients (mean age 61 years, 58% men), of whom 255 have meanwhile completed one-year follow-up. At 3- and 12-month follow-up, 194 (60%) and 155 (61%) of patients exhibited no abnormal echocardiographic findings or natriuretic peptide levels. The percentage of patients with echocardiographic abnormalities was 20.4% and 18.0%, respectively. The negative predictive value of the score for ruling out the combination of functional limitation and intermediate/high probability of pulmonary hypertension as recommended by the 2015 ESC/ERS Guidelines on Pulmonary Hypertension was 0.96 (95%CI 0.92-0.98) at 3 and 0.97 (0.92-0.99) at 12 months. The corresponding positive predictive values were 0.10 (0.06-0.17) and 0.09 (0.05-0.17), respectively.

Conclusions: The CTEPH rule-out criteria are capable of excluding functional limitation and evidence of (chronic) pulmonary hypertension 3 and 12 months after the diagnosis of acute PE.

OC 04.3 | Early Identification of Chronic Thromboembolic Pulmonary Hypertension after Pulmonary Embolism (InShape II Study)

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Background: The diagnostic delay of chronic thromboembolic pulmonary hypertension (CTEPH) after acute pulmonary embolism (PE) is >1 year and associated with worse hemodynamic profile at diagnosis and increased mortality. We have derived a non-invasive screening strategy consisting of the 'CTEPH prediction score' and 'CTEPH rule-out criteria' (Figure 1), aimed at diagnosing CTEPH earlier.

Aims: To prospectively evaluate the diagnostic accuracy of this strategy.

Methods: The InShape II study is a prospective, multicenter outcome study in consecutive PE patients. All patients are managed according to the strategy 3-6 months after PE. Main outcomes are 1) the number and yield of echocardiograms indicated at baseline and 2) the 2-year CTEPH incidence in patients in whom CTEPH was initially considered absent. The study protocol was approved by all local IRBs.

Results: Last patient was included in October 2017. We hereby report the results of the baseline application of the strategy: 424 patients were included in The Netherlands, Poland and Belgium. Mean age was 56 (SD 16), 59% of patients had unprovoked PE and 20% had recurrent PE. CTEPH was considered absent in 202 patients (48%)

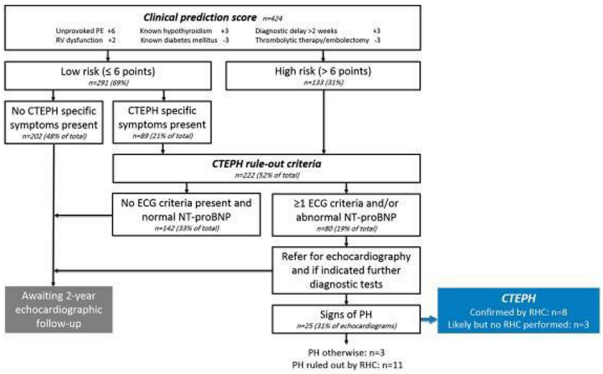

FIGURE 1 Screening algorithm for early detection of CTEPH, showing baseline results

without further diagnostic tests. Of the remaining 222 patients, 80 patients had one of the ECG requirements for right ventricular overload and/or abnormal NT-proBNP. These patients underwent echocardiography: 25/80 (31%) had signs of PH of whom 8 were diagnosed with CTEPH by right heart catheterization; 3 died before right heart catheterization was performed ('CTEPH likely') and CTEPH was ruled out in 14. The CTEPH incidence ranged between 1.9% (confirmed cases) and 2.6% (confirmed plus likely cases).

Conclusions: Echocardiograms were indicated in only 19% of patients. The CTEPH incidence in patients referred for echocardiography was 10-14%, and in the overall population 1.9-2.6%, which is within the expected range. Results of ongoing follow-up will determine whether cases of CTEPH were missed by the algorithm.

OC 04.4 | Long-term Clinical Prognosis of Massive and Non-massive Pulmonary Embolism: A Registry-based Cohort Study

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Background: Severe pulmonary embolism with hemodynamic compromise (« massive PE ») carries a substantial short-term morbidity and mortality. Its longer-term prognosis, however, is not well known, in particular with regards to its risk of recurrent venous thromboembolism (VTE). This information may help inform the decisions of duration of anticoagulation in such patients.

Aims: To compare the risk of recurrent VTE after massive and non-massive PE.

Methods: We included participants in the RIETE registry who suffered an objective diagnosis of segmental or more central PE. Massive PE was defined by a systolic hypotension at clinical presentation (< 90mmHg). The risks of recurrent VTE were compared between massive and non-massive PE, during the phase of therapeutic anticoagulation and after anticoagulation was stopped, using survival analysis regression models and competing risk models. Informed consent was obtained from participants in accordance with independent local ethics committee and institutional review board requirements.

Results: Among 34,176 participants with PE, 1197 (3.5%) were classified as massive. Compared with non-massive PE, at baseline, they had a greater proportion of syncope (43% vs. 14%), RV dysfunction (51% vs. 23%), and use of thrombolysis (17% vs. 2%). During the period of anticoagulation (mean of 10 months), there was no difference in the risk of recurrent VTE (adjusted subhazard ratio 1.17, 95%CI 0.75-1.81). After discontinuation of anticoagulation, 8,683 participants were followed for a mean of 13 months. The risk of recurrent VTE was similar between massive and non-massive PE (adjusted HR 0.81, 95%CI 0.46-1.39).

Conclusions: In this large observational registry, the risk of recurrent VTE, during and after anticoagulation, was not different between patients having suffered massive vs. non-massive PE.

OC 04.5 | The Prognostic Value of Syncope on Mortality in Patients with Pulmonary Embolism: A Systematic Review and Meta-analysis

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Background: Syncope is a presenting symptom of pulmonary embolism (PE) in 10-20%. Previous studies suggest an association with hemodynamic instability and mortality, but results are contradictory. The prognostic value of syncope in PE remains to be elucidated.

Aims: To clarify the prognostic value of syncope on short-term (in-hospital or 30-day) mortality in patients with PE and its association with hemodynamic instability.

Methods: Pubmed, EMBASE and Cochrane Library databases were searched up till July 6, 2018. Studies reporting on in-hospital or 30-day mortality of adults with PE with and without syncope were included. Quality of included studies was evaluated with the Quality In Prognosis Studies tool. Meta-analysis was conducted to derive combined odds ratios (OR) for the relation of syncope with mortality and with hemodynamic instability. To study the influence of hemodynamic instability on the association between syncope and mortality, meta-regression was performed. This study was registered in PROSPERO (CRD42018098977).

Results: Our search and selection resulted in 20 studies. The final analysis included 15 studies, involving 1111/8632 (13%) patients with syncope. Syncope was associated with higher mortality (OR 1.64,

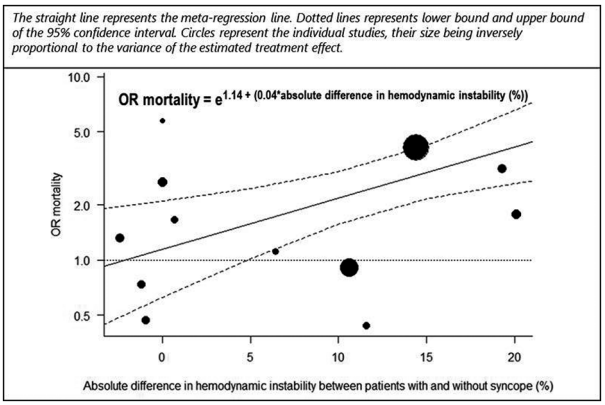

FIGURE 1 Univariate meta-regression analysis with association between syncope and mortality

95% CI 1.02 - 2.63) and higher prevalence of hemodynamic instability (OR 3.45, 95% CI 2.76-4.33). Substantial heterogeneity was present for the association with mortality (I^2 70%), but not for the association with hemodynamic instability (I^2 0%). Meta-regression showed that higher mortality in patients with syncope is at least partially explained by a higher prevalence of hemodynamic instability (Figure 1).

Conclusions: Syncope is associated with higher short-term mortality, which is linked to hemodynamic instability. In the absence of a difference in hemodynamic instability between patients with and without syncope, no significant difference in mortality is observed. This could reflect different mechanisms underlying syncope in hemodynamically stable patients. Future research is warranted to assess the added value of syncope on current risk assessment strategies.

OC 14.1 | Current Practice Patterns of Outpatient Management of Acute Pulmonary Embolism: A Post-hoc Analysis of the YEARS Study

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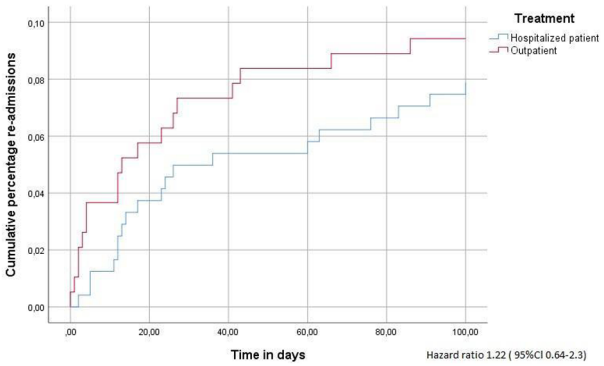

FIGURE 1 PE-related readmissions in time

Background: Over the last decade, studies have shown the safety of outpatient treatment of patients with pulmonary embolism (PE) at low-risk of adverse events. Management studies focussing on outpatient treatment have suggested that 30% to 55% of acute PE patients could be treated at home based on the HESTIA criteria, but data from day-to-day clinical practice are less established.

Aims: To determine current practice patterns of outpatient treatment of acute PE in the Netherlands.

Methods: We performed a post-hoc analysis of the YEARS study, a prospective, multicentre, diagnostic management study in 12 hospitals in the Netherlands (vd Hulle et al, Lancet 2017). The main outcomes were the proportion of patients who were discharged < 24 hours and reasons for admission if treated in hospital. Further, we compared the 3-month incidence of PE-related re-admissions between patients treated as outpatient and as inpatient.

Results: Of the 456 patients with PE included in the YEARS study, 12 were excluded because PE was diagnosed during hospitalization. Of the remaining 444 patients, 191 (43%, 95%CI 38-48) were treated as outpatient. The mean duration of admission of the hospitalized patients was 4.5 days (± 4.1 days). Reasons for hospitalization are shown in **Table 1:** mainly for oxygen administration and/or "medical or social reasons". The rate of PE-related re-admissions of patients treated as outpatient was 9.4% versus 7.9% for hospitalized patients, resulting in a crude hazard ratio of 1.2 (95%CI 0.64-2.3; **Figure 1**). The 3-month incidence of any adverse event was 4.2% in those treated at home (1 recurrent VTE, 5 major bleedings and two

TABLE 1 Reasons for hospitalization after diagnosis PE*

Reasons for hospital admission	Frequency	Proportion
1. Hemodynamically unstable	15	6.2%
2. Need for thrombolysis or embolectomy	1	0.41%
3. Active bleeding or high bleeding risk	10	4.1%
4. > 24 hr oxygen supply	78	32%
5. Need for intravenous pain medication > 24 hr	21	8.7%
6. Medical or social reasons	100	41%
7. Renal insufficiency (< 30 ml/min)	5	2.1%
8. Severe liver impairment	2	0.83%
9. New PE during anticoagulant treatment	3	1.2%
10 No risk stratification scheme such as Hestia applied	26	

* Multiple Hestia criteria for admission could be scored in one patient

deaths) compared to 9.5% in the hospitalized patients (3 recurrent VTE, 6 major bleedings and fourteen deaths).

Conclusions: In the YEARS study, 43% of patients with PE was treated at home. PE-related re-admission rates were not different between patients treated at home or in hospital.

OC 14.2 | Two Weeks of Low Molecular Weight Heparin for Isolated Symptomatic Distal Vein Thrombosis (TWISTER Study): Interim Analysis of First 200 Patients

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Background: Most deep vein thrombosis (DVT) originate from distal (calf) vein thrombi, however 80-90% of these lyse spontaneously. The management of isolated distal DVT (IDDVT) is controversial.

Aims: Examine the efficacy and safety of two weeks of anticoagulation for a first symptomatic IDDVT.

Methods: Multicentre prospective cohort study in Australia/New Zealand for patients with confirmed first symptomatic IDDVT. Exclusions included active malignancy, bleeding risk factors, pregnancy/lactation, ongoing immobility, plaster casts or non-weight-bearing. Patients received 2 weeks of therapeutic anticoagulation (enoxaparin or rivaroxaban) with repeat complete compression leg vein ultrasound (CUS) at the end of 2 weeks. If the patient was asymptomatic and there was no proximal DVT extension on CUS, treatment was stopped (group A). If the patient was still symptomatic, treatment was continued for a further 4 weeks(group B). The primary outcome is symptomatic recurrence of venous thromboembolism, VTE (DVT and pulmonary embolism, PE) within 3 months, in group A patients.

Results: 200 patients were enrolled at eleven sites; one was withdrawn on day five due to clinically relevant non-major bleeding. Of the remaining 199, 140 (70%) received two weeks anticoagulation; 56 (28%) six weeks anticoagulation; and three patients (2%) had proximal extension on 2-week CUS. Nine patients in group A were lost to follow-up. Of the remaining 131 in the two-week anticoagulation arm, three had symptomatic distal DVT recurrence - VTE recurrence rate 3/131; 2.3%(95% CI 0.77-5.07%). No patients, 0/131; 0%(95% CI 0.00-2.78) had proximal DVT or PE recurrence.

Conclusions: For patients with IDDVT, two weeks of anticoagulation is associated with a low risk of VTE recurrence (2.3%), if patients are symptom-free at two weeks and the repeat CUS at two weeks doesn't show thrombus extension into proximal veins. Based on this data, the previous standard of care, 6-12 weeks anticoagulation, isn't required for the majority of IDDVT patients.

OC 14.3 | Safety of Outpatient Management of Pulmonary Embolism: 2010 - 2018 in a Large Teaching Hospital

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Background: We have collected data on all pulmonary embolism (PE) diagnoses since 2010 reviewing treatment and demographic information. Criteria based on the modified Pulmonary Embolism Severity Index (PESI) score is used to decide if outpatient management (OP) is suitable.

Aims: Whilst most deep vein thrombosis is managed as OP, smaller numbers of PE are treated this way. We reviewed the safety of OP PE management locally covering mortality and hospital readmission over nine years.

Methods: Retrospective cohort study of all PE's diagnosed between 2010 to 2018. Using radiological records to identify PE and patient management system to compare OP with inpatient treatment. OP management, defined as discharge within 24 hours of diagnosis. All-cause mortality compared at 7 and 28 days for both groups and the number of OP managed patients readmitted within 28 days of discharge, as a result of PE.

Results: Over 9 years, there has been an increase in PE diagnoses: 355 (55% total VTE) to 485 (59%) in 2018. Over the same period there has been a significant increase in OP PE management from 111/355 (31%) to 210/485 (43%) (p=>0.001). Over the study period 29/1665 (1.7%) patients were readmitted due to complications related to their PE. We also looked at 7 day all-cause mortality post PE diagnosis; this was much greater for inpatient management at 128/2583(5%) compared to outpatients being 3/1665 (0.2%). There were similar findings at 28 days 301/2583 (12%) compared with 22/1665 (1%).

Conclusions: Over 9 years we demonstrate increases both in total PE but also OP managed PE. The safety of this approach is confirmed when appropriate criteria are followed. The increased use of direct oral anticoagulants may also have contributed to the increase in OP management. This is a safe methodology, with financial benefits, using the modified PESI saving nearly 5000 bed days over the study period.

OC 14.4 | No Predictive Role for Right Ventricular Function Measurement in Patients with Pulmonary Embolism Treated at Home Based on Absence of Hestia Criteria

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Background: There is a continuing debate on the relevance of right ventricle (RV) dysfunction in normotensive pulmonary embolism (PE) patients as a tool to identify low-risk patients eligible for outpatient treatment. According to the Hestia criteria, assessment of RV function is not required to select outpatient treatment candidates (Zondag J, Thromb Haemost, 2011).

Aims: To assess the incidence of CT-measured RV dysfunction in patients treated at home based on the application of the Hestia criteria, and its impact on clinical outcome.

Methods: This is a patient-level post-hoc analysis of the combined Hestia and Vesta studies, both multicentre prospective outcome studies evaluating the safety of outpatient treatment for PE based on the Hestia criteria (Zondag, J Thromb Haemost, 2011; den Exter, Am J Respir Crit Care Med, 2016). Primary outcome was the combined 3-month incidence of recurrent VTE, major bleeding and mortality ('adverse events') in patients with versus those without RV dysfunction, the latter defined as post-hoc assessed CT-measured RV/LV ratio >1.0.

Results: Of all 1627 consecutive patients eligible for the Hestia and Vesta studies, complete data were available for 1474 patients, of whom 752 were treated at home (51%); 225 (30%) of those had a RV/LV diameter ratio >1.0 (range 0.74-2.4). Incidence of adverse events was 3.1% in patients with RV dysfunction treated at home compared to 3.0% for those with a normal ratio, for an Odds Ratio of 1.1 (95% CI 0.44-2.7; table 1).

Conclusions: In this study, RV dysfunction did not predict poor outcome in patients selected for outpatient treatment by the Hestia criteria, challenging the concept that RV function should be used to guide management in all normotensive PE patients.

OC 14.5 | Prognostic Value of Respiratory Index (RI) in Haemodinamically Stable Patients with Acute Pulmonary Embolism: The RI-MODEL Study

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Background: The accuracy of the 2014 ESC model to predict 30-day mortality in hemodynamically stable patients with acute pulmonary embolism (PE) is relatively limited.

Aims: The aims of this study in hemodynamically stable patients with acute PE were i) to evaluate the prognostic value of a novel respiratory index (RI) (oxygen saturation in air to respiratory rate ratio) and ii) to assess the accuracy of the RI-model (simplified Pulmonary Embolism Severity Index [sPESI] + RI), both in predicting 30-day mortality.

Methods: A collaborative database of hemodynamically stable patients with PE was divided into two cohorts (derivation and validation) with equal numbers of patients, based on a temporal criterion. Study outcome was 30-day all-cause-death. Discrimination and calibration were assessed in the derivation and validation cohorts by the c-statistics and by the Hosmer-Lemeshow test, respectively.

Results: 30-day all-cause-death occurred in 7.5% of the 319 patients in the derivation cohort (mean age 72 years, females 53%). The RI was an independent predictor of 30-day mortality (p=0.004). A RI ≤3.8 was associated with an increased death rate compared to

TABLE 1 Adverse outcomes in patients with acute pulmonary embolism treated out-of-hospital stratified by RV dysfunction

	RV/LV ratio > 1.0 (n=224)	RV/LV ratio ≤ 1.0 (n=527)	OR	95% CI
1. All-cause mortality within 3 months	5 (2.2%)	5 (0.9%)	2.4	(0.68-8.3)
2. major bleeding within 3 months	1 (0.45%)	3(0.6%)	0.78	(0.081-7.6)
3. recurrent VTE within 3 months	1 (0.45%)	7 (1.3%)	0.33	(0.041-2.7)
4. Total adverse events	7 (3.1%)	15 (3.0%)	1.1	(0.44 -2.7)

higher RI values (15.4% vs 5.0%, OR 3.4, 95% CI 1.5-8.1). When the RI≤3.8 was integrated in the sPESI, the novel RI-model showed a good discriminatory power (c-statistics 0.703, 95% CI 0.603-0.803). In the 319 patients of the validation cohort (30-day mortality 6.9%, mean age 71 years, females 55%) the discriminatory power of the RI-model was confirmed (c-statistics 0.838, 95% CI 0.768-0.907).

The RI-model and the 2014 ESC model had a c-statistics of 0.772 (95% CI 0.709-0.834) and of 0.687 (95% CI 0.620-0.753) in the overall population, respectively.

Conclusions: In this study, the RI independently predicted 30-day mortality in hemodynamically stable patients with acute PE. A clinical model including RI showed a better discriminatory value than 2014 ESC model and could be used for risk stratification in these patients.

OC 24.1 | The Influence of Renal Impairment on Clinical Outcomes in Venous Thromboembolism Patients Enrolled in GARFIELD-VTE

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Background: Venous thromboembolism (VTE) patients with concomitant chronic kidney disease (CKD) represent a complex patient group, with an increased risk of thrombosis as well as serious bleeding complications. Moreover, anticoagulation is challenging because the majority of anticoagulant drugs are excreted by kidney.

Aims: Compare the baseline characteristics, treatment patterns, and 12-month outcomes between patients with moderate-to-severe (stages 3-5) CKD and patients with normal-to-mild (stages 1-2) CKD enrolled in the Global Anticoagulant Registry in the FIELD (GARFIELD)-VTE.

Methods: GARFIELD-VTE (ClinicalTrials.gov: NCT02155491) is a global, prospective, non-interventional study of real-world treatment practices. 10,685 patients with objectively confirmed VTE were enrolled between May 2014 and January 2017, 8,939 of which (normal-to-mild CKD n=6897, moderate-to-severe CKD n=2042) were eligible for analysis in this study.

Results: The distribution of VTE events was comparable between groups (moderate-to-severe CKD: 57.1% deep vein thrombosis (DVT) alone, 42.9% pulmonary embolism (PE) ± DVT; normal-to-mild: 58.9% DVT alone, 41.1% PE ± DVT). Moderate-to-severe CKD (vs. normal-to-mild CKD) was more common in females (57.0% vs. 47.0%). Moderate-to-severe CKD patients were older; mean (standard deviation) 67.4 (14.8) years vs. 56.1 (16.6) years, but BMI; mean (standard deviation) was comparable to patients with normal-to-mild

TABLE 1 Treatment at baseline (± 30 days of VTE diagnosis) in moderate-to-severe CKD patients vs normal-to-mild CKD patients enrolled in GARFIELD-VTE

Treatment	*Moderate-to-severe CKD N (%)*	*Normal-to-mild CKD N (%)*
Anticoagulation therapy only	**1877 (91.9)**	**6416 (93.0)**
Parenteral therapy only	***331 (17.6)***	***1130 (17.6)***
Parenteral therapy + VKA	***586 (31.2)***	***1658 (25.8)***
VKA only	***114 (6.1)***	***311 (4.8)***
DOAC only	***514 (27.4)***	***1956 (30.5)***
Parenteral therapy + DOAC	***286 (15.2)***	***1209 (18.8)***
Unknown	***46 (2.5)***	***152 (2.4)***
Other therapy ± anticoagulation	**123 (6.0)**	**382 (5.5)**
No therapy or compression only	**42 (2.1)**	**99 (1.4)**

TABLE 2 12-month outcomes in moderate-to-severe CKD patients and normal-to-mild CKD patients enrolled in GARFIELD-VTE

Event	Moderate-to-severe CKD (N=2,022)		Normal-to-mild CKD (N=6,860)		Hazard Ratio [95% CI]	P-value
	n	Rate per 100 person-years [95% CI]	n	Rate per 100 person-years [95% CI]		
All-cause mortality	228	12.7 [11.2-14.5]	413	6.6 [6.0-7.3]	1.92 [1.63-2.26]	<0.0001
Major bleed	58	3.3 [2.5-4.2]	104	1.7 [1.4-2.0]	1.94 [1.41-2.68]	<0.0001
Recurrent VTE	113	6.6 [5.5-7.9]	311	5.1 [4.6-5.7]	1.27 [1.02-1.57]	0.0301
Any bleeding	238	14.4 [12.7-16.3]	627	10.7 [9.9-11.5]	1.33 [1.14-1.54]	0.0002
Stroke/Transient Ischemic Attack	20	1.1 [0.7-1.7]	44	0.7 [0.5-1.0]	1.59 [0.94-2.70]	0.0858
Myocardial Infarction/Acute Coronary Syndrome	23	1.3 [0.9-1.9]	41	0.7 [0.5-0.9]	1.95 [1.17-3.26]	0.0101

CKD: 29.2 (6.9) kg/m^2 vs. 28.1 (6.5) kg/m^2, respectively. Chronic heart failure (6.8% vs. 2.2%), chronic immobilisation (7.1% vs. 5.3%) and a history of cancer (17.2% vs. 12.9%) were all more common in moderate-to-severe CKD patients compared to normal-to-mild CKD patients, respectively. No clinically meaningful differences were found between treatment patterns at baseline between groups (Table 1). Over 12-months follow-up, patients with moderate-to-severe CKD were at an increased risk (hazard ratio [95% confidence interval]) of all-cause mortality (1.92 [1.63-2.26]), major bleeding (1.94 [1.41-2.68]), and recurrent VTE (1.27 [1.02-1.57]) (Table 2).

Conclusions: VTE patients with moderate-to-severe CKD are at an increased risk of major adverse outcomes.

OC 24.2 | Clinical Utility of Measuring DOAC Levels in Patients with Major Haemorrhage or Requiring Urgent Anticoagulation Reversal - Results from the Anticoagulation Reversal and Events Study (ARES) Collaborative

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Background: The ARES Collaborative (ACTRN12613001304729) is a large ANZ observational study of patients who present to hospital with haemorrhage, thromboembolism or needing urgent anticoagulant reversal and are taking either a DOAC (dabigatran, rivaroxaban or apixaban) or warfarin. The clinical context, event severity, outcome and haemostatic strategies utilised were recorded. ARES included case records from 1218 presentations, of which 449 (36.9%) involved a DOAC.

Aims: To describe the frequency of DOAC plasma level ascertainment within 24 hours of presentation, and its association with the haemostatic intervention given in patients presenting with major haemorrhage (MH) or urgent anticoagulation reversal (UAR).

Methods: After multisite IRB approval, clinical data and DOAC plasma levels within 24 hours of presentation were extracted from the records from DOAC patients presenting with MH or UAR (n=387).

Results: A total of 213/387 (55.3%) DOAC-treated patients had levels taken within 24 hours of presentation (dabigatran n=95, rivaroxaban n=76, apixaban n=42). Most DOAC levels were within the on-therapy expected range although 38% had a low plasma concentration (< 50ng/ml) where a haemostatic or reversal agent (HRA) (Prothrombinex-VF®/FEIBA® or idarucizumab, respectively) may not be necessary. HRA were given in around a half of DOAC patients and there was little difference in HRA usage in those who had a level taken or not. In patients where the DOAC level is < 50ng/ml, around 38% were given a HRA.

Conclusions: Around half the DOAC patients admitted to hospital with MH or UAR had a DOAC level measured. Most levels were in the expected on-therapy range indicating that case selection for DOAC anticoagulation therapy is appropriate. If a DOAC level was taken, those patients with low/no level would unlikely to benefit from a HRA. These results suggest that obtaining a timely DOAC level could avoid the use of unnecessary and expensive HRA in patients with MH or UAR.

OC 24.3 | Evaluation of the Predictive Value of the Bleeding Prediction Score "VTE-BLEED" for Recurrent Venous Thromboembolism

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Background: VTE-BLEED is a validated score for identification of patients at a 3 to 5-fold increased risk of major bleeding during extended anticoagulation for venous thromboembolism (VTE; table 1). It is unknown whether VTE-BLEED high-risk patients also have an increased risk for recurrent VTE, which would limit the potential usefulness of the score.

Aims: To evaluate the association between VTE-BLEED classification and risk of recurrent VTE.

Methods: This was a *post-hoc* analysis of the randomised double-blind placebo-controlled PADIS-PE trial, in which patients with a first unprovoked pulmonary embolism (PE) initially treated for 6 months were randomised to receive an additional 18-month of warfarin versus placebo. Primary outcome of the current analysis was recurrent VTE during 2-year follow-up after anticoagulant discontinuation, i.e. after the initial 6-month treatment in the placebo arm and after 24 months of anticoagulation in the active treatment arm. This rate, adjusted on study treatment allocation, was compared between patients in the high- *versus* low-risk VTE-BLEED group.

Results: In complete case analysis (n=308; 82.4% of total population), 89 (28.9%) patients were classified as VTE-BLEED high risk. A total of 44 VTE events occurred after anticoagulant discontinuation during 668 patient-years. The cumulative incidence of recurrent VTE was 16.4% (95%CI 10.0-26.1%; 14 events) and 14.6% (95%CI 10.4-20.3%; 30 events) in the high-risk and low-risk VTE-BLEED groups, respectively, for an adjusted Hazard Ratio of 1.16 (95%CI 0.62-2.19; Figure 1).

Conclusions: In this study, patients with unprovoked PE classified at high risk of major bleeding by VTE-BLEED did not have a higher incidence of recurrent VTE after cessation of anticoagulant therapy, supporting the potential yield of the score for making management decisions on the optimal duration of anticoagulant therapy.

TABLE 1 The VTE-BLEED score with original definition of variables

Factor	Score
Active cancer	2
Male with uncontrolled arterial hypertension m(systolic blood pressure ≥140 mmHg)	1
Anaemia	1,5
History of bleeding	1,5
Age ≥60 years old	1,5
Renal dysfunction (eGRF <60 mL/min)	1,5
Classification of patients with the VTE-BLEED score	
Low bleeding risk	Total score <2
High bleeding risk	Total score ≥2

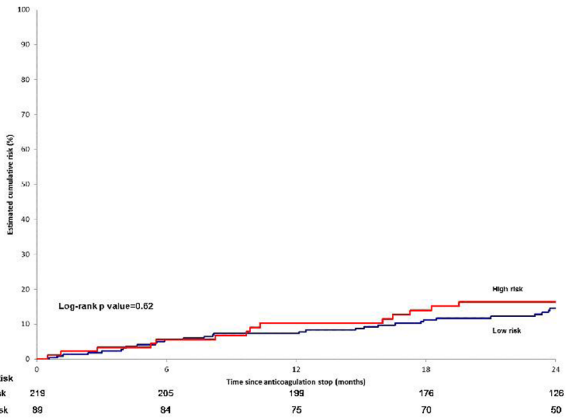

FIGURE 1 Kaplan Meyer curve showing estimated cumulative incidence of recurrent VTE

OC 24.4 | Major Bleeding Rates are Similar in Patients who Use DOACs with Interacting Antiarrhythmics as Compared with DOAC Monotherapy

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Background: Patients with atrial fibrillation (AF) frequently require oral anticoagulant therapy, combined with antiarrhythmics such as amiodarone and verapamil. These antiarrhythmics interact with direct oral anticoagulants (DOACs) which may result in major bleeding. However, data are lacking on major bleeding rates stratified by DOAC and antiarrhythmic use.

Aims: To determine incidence rates of major bleeding in AF patients who use a DOAC and an interacting antiarrhythmic compared with DOAC monotherapy users.

Methods: This nationwide cohort study included Danish patients aged 50 years or older diagnosed with incident AF who started DOAC treatment from 2011-2015. Six exposure categories (based on prescription data) were considered: dabigatran, Xa-inhibitor, dabigatran with verapamil, dabigatran with amiodarone, Xa-inhibitor with verapamil, and a Xa-inhibitor with amiodarone. Incidence rates (IRs) of major bleeds were calculated per 100 patient-years, and hazard ratios and 95% confidence intervals (CIs) were estimated by means of time-dependent Cox regression.

Results: Of 48,052 AF patients using DOACs at baseline, 43,513 (91%) used DOAC monotherapy, 2233 (5%) used a combination with amiodarone, and 2306 (5%) used a combination with verapamil. 1620 major bleeds occurred during a follow-up of 63,377 patient-years. The IR for bleeds during dabigatran monotherapy was 2.09 (95%CI 1.95-2.24) and similar for patients who also used amiodarone (2.23, 95%CI 1.62-3.01) or verapamil (2.10, 95%CI 1.56-2.78). The hazard ratios were 1.03 (95%CI 0.75-1.42) and 1.02 (95%CI 0.76-1.38), respectively (see Table 1). The IR for bleeds during Xa-inhibitor monotherapy was 3.35 (95%CI 3.11-3.60) and similar for patients who also used amiodarone (3.34, 95%CI 2.40-4.53) or verapamil (3.35, 95%CI 2.39-4.60). The hazard ratios were 0.99 (95%CI 0.71-1.37), and 1.02 (95%CI 0.73-1.43), respectively (see Table 1).

Conclusions: Major bleeding rates and risk estimates were similar in AF patients using amiodarone or verapamil with a DOAC as compared with DOAC monotherapy. Different types of DOACs had similar risk estimates.

TABLE 1

Incidence rate and hazard ratio of major bleeding associated with mono- and combination therapy				
	Outcome (no.)	Exposure time (PY)	IR per 100 PY (95% CI)	Hazard ratio (95% CI)
Dabigatran monotherapy	756	36,159	2.09 (1.95-2.24)	reference
Dabigatran with amiodarone	40	1792	2.23 (1.62-3.01)	1.03 (0.75-1.42)
Dabigatran with verapamil	46	2186	2.10 (1.56-2.78)	1.02 (0.76-1.38)
Xa-inhibitor monotherapy	704	21,028	3.35 (3.11-3.60)	reference
Xa-inhibitor with amiodarone	38	1139	3.34 (2.40-4.53)	0.99 (0.71-1.37)
Xa-inhibitor with verapamil	36	1073	3.35 (2.39-4.60)	1.02 (0.73-1.43)

OC 24.5 | Major Bleeding Rates Are High in Patients with a High INR Combined with a G1639A SNP in the VKORC1 Gene

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Background: The A variant of SNP rs9923231 (G1639A) is associated with decreased expression of VKORC1 and increases sensitivity to vitamin K antagonists (VKAs). Whether this results in a higher major bleeding risk is not well-established. It is also unknown whether the increased VKA-sensitivity combined with other risk factors affects major bleeding risk.

Aims: Determine the association of VKORC1 G1639A with(out) additional risk factors with major bleeding during VKA treatment.

Methods: DNA was collected from the BLEEDS cohort of 16,570 patients starting VKA treatment between January 2012 and July 2014. Patients were followed until major bleeding, end of VKA therapy, or December 31st 2014, whichever came first. From the

TABLE 1 VKORC1 G1639A genotype in association with major bleeding during VKA treatment

Follow-up period	VKORC1 genotype	Cases	Person years	Incidence rate (per 100 PY; 95%CI)	HR (95%CI)
Whole period	GG	81	5408	1.5 (1.2-1.9)	reference
Whole period	GA	110	6436	1.7 (1.4-2.1)	1.1 (0.8-1.6)
Whole period	AA	48	2151	2.2 (1.7-2.9)	1.5 (1.0-2.3)
1 month	GG	13	386	3.4 (1.9-5.6)	reference
1 month	GA	17	463	3.7 (2.2-5.8)	1.1 (0.5-2.3)
1 month	AA	11	154	7.1 (3.8-12.4)	2.1 (0.9-4.9)
6 months	GG	40	2176	1.8 (1.3-2.5)	reference
6 months	GA	56	2548	2.2 (1.7-2.8)	1.2 (0.8-1.9)
6 months	AA	30	824	3.6 (2.5-5.1)	1.9 (1.2-3.3)

TABLE 2 Estimated incidences and risk estimates of major bleeding, according to VKORC1 G1639A genotype and INR

Follow-up period	VKORC1 genotype	INR	IR per 100 PY (95%CI)	HR (95%CI)
1 Month	GG	Low (<4)	3.4 (1.8-5.7)	reference
1 Month	GG	High (≥4)	3.1 (0.2-15.4)	1.4 (0.2-11.1)
1 Month	AA	Low (<4)	3.9 (1.4-8.6)	1.3 (0.5-3.7)
1 Month	AA	High (≥4)	18.8 (7.6-39.0)	5.3 (1.9-15.3)
6 Months	GG	Low (<4)	1.9 (1.3-2.5)	reference
6 Months	GG	High (≥4)	1.6 (0.3-5.3)	0.9 (0.2-3.7)
6 Months	AA	Low (<4)	1.5 (0.8-2.6)	0.8 (0.4-1.7)
6 Months	AA	High (≥4)	20.2 (12.5-31.0)	9.8 (5.1-18.9)

cohort, a case-cohort study was assembled that included all cases with a major bleeding (326) and a random sample of 978 patients at baseline (subcohort). Incidence rates (IRs) per 100 patients years were calculated, and hazard ratios (HRs) and 95% confidence intervals (CIs) were estimated by weighted Cox regression, stratified by treatment period, and high international normalized ratio (INR) (≥4).

Results: Results were available from 246 cases and 834 subcohort members. The AA genotype was associated with a 1.5-fold (95%CI 1.0-2.3) increased risk for major bleeding compared with GG carriers. Risk estimates of AA carriers during the first treatment month were higher (HR 2.1, 95%CI 0.9-4.9) compared with the whole period, and decreased with elapsing time (Table 1). GG carriers with high and low INRs had similar IRs of major bleeding (IR 1.6-3.1 versus 1.9-3.4 respectively). In sharp contrast, high INR GA carriers had high IRs (8.0-10.2), and high INR AA carriers had very high IRs (18.8-20.2; Table 2).

Conclusions: The AA genotype of VKORC1 G1639A is associated with an increased risk for major bleeding. Very high major bleeding rates among AA carriers with a high INR warrant monitoring and consideration of VKA therapy in these patients.

OC 33.1 | Negative D-dimer Not Sufficient to Stop Anticoagulation in Men

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Background: Patients with unprovoked VTE have high recurrence risk if anticoagulation is stopped after initial treatment. How to determine the need for long-term anticoagulation is unclear. D-dimer results have been proposed as a risk stratification tool, with low values suggesting lower risk of recurrence.

Aims: To evaluate risk of recurrent VTE in patients with first unprovoked proximal DVT or PE and negative D-dimer test on anticoagulants and 1 month after stopping.

Methods: After 3 to 7 months of anticoagulation, 410 patients had D-dimer levels on and at 1 month off anticoagulation. If both were negative patients remained off anticoagulation and were followed for median of 5.0 years; longest duration of follow up 7.5 years. Primary outcome was symptomatic recurrent VTE. We describe time to first recurrent VTE, the overall rate of recurrent VTE, and assessed changes in recurrence rates over time.

Results: In 319 patients remaining off anticoagulants, recurrent VTE was 5.1% (60/319) per patient-year (95% CI 3.6 to 6.5); 7.5% per patient-year (95% CI 5.5 to 10.0) in men, 3.8% per patient-year (95% CI 2.0 to 6.6) in women with VTE not associated with estrogens, and 0.4% per patient-year (95% CI 0.0 to 2.3) in women with estrogen associated VTE (p< 0.001 for 3-group comparison). The cumulative risk of recurrence at 5 years was 21.5% (95% CI 16.4 to 26.5) overall, 29.7% (95% CI 22.1 to 37.3) for men, 17.0% (95% CI 8.1 to 25.9) for non-estrogen women, and 2.3% (95% CI 0.0 to 6.8) for estrogen-women. Annualized rate of recurrent VTE in non-anticoagulated patients was 7.8% in year 1, 7.5% in year 2, 2.9% in year 3, 3.8% in year 4, 2.1% in year 5 and 1.9% in year 6 or later.

Conclusions: Negative D-dimer results in men with unprovoked VTE are not associated with low rates of recurrent VTE over time.

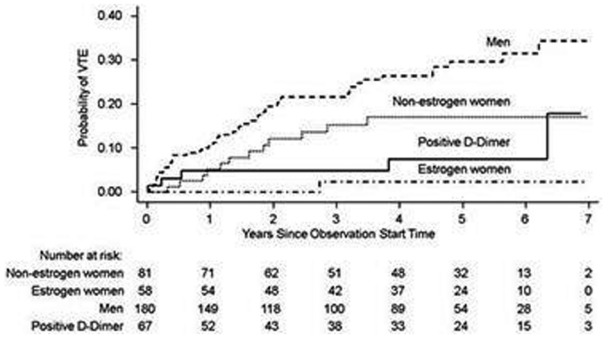

FIGURE 1 Cumulative risk of recurrent venous thromboembolism during follow-up

OC 33.2 | Recurrence Risk after First Symptomatic Distal versus Proximal Deep Vein Thrombosis According to Baseline Risk Factors: A Cohort Study

L. Valerio[1]; C. Ambaglio[2]; M. Barone[3]; M. Ciola[2]; S.V. Konstantinides[1]; S.H. Mahmoudpour[1]; C. Picchi[2]; C. Pieresca[2]; A. Trinchero[1]; S. Barco[1]

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Background: It remains unclear whether the distal versus proximal location of deep vein thrombosis (DVT) is independently associated with a lower risk of recurrence in all patients, or represents a marker of the presence and severity of concomitant provoking risk factors for venous thromboembolism (VTE).

Aims: To investigate the prognostic role of DVT location across groups of patients classified according to baseline risk factors.

Methods: We investigated the impact of distal (versus proximal) DVT on the risk of symptomatic recurrence in 831 patients with a first acute DVT not associated with pulmonary embolism (PE), who were stratified according to concomitant transient or persistent risk factors. The primary outcome was symptomatic, objectively diagnosed recurrent VTE, including proximal DVT and PE.

Results: A total of 205 (24.7%) patients presented with a transient risk factor for VTE, 189 (22.7%) with a minor persistent risk factor, whereas 202 (24.3%) had unprovoked DVT and 235 (28.3%) had

cancer. Recurrence occurred in 125 (15.0%) patients during a median follow-up time of 4.5 years. The largest relative difference between patients with distal (versus proximal) DVT was observed in the absence of identifiable risk factors (HR 0.11 [95%CI 0.03-0.45] adjusted for age, sex, length of anticoagulant treatment, in-hospital diagnosis of DVT) (Figure 1). These results were confirmed both for the whole observation period and after anticoagulant discontinuation. In patients with cancer, distal and proximal DVT had a similar risk of recurrence (adjusted HR 0.70 [95%CI 0.28-1.78]) and death (HR 1.02 [95% CI 0.69-1.50] adjusted for age, sex, in-hospital status, presence of metastasis, and relevant comorbidities).

Conclusions: The distal (versus proximal) location of first acute symptomatic DVT represents, in the absence of identifiable risk factors, a favourable prognostic factor for recurrence. In contrast, the prognostic impact of DVT location was weaker if persistent provoking risk factors for VTE were present, notably cancer.

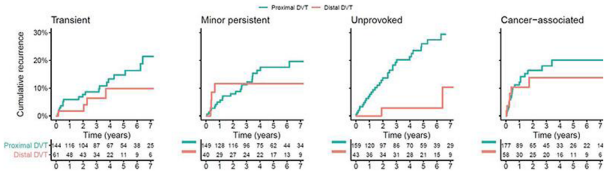

FIGURE 1 Cumulative recurrence rate after first distal (vs proximal) DVT by baseline risk factors

OC 33.3 | Validation of a Tool for Patients' Assessment of Villalta Scale

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Background: Post-thrombotic syndrome (PTS) is a long-term complication of deep vein thrombosis (DVT), affecting about 50% of DVT patients. The Villalta scale is the recommended tool for diagnosing PTS. The tool comprises 5 symptoms to be scored by patients and 6 clinical signs plus venous ulcer assessed by healthcare personnel. We previously developed a tool for patients' reporting of the villalta scale (PRV), which showed very good agreement with the original Villalta scale (kappa 0.81).

Aims: The present study aimed to validate the PRV in a new cohort of patients and evaluate the agreement between individual clinical signs in the PRV as compared to the original Villalta scale.

Methods: One hundred and sixty-nine patients diagnosed with DVT between 2011 and 2014 were recruited from three Norwegian centers 24 (±6) months after DVT. Patients completed the PRV before a scheduled visit. PTS diagnosed by the original Villalta scale served as the reference method. PTS was diagnosed if Villalta score was >4 points. Interobserver agreement was measured using kappa and weighted kappa coefficient and 95% CI.

Results: Sixty-nine (40.8%) patients were diagnosed with PTS using the original Villalta scale compared to 77 (45.6%) using PRV. The PRV showed good agreement with the original Villalta scale in diagnosing PTS (kappa agreement 0.78, 95% CI 0.69-0.88). The sensitivity and specificity was 93% (95% CI 87-99) and 87% (95% CI 80-94), respectively. Regarding individual items, good agreement between the scales was found in "pain on compression" and "hyperpigmentation" (weighted kappa 0.76; 95% CI 0.67-0.85 and 0.73; 95% CI 0.63-0.84, respectively). Good to fair agreement was found in assessment of the remaining clinical signs.

Conclusions: Validation of the PRV confirmed the tool as a valid and sensitive tool for diagnosing PTS.

OC 33.4 | Tailoring of Anticoagulant Therapy by a Recurrence Risk Prediction Model in Patients with Venous Thrombo-embolism as Compared to Care-as-Usual: The Vista Randomized Controlled Trial

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Background: After 3 to 6 months of anticoagulant treatment, patients with unprovoked venous thrombo-embolism (VTE) have an increased recurrence risk if treatment is then stopped. Prolonging anticoagulants reduces recurrence risk. Yet, given the inherent bleeding risk, ideally this is only considered in those with a high recurrence risk. For this purpose, the Vienna prediction model (VPM) was developed to predict recurrence risk and tailor treatment accordingly.

Aims: To evaluate the impact of VPM-guided treatment on recurrent VTE risk, and compare this effect on recurrence risk with usual care.

TABLE 1 Agreement between different grades of PTS by PRV and original Villalta scale

Grading of PTS	Category		Agreement	
	PRV	Villalta	Kappa (95% CI)	% Agreement
Any PTS, n (%)	77 (45,6)	69 (40,8)	0,78 (0.69-0.88)	78
Mild PTS, n (%)	41 (24,2)	46 (27,2)	0,52 (0,37-0,67)	82
Moderate PTS, n (%)	24 (14,2)	17 (10,0)	0,48 (0,28-0,68)	89
Severe PTS, n (%)	12 (7,1)	6 (3,6)	0,65 (0,39-0,91)	96

Methods: In a randomized, controlled trial, 441 patients with an unprovoked VTE that completed an initial treatment of 3 to 6 months of vitamin K antagonists were assigned to VPM-guided management (i.e. prolonging treatment if the predicted risk of recurrence exceeded an annual rate of 5%), and 442 patients were assigned to receive usual care (i.e. prolonging or stopping anticoagulants left at the discretion of the physician). The primary outcome of the trial was recurrence risk at 24 months.

Results: A total 96 recurrent VTE events (10.9%) were observed during 24 months of follow-up, 46 in the intervention arm and 50 in the control arm respectively, yielding a risk ratio of 0.92 (95% CI 0.36 to 1.35). Additional analyses confined to only those compliant to the intervention (i.e. 76%) yielded similar results. Observed recurrence risk in patients identified as low-risk by VPM in the index group (i.e. a predicted risk of 5% or lower, occurring in 225 patients in the index group) was 4.4 % (95% CI 2.7 to 6.9).

Conclusions: Application of VPM-guided management in patients with unprovoked VTE is unlikely to reduce recurrence risk, compared to usual care. However, in those with a low-predicted risk, a substantial part may safely discontinue antithrombotic treatment given the satisfactory low observed recurrence risk in those patients.

OC 33.5 | Definition of Major Bleeding: Prognostic Re-classification

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Background: The definition of major bleeding (MB) released by ISTH is widely used in clinical trials. However, the prognostic impact of individual MBs seems heterogeneous.

Aims: 1) to assess the prognostic value of ISTH criteria for MB; 2) to derive a definition of MB with prognostic value ('disabling', 'severe' and 'life-threatening' MB); 3) to validate the prognostic value of the new definition of MB.

Methods: Consecutive patients admitted for MB (according to ISTH criteria) while on treatment with VKAs or DOACs were included in the study and divided into i) a derivation cohort to assess the aims 1 and 2; ii) a validation cohort to assess the aim 3. Death at 30 days was the primary outcome.

Results: Overall, 517 and 560 patients were included in the derivation and validation cohorts, respectively. Prevalence of individual bleeding sites was similar in the two cohorts. Death occurred in 11% and 12% of patients in the derivation and validation cohort, respectively.

Among ISTH criteria for MB, the critical site was an independent predictor of death in the derivation cohort while reduction in hemoglobin ≥2 g/L, or transfusion of ≥2 units blood/red-cells were not associate with death. Among patients with ICH, GCS< 14 was an independent predictor of death (HR 6.44, 95% CI 3.74-11.10). Among patients with non-ICH MB, shock (HR 4.39, 95% CI 2.27-8.48) and pericardial bleeding (HR 8.64, 95% CI 2.39-31.21) were associated with death.

MB were re-classified as 'disabling' (e.g. ocular and articular), 'severe' and 'life-threatening' (ICH with GCS< 14 or non-ICH MB with shock). The prognostic value of this new classification was confirmed in the validation cohort.

Conclusions: ISTH definition of MBs can be modified by the inclusion of GCS and shock to acquire prognostic value. The new classification should include 'disabling', 'severe' and 'life-threatening' MBs based on the risk for death at 30 days.

OC 45.4 | Low Molecular Weight Heparin Reduces Tumour Formation, Invasion and Vascularisation

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Background: It has been suggested that the treatment of cancer patients with low molecular weight heparin (LMWH) may have additional benefits that extend beyond anticoagulation.

Aims: This study aimed to explore the potential of a number of anticoagulants to influence tumour formation, growth and invasion *in vitro*, and to examine the influence on tumour vascularisation using a Chorio-allantoic membrane (CAM) based model.

Methods: WM-266-4 melanoma and AsPC-1 pancreatic cancer cell lines were treated with LMWH, Tinzaparin and Dalteparin (0-2 IU/ml), and DOAC; Apixaban (0-1 µg/ml) and Rivaroxaban (0-0.6 µg/ml), and the rate of tumour formation, growth and invasion were measured *in vitro*. In addition, the influence of pharmacological concentrations of the anticoagulants on the growth, invasion and vascularisation of tumours derived from WM-266-4 and AsPC-1 cells was also measured using the CAM model.

Results: Tinzaparin and Dalteparin (2 IU/ml) significantly reduced tumour formation and invasion by the cell lines *in vitro*, but with dissimilar potencies. In addition, treatment of CAM with LMWH reduced the local vascular density and the average vessel diameter beyond that achievable with Bevacizumab (12.5 µg/ml). Treatment with DOAC had little effect on CAM vasculature formation. Treatment of CAM-implanted WM-266-4 and AsPC-1 tumours with Tinzaparin (2 IU/ml) also reduced tumour invasion and vascularisation, but had little effect on tumour size. In contrast, Treatment with DOAC was seen to reduce CAM-implanted tumour, whilst having little effect of tumour vascularisation.

Conclusions: LMWH and DOAC may have anti-cancer properties that are exerted through different mechanisms. LMWH may have

anti-cancer properties by reducing the formation, invasion and vascularisation of the tumours, whilst DOACs reduce tumour growth in CAM-implanted tumours.

OC 49.1 | Molecular and Biological Characterization of Transitory Antithrombin Deficiency: A New Concept in Congenital Thrombophilia

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Background: The low incidence in thrombosis of antithrombin deficiency (ATD), the strongest thrombophilia, suggests that ATD might be underestimated. As full characterization of ATD is usually restricted to cases with consistent deficiency, clinically relevant cases with congenital ATD but fluctuating expression of AT levels, what we call transitory ATD, might be missed.

Aims: To identify and characterize transitory ATD.

Methods: We studied a cohort of 344 unrelated cases with potential ATD (< 80%) recruited during 20 years (1998-2018). Plasma AT was evaluated by functional methods at least in two samples. Transitory ATD was defined by the presence of normal AT activity in at least one sample. Hypoglycosylation was studied by electrophoresis and HPLC. Molecular analysis of *SERPINC1* (AT codifying gene), or 74 genes involved in N-glycosylation, was done by sequencing (Sanger, NGS) and MPLA. Recombinant expression of specific *SERPINC1* variants was done in HEK-EBNA cells.

Results: 227 cases had consistent ATD: 189 (83.3%) had 103 different *SERPINC1* defects and 5 (2.2%) N-glycosylation defects. The remaining 117 cases had transitory ATD. Interestingly, 44 of these cases (37.6%), all with thrombosis, carried pathogenic mutations: 33 in *SERPINC1*, with two recurrent mutations (p.Ala416Ser-AT Cambridge II, N=12; p.Val30Glu-AT Dublin, N=9) and 12 in N-glycosylation genes (10.3%) (Figure). Two main mechanisms explained transitory ATD: the limitation of current functional methods to detect some variants and the influence of external factors (conformational stress, generation of thrombin, or alcohol) on the pathogenic consequences of these mutations.

Conclusions: Our study reveals a genetic defect in one third of the cases with transitory ATD. This finding changes the paradigm of congenital thrombophilia, as the pathogenic effect of a mutation might depend on external factors and be present only at certain time points. Our data support that ATD is underestimated and might be screened, by molecular methods, in cases with a positive finding.

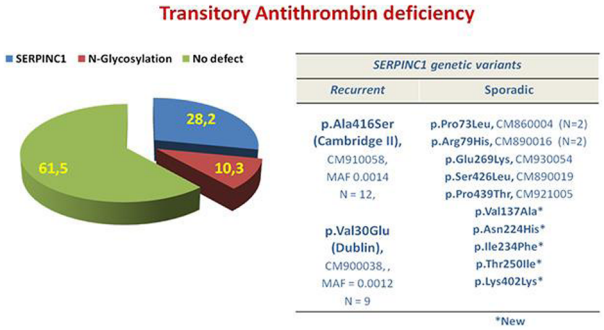

FIGURE 1 Molecular analysis of transitory antithrombin deficiency

OC 49.2 | Genomic and Transcriptomic Association Studies Identify 17 Novel Susceptibility Loci for Venous Thromboembolism: The INVENT Consortium

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Background: Venous thromboembolism (VTE) is a significant contributor to morbidity and mortality in adults.

Aims: To conduct a genome-wide association study (GWAS) meta-analysis of VTE in participants from the INVENT Consortium as well as a transcriptome-wide association study (TWAS) based on imputed gene expression from whole blood and liver.

Methods: Data from 18 cohort and case-control GWAS studies, which included adult men and women of either European or African-American ancestry, were meta-analyzed. In total, there were 30,234 VTE cases and 172,122 controls. We tested the association of 12,923,718 genetic variants and VTE. We imputed gene expression from whole blood and liver tissue into our GWAS to assess the association between gene expression and VTE. Mendelian randomization (MR) analyses were conducted for traits genetically associated with VTE.

Results: We identified 35 independent genetic signals for VTE risk from GWAS meta-analysis of which 15 are newly-reported associations. These included 12 newly-associated genetic loci (C1orf198, *PLEK*, Chr3.q26.1, *OSMR-AS1*, *NUGGC/SCARA5*, *GRK5*, *MPHOSPH9*,

ARID4A, *PLCG2*, *SMG6*, *EIF5A*, *STX10*) and 3 new independent signals in 3 known genes. Further, TWAS identified 5 additional genetic loci with gene expression levels differing between cases and controls in whole blood (*SH2B3*, *SPSB1*, *RP11-747H7.3*, *RP4-737E23.2*) and in liver (*ERAP1*). In many of the loci, we found evidence that the association signal for both novel and previously known regions is likely mediated through gene expression of nearby genes. Furthermore, MR analyses suggested that platelet mass and count contribute to the underlying biology of VTE risk.

Conclusions: We identify 17 novel susceptibility loci for VTE and show that in many of the novel and known loci the associations are likely mediated through gene expression of nearby genes. The data also supports the role of platelet biology in the risk of VTE.

OC 49.3 | Postmenopausal Hormone Use and Venous Thromboembolism Risk in a Prospective Cohort Study: Exploring Effect Modification by Genetic Risk Factors

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Background: Previous studies have examined effect modifications mostly with candidate genes in the association between hormone therapy (HT) use and the risk of venous thromboembolism (VTE). However, effect modification of a genetic risk score (GRS) for VTE remains unknown.

Aims: We aimed to evaluate associations between HT use (past or current use compared to never use) and incident VTE risk and to evaluate interactions between HT use and GRS.

Methods: Eligible women were participants of the Nurses' Health Study (NHS I) without past or current HT use, history of VTE, breast cancer, or cardiovascular disease at baseline in 1976 (n=120,470). HT use data was collected from 1976 and updated with biennial questionnaires. Incident VTE events (deep vein thrombosis and pulmonary embolism) were identified by self-report between 1976-2012. We used multivariable Cox proportional hazards models to estimate hazard ratio (HR) and 95% confidence intervals (CIs) adjusting for time-varying covariates (e.g., age, body mass index, physical activity, smoking, alcohol drinking, and comorbidities), censoring at death, loss of follow-up, or incident VTE. For interaction analyses, eligible women were an NHS subset with available genotype data (n=18,499). We calculated a GRS using 16 VTE-associated SNPs and classified it as high risk, top one third, and low risk, the others, on the basis of thirds of the GRS. Interactions were evaluated on the multiplicative scale.

Results: During ≥ 30 years of follow-up, 2,380 incident VTE cases were identified. Compared to never HT use, VTE risk was greater with current use (HR=1.3, 95%CI=[1.2-1.5]) and further increase in the risk was observed with the high GRS (HR=2.2, 95%CI=[1.5-3.2]). But we did not find significant multiplicative interactions between GRS and any forms of HT use.

Conclusions: Our study found that current HT use was significantly associated with elevated risk of VTE, as compared to never use, but no multiplicative interactions with GRS.

OC 49.4 | A Novel Gain of Function Variant of Prothrombin (p.R541W) Causes Dominant Heritable Thrombosis and Is Associated with Antithrombin Resistance

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Background: We identified a family with autosomal dominant inheritance of venous thrombosis (VT). The index was under investigation because of recurrent unprovoked VT from the age of 15 years. Family members with and without thrombosis were consented to the NIHR BioResource and whole genome sequencing was performed through the ThromboGenomics platform. Five variants co-segregated with the phenotype, of which the likely pathogenic was a novel heterozygous c.1621C>T mutation in *F2* causing a p.R541W substitution. Interestingly, R541 is located within the sodium binding region of prothrombin (Figure 1), an area of critical importance for stabilising the thrombin/antithrombin (TAT) complex and promoting efficient thrombin inhibition. We hypothesised that p.R541W could be a novel prothrombin variant associated with antithrombin resistance.

Aims: Characterise the molecular mechanisms inducing the prothrombotic phenotype of p.R541W.

Methods: Plasma from family members, with and without the p.R541W variant and not on anticoagulant medication, were characterised using thrombin generation and prothrombin activity/antigen ratios. Recombinant wild-type and prothrombin p.R541W were produced and analysed for antithrombin resistance and TAT complex formation.

Results: Quantitative and qualitative measurement of prothrombin in plasma samples showed similar levels between the wild-type family control and p.R541W carrier as well as unchanged activity/antigen ratios indicating that intrinsic catalytic activity of the variant is

unmodified. However, thrombin generation time revealed a significantly increased Endogenous Thrombin Potential with the p.R541W variant as well as increased time to tail indicating a reduced thrombin inhibition.

The resistance of recombinant p.R541W to inhibition by antithrombin was compared with wild-type prothrombin. The p.R541W variant had statistically significant reduced antithrombin-mediated inhibition of activity associated with reduced TAT complex formation (Figure 2).

Conclusions: In this family with inherited VT, we have identified a causal prothrombin variant, previously unobserved in humans, with a gain-of-function mutation. The p.R541W substitution disrupts thrombin-antithrombin interaction and causes this variant to display pathological antithrombin resistance properties.

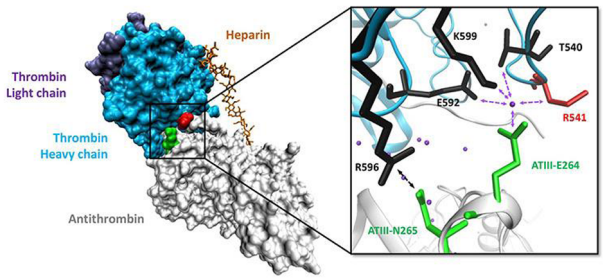

FIGURE 1 Crystal representation of thrombin/antithrombin complex with Arg541 in the sodium binding area

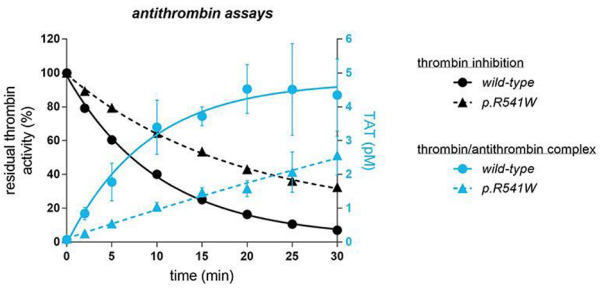

FIGURE 2 Characterisation of antithrombin resistance using purified recombinant proteins

OC 49.5 | Type of Combined Contraceptives and the Risk of Venous Thromboembolism in Antithrombin, Protein C and Protein S Deficient Women: A Prospective Cohort Study

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Background: Antithrombin and protein C and S defects, are well-recognized major thrombophilic risk factors for venous thromboembolism (VTE). The relevance of these defects in predisposing wome under combined hormonal contraception (CHC) to VTE is recognized.

Aims: To evaluate the incidence of VTE, CHC related, in asymptomatic women who were family members of a proband with an objectively diagnosed venous thromboembolic event and a documented inherited thrombophilic abnormality.

Methods: In a prospective cohort study we assessed the incidence of VTE, CHC related, in asymptomatic women who were family members of a proband with a documented inherited thrombophilic abnormality.

Results: We enrolled 197 women of child-bearing age from 88 families.

Although we had discouraged the use of estrogen-progestin therapy once diagnosed a thrombophilic condition, 19 women carriers of inherited thrombophilia used this therapy compared with 17 non carriers.

Preparations based on cyproterone acetate, desogestrel, gestodene, etonogestrel and drospirenone were used in the two groups.

Twelve thrombotic events occurred in 279 months of CHC use in the carriers group and two in 992 months of use in the non-carrier group (one heterozygous for factor V Leiden). The incidence of VTE in the two groups was 63.2 (95% CI 38 to 84) and 11.8 (95% CI 1.5 to 36.4) respectively.

Considering the months of use for women of CHC the relative risk for VTE was 21.3 (95% CI 5 to 95): considering the number of women who have used CHC the relative risk for VTE was 5.37 (95% CI 1.5 to 21).

Conclusions: The thrombotic risk in women with an identified major thrombophilic defect appears to be definitely high. This risk appears independent of the type of CHC used. Screening for thrombophilia in women who belong to families with inherited defects seems justified as well as the strong recommendation not to use estroprogestin therapy.

OC 63.1 | Diagnosis of Pulmonary Embolism Using D-Dimer Thresholds Selected According to Clinical Pre-Test Probability

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[1]*McMaster University and Thrombosis and Atherosclerosis Research Institute, Hamilton, Canada,* [2]*McMaster University, Hamilton, Canada,* [3]*McMaster University and the Thrombosis and Atherosclerosis Research Institute, Hamilton, Canada,* [4]*McGill University, Montreal, Canada,* [5]*Universite de Sherbrooke, Sherbrooke, Canada,* [6]*Ottawa Hospital Research Institute, Ottawa, Canada,* [7]*Western University, London, Canada,* [8]*University of Alberta, Edmonton, Canada*

Background: D-dimer < 500 (ng/mL FEU) in combination with Low clinical pretest probability (C-PTP) is widely used to exclude PE. Retrospective analyses suggest that D-dimer < 500 with Moderate C-PTP, and D-dimer < 1,000 with Low C-PTP, also excludes PE.

Aims: To test the safety of considering PE excluded if there is Low C-PTP and D-dimer < 1,000 or Moderate C-PTP and D-dimer < 500.

Methods: Consenting patients were enrolled in a prospective multicentre management study. No chest imaging was done in patients with Low C-PTP and D-dimer < 1,000, or Moderate C-PTP and D-dimer < 500 (Figure). All others had chest imaging. If PE was not diagnosed, patients were not anticoagulated. All patients were followed for 3 months to detect venous thromboembolism (VTE).

Results: 2017 patients were enrolled and analyzed; mean age 52 years, 66% women, and 9% malignancy. There was: Low C-PTP in 1752 (86.9% of total) and 5.0% of these had PE at initial testing; Moderate C-PTP in 218 (10.8%) and 19.7% had PE; High C-PTP in 47 (2.3%) and 40.4% had PE. Overall, 7.4% had PE at initial testing. D-dimer was negative in 1325 patients with Low or Moderate C-PTP, and none had VTE during follow-up (95% CI 0.00 to 0.29%) (Figure, Table). Of all 1863 not diagnosed with PE initially and not anticoagulated, 1 (0.05%; 95% CI 0.01 to 0.30%) had VTE during follow-up. Compared with only excluding PE if there was Low C-PTP and D-dimer < 500, the current diagnostic strategy avoided the need for chest imaging in 66% rather than 48% of all patients (Table).

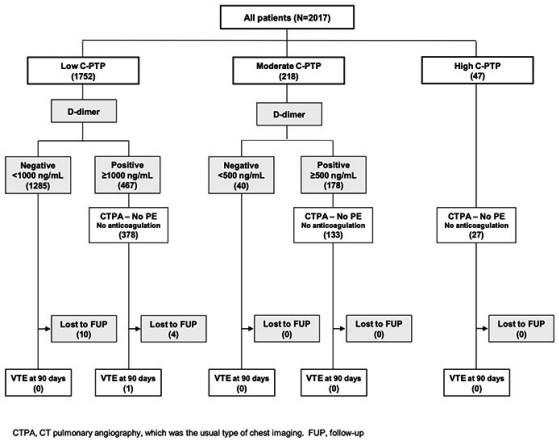
Conclusions: Low C-PTP and D-dimer < 1,000, and Moderate C-PTP and D-dimer < 500, excludes PE and avoids the need for chest imaging.

TABLE 1

Grouping	Patients	VTE Events	Percentage (95% CI)
No PE and no anticoagulation on initial testing	**1863**	**1**	**0.05 (0.01, 0.30)**
Low or moderate C-PTP and D-dimer negative:	**1325**	**0**	**0.0 (0.00, 0.29)**
Low C-PTP and D-dimer negative	1285	0	0.0 (0.00, 0.30)
Low C-PTP and D-dimer <500 ng/mL FEU	970	0	0.0 (0.00, 0.39)
Low C-PTP and D-dimer 500 to 999 ng/mL	315	0	0.0 (0.00, 1.20)
Moderate C-PTP and D-dimer negative	40	0	0.0 (0.00, 8.76)
Low or moderate C-PTP and D-dimer positive:	**511**	**1**	**0.20 (0.03, 1.10)**
Low C-PTP and D-dimer positive	378	1	0.26 (0.05, 1.50)
Moderate C-PTP and D-dimer positive	133	0	0.0 (0.00, 8.76)
High C-PTP	**27**	**0**	**0.0 (0.00, 12.5)**

VTE = venous thromboembolism; PE = pulmonary embolism; C-PTP = clinical pretest probability, which was assessed using the Wells score; CI = confidence interval.

Figure: Patient flow, results of initial diagnostic testing and VTE outcomes during follow-up



CTPA, CT pulmonary angiography, which was the usual type of chest imaging. FUP, follow-up

FIGURE 1 Patient flow, results of initial diagnostic testing and VTE outcomes during follow-up

OC 63.2 | Predicting Venous Thromboembolism Risk after Immobilization of the Lower-limb for Trauma: Update and Validation of a Clinical Risk Assessment Model, the TRiP(Cast) Score

D. Douillet[1,2]; P.-M. Roy[1,2]; B. Nemeth[3]; S. Le Cessie[3]; A. Penaloza[4]; T. Moumneh[1,2]; S. Cannegieter[3]

[1]*Centre Hospitalier Universitaire d'Angers, Département de Médecine d'Urgence, Angers, France,* [2]*Université d'Angers, Institut MITOVASC, UMR CNRS 6015 UMR INSERM 1083, Angers, France,* [3]*Leiden University Medical Center, Leiden, the Netherlands,* [4]*Cliniques Universitaires Saint-Luc, Bruxelles, Belgium*

Background: Patients with lower-limb trauma requiring immobilization have an increased risk of venous thromboembolism. While thromboprophylaxis in all patients seems not effective in the whole population, targeted thromboprophylaxis in high risk patients may be an appropriate alternative.

Aims: The aim of this study was to develop and validate a single and simplified score from two earlier developed risk assessment models: the TIP score and the L-TRiP(cast) score.

Methods: Model discrimination was assessed in the MEGA study (case-control study of risk factors for venous thrombosis) and the POT-CAST trial (randomized trial on the effectiveness of thromboprophylaxis following cast immobilization). The Area Under the Curve (AUC) was calculated by plotting a Receiver Operating Characteristic. For model calibration, predicted risks were calculated by obtaining a new intercept and slope using logistic regression analysis in the POT-CAST trial. A calibration plot was made to compare observed and predicted risks.

Results: The combined TRiP(cast) score (Thrombosis Risk Prediction following cast immobilization) includes 14 items; 1 item for trauma severity (or type of trauma), 1 for type of immobilization

and 12 items related to patients' characteristics.The AUC was 0.74 (95%CI 0.61 to 0.87) in the complete dataset (n=1250) and 0.72 (95% CI 0.60-0.84) in the imputed data set (n=1435). The calibration plot showed very good agreement between the observed and predicted risks *(Figure 1)*.Using a cut-off score of 7 points in the POT-CAST trial (incidence 1.6%), the sensitivity, specificity, positive and negative predictive values were 76.1%, 51.2%, 2.5%, and 99.2%, respectively.

Conclusions: The TRiP(cast) score provides a helpful tool in daily clinical practice to accurately stratify patients in high versus low-risk categories in order to guide thromboprophylaxis prescribing. To accommodate implementation in clinical practice a mobile phone application has been developed.

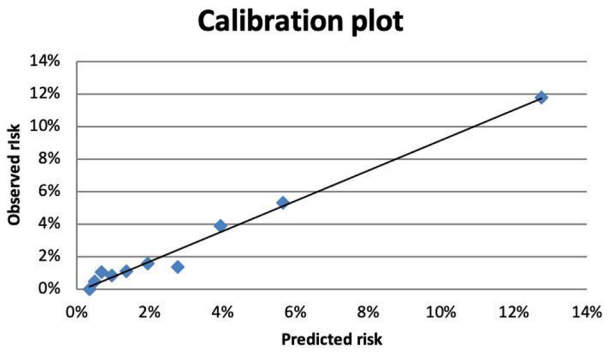


FIGURE 1 Calibration plot, showing the observed versus predicted risks for VTE for TRiP(cast) score

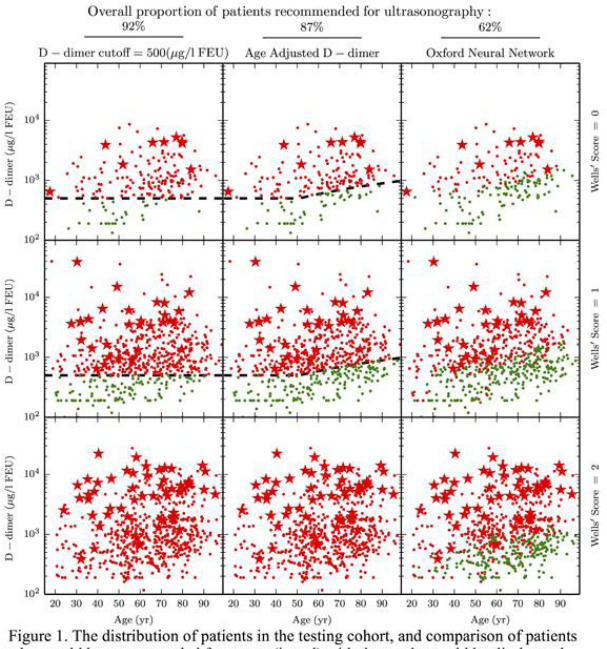


Figure 1. The distribution of patients in the testing cohort, and comparison of patients who would be recommended for a scan (in red) with those who could be discharged without a scan (in green). Patients shown not to have a deep vein thrombosis (DVT) are shown as dots, whereas the star symbols represent patients found to have a DVT. The columns each use a different strategy to determine whether or not compression ultrasonography is required; Column 1 uses a fixed cut-off of a Wells' score >1 or a D-dimer >500 μg/l, Column 2 uses the age-adjusted strategy (scan if Wells' score >1, D-dimer > [Age (years) x 10) μg/l or >500 μg/l if less than 50 years old], and Column 3 uses the machine-learning algorithm with a cut off "Oxford neural network" score of 0.1 above which a scan is recommended. The rows correspond to patients with a different total Wells' score: Row 1 shows those with a Wells' score of zero, Row 2 shows those with a Wells' score of 1, and Row 3 shows those with a Wells' score of 2. The proportion of patients recommended for scanning by each method of risk assessment is shown at the top of each column. FEU, fibrinogen equivalent unit.

FIGURE 1

OC 63.3 | Machine-learning Algorithms Can Improve upon Current Methods of Risk-stratification of Patients Presenting with Suspected Deep Vein Thrombosis

J. Willan[1,2]; H. Katz[3]; D. Keeling[1]

[1]*Oxford University Hospitals, Haemophilia and Thrombosis, Oxford, United Kingdom,* [2]*Frimley Park NHS Foundation Trust, Department of Haematology, Wexham, United Kingdom,* [3]*University of Oxford, Astrophysics, Oxford, United Kingdom*

Background: Machine-learning algorithms analyse complex data without a pre-existing hypothesis, to identify underlying patterns. Unlike many conventional methods, they do not require linear relationships between the variables.

Current risk assessment of patients with suspected deep vein thrombosis (DVT) is by a points-based clinical score, typically the Wells' Score, which may be combined with a D-dimer blood test. We hypothesised that use of machine-learning algorithms may enable the safe exclusion of DVT in a larger number of patients without the requirement for ultrasonography.

Aims: To develop a machine-learning algorithm that is better able to risk-stratify the presence of DVT than current approaches.

Methods: Retrospective data from 11,490 patients presenting with suspected DVT to a single diagnostic clinic were analysed. 7,080 patient records contained all fourteen required data points (the individual components of the Wells' score, age, sex, D-dimer level and an ultrasound) and were included in the study. These records were divided into a training set of 5,270 patients used to develop the algorithm, and a testing set of 1,810 to assess the performance of the trained algorithm. 12.5% of the population was found to have DVT.

Results: A machine-learning algorithm was developed which was able to exclude DVT in 37.5% of patients without the need for ultrasound (compared to 8.3% using the Wells' score and D-dimer), whilst maintaining a false negative rate of only 0.22% (Figure 1).

Factors identified by the algorithm as carrying the highest risk for DVT included young age, and male sex. Intriguingly the presence of cancer was a relatively low risk factor, perhaps reflecting a tendency for over-referral of this patient group to such clinics (Figure 2).

Conclusions: Machine-learning algorithms offer the potential to improve upon current risk-assessment strategies for patients presenting with suspected DVT. We hope to validate this system prospectively, then proceed to an interventional study.

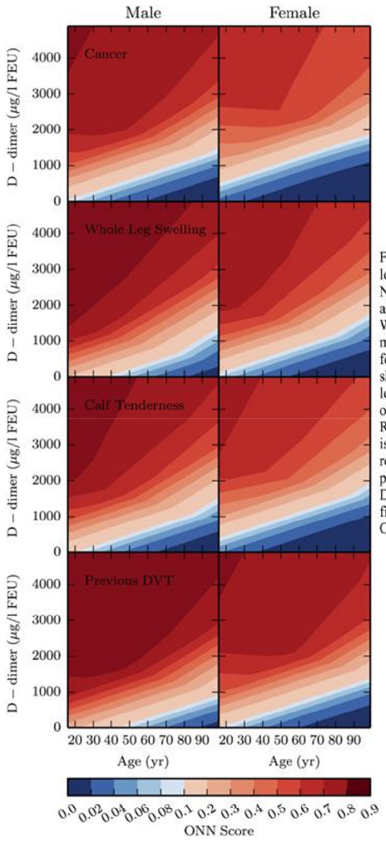


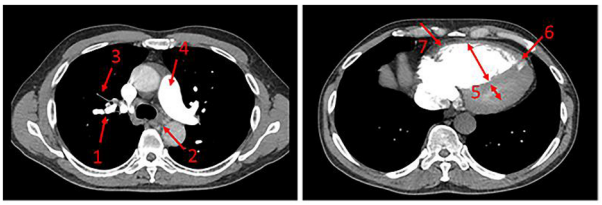
Figure 2. Heat maps of the machine learning algorithm "Oxford Neural Network" risk score as a function of age and D-dimer for patients with a Wells' score of 1. Column 1 shows male patients, and column 2 shows female patients. The different rows show the single Wells' criteria which led to the score of 1; Row 1 is cancer only, Row 2 is whole leg swelling, Row 3 is calf tenderness and Row 4 is previous DVT. The white lines represent the threshold above which patients are recommended for a scan. DVT, deep vein thrombosis; FEU, fibrinogen equivalent unit; ONN, Oxford neural network.

FIGURE 2

OC 63.4 | External Validation of Radiological Signs of Chronic Thromboembolic Pulmonary Hypertension on Computed Tomography Pulmonary Angiography at the Time of Suspected Pulmonary Embolism

G.J.A.M. Boon[1]; Y.M. Ende-Verhaar[1]; L.F.M. Beenen[2]; J. Coolen[3]; M. Delcroix[4]; M. Golebiowski[5]; M.V. Huisman[1]; L.J.M. Kroft[6]; A.T.A. Mairuhu[7]; L.J. Meijboom[8]; S. Middeldorp[9]; P. Pruszczyk[10]; C.J. van Rooden[11]; A. Vonk Noordegraaf[12]; F.A. Klok[1]

[1]Leiden University Medical Center, Thrombosis and Hemostasis, Leiden, the Netherlands, [2]Amsterdam UMC, University of Amsterdam, Department of Radiology, Amsterdam, the Netherlands, [3]University Hospitals Leuven, Department of Imaging and Pathology, Leuven, Belgium, [4]University Hospitals Leuven, Department of Pneumology, Leuven, Belgium, [5]Medical University of Warsaw, Department of Clinical Radiology, Warsaw, Poland, [6]Leiden University Medical Center, Department of Radiology, Leiden, the Netherlands, [7]Haga Teaching Hospital, Department of Internal Medicine, The Hague, the Netherlands, [8]Amsterdam UMC, VU University Medical Center, Department of Radiology and Nuclear Medicine, Amsterdam, the Netherlands, [9]Amsterdam UMC, University of Amsterdam, Department of Vascular Medicine, Amsterdam Cardiovascular Sciences, Amsterdam, the Netherlands, [10]Medical University of Warsaw, Department of Internal Medicine and Cardiology, Warsaw, Poland, [11]Haga Teaching Hospital, Department of Radiology, The Hague, the Netherlands, [12]Amsterdam UMC, VU University Medical Center, Department of Pulmonology, Amsterdam Cardiovascular Sciences, Amsterdam, the Netherlands



1. Webs
2. Dilated bronchial arteries
3. Arterial retraction
4. Dilated pulmonary artery
5. RV/LV diameter ratio >1
6. Flattening of the interventricular septum
7. RV hypertrophy

FIGURE 1 CTPA image showing the 6 radiological predictors of CTEPH in addition to RV/LV ratio of >1

Background: Close reading of computed tomography pulmonary angiography (CTPA) images in patients with acute pulmonary embolism (PE) may identify patients at high risk of chronic thromboembolic pulmonary hypertension (CTEPH). Six independent radiological predictors for CTEPH (in addition to RV/LV ratio >1; Figure 1) were derived on CTPA scans performed to diagnose acute PE (*Klok FA, Eur Heart J, volume 39, suppl 2018, ehy565.P1625*). The presence of ≥3 predictors had a sensitivity of 70% (95%CI 55-82) and a specificity of 96% (95%CI 86-100) for a future CTEPH diagnosis.

Aims: To validate the predictive value of these six radiological predictors for CTEPH.

Methods: 339 consecutive patients with acute PE were prospectively included in 6 European hospitals, and followed for development of CTEPH. The index CTPA scan performed to diagnose PE was analysed post-hoc by 8 independent expert radiologists by scoring the 6 radiological predictors and giving an overall adjudication on the presence of CTEPH conform the derivation study.

Results: Signs of chronic thrombus remnants, e.g. webs and bands, were present in 22% of patients on the index CTPA scan, which was not associated with a previous VTE diagnosis (OR 1.3; 95%CI 0.73-2.5). Eight patients (2.4%) were diagnosed with CTEPH during follow-up. A total of 37 patients (11%) were found to have ≥3/6 radiological predictors, of whom 5 (14%) were diagnosed with CTEPH during follow-up (sensitivity 63%, 95CI% 24-91; specificity 90%, 95%CI 87-93; NPV 99%, 95%CI 98-100; PPV 14%, 95%CI 8-23). The expert radiologists suspected CTEPH in 27 patients of whom 7 were diagnosed with CTEPH (sensitivity 88%, 95%CI 47-100; specificity 94%, 95%CI 91-96).

Conclusions: The predictive accuracy of the objective radiological predictors proved comparable to the derivation study and within the confidence interval of the experts' overall judgment. Routine assessment of these radiological signs in PE patients may help reducing the current long diagnostic delay of CTEPH.

OC 63.5 | Acute Submassive Pulmonary Embolism is Not Associated with an Increased Rate of Chronic Thromboembolic Pulmonary Hypertension

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[1]Alfred Health, Melbourne, Australia, [2]Monash University, Melbourne, Australia

Background: Chronic thromboembolic pulmonary hypertension (CTEPH) is an uncommon but serious complication of pulmonary embolism (PE) with reported rates of up to 4% in patients with PE.

Aims: To compare the rate of CTEPH diagnosed following acute submassive PE and non-submassive PE.

Methods: A retrospective single-centre study reviewing consecutive admissions of patients with newly diagnosed PE between January 2012 and July 2017. Exclusion criteria included pre-existing right heart dysfunction or pulmonary hypertension, massive PE or < 6 months follow-up, unless there was earlier radiological imaging showing complete resolution of PE. Submassive PE was defined as right heart dysfunction on transthoracic echocardiogram (TTE) or right ventricular enlargement on computed tomography (CT). Repeat radiological imaging and TTE were performed for persistent dyspnoea or clinician preference. CTEPH was suspected if there was residual pulmonary obstruction on imaging (CT or ventilation-perfusion scan) and TTE demonstrating right ventricular systolic pressure (RVSP) >36mmHg. Confirmation of CTEPH required right heart catheterisation (RHC). Secondary outcomes included anticoagulation type and duration, recurrent venous thromboembolism (VTE), bleeding and mortality rates. Ethics approval was granted by the local Ethics Committee.

Results: Records of 1231 patients were reviewed and 415 patients with PE were included; 117 with submassive PE and 298 with non-submassive PE. The median follow-up of survivors was 30.8 months (range, 1 - 80 months). Baseline demographics are shown in table 1. CTEPH was suspected in three patients with submassive PE, and three patients with non-submassive (2.6% versus 1.0%, p=0.36). No patients in either group were investigated with RHC. Submassive PE was associated with increased residual pulmonary obstruction on repeat imaging (42% versus 18.5%, p=0.002) but no difference in persistent right heart dysfunction, major bleeding, recurrent VTE or all-cause mortality (table 2).

Conclusions: There is no difference in rates of CTEPH when comparing submassive and non-submassive PE despite increased residual pulmonary obstruction on radiological imaging.

TABLE 1 Baseline demographics

	Submassive PE, n = 117 (%)	Non-submassive PE, n = 298 (%)	Comparison (P value)
Age, years	63.3	59.9	0.08
Weight, kg	86.5	80.4	0.01
Male sex	66 (56.4)	168 (56.4)	1.0
Active malignancy	22 (18.8)	58 (19.5)	1.0
Prior venous thromboembolism	24 (20.5)	47 (15.8)	0.25

TABLE 2 Outcomes

	Submassive PE, n = 117 (%)	Non-submassive PE, n = 298 (%)	Comparison (P value)
Suspected CTEPH*	3/117 (2.6)	3/298 (1.0)	0.36
Residual pulmonary obstruction on imaging	21/50 (42)	25/135 (18.5)	0.002
Persistent right heart dysfunction on TTE	13/67 (19.4)	10/86 (11.6)	0.25
Warfarin use	45 (38.5)	143/298 (48)	0.08
Direct oral anticoagulant use	47/117 (40.2)	71/298 (24)	0.001
Recurrent VTE	6/117 (5.1)	26/298 (8.7)	0.31
Major bleeding**	8/117 (6.8)	19/298 (6.4)	0.83
All-cause mortality at 30 days	11/117 (7.5)	18/298 (6)	0.28

*Residual pulmonary obstruction on imaging and right ventricular systolic pressure >36mmHg **Based on ISTH definition

OC 73.1 | DOAC Plasma Concentrations in High Weight Patients: An Observational Study

A.C. Martin[1]; Z. Mahir[2]; M. Crowley[2]; T. Dowling[2]; K. Breen[2]; V. Collings[2]; B. Hunt[2]; G. Moore[2]; W. Thomas[3]; A.T. Cohen[2]

[1]INSERM UMRS 1140 - University Paris Descartes, Department of Cardiology, Paris, France, [2]Guy's & St Thomas' NHS Foundation Trust, Department of Haematology, London, United Kingdom, [3]Cambridge University Hospitals NHS Foundation Trust, Department of Haematology, Cambridge, United Kingdom

Background: Obesity is associated with an increased risk of cardiovascular diseases including atrial fibrillation (AF) and venous thromboembolism (VTE), both treated with direct oral anticoagulants (DOACs). However, outcomes from trials and expected peak and trough drug levels were based on few individuals ≥115 kg. There is uncertainty whether weight affects DOAC levels.

Aims: To describe the relationship between DOAC levels and high body weight, and to compare DOAC levels in high weight patients with those in the ICSH recommendations[1].

Methods: All patients in two centres in the UK, weighing ≥115kg receiving rivaroxaban or apixaban for AF or VTE and having DOAC levels were included. Peak or trough DOAC plasma concentrations

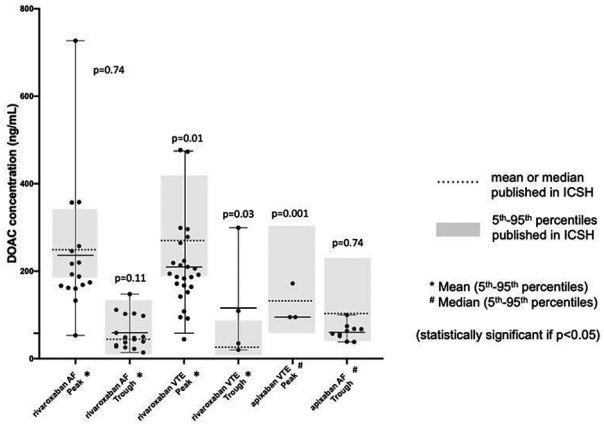

FIGURE 1 DOAC concentrations in high weight patients and expected peak and trough levels

were assessed and measured using specific chromogenic assays. They were expressed in mean or median (5^{th}-95^{th} percentiles) depending on ICSH values. T-test or Wilcoxon test were used for comparisons.

Results: 78 high weight patients with 84 samples were included. Age range: 23-78, 37% were women, 51% had AF, creatinine clearance range: 67-474 mL/min. Median weight was 138kg, and 78.9% had BMI ≥40kg/m^2. Of 84 samples, 75 assessed "full dose" i.e. apixaban 5mg bid and rivaroxaban 20mg od. There was no linear relationship between DOAC levels at peak or trough and body weight (p=0.28, p=0.98) or BMI.

In high weight patients, rivaroxaban concentrations at peak were widely distributed: 62.5% (10/16) of the samples in AF patients and 46% (14/25) in VTE patients were out the expected levels (figure), most values (19/24) were below. Conversely, all but one trough value for AF were within the ranges. Only few apixaban samples were available however all were within the expected levels.

Conclusions: No linear relationship existed between DOAC levels and body weight. However, half of the rivaroxaban peak levels measured in high weight patients remained below the range expected in general AF and VTE populations.1-Gosselin. ..Thromb Haemost 2018.

OC 73.2 | Association of Body Weight with Efficacy and Safety of Apixaban and Rivaroxaban in Relation to Low Molecular Heparin and Vitamin K Antagonists in Acute Venous Thromboembolism

W. Wysokinski[1]; R. McBane[1]; D. Froehling[1]; D. Vlazny[1]; D. Bott-Kitslaar[1]; D. Houghton[1]; W. Kuczmik[2]; K. Sutkowska[3]; K. Bator[3]; D. Hodge[4]; L. Peterson[1]; A. Casanegra[1]

[1]Mayo Clinic Rochester, Rochester, United States, [2]Medical University of Silesia, Katowice, Poland, [3]University Medical School of Wroclaw, Wroclaw, Poland, [4]Mayo Clinic Jacksonville, Jacksonville, United States

Background: The use of fixed-dose of direct oral anticoagulant(s) (DOACs) in patients with extremes of body weight raises concern for their efficacy and safety.

Aims: To investigate the association of body weight and outcomes in patients treated for acute venous thromboembolism (VTE) with apixaban, rivaroxaban, low molecular weight heparin, or warfarin, VTE recurrence, major bleeding, clinically relevant non-major bleeding (CRNMB), and mortality rates were compared between patients with body weight < 60kg, 60-120kg, and > 120kg.

Methods: Consecutive patients seen at the Mayo Thrombophilia Clinic Registry (March 1, 2013-December 31, 2018) with acute VTE were followed prospectively by in person assessment, mailing a questionnaire, or by a scripted phone interview.

Results: Among 2133 patients with acute VTE, 184 had weight < 60kg, 1757 had weight 60-120kg, and 192 had weight >120kg. Patients with the weight < 60kg were more often women, had cancer, but less often pulmonary embolism. Obese patients were younger, more often men, with pulmonary embolism, but less often had cancer (Table 1). Patients with body weight < 60kg had a higher mortality (p=0.002) while patients with body weight >120kg had a lower mortality (p < 0.006) compared to 60-120kg weight group when treated with any anticoagulant. Patients without cancer and weight < 60kg showed higher rate of VTE recurrence (p=0.01).

Within the group of VTE patients receiving apixaban or rivaroxaban (Table 2) those with the weight < 60kg had a higher major bleeding rate (p=0.04) while patients with weight >120kg had better survival (p=0.05) compared to 60-120kg group. All other outcomes did not differ according to body weight groups.

TABLE 1 Patient demographics

Variables	Patients with acute VTE by weight (kg)			
	< 60 (n=184)	60-120 (n=1757)	> 120 (n=192)	p value
Age, mean (SD)	61.9 (16.1)	61.6 (14.2)	58.3 (12.8)	<0.001
Weight, mean (SD)	52.9 (5.7)	87.4 (15.2)	140.3 (22.8)	<0.001
Weight, range	(30.1-59.9)	(60.0-120.0)	(120.2-263.2)	
Female, n (%)	160 (87.0%)	728 (41.3%)	58 (30.2%)	<0.001
Symptomatic VTE, n (%)	29 (47.5%)	417 (56.0%)	77 (77.0%)	0.001
Deep vein thrombosis, n (%)	135 (73.4%)	1336 (76.0%)	142 (74.0%)	0.62
Pulmonary embolism, n (%)	62 (33.7%)	758 (43.1%)	102 (53.1%)	0.001
Provoked VTE, n (%)	163 (90.6%)	1410 (81.4%)	144 (75.4%)	0.001
Active Cancer, n (%)	104 (56.5%)	813 (46.3%)	64 (33.3%)	<0.001
Chemotherapy, n (%)	66 (66.0%)	516 (64.0%)	37 (58.7%)	0.60
Previous History of VTE, n (%)	40 (22.7%)	375 (22.0%)	60 (31.7%)	0.01

TABLE 2 Clinical outcome of patients with weight based groups treated with either apixaban or rivar

Outcome	Variable	Weight			P-value	P-value
		<60 kg	60-120 kg	>120 kg	<60 vs.	60-120
		n=63	n=726	n=75	60-120	vs. >120
VTE recurrence	Rate per 100 person-years (95%CI)	0	3.5 (1.7-5.2)	5.1 (0-10.9)	---	0.51
	No of events		15	3		
	Total person-years		434.5	58.8		
Major bleeding	Rate per 100 person-years (95%CI)	10.3 (0.2-20.5)	3.5 (1.7-5.2)	3.4(0-8.1)	0.04	0.96
	No of events	4	15	2		
	Total person-years	38.7	433.9	58.8		
CRNMB	Rate per 100 person-years (95%CI)	7.9 (0-16.8)	7.8 (5.1-10.4)	3.4 (0-8.2)	0.99	0.32
	No of events	3	33	2		
	Total person-years	38.1	423.8	58.6		
Death	Rate per 100 person-years (95%CI)	35.5 (19.6-51.5)	21.5 (17.8-25.2)	9.9 (2.6-17.3)	0.09	0.05
	No of events	19	132	7		
	Total person-years	53.5	613.9	70.5		

Conclusions: Patients with weight >120kg have clinical outcome similar to 60-120kg group with the exception of better survival reflecting lower cancer prevalence. Patients with < 60kg of body weight treated with DOACs have a higher risk of major bleeding compared to 60-120kg group.

OC 73.3 | Use of Direct Oral Anticoagulants in Patients with Different Types of Hereditary Thrombophilia: A Retrospective Cohort Study

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Background: Direct oral anticoagulants (DOACs) for venous thromboembolism (VTE) in patients with hereditary thrombophilia have been evaluated only in a post-hoc analysis and in some case reports.
Aims: This retrospective cohort study evaluated the efficacy and safety of DOACs for the acute treatment and secondary prevention of VTE in hereditary thrombophilia (natural anticoagulants deficiencies, FV Leiden, prothrombin G20210A, antithrombin resistance).
Methods: Consecutive adult patients with a first acute VTE between January 2014-2017 identified as thrombophilia carriers treated with DOACs for at least 3 months were enrolled. A cohort of thrombophilic patients treated for at least 3 months with conventional anticoagulants (heparin and warfarin) between January 2007-2015 acted as controls, matched for age, sex, ethnic origin and thrombophilia with cases. Endpoints were incidence rate (IR) of VTE recurrence, any bleeding complication, residual vein thrombosis (RVT) and post-thrombotic syndrome (PTS) in the two cohorts.
Results: 197 patients with hereditary thrombophilia and VTE treated with DOACs (mean age 53, F 45%, severe thrombophilia 42%) and 211 controls (mean age 56, F 47%, severe thrombophilia 36%) were considered. During anticoagulation we documented 3 (1.5%) VTE recurrences in cases and 9 (4.2%) in controls, with IR of 5.3 per 1000PY and 10.3 per 1000PY, respectively, and hazard ratio (HR) 0.66 [95%CI 0.20-2.20]. Additionally, we found 14 (7.1%) bleeding complications in cases and 9 (4.3%) in controls, IR 24.9 per 1000PY and 10.3 per 1000PY, respectively, HR 1.94 [95%CI 0.85-4.43] (Fig.1). After stopping anticoagulant treatment, we documented 6 (6%) VTE recurrences in cases and 22 (18%) in controls over the course of 12 months, HR 0.41 [0.19-0.86] (Fig.2). No significant differences in RVT and PTS were detected.
Conclusions: DOACs appear equally effective and safe as standard therapy to treat VTE in patients with hereditary thrombophilia. Additionally, DOACs showed less recurrent events in the 12 months following anticoagulation discontinuation.

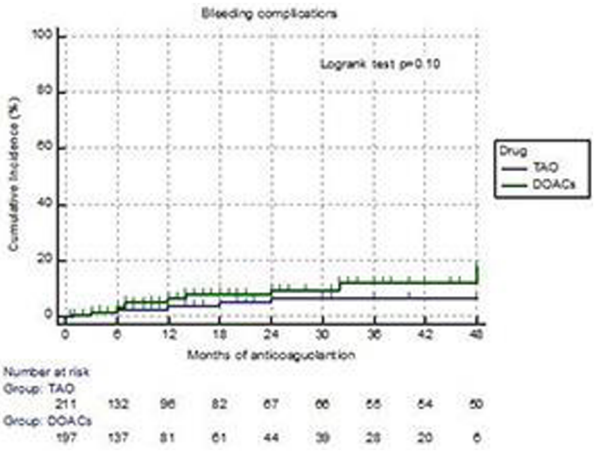

FIGURE 1 Kaplan-Meier survival analysis of bleeding complications during anticoagulant treatments

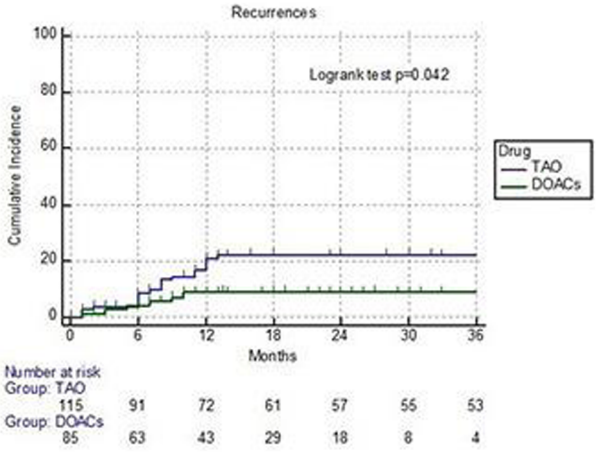

FIGURE 2 Kaplan-Meier survival analysis of 12-months VTE recurrence after stopping anticoagulation

OC 73.4 | Thrombus Resolution in Splanchnic Vein Thrombosis

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Background: Direct oral anticoagulants (DOACs) are commonly used in clinical practice to treat splanchnic vein thrombosis. Registries suggest similar rates of complications compared with warfarin but recanalization has not been evaluated.

Aims: Determine the rate of recanalization of the splanchnic (portal) veins in patients treated with DOACs vs other anticoagulants.

Methods: Consecutive patients with acute splanchnic vein thrombosis enrolled in the Mayo Thrombophilia Clinic Anticoagulants Registry (3/1/2013 -8/30/2018) were followed forward in time. Patients were included if they had anticoagulants started within the first 14 days of diagnosis and completed at least 3 months of therapy. Recanalization was evaluated at 3 months or later by independent review of available CT or ultrasound.

Results: Among 75 patients, 72% had portal vein thrombosis, 37% mesenteric, 1% splenic, and more than one vein (usually portal and superior mesenteric vein) was involved in 11%. Half of the patients were males (53%). Half of the patients had active cancer (57%). The most common cancers were pancreatic (30%) and gastrointestinal tract (33%). DOACs were the most common anticoagulants (63%), followed by low molecular weight heparin (LMWH) (29%) and warfarin(8%)

Follow up imaging was obtained on average at 108 days (range 13-466) and was done by CT scan in 81% of the patients and was missing in 13%. Recanalization was complete in 32% of the patients, partial in 21%. There was no improvement in 21% and progression in 1%. Recanalization was more likely in patients without cancer (83% vs 44%, p=0.002). There was no statistical difference in recanalization by anticoagulant in patients with cancer (p=0.14 LMWH vs Doacs). Recanalization was more likely in patients treated with DOACs than LMWH or warfarin (p< 0.001) but cancer could be a confounding factor.

Conclusions: Malignancy associated splanchnic thromboses are less likely to recanalize. Recanalization is not statistically significant improved with DOACs.

OC 73.5 | Australian and New Zealand Registry of Anticoagulation in the Obese

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Background: There is limited data to guide prescribing of anticoagulants in the obese, in particular in those weighing > 150kg or with a BMI > 40kg/m^2.

Aims: To examine anticoagulant prescribing in the obese in Australia and New Zealand; determine an appropriate LMWH dosing strategy; evaluate whether obese patients achieve appropriate DOAC levels and evaluate the efficacy and safety of anticoagulation in the obese.

Methods: We have prospectively registered patients with a BMI >35kg/m^2 or weight >120kg receiving anticoagulants at sites in Australia and New Zealand from August 2017. 3 years of follow-up is planned.

Results: Currently 91 patients have been recruited across 6 sites with a further 3 sites awaiting approval. Cross-over between agents occurred during the follow-up period.

Interpretation of DOAC levels varies depending on which of the published 'on therapy' ranges are used. According to one publication, 5/17 peak rivaroxaban, 3/11 peak apixaban and 5/9 trough apixaban levels fell outside range; while using another published range all apixaban levels were within the expected ranges. Median enoxaparin dose to achieve therapeutic Anti-Xa (0.5-1.2U/ml) (n=12) was 0.88mg/kg (range 0.52-1.01), all patients (n=5) weighing ³200kg achieved therapeutic Anti-Xa on 150mg BD (0.52-0.75mg/kg). A confirmed recurrence occurred on therapeutic warfarin and on

TABLE 1 Demographics

Drug	Number of Patients	Age (median, range)	Weight (kg) (median, range)	BMI (kg/m2) (median, range)	Indication
Apixaban	25	49 (27-85)	124 (76-184)	44.4 (35.3-70.0)	DVT (14), PE (12).
Dabigatran	2	40 (41-42)	113 (108-118)	36.8 (35.2-38.3)	AF (1), PE (1)
Rivaroxaban	37	55 (27-81)	120 (90-171)	38.5 (35-51.6)	DVT (26), PE (10), other (1)
Enoxaparin	22	51 (29-78)	130 (90-290)	45.1 (35.2-87.5)	DVT (6), PE (15), other (1)
Warfarin	18	55 (33-79)	153 (110-229)	49.5 (36.3-74.5)	DVT (6), PE (12)

TABLE 2 DOAC Levels

Anticoagulant	Dose	Median peak (ng/ml) (range)	Median trough (ng/ml) (range)
Rivaroxaban	20mg daily	281 (165-561) (n=17)	31 (<10-122) (n = 17)
	15mg BD	338 (n=1)	37 (25-55) (n=3)
Apixaban	5mg BD	122 (55-241) (n=11)	40 (<25-115) (n=9)
	2.5mg BD	85 (61-130) (n=3)	55 (33-63) (n=3)

apixaban (suspected non-compliance), an equivocal recurrence occurred on enoxaparin and 1 DVT occurred on dabigatran for a cardiac indication. 3 patients were changed from rivaroxaban due to low drug levels (peak 165 and troughs < 25ng/ml), no patient ceased apixaban for this reason.

Conclusions: Interpretation of DOAC levels is difficult due to a lack of well validated therapeutic ranges with clinical correlates. Further data regarding the clinical utility of DOAC levels, appropriate dosing strategy for enoxaparin, and clinical outcomes in this population is required. Our registry adds to recently published data by providing data on drug levels and how this may influence prescribing.

WOMEN'S HEALTH

OC 19.2 | The Experiences of Hemophilia Carriers Surrounding Pregnancy: A Qualitative Systematic Review

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Background: Hemophilia carriers (HCs) face specific psychosocial and physical challenges related to their inherited bleeding disorder: from reproductive decision-making, prenatal diagnosis, pregnancy, childbirth to puerperium. Therefore, optimal support and advice from health care providers is needed. This can only be realized by exploring the healthcare requirements of these women.

Aims: To review all available evidence in the published literature on the experiences and attitudes of being a HC surrounding pregnancy.

Methods: Cochrane library, PubMed/MEDLINE, EMBASE, CINAHL and PsycINFO were searched up to 30-11-2018 for publications on experiences and needs of HCs concerning these reproductive stages. Only papers in Dutch or English, containing original qualitative data were eligible for inclusion. Two independent authors conducted study selection. Data extraction was performed using a standardized form, meta-summary was used for data analysis. The Critical Appraisal Skills Programme Qualitative Checklist was used to assess risk of bias.

Results: 3302 publications were identified, of which 11 studies were included. All papers focused on reproductive decision-making or prenatal diagnosis and two papers on pregnancy or puerperium (Table 1). The following primary findings were reported:1) Genetic counseling was generally considered useful;2) The development of a specialized clinic focusing on HCs to enable informed reproductive decision-making would be considered as valuable;3) HCs considered prenatal diagnosis as beneficial, however it was experienced as psychosocially challenging;4) The experiences of non-invasive prenatal diagnosis (NIPD) and the attitudes towards preimplantation genetic diagnosis (PGD) were predominantly positive. Overall quality of studies was moderate.

Conclusions: This review highlights the lack of literature on psychosocial challenges surrounding pregnancy in HCs. The available literature focused on attitudes and experiences during genetic counseling and prenatal diagnosis in HCs and the importance of specialized psychosocial support during these phases. Future studies are needed to gain insight into the experiences and needs of HCs, especially during pregnancy and puerperium.

TABLE 1 Main themes according to reproductive stage and number of studies per theme

Main themes according to reproductive stage		Number of studies
Reproductive decision-making	Attitudes towards and experiences of genetic counseling	6
	Structure of genetic counseling	5
	Attitudes and experiences in reproductive decision making	5
Prenatal diagnosis	Attitudes towards and experiences of prenatal diagnosis (decision-making, result)	8
	Attitudes towards and experiences of noninvasive prenatal diagnosis (NIPD), preimplantation genetic diagnosis (PGD) and invasive testing	4
	Attitudes towards and experiences of termination of pregnancy	6
Pregnancy	Experiences of women whose pregnancies continued after prenatal diagnosis	1
Childbirth	None	0
Puerperium	Experience of hearing the diagnosis in offspring	1

OC 19.3 | Prepartum Factor XIII Has Significant Impact on Postpartum Blood Loss

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Background: Postpartum hemorrhage (PPH) is a major cause of maternal mortality and besides several known risk factors occurs often unexpectedly.

Aims: To assess the possible influence of prepartum activity of F. XIII on postpartum blood loss.

Methods: In this monocentric, prospective study, prepartum activity of coagulation factors I, II and XIII were analyzed in 1300 patients. Blood samples were drawn within 36 hours prior to onset of delivery. To avoid treatment paradox, all samples were assessed in a batch weeks after the delivery of the last patient. Thus, peripartum management was not biased. Correlations of coagulation factors with measured blood loss (MBL) were assessed by continuous outcome logistic regression. Furthermore, the impact of prepartum and / or postpartum available effect modifiers was evaluated.

Results: F. XIII demonstrated a high correlation with MBL: for every unit increase of F. XIII, the odds ratio to remain below any cut-off for MBL was 1.011 (95% CI, 1.006 to 1.015; p< 0.001, Table 1). Hence, a hypothetical increase of F. XIII activity by 30% would result in a 38.9% increase of the probability to not suffer from PPH. This effect was seen independently of the statistical model applied, after stratification for delivery mode, for any cut-off for MBL, and likewise when considering other risk factors. The effect size of F. XIII on blood loss was evaluated in different clinical subgroups, thus preparing the ground for further clinical trials. Interestingly, prepartum F. I did not have any effect on postpartum blood loss.

Conclusions: In this up to date largest analysis of the impact of F. XIII activity on postpartum blood loss, prepartum F. XIII activity was highly correlated with blood loss irrespective of the statistical model or clinical subgroup applied. Interventional studies are required to assess whether increasing F. XIII is effective to lower the probability of PPH.

TABLE 1 OR to remain below any cut-off for MBL, induced by a one-unit increase in one of the 4 prepartum blood parameters (Continuous outcome log regression)

Blood Parameter	Odds Ratio (OR)	95% CI	p
Hb	1.008	0.999 - 1.018	0.07
F. I	0.930	0.828 - 1.044	0.22
F. II	1.007	1.001 - 1.013	0.02
F. XIII	1.011	1.006 - 1.015	<0.001

OC 19.4 | Postpartum Hemorrhage in Women with von Willebrand Disease and Carriers of Hemophilia: A Retrospective Analysis

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Background: A large Dutch retrospective study reported a prevalence of 34% postpartum hemorrhage (PPH) in women with von Willebrand disease (VWD) and hemophilia A/B (HA, HB) carriers between 2002-2012. This high PPH prevalence might have declined in recent years.

Aims: To analyze the recent prevalence of PPH in hemophilia carriers (HCs) and women with VWD.

Methods: We conducted a multicenter retrospective cohort study in two Dutch hemophilia treatment centers. After ethical approval, women with VWD and HCs who delivered between 2012 and 2017 were selected through analysis of the hospitals´ patient registry and by

searching lab reports and letters of discharge in both hospitals' medical file databases. Patient and disease characteristics, peripartum hematologic/obstetric management and outcomes were collected. The primary outcome parameter was primary PPH, defined as ≥500 ml of blood loss from the genital tract ≤24 hours after childbirth.

Results: 112 deliveries were included: 45 in VWD, 60 in HA and 7 HB carriers. The overall PPH incidence was 36.6% (41/112): 44.4% (20/45) of VWD women, 28.3% (17/60) of HA carriers and 57.1% (4/7) of HB carriers. The risk of PPH was higher in women who received clotting factor correction compared to those who did not (21/44 vs. 20/68, OR 2.2, 95% CI: 1.0-4.8, p=0.05), partly explained by uterus atony (OR 1.9, 95% CI: 0.7-4.7, p=0.19). Third trimester clotting factor levels < 50IU/dL were not clearly associated with PPH (8/18 vs. 24/72, OR 1.6, 95% CI: 0.6-4.6, p=0.38).

Conclusions: The need for changes in peripartum management is evident as the PPH prevalence over the last 5 years remains high in women with VWD and hemophilia carriers despite treatment to correct clotting factor levels >50IU/dL. Future prospective studies should reveal whether more aggressive treatment to correct the clotting factor deficiency aids in minimizing PPH occurrence.

OC 19.5 | Maternal Death and Post-partum Hemorrhage in sub-Saharan Africa - A Pilot Study in Metropolitan Mozambique

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Background: Maternal mortality in Sub-Saharan Africa is ≈500-1000/100,000 births (vs ≈5-20 in developed countries), and post-partum hemorrhage (PPH) is a leading cause, responsible for 30-50% of deaths.

Aims: To study PPH, its risk factors and mortality in metropolitan Mozambique to inform future studies and intervention strategies.

Methods: Retrospective data extraction from a non-random sample of 495 patients' charts who delivered between January and June 2018 at Maputo Central Hospital, Mozambique. Data included age, survival, HIV status, parity, delivery mode, complications, vital signs, laboratory values and maternal/fetal data. PPH was determined by charted diagnosis, interventions for hemorrhaging, placental abruption, transfusion or abnormal blood loss. Autopsy reports from all deceased patients were examined.

Results: Median age and parity were 29 years (range 15-45) and 1 (range 0-10). Approximately half experienced C-sections or complications; 35 died. PPH frequency was 12% and strongly associated with mortality (40%; p< 0.0001). Prominent risk factors for PPH were HIV (prevalence 17%; OR = 11.7 [4.7,28.8]), elevated body temperature (OR = 1.1 [1.4,4.3] per 0.1 C°), high parity (OR for parity of 3+ = 7.2 [2.4,4.3]) and short gestation (OR = 0.86 [0.81,0.92]). Available labs from 241 distressed patients revealed anemia, thrombocytopenia, and hypoalbuminemia in 54%, 31%, and 85%, respectively, all strongly associated with PPH (all p-values ≤ 0.003). Medians were lower in PPH patients compared to other distressed patients: (hemoglobin 8.6 vs 11.1 gm/dL; platelets 148,000 vs 196,000/uL; albumin 2.55 vs 2.95 g/dL; all p-values < 0.001). Protocols to estimate peri-partum blood loss were not routinely used; anti-fibrinolytics and/or cryoprecipitate were unavailable. A simple model using parity, temperature and gestation length to predict PPH risk was developed.

Conclusions: PPH is a serious problem even in metropolitan areas of Sub-Saharan Africa. To address this problem, it is critical to raise awareness and develop simple prediction and treatment protocols.

OC 48.1 | Thrombosis Risk after Switching Oral Contraceptive Type

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Background: Combined oral contraceptives (COCs) increase the risk of venous thromboembolisms (VTEs) 2 to 4-fold, depending on the type of COC. This risk is highest in the first 3-12 months of use (starters-effect), due to redistribution of clotting factors and attrition of susceptibles. The effect of switching COCs on VTE risk is yet unknown. Potentially, it could induce a renewed starters-effect.

Aims: We aim to study the effect of switching COC type on VTE risk.

TABLE 1 VTE events and switches within starters per COC type

COC type	Lev	Noret	Norges	Des	Gest	Dros	Norm	Dien	Cyp
Starters	3,931,755	44,873	27,552	523,900	258,518	272,077	7,836	7,585	472,393
FU years	2,463,287	28,868	17,221	352,832	172,620	148,148	3,570	3,594	266,129
VTE (% of starters)	3,678 (0.09)	56 (0.12)	20 (0.07)	819 (0.16)	318 (0.12)	240 (0.09)	1 (0.01)	2 (0.03)	429 (0.09)
Switches (% of starters)	137,178 (3.49)	3,925 (8.75)	2,303 (8.36)	27,744 (5.30)	16,944 (6.55)	20,312 (7.47)	793 (10.12)	849 (11.19)	39,017 (8.26)

Cyp: cyproterone, Des: desogestrel, Dien: dienogest, Dros: drospirenone, FU: follow-up, Gest: gestodene, Lev: levonorgestrel, Noret: norethisterone, Norges: norgestimate, Norm: normegestrol, VTE: venous thromboembolism

Methods: We conducted a cohort study using data from the Dutch Foundation for Pharmaceutical Statistics (SFK) including women < 50 years starting COC use between 2000-2016. We will calculate hazard rates per day (incidence rates) after starting or switching COC, per type of COC and per type of switch, up to a total follow-up of 365 days. The development of the risk over time after starting will be modelled as function of time, taking current type of COC, and age into account. When comparing the risk over time after switching with that after starting with a similar COC, we can assess whether the risk in switchers changes only because a different risk associated COC preparation is initiated or whether there is a new starters effect (Figure 1). As the database does not provide definite diagnoses of VTE, we used prescriptions of oral anticoagulants as a proxy for VTE.

Results: The number of starters per COC type, the number of subsequent switchers, and the number of VTE events within starters are shown in Table 1. Calculation of hazard rates is still ongoing and these risk profiles will be available to present at the ISTH meeting in July 2019.

Conclusions: The large amount of starters and switchers in our database allows us to reliably estimate the risk of VTE over time after starting and switching COC.

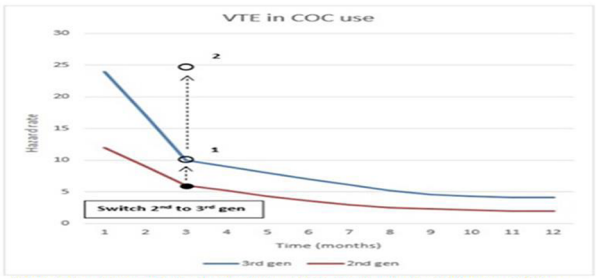

FIGURE 1 Graph demonstrating the modelling of VTE risk after starting COC use per COC type

OC 48.2 | Preventing Postpartum Venous Thromboembolism; Impact of Systematic VTE Risk Assessment

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Background: Venous Thromboembolism (VTE) is a leading cause of direct maternal death(1). Formal assessment of VTE risk is recommended in all women(2-4). In 2014, a postpartum VTE risk assessment tool (VTERA), *'Thrombocalc'*, was introduced at our institution to streamline VTERA in a high-throughput environment(5).

Aims: To assess the impact of systematic postpartum VTERA.

Methods: A before and after study design was used. To assess the impact of Thrombocalc on VTE prevention practices and use of risk-appropriate thromboprophylaxis, a retrospective chart review was undertaken in a random sample of women (N=599) who gave birth at our institution before or after Thrombocalc(July´12-June´14&July´15-June´17 respectively). Differences were assessed using chi-squared test.

We also undertook a preliminary analysis of VTE events occurring at a hospital level in the 3 calendar-years before and after Thrombocalc implementation(Jan´11-Dec´13&Jan´15-Dec´17). Data on VTE events occurring during the delivery admission or postpartum were ascertained from our hospital discharge administration

TABLE 1 Impact of Thrombocalc VTE risk assessment on VTE prevention strategies

VTE prevention strategy	Pre-Thrombocalc			Post-Thrombocalc			P-value (All)	P-value (Operative)	P-Value (vaginal)
	All (N=300)	Caesarean delivery (N=100)	Vaginal delivery (N=200)	All (N=299)	Caesarean delivery (N=132)	Vaginal delivery (N=167)			
Full documented risk assessment*	7 (2%)	5 (5%)	2 (1%)	293 (98%)	128 (97%)	165 (99%)	<0.001	<0.001	<0.001
Appropriate VTE prevention strategy †	255 (85%)	92 (92%)	163 (82%)	275 (92%)	125 (95%)	150 (90%)	0.008	0.408	0.025
Use of risk-appropriate pharmacological thromboprophylaxis	96 (68%) (N‡=141)	92 (92%) (N‡=100)	4 (10%) (N‡=41)	161 (89%) (N‡=182)	125 (95%) (N‡=132)	36 (72%) (N‡=50)	<0.001	0.408	<0.001

*Partial risk assessment, whereby the postpartum thromboprophylaxis was recommended in the absence of a documented individualised risk assessment, was present in 67 and 3 postpartum women, in the Pre- and Post-Thrombocalc period respectively. † Appropriate VTE prevention: Pharmacological thromboprophylaxis appropriately indicated and prescribed, or thromboprophylaxis appropriately not indicated and not prescribed. ‡ Total indicated for postpartum thromboprophylaxis according to local guidelines.

TABLE 2 VTE events occurring during the delivery admission or in the postpartum period recorded in the hospital discharge administration system

	Pre-Thrombocalc (Jan 2011-Dec 2013)				Post-Thrombocalc (Jan 2015-Dec 2017)				Risk Ratio (95%CI, p-value)
Year	2011	2012	2013	Total	2015	2016	2017	Total	
Total women giving birth to infant >500 grams	9116	8846	8648	26610	8361	8405	8226	24992	
VTE events* (%)	8 (0.09%)	9 (0.1%)	5 (0.06%)	22 (0.08%)	2 (0.02%)	1 (0.01%)	1 (0.01%)	4 (0.02%)	0.19 (0.07-0.56, <0.001)

* VTE events occurring during the delivery admission or postpartum period as recorded in our hospital discharge administration system

system. Absolute differences and relative risk of VTE (with 95%CI) were calculated.

Results: The impact of Thrombocalc on VTE prevention strategies in our review of 599 postpartum women is shown in Table1. Introduction of our postnatal VTERA resulted in a statistically significant increase in use of risk-appropriate pharmacological thromboprophylaxis (RA-TPX) from 68% to 89%(p< 0.001)). This was primarily driven by increased use of RA-TPX after vaginal delivery (10% to 72%) following the introduction of Thrombocalc(p< 0.001).

In this preliminary sample, hospital-level VTE events recorded at our institution during the delivery admission or in the postpartum period reduced from 22 (0.08%) in the 3-year period before Thrombocalc, to 4 (0.02%) in the 3-year period after Thrombocalc (RR 0.18 (95%CI 0.06-0.53, p< 0.001)).

Conclusions: Systematic implementation of a postpartum VTE risk assessment tools has the potential to increase use of RA-TPX and may reduce postpartum VTE events. Validation of these data is ongoing.

OC 48.3 | Performance of Standard Dose Enoxaparin in Preventing Pregnancy-associated Venous Thromboembolism

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Background: Pregnancy-associated venous thromboembolism (PAVTE) which includes deep venous thrombosis (DVT) and pulmonary embolism (PE) is a major cause of morbidity and mortality. Low molecular weight heparins such as enoxaparin are commonly prescribed to reduce this risk. Maternal obesity is an increasing problem. However, data on optimal dosing in the obese maternal population is lacking.

Aims: To evaluate the efficacy and safety of enoxaparin 40mg daily antepartum (AP) and postpartum (PP) in preventing PAVTE in obese patients (body mass index ≥ 30kg/m^2 or weight > 90kg) compared to their non-obese counterparts while accounting for underlying risk factors.

Methods: A retrospective study was conducted consisting of all women (obese and non-obese) cared for at Monash Health who received enoxaparin 40mg subcutaneously daily for VTE prophylaxis while pregnant and/or postpartum (up to 6 weeks following delivery). The study period was 1/1/2013 to 30/6/2018. The AP and PP periods were analysed separately. Data was obtained from hospital records. The primary efficacy outcome was new radiologically-proven VTE (i.e. DVT and/or PE).

The primary safety outcome was a composite bleeding measure (i.e. adaptation of ISTH bleeding scale and blood transfusion requirements) and blood loss within the first 24 hours postpartum (for AP patients only).

Results: Results are summarised in Tables 1 and 2. No new VTE events occurred in AP period. Two events occurred in PP period - one obese (1/53, 1.9%) and 1 non-obese (1/93, 1.1%). Both patients had a history of prior VTE. Bleeding rates were comparable among the groups.

Conclusions: Within the limitations of the study, our findings suggest that enoxaparin 40mg daily is effective at preventing PAVTE for most obese patients in AP period. VTE risk appeared higher in PP period, especially in patients with prior VTE. No significant difference was detected in the performance of 40mg daily enoxaparin between the obese and non-obese groups.

TABLE 1 Results from antepartum phase (N=76)

Number of patients receiving antepartum prophylaxis	Obese = 28 patients N (%)	Non-obese = 48 patients N (%)
Prior history of VTE	15 (53.6)	27 (56.3)
Efficacy outcome: Any new VTE	0	0
• DVT	0	0
• PE	0	0
1. Composite bleeding	4 (14.3)	12 (25.0)
• Major bleeding	2 (7.1)	5 (10.4)
• CRNMB	2 (7.1)	7 (14.6)
• Number of patients who received blood transfusion	0	1 (2.1)
2. Mean blood loss ≤ 24 hours after delivery (mL)	471	475

TABLE 2 Results from postpartum phase (N=146)

Number of patients receiving postpartum prophylaxis	Obese = 53 N (%)	Non-obese = 93 N (%)
Prior history of VTE	30 (56.6)	44 (47.3)
Efficacy outcome: Any new VTE	1 (1.9)	1 (1.1)
• DVT	1 (1.9)	0
• PE	0	1 (1.1)
1. Composite bleeding	7 (13.2)	16 (17.2)
• Major bleeding	3 (5.7)	3 (3.2)
• CRNMB	4 (7.5)	11 (11.8)
• Number of patients who received blood transfusion	2 (3.8)	2 (2.2)
2. Mean blood loss ≤ 24 hours after delivery (mL)	NA	NA

OC 48.4 | Enoxaparin Prescription during Pregnancy. Are We Using it According to International Guidelines

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Background: Pregnancy is a prothrombotic state. International guidelines recommend using aspirin plus enoxaparin in patients with Antiphospholipid syndrome (APS). It is recommended thromboprofilaxis during puerperium when one high risk factor o two low risk factor for thromboembolic disease (TED) are present. The use of enoxaparin is also recommended during pregnancy and puerperium if there is history of TED during pregnancy or estrogens treatment or idiopatic thrombosis. When an inherited high risk thrombophilia is present without thrombosis but there is family history of TED, thromboprofilaxis during pregnancy and puerperium is recommended. (Guidelines ECP. 9th ed : American College of Chest Physicians. 2012).

Aims: To describe the proportion of patients with enoxaparin prescriptions during pregnancy planning, actual pregnancy and/or puerperium made according to international guidelines.

Methods: Descriptive, retrospective study of consecutive patients with enoxaparin prescription due to TED or thromboprophylaxis due to any cause, evaluated in the Hematology and obstetrics outpatient clinic, from March 2013 to March 2018. Acute events were excluded.

Results: Ninety five patients were included; six where preconceptional consults. Median age was 37 (IQR 34-40). Thirty seven percent of prescriptions made were according to international recommendations. Fifty percent used enoxaparin since the first trimester till puerperium. Thirty one percent used it only during pregnancy. History of miscarriages was the most frequent reason of prescription (Figure 1). Multivariate analysis shows that age > 37, history of miscarriages, incomplete APS criteria or PAI 1/ MTHFR mutation or prolonged euglobulin lysis test were associated with an incorrect prescription (Table 1). 34/95 had thrombophilia diagnosis, 50% of those prescriptions were made according to international guidelines.

Conclusions: There is a significant misuse of enoxaparin prescription in this clinical setting. Erroneous diagnosis along with low quality evidence-based indications generates indiscriminate use of enoxaparin. Prospective clinical studies are needed to generate clear indications to update current guidelines.

TABLE 1

	Odds Ratio	CI	p
Age > or = 37	2.93	0.99-8.64	0.05
Miscarriages	9.81	2.67-36	0.001
Incomplete APS criteria, PAI 1/ MTHFmutation, prolonged euglobulin lysis tests	10.68	2.33-48.97	0.002
Thrombophilia	0.38	0.10-1.33	0.133

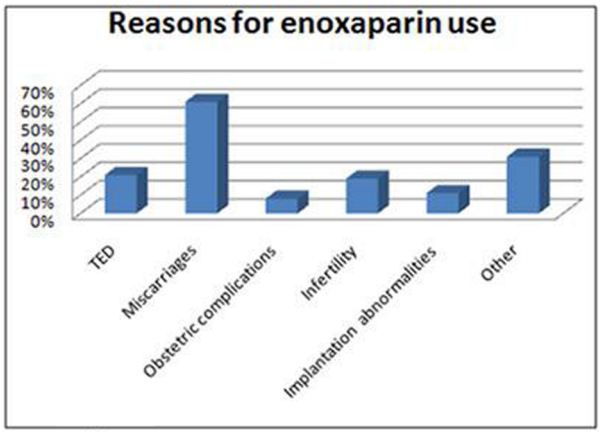

FIGURE 1

OC 48.5 | Promising Maternal and Fetal Therapeutic Outcomes from Sildenafil and Dalteparin Treatments in a Murine Pregnancy Loss Model

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Background: Sildenafil (Viagra®) is a PDE5 inhibitor with an excellent tolerability and safety profile. It was recently shown to improve pregnancy outcomes in LPS-induced experimental models. Low Molecular Weight Heparin (LMWH) is a standard anti-coagulation therapy for DVT and thrombophilia in pregnancy with some success in pre-eclampsia and recurrent pregnancy loss (RPL). However outcomes remain ambiguous and still carry bleeding risk. Indeed, alternative treatments that may supplement or substitute heparin will be valuable.

Aims: To evaluate and compare the effects of Sildenafil+LMWH to either drug alone or placebo on fetal survival and coagulation profile in experimentally-induced murine pregnancy failure.

Methods: Twenty-five pregnant Swiss-Albino mice and blood from 6 healthy non-pregnant women were studied. Pregnant Mice were treated daily with oral Sildenafil (50mg/kg) and Heparin injection (500IU/kg) or either drug alone or placebo. LPS was administered intraperitoneally (100 µg/kg; GD15) and pregnancy outcomes were evaluated after 2h. Human blood was evaluated using Thromboelastography (TEG) after drugs incubation *in vitro*. Mouse blood was analysed by complete blood count (CBC) and TEG. Mouse implant sites were scored. Placentas were analysed by immunohistochemistry for endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS) and placental growth factor (PGF) together with amniotic fluid multiplex inflammatory cytokines assays.

Results: Sildenafil improved fetal health; increased implantation sites and fetal sizes. No significant differences between Sildenafil+LMWH group and normal pregnant without LPS. Placentas from Sildenafil+LMWH group showed higher staining for eNOS but similar iNOS and PGF expression compared to control. LPS treatment without prophylaxis lowered platelets and TEG displayed hypercoagulability with secondary fibrinolysis. Sildenafil alone did not change coagulability of human blood and had no additive effect to the LMWH-induced hypo coagulation.

Conclusions: Sildenafil appears to be a safe prophylactic treatment for RPL. When used as an adjuvant to LMWH treatment or alone, it maintains fetal health even after an abortifacient challenge.