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Oral communication abstracts

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OC - 1

Novel inhibitory GPVI antibodies block the GPVI ligand binding domain

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

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

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

Results:
Four mAbs were tested; one blocked GPVI-mediated aggregation and another reduced this by ~50%. Three of the Fabs completely inhibited fibrinogen binding and P-selectin exposure in response GPVI agonists while the response to PAR agonists was normal. All mAbs bound to monomeric and dimeric GPVI with similar affiminities (KD ~1-5 nM), which suggests that they do not induce receptor clustering. Recombinant chimeric GPVI showed binding to the D1 domain, the same domain where collagen binds. This may prevent collagen binding or receptor clustering and explain the inhibition.

Conclusions:
GPVI-mediated platelet activation was inhibited by multiple Fab fragments and one mAb suggesting these have potential as a novel α-GPVI therapy. BLI suggested that antibodies do not induce receptor clustering. Using GPIV chimeras we showed that antibodies bind to the collagen binding D1 domain and therefore they may be inhibiting to the collagen binding area or inhibiting clustering.

OC - 2

Anti-GPVI Nanobody 2 disrupts Collagen and Atherosclerotic Plaque induced GPVI Signalling and prevents Receptor Clustering under Flow

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Background:

The collagen receptor glycoprotein VI (GPVI) is an attractive target for novel antiplatelet therapies due to its critical role in platelet activation after atherosclerotic plaque rupture and minor role in haemostasis. GPVI clustering is considered to determine the strength and duration of signalling leading to increased platelet activation. We raised and characterized 54 novel anti-GPVI nanobodies (Nbs), and identified Nb2 as the most potent antagonist of the GPVI-collagen interaction. Further, Nb28 was shown to bind strongly to GPVI but not affect its binding to collagen.

Aims:

Here, we set out to investigate the effects of inhibitory Nb2 on collagen and plaque-mediated GPVI signalling. In addition, we characterise and develop the non-inhibitory anti-GPVI Nb28 for molecular localization and imaging studies of GPVI in thrombus formation.

Methods:

GPVI downstream phosphorylation was assessed in collagen and human pooled plaque homogenate stimulated washed platelets, preincubated with inhibitory Nb2. Fluorescently labelled Nb28 was used to assess GPVI localisation and clustering in thrombi formed on collagen or plaque material at arterial shear.

Results:

Nb2 suppressed collagen- and plaque-induced thrombus formation, along with platelet activation. With either agonist, Nb2 reduced the phosphorylation level of Syk-Y525/526, LAT-Y200 as well as PLCγ2-Y1217. Labelled Nb28 revealed complete disruption of GPVI clustering by Nb2 in platelets adhered to collagen fibres under flow. None of the investigated parameters were affected by a negative control Nb.

Conclusions:

These data not only stress the effectivity of Nb2 to inhibit atherosclerotic plaque mediated GPVI signalling, but further, underline the physiological relevance of collagen-induced GPVI clustering for signalling. Highlighting the usefulness of Nbs as imaging tools (Nb28) and as potential anti-thrombotic agents (Nb2).

OC - 3

A differential effect of CLEC-2 divalent ligands on human and mouse platelets

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Background: The platelet C-type lectin-like receptor 2 (CLEC-2) has been identified as a candidate for a new class of anti-thrombotic drugs due to its role in driving occlusive thrombosis and its minor role in haemostasis. Activation of CLEC-2 is mediated by clustering but its membrane organisation is controversial and its dependency on ligand valency is not known. Previous results have shown that divalent ligands to CLEC-2 activate mouse platelets.

Aims: The aims were to determine whether CLEC-2 is expressed as monomers or dimers in the membrane and investigate the dependency on ligand valency for activation of human platelets and humanised mouse platelets.

Methods: We used fluorescence correlation spectroscopy (FCS), photobleaching and non-detergent membrane extraction to determine the stoichiometry of CLEC-2 in cell lines and platelets. We have investigated the ability of divalent and tetravalent ligands (including novel crosslinked nanobodies) to induce activation of platelets and transfected cell lines with aggregometry and an NFAT reporter assay, respectively. We tested whether the ligands cause clustering of CLEC-2 by FCS.

Results: Contrary to previous reports, we show that CLEC-2 is expressed as a mixture of monomers and dimers in platelets and cell lines. Using human and mouse platelets, and cell lines transfected with low and high levels of CLEC-2, we report that divalent ligands serve as antagonists in human platelets and low-expressing cells lines and as agonists in mouse platelets and high-expressing cell lines. Further, we show that tetravalent nanobodies cause aggregation of human platelets and using FCS that multivalent ligands cause clustering of CLEC-2 In cell lines.

Conclusions: These results provide evidence that CLEC-2 is expressed as a mixture of monomers and dimers and that dimerisation is governed by receptor density. Divalent ligands can act as agonists and antagonists depending in part on receptor density. We therefore propose that divalent ligands should be considered as partial agonists.

OC - 4

Galectin-9 activates platelet ITAM receptors Glycoprotein VI and C-type Lectin-Like Receptor-2

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Background:

Platelets are multifunctional cellular mediators in many physiological and pathophysiological processes such as thrombosis, angiogenesis, and inflammation. Several members of galectins, a family of carbohydrate-binding proteins with a broad range of immunomodulatory actions, have been reported to activate platelets.

Aim:

In this study, we investigated the role of Galectin-9 (Gal-9) as a novel ligand for platelet glycoprotein VI (GPVI) and C-type lectin-like receptor-2 (CLEC-2).

Methods:

Platelet spreading, aggregation and P-selectin expression in response to Gal-9 were measured in washed platelet suspensions via static adhesion assay, light transmission aggregometry and flow cytometry, respectively. Solid-phase binding assay and protein phosphorylation studies were utilised to validate the interaction between Gal-9 and GPVI, and immunoprecipitation for detecting CLEC-2 phosphorylation. Wild-type (WT), GPVI-knockout (*Gp6*$^{-/-}$) and GPVI and CLEC-2-double knockout (*Gp6*$^{-/-}$*/Gp1ba-Cre-Clec1b*$^{fl/fl}$) mice were employed.

Results:

We have shown that recombinant Gal-9 stimulates aggregation in human and mouse washed platelets dose-dependently. Platelets from both species adhere and spread on immobilised Gal-9 and express P-selectin. Gal-9 competitively inhibited the binding of human recombinant D1 and D2 domains of GPVI to collagen. Gal-9 stimulated tyrosine phosphorylation of CLEC-2 and proteins known to lie downstream of GPVI and CLEC-2 including Syk and LAT in human platelets. GPVI-deficient murine platelets exhibited significantly impaired aggregation in response to Gal-9 which was further abrogated in GPVI and CLEC-2-double-deficient platelets.

Conclusions:

We have identified Gal-9 as a novel platelet agonist which induces activation through interaction with GPVI and CLEC-2.

OC - 5

The Btk inhibitor AB-95-LH34 potently inhibits atherosclerotic plaque-induced thrombus formation and platelet procoagulant activity

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Background:

New anti-thrombotic therapies that do not cause bleeding are needed to treat coronary artery disease. The Btk inhibitor ibrutinib has been shown to block atherosclerotic plaque-mediated thrombus formation *in-vitro*. However, is associated with increased bleeding due to off-target effects. Btk-deficient individuals do not bleed, suggesting selective Btk inhibition is a viable anti-thrombotic strategy.

Aims:

To compare the effect of ibrutinib with the highly selective Btk inhibitor AB-95-LH34 (remibrutinib analogue, compound 14, Angst *et al.*, 2020) on plaque-mediated platelet function and signalling.

Methods:

GPVI and GPCR-mediated platelet function and signalling were analysed in human donor platelets by lumi-aggregometry, flow adhesion and western-blot following 1-hour *ex-vivo* inhibitor treatment.

Results:

AB-95-LH34 showed similar inhibition of Btk-Y223 phosphorylation as ibrutinib, but had no off-target inhibition of Src-Y418 or LAT-Y200 phosphorylation to both atherosclerotic plaque and collagen-related-peptide (CRP). Similar dose-dependent inhibition of aggregation to plaque was observed for both inhibitors. However, in response to collagen, ibrutinib exhibited stronger aggregation inhibition than AB-95-LH34. Neither inhibitor affected PAR1 or thromboxane-A2 receptor-mediated platelet function. In whole blood perfused over plaque at arterial shear ($1000s^{-1}$), platelet adhesion and aggregate formation was reduced by both Btk inhibitors. Ibrutinib demonstrated the most potent effect, with complete blockade at 5 µM. On collagen, 5 µM concentrations of both inhibitors were required to inhibit aggregate formation, with ibrutinib again displaying the strongest inhibition and also inhibiting platelet adhesion. Platelet activation (P-selectin) and procoagulant platelet formation (phosphatidylserine exposure) in thrombi were inhibited by AB-95-LH34, but completely blocked by ibrutinib (5 µM). Furthermore, thrombin generation in response to plaque was reduced by 5 µM AB-95-LH34 and ibrutinib. Ibrutinib, but not AB-95-LH34, also inhibited tissue factor induced thrombin generation.

Conclusion:

AB-95-LH34 shows potent inhibition of atherosclerotic plaque-induced thrombus formation and procoagulant platelet activity, with less off-target inhibition than ibrutinib, suggesting it is a promising anti-platelet therapy with reduced bleeding side-effects.

Acknowledgement: This work was funded by Novartis.

References: Angst, D. *et al.* (2020) 'Discovery of LOU064 (Remibrutinib), a Potent and Highly Selective Covalent Inhibitor of Bruton's Tyrosine Kinase', *Journal of Medicinal Chemistry*, 63(10), pp. 5102–5118. doi: 10.1021/acs.jmedchem.9b01916.

OC - 6

Impaired fibrin α-chain cross-linking in a new genetically modified murine model

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Introduction:
Factor XIII (FXIII) cross-links fibrin and incorporates fibrinolysis inhibitors into the clot, increasing resistance to mechanical strain and proteolytic degradation. We have recently used a mouse strain (FGG3X) where γ-chain cross-linking is abolished, and demonstrated a key role of fibrin γ-chain cross-linking in reducing thromboembolisation *in-vivo*, by increasing fibre toughness. However, the role of α-chain cross-linking in clot formation and stability *in-* and *ex-vivo* is not yet known.

Aims:
The aims of this study were to generate and characterise a genetically modified murine model of impaired fibrin α-chain cross-linking.

Methods:
Genetically modified FGA4X mice were designed and produced by mutating the conserved fibrin α-chain glutamine residues involved in cross-linking by FXIII (αQ241N/Q243N/Q257N/Q518N). Mice phenotype was characterised, and clot properties were analysed by ROTEM and clot contraction assay.

Results:
Compared to WT (C57BL/6), FGA4X mice showed no differences in haematological parameters, however α-α cross-linking was significantly reduced (35.6±6.2 vs 83.7±4.0 %, $p<0.01$), whilst γ-γ cross-linking remained normal. ROTEM analysis of whole blood showed that clotting time (37.0±4.0 and 47.0±6.0 sec) and maximum clot firmness (61.6±4.0 and 61.8±3.0 mm) were not significantly different. However, lysis time was significantly prolonged in FGA4X mice (42.8±2.6 vs 30.8±1.6 min, $p<0.01$) compared with WT. Clot contraction experiments showed that FGA4X mice formed larger (46.7±1.4 vs 30.8±1.5 μl) and heavier (44.3±0.7 vs 31.6±1.8 mg) clots than WT, containing more red blood cells (+21%).

Conclusions:
Initial analysis of this new murine model of impaired fibrin α-chain cross-linking shows that α-α cross-links play a role in determining resistance to fibrinolysis and thrombus size. Further work will allow to investigate the role of α-chain cross-linking on thrombosis and thromboembolism *in-vivo*. This FGA4X strain, combined with our FGG3X mice, will enable in-depth understanding of the role of fibrin cross-linking in thromboembolic diseases, and possibly point to new therapeutic targets.

OC - 7

Rare missense variants in Tropomyosin-4 (TPM4) are associated with platelet dysfunction, cytoskeletal defects and excessive bleeding

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Background:

Diagnosing inherited platelet disorders is notoriously challenging where candidate variants can be found in over 100 bleeding, thrombotic and platelet related genes. This becomes particularly apparent within families where there is both normal and low platelet counts. Genetic variants of unknown significance (VUS) are found in a significant proportion of patients, therefore functional investigation is required to prove pathogenicity.

Aims:

To identify the genetic cause in patients with a suspected platelet disorder and perform in depth platelet function analysis of the candidate variants found.

Methods:

Genetic and functional studies were undertaken in three patients from two unrelated families with a suspected platelet disorder and excessive bleeding. A targeted gene panel of known bleeding and platelet related genes was used to identify plausible genetic variants. Platelet phenotyping was performed using platelet spreading, transmission electron microscopy, immunofluorescence, lumiaggregometry and flow cytometry.

Results:

Rare conserved missense variants (R182C and A183V) were identified in all three patients within *TPM4* encoding Tropomyosin-4. Tropomyosin-4 is a member of the tropomyosin family of actin-binding proteins involved in the cytoskeleton of non-muscle cells. Deep platelet phenotyping revealed platelet function defects across all three patients including reduced aggregation, ATP secretion, spreading and disordered tropomyosin staining by immunofluorescence. Platelet TPM4 protein expression was not affected by the missense mutations as assessed by western blot.

Conclusions:

Variants within *TPM4* are responsible for platelet function defects and inherited bleeding in three patients from two unrelated families. This highlights the importance of including *TPM4* as a grade tier 1 gene in diagnostic screening for patients with inherited bleeding and undiagnosed platelet disorders, particularly in those with a normal platelet count.

OC - 8

A simple in vitro model of thrombosis to assess the impact of endothelial dysfunction on antiplatelet efficacy

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Background:

Current *in vivo* models of arterial thrombosis do not reflect the local environment observed in human atherothrombosis. This study aimed to develop a simple endothelialized *in vitro* model of human atherothrombosis to replace animal models.

Method:

Human whole blood labelled with $DIOC_6$ was perfused through microfluidic chambers lined with human umbilical vein endothelial cells (HUVECs) or human coronary artery endothelial cells (HCAECs) immediately adjacent to exposed collagen (100 µg/ml), at venous (5 dyne/cm^2) or arterial (15 dyne/cm^2) wall shear stress (WSS). The antithrombotic capacity of the endothelial cell types was compared and the contribution of nitric oxide, prostacyclin and CD39 assessed. Endothelial dysfunction was stimulated by TNF-alpha (10ng/ml, 24h), and the effect of this on platelet thrombus formation and antiplatelet efficacy determined.

Results:

Incorporation of HUVECS into the flow model similarly reduced thrombus size (20.61%; $P<0.05$) and thrombus surface coverage (32.77%; $P<0.001$), in a nitric oxide dependent manner. The antithrombotic effects of endothelial cells was diminished by TNF-alpha resulting in significantly larger thrombi formed on the immobilized collagen ($P<0.05$). This was accompanied by an increase in cell adhesion molecules VCAM-1 ($P<0.05$) and ICAM-1 ($P<0.05$) and a reduction in eNOS expression. Aspirin and Clopidogrel both significantly decreased average thrombus size ($P<0.05$) and the extent of this inhibition was altered by the presence of healthy and dysfunctional endothelial cells. The reduction in thrombus size achieved by HUVECS and HCAECs at an arterial shear rate was comparable, indicating that HUVECS are suitable and cost-effective option for the model.

Conclusion:

Here we present a simple and easily accessible alternative to animal models to investigate the critical role endothelial dysfunction in the regulation of thrombosis and antiplatelet efficacy.

OC - 9

Anti-Thrombotic Effects of the Proteasome Inhibitor Carfilzomib on the Endothelium

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Background

Carfilzomib (CFZ) is a second-generation proteasome inhibitor used to treat multiple myeloma. Potent inhibition of the proteasome results in chronic proteotoxic endoplasmic reticulum (ER) stress, leading to apoptosis. While CFZ has improved survival rates in multiple myeloma, it is associated with an increased risk of cardiovascular adverse effects. While this has been putatively linked to cardiotoxicity, CFZ could potentially also exhibit adverse effects on the endothelium.

Aim

To investigate the effects of CFZ on the endothelium

Methods

HUVECS were treated with CFZ and expression of relevant markers of ER stress, inflammation and thrombosis measured. In some experiments cells were stimulated with TNFα following CFZ treatment. Immunofluorescent staining was used to determine VWF string formation following drug treatment and turbidity and Factor X activation assays used to assess clot formation on endothelial cells.

Results

CFZ treatment of sub-confluent HUVECS induced BIP and ubiquitin expression indicating induction of ER stress, however while CFZ treatment of confluent HUVECS increased ubiquitin expression after 24 hours, expression returned to normal after 48 hours and there was minimal effect on the expression of BIP and other ER stress markers, indicating CFZ cannot induce ER stress in resting HUVECS. Interestingly, CFZ treatment induced the expression of eNOS, tissue plasminogen activator and thrombomodulin and significantly, CFZ was able to reduce TNFα induced ICAM-1 and tissue factor expression, suggesting a potential protective effect on the endothelium. Consistent with these observations, CFZ reduced factor Xa generation and fibrin clot formation on the endothelium following TNF treatment and prevented the formation of VWF-platelet strings and leukocyte adhesion under shear stress.

Conclusions

This data demonstrates that CFZ is unable to induce ER stress in confluent resting endothelial cells and can conversely attenuate the pro-thrombotic effects of TNF on the endothelium. This study suggests that CFZ does not negatively alter HUVECS, and proteasome inhibition of the endothelium may offer a potential way to prevent thrombosis. Further studies are now required to establish the mechanisms behind these effects.

OC - 10

Cryoprecipitate transfusion in trauma patients attenuates hyperfibrinolysis and restores normal clot structure by elevating PAI-1; results from a sub-study of the FEISTY trial

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Background:

Fibrinogen is the first coagulation protein to reach critically low levels during traumatic haemorrhage. Hyperfibrinolysis is common and exacerbates hypofibrinogenaemia. The Fibrinogen Early in Severe Trauma studY (FEISTY; NCT02745041) is the first randomised controlled trial (RCT) to compare the clinical effects of cryoprecipitate and fibrinogen concentrate (Fg-C) in these patients.

Aims:

To compare the effect of Fg-C or cryoprecipitate on the fibrinolytic pathway in severely injured patients enrolled to the FEISTY RCT.

Methods:

Paired plasma samples pre- and post-fibrinogen replacement from patients recruited to the FEISTY study were examined for plasmin and PAI-1 activity using chromogenic activity assays. PAI-1 antigen levels were quantified by ELISA. Fibrin clot structure was analysed using confocal microscopy by addition of Alexa Fluor 488 fibrinogen.

Results:

Plasmin generation was significantly reduced in trauma patients treated with cryoprecipitate, but remained unchanged with Fg-C. This was observed as a 1.5-fold decrease in the rate of plasmin generation pre- and post-transfusion of cryoprecipitate (p <0.01). PAI-1 activity and antigen levels were increased post-treatment with cryoprecipitate, but not Fg-C, transfusion. A strong correlation was observed between PAI-1 activity and antigen (r^2= 0.7, p <0.001). Confocal microscopy analysis of fibrin clots revealed that upon hospital admission trauma patients formed a significantly lower number of fibrin fibres and that these fibres were shorter when compared to normal plasma (p <0.01 and p <0.0001, respectively). Cryoprecipitate transfusion led to restoration of the fibrin structure with fibres comparable to those observed in normal plasma. In contrast, infusion with Fg-C did not restore normal clot structure.

Conclusion:

In severely injured and bleeding trauma patients, infusion of cryoprecipitate restores normal clot structure and increases PAI-1 activity thereby down-regulating plasmin activity. These effects were not observed with Fg-C. Our data indicate that cryoprecipitate has anti-fibrinolytic activity and is a superior source of fibrinogen to manage bleeding in trauma coagulopathy.

OC - 11

Fatty acids modulate zinc-dependent coagulation through albumin binding in type 2 diabetes

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Background:

Zn^{2+} is an essential regulator of coagulation following release from activated platelets and injured cells. In plasma, Zn^{2+} availability is fine-tuned through buffering by human serum albumin (HSA). Non-esterified fatty acids (NEFAs; also transported by HSA) reduce its ability to bind/buffer Zn^{2+}. Several disease states, include obesity and type 2 diabetes mellitus (T2DM), are associated with elevated plasma NEFA levels and with an increased risk of developing thrombotic complications. We hypothesise that elevated plasma NEFAs impact on haemostasis, where Zn^{2+}-mediated interactions are critical.

Aims:

These are to: 1). Examine the extent to which NEFAs reduced the Zn^{2+}-binding ability of HSA. 2). Determine the impact of NEFAs on fibrin clotting and platelet aggregation. 3). Measure the concentrations of plasma NEFAs in individuals with T2DM and age-matched controls and correlate these with fibrin clot parameters.

Methods:

Zn^{2+}-binding to HSA in the presence of different NEFAs at various concentrations was assessed using isothermal titration calorimetry. Fibrin clotting and platelet aggregation in plasma and platelet-rich plasma, respectively was measured spectrophotometrically. In plasma taken from T2DM and control individuals, fibrin clotting was quantified as above and NEFA concentrations measured by GC-MS.

Results:

Most NEFAs assayed were effective at suppressing zinc-binding to HSA. Assessment of platelet aggregation and fibrin clotting parameters in purified systems and in pooled plasma suggested that the HSA-mediated impact of the model NEFA myristate on zinc availability intensified the effects of Zn^{2+} alone. The effects of Zn^{2+} alone on fibrin clot density were mirrored in samples from T2DM patients. Crucially, the T2DM patients had increased total plasma NEFAs compared to controls, with the concentrations of key saturated and mono-unsaturated NEFAs positively correlating with clot density.

Summary/conclusions:
Collectively, these data strongly support the concept that elevated NEFA levels contribute to an increased thrombotic risk in T2DM through dysregulation of plasma zinc speciation.

OC - 12

Platelet factor XIII-A regulates platelet function and promotes clot retraction intracellularly and functions extracellularly by enhancing fibrinolytic stability of clots via procoagulant platelet formation

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Background:

Platelet FXIII-A is externalised upon activation, via an unknown mechanism, to cross-link extracellular fibrin and fibrinolytic inhibitors into thrombi to stabilise them against fibrinolysis.

Aims:

To determine if FXIII-A regulates platelet function and define how FXIII-A is delivered into thrombi.

Methods:

Platelets from FXIII-A-deficient patients and normal donors were assessed using flow cytometry and included in thrombi formed from FXIII-depleted plasma. FXIII-A activity was quantified using an in-house assay. FXIII-A was inhibited using a cell-permeable transglutaminase inhibitor (TGI). Various inhibitors were incorporated including, aIIbb3 inhibitor, tirofiban, SRC-family kinase(SFK) inhibitor, dasatinib and a caspase inhibitor.

Results:

FXIII-A-deficient and TGI-treated normal platelets showed reduced sensitivity to agonist stimulation and attenuated fibrinogen binding (P<0.05). Spreading on collagen (P<0.01) and fibrinogen (P<0.05) was attenuated in TGI-treated and FXIIIA-deficient platelets compared to normal platelets. Similarly, FXIII-A-deficient and TGI-treated normal platelets demonstrated reduced adherence to fibrinogen under flow (P<0.05). Retraction of FXIII-A-depleted plasma clots was significantly reduced when TGI-treated (P<0.05) or FXIII-A-deficient platelets were incorporated compared to normal platelets.

Inhibition of aIIbb3, caspase or SFK before CVX/thrombin stimulation significantly reduced procoagulant platelet formation (P<0.01) and FXIII-A exposure (P<0.01) on the platelet surface. Unlike normal platelets FXIII-A-deficient platelets were unable to stabilise FXIII-depleted thrombus lysis. This antifibrinolytic effect was abolished upon inhibition of aIIbb3 (P<0.01), caspases (P<0.05), and SFK (P<0.01). In line with this, FXIII-A activity was dramatically reduced upon inhibiting aIIbb3 (P<0.001), caspases (P<0.001), and SFK (P<0.001).

Conclusions:

Platelet activation and clot retraction are attenuated in TGI-treated and FXIII-A-deficient platelets, suggesting that platelet FXIII-A plays intracellular roles in facilitating these processes. Platelet-FXIII-A has extracellular roles in stabilising thrombi against fibrinolysis but this activity is attenuated upon inhibition of pathways that promote procoagulant platelet and microparticle formation. These data suggest platelets must be highly activated to deliver FXIII-A to the extracellular environment to stabilise thrombi.

OC - 13

BTK inhibitors, platelet inhibition and bleeding risk: How low can you go?

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Background

Bruton Tyrosine Kinase inhibitors (BTKi) changed the management landscape of a number of chronic B cell malignancies but are associated with development of resistance and a range of toxicities including bleeding (Any grade bleeding: 51% ibrutinib, 38% acalabrutinib). BTKi-associated bleeding is attributed to a mixture of on and off-target effects on platelet kinases that result in dysfunction. Successive generations of BTKi have improved kinase specificity, reduced antiplatelet effects and improved safety. The third generation BTKi Pirtobrutinib circumvents BTKi resistance caused by C481-mutation and is also associated with lower haemorrhagic adverse events (13% any grade).

Aims

To investigate the relative antiplatelet effects of three generations of BTKi to understand if differences in platelet inhibition might explain the observed frequency of bleeding events.

Methods

The effects of the drugs on Ca2+ release, PKC and Src Family Kinase (SFK) activity, aggregation and thrombus formation at arterial shear were studied in vitro. Patients receiving pirtobrutinib (n = 20), acalabrutinib (n = 10) or ibrutinib therapy (n = 10) were recruited to the study after providing informed consent. Platelet function was investigated using light transmission aggregometry, PFA-200 and thrombus formation under flow. Reversal of antiplatelet activity was analysed by flow cytometry.

Results

Pirtobrutinib and ibrutinib potently inhibit aggregation, thrombus formation under arterial shear, Ca2+ release and PKC activity, while acalabrutinib was less potent. However, Pirtobrutinib was highly specific with regard to off-target inhibition of SFKs. Platelet function was increased after wash-off of Pirtobrutinib but not acalabrutinib or ibrutinib.

Summary/conclusions

The superior safety profile of pirtobrutinib with regard to haemorrhagic adverse events is not reflected by the platelet function of patients receiving pirtobrutinib therapy compared to ibrutinib or acalabrutinib. This raises questions about the ability of standard platelet function tests, particularly aggregometry, to characterise and understand BTKi-induced platelet dysfunction and bleeding risk. If new BTKi can achieve high levels of platelet inhibition without significant bleeding AEs, their therapeutic uses might extend to cardiovascular disease.

OC - 14

The efficacy of fibrinogen concentrates in restoring clot integrity and stability against lysis

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Background
Trauma induced coagulopathy (TIC) is characterised by various haemostatic abnormalities and loss of coagulation factors, particularly fibrinogen, leading to major haemorrhage. Supplementation with fibrinogen has gained interest as treatment option.

Aim
To compare the efficacy of two commercially available purified fibrinogen sources, RiaSTAP® (CSL Behring) or FibCLOT® (LFB Biotechnologies) to standard cryoprecipitate therapy in restoring clot integrity and stability in TIC models.

Methods
Fibrinogen-deficient plasma (FDP) or 40% haemodilution were used to mimic fibrinogen loss in TIC. Reconstitution with the fibrinogen sources (0.5 – 3 mg/ml) was examined and compared to pooled normal plasma (PNP). Maximal clot absorbance and subsequent lysis, on inclusion of tissue plasminogen activator was monitored by turbidity. ROTEM analysis was performed using both models. Clot structure was visualised by confocal microscopy with fluorescently labelled-fibrinogen. Factor XIII (FXIII) was measured by ELISA.

Results
Reconstitution of FibCLOT or cryoprecipitate into FDP resulted in a fibrin network structure similar to PNP. In contrast, RiaSTAP produced shorter more branched fibres. Turbidity assays revealed higher maximum absorbance with FibCLOT compared to RiaSTAP, however, both demonstrated similar lysis profiles. ROTEM analysis revealed a strong concentration dependent increase in maximum clot firmness (MCF) with all fibrinogen preparations in the FDP model. Interestingly, fibrinogen concentrates enhanced MCF more than cryoprecipitate. Haemodiluted blood demonstrated reduced MCF which was normalised upon fibrinogen supplementation. Haemodiluted clot firmness was normalised with 1 mg/ml of fibrinogen concentrate, whereas 2 mg/ml cryoprecipitate was required. Haemodiluted clots lysed more readily than whole blood clots,but were stabilised against fibrinolytic degradation upon addition of fibrinogen concentrate, with FibCLOT superior at increasing resistance. FibCLOT contained approximately 3-fold more FXIII/mg of fibrinogen than RiaSTAP.

Summary
Addition of 1 mg/ml fibrinogen, a clinically achievable concentration, restored adequate clot formation. FibCLOT, which contained more FXIII, was superior to RiaSTAP in normalising clot structure and stabilising haemodiluted clots.

OC - 15

A pilot, open-label, phase II clinical trial of nebulised recombinant tissue-plasminogen activator in patients with COVID-19 acute respiratory distress syndrome: the PACA trial

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Background: Alveolar fibrin deposition and pulmonary microthrombi are pathophysiological features of COVID-19-induced respiratory failure. Nebulised thrombolysis offers locally targeted therapy with potentially lower bleed risk than systemic administration.

Aims: To test the safety and investigate the potential for clinical efficacy of nebulised recombinant tissue plasminogen activator (rt-PA) for improving oxygenation in patients hospitalised with COVID-19 complicated by mild to severe acute respiratory distress syndrome (ARDS).

Methods: Patients hospitalised with severe COVID-19 and a PaO_2/FiO_2 (P/F) ratio of <300 (units), requiring invasive mechanical ventilation (IMV) or non-invasive respiratory support (NIRS) received 40–60mg per day of rt-PA, dosed for ≤14 days in a phase II, open-label, single-centre pilot interventional study. Efficacy was assessed via improved oxygenation. Safety was assessed by treatment-related serious adverse event bleeding and reduction of fibrinogen to <1.0–1.5 g/L.

Results: Nine (Cohort 1 [C1]; 6/9 IMV, 3/9 NIRS) and 26 adults (Cohort 2 [C2]; 12/26 IMV, 14/26 NIRS) received nebulised rt-PA alongside standard of care. Matched historical controls (HC) (n=18) were used for comparison in C1. Mean P/F ratio increased in both C1 groups from baseline (BL) to end of study (EOS) (rt-PA; 154.44 to 298.78 vs. HC; 154.11 to 211.56); a linear mixed effect model confirmed higher P/F ratios in the rt-PA group. Among C2 groups, greater improvements in mean P/F ratio from BL to EOS were seen in the NIRS group (NIRS; 125.53 to 239.39 vs. IMV; 120.30 to 188.22). No treatment-related bleeds or clinically significant fibrinogen reductions were reported. Lower mortality was observed in the C1 rt-PA group (11.1%) vs. the HC group (55.6%) and in the C2 NIRS group (21.4%) vs. the IMV group (41.7%).

Summary/Conclusions: Nebulised rt-PA is well-tolerated, improves oxygenation in patients with COVID-19-related ARDS, and merits a randomised controlled trial to confirm efficacy and potentially identify a subgroup of interest.

OC - 16

7 month follow up of cerebral venous sinus thrombosis following ChAdOx1 nCov-19 Vaccination: a single centre experience

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Background

In April 2021, the MHRA and the EMA reported an association between ChAdOx1 nCoV-19 (AZV) and a rare syndrome of unusual site thrombosis combined with thrombocytopenia, termed vaccine-induced immune thrombotic thrombocytopenia (VITT).

Aims/Methods

We report 7-month outcomes of four female patients, aged 41-46 years who developed cerebral venous sinus thrombosis (CVST) with or without thrombosis at other sites as a result of VITT. Each presented with headache and neurological deficit. Uniquely, all four patients received a uniform aggressive management approach with immediate plasma exchange, IV immunoglobulins, high dose steroids and argatroban with two patients also receiving Rituximab.

Results

All four patients survived with complete resolution of symptoms and laboratory markers and were discharged on apixaban 5mg bd. At 7-months follow-up, platelet count, D-dimer and fibrinogen levels remain normal in all patients with no signs of relapse. Median time to anti-PF4 antibody normalisation was 5 months. Patient 4 still has detectable a strongly antibody level at 1.66 optical density (Figure 1). At 7-months follow-up, factor VIII (FVIII), von Willebrand factor (VWF) antigen and VWF: RCo which were elevated at acute presentation had normalised except in Patient 4 who has persistent elevation of FVIII, VWF:Ag and VWF:RCo.

Patient 4 has no other clinical or laboratory features of relapse disease and patient serum failed to activate donor platelets in the presence or absence of heparin (low or high dose). Notably, there was no demonstrable anti-spike antibody response following the ChAdOx1 nCoV-19 vaccine in any of the patients, but all showed a good response following Pfizer vaccination 3 months after the diagnosis of VITT.

Conclusions

The four cases presented represent an aggressive approach to VITT that we believe played a central role in their favourable 7 month clinical and laboratory outcomes. The significance of ongoing detectable anti-PF4 antibody remains unknown.

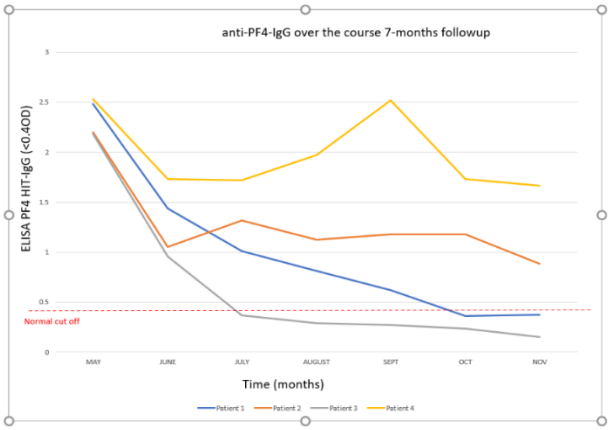

Figure 1

OC - 17

Metabolic reprogramming is essential for myeloid cell-dependent haemostatic activity

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Background:
Myeloid cell activation leading to enhanced tissue factor (TF) expression, decryption and procoagulant activity represents a key event in immunothrombotic disease development. Macrophage polarisation causes rewiring of cellular metabolism, causing M1-skewed macrophages and activated peripheral monocytes to favour aerobic glycolysis in order to generate the energy required for phagocytosis and cytokine production.

Aim:
In this study, we assessed whether metabolic reprogramming in response to inflammatory stimuli may impact myeloid cell procoagulant and antifibrinolytic activity to promote thrombosis.

Methods & Results:
To achieve this, we developed a new myeloid cell-based plasma thrombin generation assay to enable assessment of isolated human or murine innate immune cell procoagulant activity. We demonstrate that inhibition of metabolic reprogramming ablates activated myeloid cell TF-dependent thrombin generation. Specifically, treatment of murine macrophages or activated human monocytes with 2-Deoxy-D-glucose (2-DG; hexokinase inhibitor) or TEPP-46 (a pyruvate kinase M2 activator) reduced TF gene expression >15-fold and significantly extended myeloid cell TF-dependent thrombin generation lag-time to mimic lag-times observed in the presence of naive macrophages. To evaluate how glycolysis inhibition prevents TF-dependent procoagulant activity, we assessed the impact of 2-DG on cellular determinants of TF decryption. Notably, flow cytometric assessment of lactadherin binding to M1-skewed macrophages revealed significantly reduced phosphatidylserine externalisation upon 2-DG treatment. In addition, membrane localisation of acid sphingomyelinase, which degrades membrane sphingomyelin to promote TF procoagulant decryption, was significantly impaired by 2-DG, suggesting a new role for activated macrophage metabolic reprogramming in facilitating TF decryption. Finally, we examined the role of glycolysis in controlling macrophage-dependent plasmin generation. M1-skewed macrophage plasminogen activator inhibitor-1 (PAI-1) gene expression was significantly reduced by both 2-DG and TEPP-46, leading to near-total restoration of plasmin generation in M1-skewed macrophages.

Conclusion:
Collectively, the discovery of immunometabolic regulation of myeloid cell pro-thrombotic activity opens new therapeutic possibilities for the mitigation of thrombo-inflammatory disease.

OC - 18

Anti-PF4 IgG levels in patients with VITT remain high at 6 months following vaccination

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Background:

Vaccine-induced immune thrombocytopenia and thrombosis (VITT) is a new syndrome that occurs 4-30 days following vaccination predominantly with Oxford-AstraZeneca ChAdOx1 nCoV-19 (AZD1222) VITT is characterised by thrombocytopenia, high D-dimer levels and aggressive thrombosis. Anti-platelet factor 4 (PF4) antibodies from patients with VITT activate platelets via FcγRIIA. However, the evolution of these antibodies and their ability to activate platelets after initial treatment remains unknown.

Aims:

To determine how anti-PF4 antibody levels in VITT patients change following recovery and the ability of patient serum to activate platelets.

Methods:

We followed-up seven discharged VITT patients from diagnosis up to 221 days (range: 108-221) post vaccination and measured anti-PF4 antibodies and PF4 levels in patient serum. We tested the ability of patient serum to activate healthy and patient platelets over time using light transmission aggregometry. We also assessed platelet function of patients' platelets at the latest follow-up timepoint.

Results:

Anti-PF4 IgG antibody levels remained high in 6 out of 7 patients up to 6 months post vaccination. The other patient received rituximab. Diagnostic patient serum strongly activated control (n=3) and patient platelets, either alone or with PF4. However, most follow-up serum was weaker at stimulating platelets, despite similar PF4 antibody levels, although this was not the case in sera from two patients. Patients' PF4 serum levels were reduced at diagnosis compared to follow-up levels ($p<0.0001$, n=7) but returned to the same level in healthy controls during follow-up. Patients' platelet responses and FcγRIIA levels at follow-up were similar to healthy controls.

Conclusions:

The reduction in serum-mediated platelet activation during follow-up, despite similar PF4 antibody levels remains unexplained. Further assessment is required to determine if levels of high affinity anti-PF4 antibodies are reduced during follow-up. Additional understanding is also required to assess duration of anticoagulation for VITT patients and reduce the risk of relapse.