Maastricht University and University of Reading

Novel mechanisms of platelet activation and sustained signalling through GPVI and PAR1

A thesis submitted for the joint degree of Doctor of Philosophy

By

Ilaria De Simone

Born on June 5th 1994, in Lier, Belgium

Cardiovascular Research Institute Maastricht, Maastricht University

and

Institute for Cardiovascular and Metabolic Research, University of Reading

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Declaration

I can confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

Signed: Ilaria De Simone

Date: 21 September 2022

Abstract

Background. The Glycoprotein VI (GPVI) receptor and the protease-activated receptor 1 (PAR1) are key platelet receptors and are activated by collagen and fibrin, or via thrombin, respectively. GPVI and PAR1 play a role in platelet activation and thrombus formation, two crucial processes for thrombosis and haemostasis. Furthermore, they are currently interesting targets for novel antiplatelet therapy. There is, therefore, a need to increase the understanding of the working mechanisms and the potential ligands for GPVI and PAR1.

Aims. The goal of this thesis was to increase the understanding of acute and persistent effects of platelet activation mediated through GPVI and PAR1.

Results. The role of GPVI and Syk in thrombus formation on collagens and collagen-like peptides was studied. Using the Syk inhibitor PRT060318, we showed that Syk supports platelet activation induced by collagens and collagen-like peptides, regardless of the presence of the GPVI binding motif GPO. More pronounced platelet activating effects of collagens were observed after immobilization, compared to when collagens were soluble. Since GPVI is also known to be the receptor for the coagulation-generated product fibrin, the role of GPVI in other coagulation proteins was studied. Through the use of GPVI and Syk inhibitors, it was evident that GPVI and Syk were involved in platelet activation induced by FXIIIa. Moreover, we found that APC induced platelet spreading through PAR1, which had previously only been shown for endothelial cells. In line with platelet activation induced by collagens, effects induced by FXIIIa and APC were more pronounced when the proteins were immobilized on a surface compared to in solution. Short- and long-term effects through the GPVI and the GPCR receptors PAR1 and $P2Y_{1/12}$ were compared. Platelet activation through GPVI was more persistent, while responses evoked by PAR1 or $P2Y_{1/12}$ stimulation were rather transient. Interestingly, pre-activated platelets, which started to return to a resting state (integrin inactivation,

morphological change), but still expressed P-selectin on their surface, could be activated again upon restimulation.

Conclusions. Taken together, the data presented in this thesis increase our understanding of how platelet functions are regulated through GPVI and PAR1. FXIIIa and APC were identified as novel ligands for GPVI and PAR1, respectively. Further, stimulation through GPVI was persistent, while stimulation through GPCRs (PAR1 or $P2Y_{1/12}$) was transient. In addition, when platelets reverse, they can be reactivated. Since platelet function is highly regulated through GPVI and PAR1, the increased knowledge of the working mechanisms of these receptors, as well as on short- and long-term effects after activation, will contribute to an enhanced understanding of pre-activated circulating platelets in vivo in patients who are continuously exposed to activating components and will contribute to the improvement and development of more effective antiplatelet therapy.

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If I have seen further, it is by standing on the shoulders of giants. (Isaac Newton)

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Publications

Full papers

1. Jooss NJ*, **De Simone I***, Provenzale I*, Fernández DI*, Brouns SLN, Farndale RW, Henskens YMC, Kuijpers MJE, ten Cate H, van der Meijden PEJ, Cavill R, Heemskerk JWM. Role of Platelet Glycoprotein VI and Tyrosine Kinase Syk in Thrombus Formation on Collagen-Like Surfaces. **Int J Mol Sci.** 2019;20:2788. *Equal contribution

2. Veninga A*, **De Simone I***, Heemskerk JWM, ten Cate H, van der Meijden PEJ. Clonal haematopoietic mutations linked to platelet traits and the risk of thrombosis or bleeding. **Haematologica.** 2020;105:2020-2031. *Equal contribution

3. Huang J, Swieringa F, Solari FA, Provenzale I, Grassi L, **De Simone I**, Baaten CCFMJ, Cavill R, Sickmann A, Frontini M, Heemskerk JWM. Assessment of a complete and classified platelet proteome from genome-wide transcripts of human platelets and megakaryocytes covering platelet functions. **Sci Rep.** 2021;11:12358.

4. **De Simone I**, Jones CI, ten Cate H, van der Meijden PEJ. Glycoprotein VI as target for novel antiplatelet therapy. **Ned Tijdschr Hematol.** 2021;18:266-71

5. Veninga A, Baaten CCFMJ, **De Simone I**, Tullemans BME, Kuijpers MJE, Heemskerk JWM, van der Meijden PEJ. Effects of platelet agonists and priming on the formation of platelet populations. **Thromb Haemost.** 2022;122:726-738.

6. **De Simone I**, Baaten CCFMJ, Jandrot-Perrus M, Gibbins JM, ten Cate H, Heemskerk JWM, Jones CI*, van der Meijden PEJ*. Coagulation factor XIIIa and activated protein C activate platelets via GPVI and PAR1. **Int J Mol Sci.** 2022;23:10203. *Equal contribution

7. **De Simone I** Baaten CCFMJ, Gibbins JM, ten Cate H, Heemskerk JWM, Jones CI*, van der Meijden PEJ*. The potential of previously activated platelet to contribute to thrombus formation – Submitted, 2022. *Equal contribution

8. Mohammed Y, Dunster JL, **De Simone I**, Kriek N, Sage T, Brunton M, Mark S, Whyte A, Ruparelia N, Mckenna C, Jones CI, Gibbins JM. Inter-individual variation in platelet function and its impact on the effectiveness of antiplatelet medications in health and disease - in preparation

Abstracts

1. Jooss NJ, **De Simone I**, Provenzale I, Fernández DI, Brouns SLN, Farndale RW, Henskens YMC, Kuijpers MJE, ten Cate H, van der Meijden PEJ, Cavill R, Heemskerk JWM. Role of Platelet Glycoprotein VI and Tyrosine Kinase Syk in Thrombus Formation on Collagen-Like Surfaces. UK Platelet Society Meeting, Cambridge, UK, 2019

2. **De Simone I**, Kiss B, Csanyi MC, Kellermayer M, Heemskerk JWM, ten Cate H, van der Meijden PEJ, Harsfalvi J. Topology of platelets on fibrinogen, thrombin and fibrin surfaces. 250 Semmelweis Symposium, Budapest, Hungary, 2019

3. **De Simone I**, Jones CI, Gibbins JM, ten Cate H, Heemskerk JWM, van der Meijden PEJ. The role of coagulation-generated components on GPVI-mediated platelet activation. Platelet Society ECR Meeting, Queen Mary University, London, UK, 2020 (Accepted but event cancelled due to Covid)

4. **De Simone I**, Jones CI, Gibbins JM, ten Cate H, Heemskerk JWM, van der Meijden PEJ. The role of coagulation-generated components on GPVI-mediated platelet activation. ISTH 2020 Virtual Congress, Milan, Italy, 2020 (Virtual)

5. **De Simone I**, Gibbins JM, ten Cate H , Heemskerk JWM, Jones CI, van der Meijden PEJ. Coagulation and anticoagulation factors affect agonist-induced platelet activation independently of thrombin and fibrin. UK Platelet Society Meeting, Keele, UK, 2021 (Virtual)

6. **De Simone I**, Gibbins JM, ten Cate H , Heemskerk JWM , Jones CI, van der Meijden PEJ. Coagulation and anticoagulation factors affecting platelet responses independently of thrombin and fibrin. ISTH 2021 Virtual Congress, Philadelphia, USA, 2021 (Virtual)

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8. Mohammed Y, Dunster JL, **De Simone I**, Kriek N, Sage T, Brunton M, Mark S, Whyte A, Ruparelia N, Mckenna C, Jones C, Gibbins JM. Inter-individual variation in platelet function and its impact on the effectiveness of antiplatelet medications. UK Platelet Society Meeting, Hull, 2022

9. **De Simone I**, Baaten CCFMJ, Gibbins JM, ten Cate H, Heemskerk JWM, Jones CI and van der Meijden PEJ. The potential of pre-activated platelets to contribute to thrombus formation. ISTH 2022, London, UK, 2022

Presentations

1. **De Simone I**, Kiss B, Csanyi MC, Kellermayer M, Heemskerk JWM, ten Cate H, van der Meijden PEJ, Harsfalvi J. Topology of platelets on fibrinogen, thrombin and fibrin surfaces. 250 Semmelweis Symposium, Budapest, Hungary, 2019 (Poster)

2. **De Simone I**, Jones CI, Gibbins JM, ten Cate H, Heemskerk JWM, van der Meijden PEJ. The role of coagulation-generated components on GPVI-mediated platelet activation. Platelet Society ECR Meeting, Queen Mary University, London, UK, 2020 (Accepted for poster but event cancelled due to Covid)

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List of abbreviations

ABCB6	ATP binding cassette subfamily B member 6
ABL1	Abelson murine leukaemia viral oncogene homolog 1
ACD	Acid-citrate glucose
ACS	Acute coronary syndrome
ADP	Adenosine diphosphate
AF	Alexa Fluor
ALL	Acute lymphocytic leukaemia
AMKL	Acute megakaryoblastic leukaemia
AML	Acute myeloid leukaemia
APC	Activated protein C
ApoER2	Apolipoprotein E receptor 2
APT	antiplatelet therapy
ASXL1	Additional sex combs like 1
ATP	Adenosine triphosphate
BCR	Breakpoint cluster region
BSA	Bovine serum albumin
Btk	Bruton's kinase
CAD	Coronary artery disease
cAMP	Cyclic adenosine monophosphate
CHIP	Clonal haematopoiesis of indeterminate potential
CML	Chronic myeloid leukaemia
CMML	Chronic myelomonocytic leukaemia
COMPASS	Cardiovascular Outcomes for People Using Anticoagulation Strategies
CRP-XL	Collagen-related peptide – cross-linked
CYP450	Cytochrome p450
DAG	Diacylglycerol
DAPT	Dual antiplatelet therapy
DiOC6	3,3'Dihexyloxacarbocyanine iodide
DNA	Deoxyribonucleic acid
DNMT3A	DNA methyltransferase 3α
ET	Essential thrombocythemia

ETV6	E26 transformation-specific variant 6
F(X)	Factor (X)
FANC(A)	Fanconi anaemia complementation (group A)
FITC	Fluorescein isothiocyanate
FLI1	Friend leukaemia virus integration 1 (FLI1)
GATA(1)	GATA-binding protein (1)
GFI1B	Growth factor independent 1B transcription repressor
GP(VI)	Glycoprotein (VI)
GPCR	G protein-coupled receptor
HCL	Hairy cell leukaemia
HSC	Hematopoietic stem cell
ICAM-1	Intercellular adhesion molecule 1
IDH2	isocitrate dehydrogenase NADP+ 2
IL-(6)	Interleukin-(6)
IP3	Inositol 1,4,5-trisphosphate
ITAM	Immunoreceptor tyrosine-based activation motif
ITP	Immune thrombocytopenia
JAK2	Janus kinase 2
LAT	Linker for activation of T cells
LTA	Light transmission aggregometry
MDS	Myelodysplastic syndrome
MPN	Myeloproliferative neoplasm
PAR	Protease activated receptor
PBA	Plate-based aggregation
PCA	Principal component analysis
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol-(4,5)-bisphosphate
PK(C)	Protein kinase (C)
PLC(γ)	Phospholipase
PLS	Partial least square
PPACK	D-Phenylalanyl-L-prolyl-L-arginine chloromethyl ketone
PPP	Platelet-poor plasma

PRP	Platelet-rich plasma
PS	Phosphatidylserine
PV	Polycythaemia vera
RhoGEF	Rho guanine nucleotide exchange factor
RNA	Ribonucleic acid
SAC	Surface area coverage
SCP	Supernatant of (hirudin-treated) coagulated plasma
SEM	Scanning electron microscopy
SF3B1	Splicing factor 3b subunit 1
SFK	Src family kinases
SH2B3	Src homology 2 B3
SMAD4	SMAD family member 4
Syk	Spleen tyrosine kinase
T2DM	Type 2 diabetes mellitus
TEM	Transmission electron microscopy
TET2	Tet oncogene family member 2
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TMEM16F	Transmembrane protein 16F
TP53	Tumour protein p53
TPO	Thrombopoietin
TRAP(6)	Thrombin receptor activating peptide
VWF	Von Willebrand factor
VWF-BP	Von Willebrand factor binding peptide
WAS	Wiskott-Aldrich syndrome

Chapter 1

General introduction

Platelets in haemostasis and thrombosis

Platelets, also called thrombocytes, are anucleated blood cells (2-4 µm in diameter) and are key elements in haemostasis and thrombosis.[1] They are formed from megakaryocytes in the bone marrow. The development of megakaryocytes from hematopoietic stem cells is regulated by the growth factor thrombopoietin (TPO), and its binding to the thrombopoietin receptor.[2] The formation of platelets from megakaryocytes occurs in two phases. During the first phase, megakaryocytes become polyploid and the cytoplasm is enlarged, as well as cytoskeletal proteins and granules, so that sufficient components are present to assemble platelets. In the next phase, the megakaryocytes start to extend proplatelets, which are thin protrusions, from which platelets eventually form.[3] The lifespan of platelets, once released in the blood stream, is 7-10 days. Upon vascular injury, platelets adhere to the damaged site and become activated by interactions with the subendothelial matrix (adhesive ligands) and soluble ligands.[4] Eventually they form a haemostatic plug, to minimize blood loss, a process which is referred to as haemostasis (Figure 1).

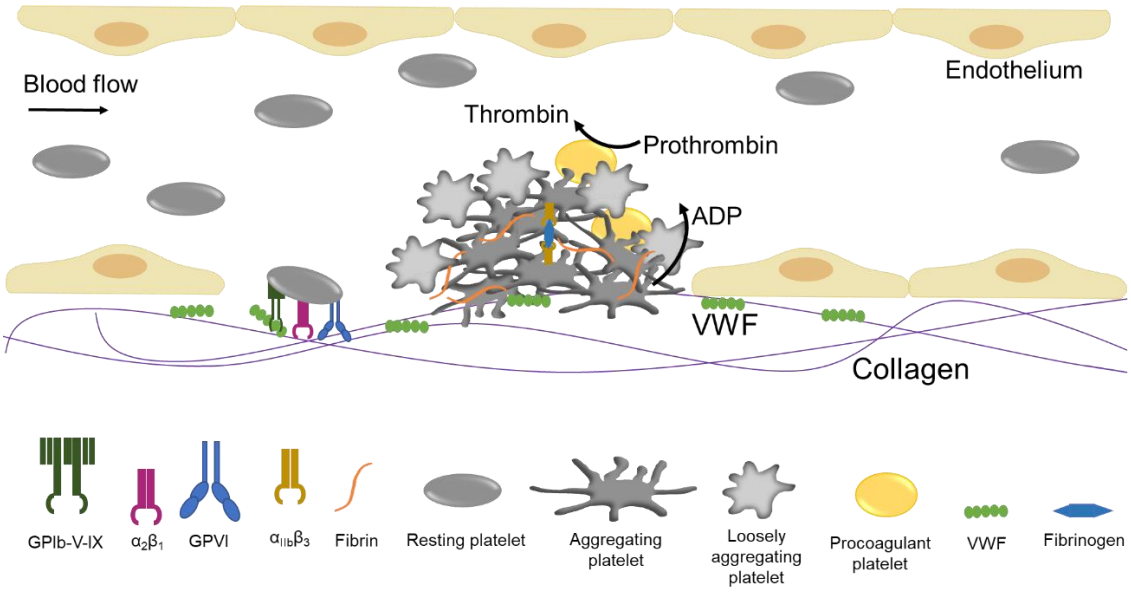

Figure 1. Schematic overview of platelet adhesion, activation and thrombus formation upon vascular injury. Upon vascular injury, extracellular matrix proteins become exposed to platelets in the circulation. Resting platelets flowing over an injured site of the endothelium are captured by von Willebrand factor (VWF), through the GPIb-V-IX receptor complex. Next, platelets interact with collagen through the platelet receptors GPVI and integrin $\alpha_2\beta_1$, which induces platelet activation, leading to platelet aggregation, secretion (ADP) and procoagulant states. More platelets are recruited to form the platelet plug, which is eventually stabilized by fibrin. The thrombus contains a core of aggregating platelets, sustained by integrin $\alpha_{IIb}\beta_3$-fibrinogen bonds, and a shell of loosely aggregating platelets. Procoagulant platelets are formed upon potent stimulation and support thrombin generation.

VWF is a large multimeric adhesive glycoprotein, released from endothelial cells and platelets. It binds to exposed collagen via its A3 domain, and is able to capture platelets from the circulation.[5] When released from endothelial cells in soluble form, the A1 domain of VWF is shielded by its globular conformation.[6] After immobilization, the VWF A1 domain becomes exposed for interaction with the GPIbα receptor (25000 copies/platelet; part of the GPIb/IX/V complex) and allows the initial tethering and rolling of the circulating platelet.[7] Subsequently, platelet receptors glycoprotein VI (GPVI) (3000-4000 copies/platelet) and integrin $\alpha_2\beta_1$ (2000-4000 copies/platelet) interact with collagen, leading to platelet activation.[8, 9]

Platelet activation can result in granule secretion, an activating conformational change of integrin $\alpha_{IIb}\beta_3$, and phosphatidylserine (PS) exposure on the platelet surface. During secretion, cargo is released from α (alpha) or δ (dense) granules.[10] Alpha granules store diverse proteins, such as VWF, fibrinogen and cytokines, while dense granules contain rather small molecules like ADP, ATP, polyphosphate and calcium.[11] Overall, the secreted content has a potent role in amplifying haemostasis, thus enforcing the initial triggering.

Another consequence of platelet activation is integrin $\alpha_{IIb}\beta_3$ activation. Integrin $\alpha_{IIb}\beta_3$ is a key receptor for the adhesive functions of platelets and is expressed on the platelet surface with a high abundance (60000-80000 copies/platelet).[12, 13] Despite its high abundance on resting platelets, integrin $\alpha_{IIb}\beta_3$ has a low affinity for fibrinogen. Upon

platelet activation follows an activating conformational change of integrin $\alpha_{IIb}\beta_3$ by inside-out signalling, whereafter affinity of this receptor for fibrinogen is increased.[13-15] During inside-out signalling, intracellular activators, such as kindlin and talin bind to the integrin $\alpha_{IIb}\beta_3$ tails. The binding of talin to the β_3 tail allows integrin $\alpha_{IIb}\beta_3$ activation by disrupting the salt bridge between α_{IIb} and β_3.[14, 16] Following inside-out signalling, fibrinogen bridges between platelets are formed, which lead to platelet aggregates. The fibrinogen binding also triggers outside-in signalling of the integrin $\alpha_{IIb}\beta_3$ on platelets, leading to activation of a cascade of intracellular signalling events, which support thrombus formation by mediating adhesion, spreading and cytoskeletal organisation.[12, 14] Calpain plays a major role during outside-in signalling by cleaving the integrin β_3 cytoplasmatic tail, which leads to dissociation of Src from this tail. Src then phosphorylates and activates amongst others spleen tyrosine kinase (Syk), talin and kindlin. Kindlin eventually links integrin β_3 to the actin cytoskeleton.[14] The conformational change of $\alpha_{IIb}\beta_3$, is not a unidirectional process, but can be reversible.[17] Within a thrombus, aggregating and secreting platelets, which are tightly packed with high levels of activated integrins and P-selectin exposure, form the thrombus core. The core is surrounded by loosely aggregating platelets, which have a lower activation state.[18] Also balloon-shaped, PS exposing platelets are present in the thrombus, but their shape suggests a controlled mechanism of platelet detachment from the thrombus core. In resting platelets, PS is located in the inner leaflet, but becomes exposed at the platelet surface in response to potent Ca^{2+}-mobilising ligands, such as (combined) collagen and thrombin.[19, 20] Scott syndrome patients, in which PS exposure is reduced or abolished, carry mutations causing defective TMEM16F, illustrating a critical role for the TMEM16F in PS exposure.[21] PS leads to a procoagulant phenotype of the platelet, since it provides a docking site for assembly of the tenase and prothrombinase complexes, which support the activation of factor (F)X (noted FXa) and of thrombin, respectively.[22]

The Glycoprotein VI (GPVI) receptor

Ligands and signalling pathway

GPVI is a type I transmembrane glycoprotein of ~60kDa and is only expressed on platelets (3000-4000 copies/platelet) and megakaryocytes.[23] Physiological ligands for GPVI are amongst others collagen and fibrin(ogen).[24-26] Over the years, fibrillar and synthetic peptides have been generated, mimicking collagens. In clinical laboratories, Horm Collagen type I is commonly used for diagnostics. Since collagens bind to both GPVI and integrin $\alpha_2\beta_1$, peptides have been designed, with different binding affinities, so that platelet collagen receptors can be triggered separately. For example, the $(GPO)_n$ binding motif, is a motif with a strong affinity for GPVI, while $(GPP)_n$, in which the hydroxyproline (O) is substituted by a proline (P), is an inactive motive. GFOGER selectively binds $\alpha_2\beta_1$, but the substitution of phenylalanine in GFOGER by alanine in GAOGER, decreases the affinity for integrin $\alpha_2\beta_1$. The combined peptide, GFOGER-GPO, thus binds with a high affinity to GPVI and integrin $\alpha_2\beta_1$,[27] while the cross-linked collagen-related peptide (CRP-XL) existing of a $(GPO)_{10}$ sequence, acts as potent and selective GPVI agonist.[28] Ligands interact with GPVI dimers, which are present on resting cells (29% of GPVI on platelets are dimeric, others are monomeric, as shown by using dimer-specific Fabs).[29] The ligand-receptor interaction results in clustering of GPVI, after which the intracellular signalling is initiated. GPVI clustering is thus necessary for efficient platelet activation and thrombus formation.[30]

Upon GPVI triggering, intracellular signalling starts with Src family kinases (SFK) phosphorylating the immunoreceptor tyrosine-based activation motif (ITAM) on the FcRγ chain.[23, 24, 31, 32] Syk then binds the phosphorylated sites via its SH2 domains, and is phosphorylated by SFK, whereafter phosphorylation of LAT (Linker for activation of T cells) follows. Several signalling proteins are then recruited, among which Btk, which undergoes autophosphorylation and phosphorylates phospholipase Cγ2 (PLCγ2).[23, 33, 34] PLCγ2 then induces an increase of the intracellular Ca^{2+} concentration and through

the generation of diacylglycerol (DAG), results in the activation of protein kinase C, leading to secretion and integrin activation, whereafter aggregation follows (Figure 2).

GPVI as potential new target for antithrombotics

In patients at high risk of thrombosis, such as patients with coronary artery disease (CAD) undergoing coronary interventions, or subjects with acute ischemic stroke, dual antiplatelet therapy (DAPT) is the standard of care.[35] However, the use of DAPT, particularly when aspirin is combined with potent $P2Y_{12}$ inhibitors in patients with ACS, is limited in duration because of the fear of major bleeding complications. To reduce the bleeding risk, either the duration of DAPT, or the $P2Y_{12}$ inhibitor (clopidogrel instead of ticagrelor or prasugrel), or both, must be tailored in the individual patient. Either way, for many patients with high thrombosis burden or high risk of (recurrent) thrombosis, effective antithrombotic management is limited due to risk for bleeding.[36] This clinical dilemma illustrates the need for effective but safer antiplatelet agents. Currently, GPVI is a promising target for novel antiplatelet therapy, since this receptor has minimal roles in haemostasis, but is involved in the collagen-induced pathogenesis of thrombosis.[37] Next to collagen, other ligands for GPVI are laminin, vitronectin, globular adiponectin, fibronectin, EMMPRIN (CD147), β-amyloid and fibrin(ogen).[25, 38-43] Human, but not murine platelets, have been shown to spread on fibrinogen through GPVI and Syk. The explanation for this is unclear. However, as human and murine GPVI share only 64% homology, possibly murine GPVI does not bind fibrinogen.[43, 44] The limited role of GPVI in haemostasis was revealed in very rare patients with GPVI deficiencies that only showed a limited bleeding risk.[45, 46] In vitro studies with platelets of heterozygous GPVI deficient patients (an adenine insertion in the *gp6* gene generates a truncated protein that is retained in the cytosol), showed normal thrombus formation, but deficient phosphatidyl serine exposure, while platelets of patients with homozygous GPVI deficiency showed defective thrombus formation and also lacked the procoagulant response.[46] GPVI deficiency can be inherited or can be acquired as a consequence of

auto-antibodies against GPVI.[47, 48] Based on these observations it was postulated that targeting GPVI would provide antithrombotic efficacy with less impact on haemostasis.

Currently, there are several strategies to inhibit GPVI activity (Figure 2), including antibodies, Fab-fragments, small molecules, GPVI mimetics and GPVI signalling inhibitors. The Fab fragment 9O12 (Glenzocimab, ACT017) binds GPVI with high affinity and in this way prevents the interaction with collagen and fibrin.[49] Further, the GPVI mimetic Revacept, is a dimeric fusion-protein, which locally inhibits platelet activation, by binding collagen with a high affinity.[50] There is, in this case no direct contact with the GPVI receptor or the platelets, hence the platelet function is unaffected. Then, the small molecules Losartan and Honokiol also suppress collagen-induced aggregation and thrombus formation and are therefore also considered as potential new GPVI inhibitors.[51]

Another way to block GPVI activity is by using a tyrosine kinase inhibitor. In platelets the tyrosine-kinase Syk is involved in the signalling by GPVI.[27, 52] The Syk inhibitor Fostamatinib is currently already used in refractory immune thrombocytopenia (ITP) patients.[53] However, other pathways than only GPVI (ITAM receptor) signalling are affected upon Syk inhibition, for instance Syk is also involved in hemi-immunoreceptor tyrosine-based activation motif (hemITAM) receptor signalling.[54] Furthermore, Syk is also involved in integrin $\alpha_{IIb}\beta_3$ (outside-in) signalling, after fibrinogen binding.[55] Other tyrosine kinases which are targeted already in clinical settings and are involved in GPVI signalling, are Src and Btk.[56] The Src inhibitor bosutinib and Btk inhibitor ibrutinib are used in the treatment of leukemia. Patients treated with these inhibitors experienced mild to severe bleeding phenotypes.[57, 58]

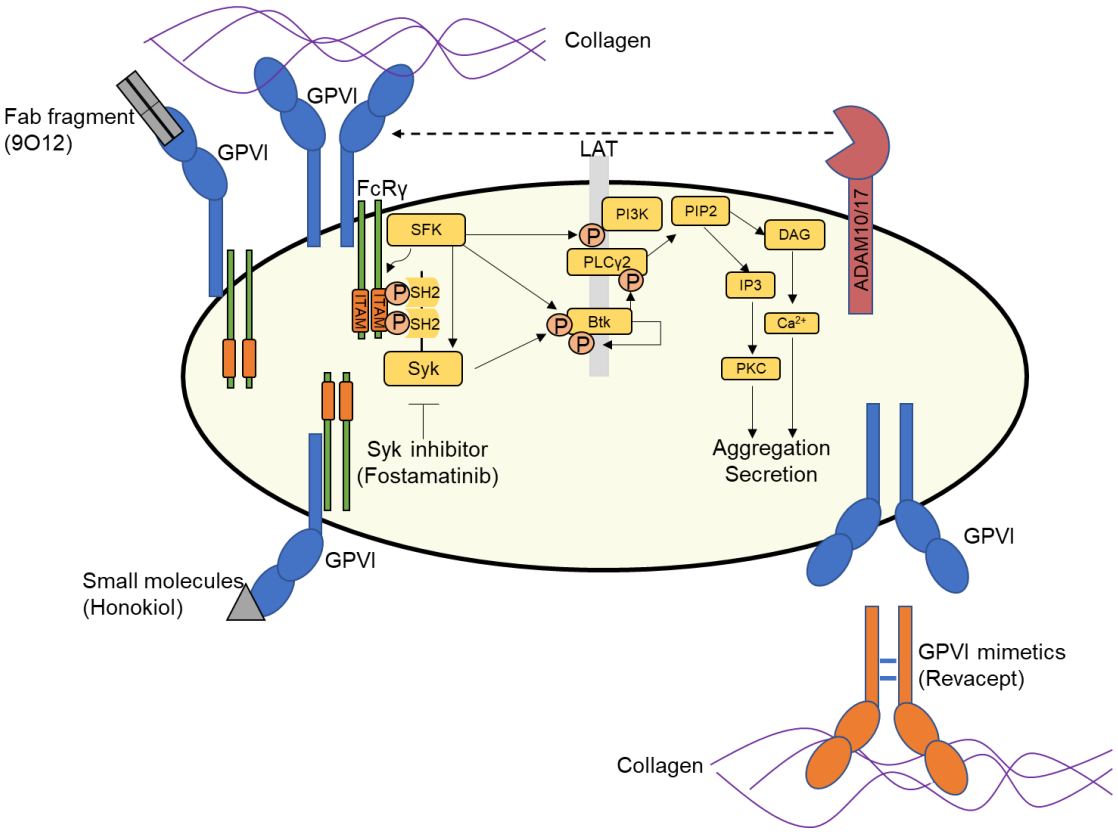

Figure 2. GPVI-dependent ssignalling and potential targets to inhibit GPVI-induced platelet activation. Collagen binds the extracellular domain of GPVI, whereafter the ITAM ('immunoreceptor tyrosine-based activation motif') domain is phosphorylated by SFK (Src family kinases). Syk then binds ITAM via its SH2 domains, is phosphorylated by SFK, and phosphorylates LAT (Linker for activation of T cells), leading to the recruitment of several signalling proteins. Btk then undergoes autophosphorylation and phosphorylates PLCγ2 (Phospholipase Cγ2), which induces the production of IP3 (inositol 1,4,5-trisphosphate) and DAG (1,2-diacylglycerol), whereafter activation of PKC (Protein kinase C) and mobilisation of intracellular Ca^{2+} follows. Subsequently, platelets aggregate and secrete. The activation process can be inhibited by inhibitors which target GPVI (pathways) in several ways. Schematically shown are targets for Fab fragments (9O12), small molecules (Honokiol) and GPVI mimetica (Revacept). Btk, Bruton's kinase; PI3K, Phosphatidylinositol 3-kinase; PIP2, Phosphatidylinositol 4,5-bisphosphate. (Figure was adapted from De Simone I et al., Ned tijdschr hematol 2021;18:266-71).

The G protein coupled receptors PAR1/4 and $P2Y_{1/12}$

G protein-coupled receptors (GPCR) on platelets mediate platelet responses induced by several soluble agonists, including ADP and thrombin (Figure 3).[59] Platelet activation by ADP is mediated by two G protein-coupled receptors, $P2Y_1$ and $P2Y_{12}$.[60] Upon stimulation of the $P2Y_1$ receptor, G13 is activated, resulting in the activation of the Rho guanine nucleotide exchange factor (RhoGEF), leading to the activation of RhoA and its

effector kinases, followed by reorganisation of the cytoskeleton and shape change of the platelet.[61, 62] $P2Y_1$ also couples to Gq which stimulates phospholipase C isoform β (PLCβ), inducing the formation of diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) from phosphatidylinositol-(4,5)-bisphosphate (PIP_2). Ca^{2+} is released from intracellular stores by the second messenger IP_3, while DAG activates protein kinase C (PKC) and the Rap1b-exchange factor, CalDAG-GEF. CalDAG-GEF activates the small GTPase Rap1, which is a molecular switch that induces platelet activation by regulating integrin-mediated aggregation and platelet secretion.[63] Instead, the $P2Y_{12}$ receptor upon stimulation couples to Gi which on the one hand inhibits adenylyl cyclase, which produces cAMP and activates protein kinase A (PKA) and on the other hand $P2Y_{12}$ receptor coupling to Gi results in the activation of phosphoinositide 3-kinase (PI3-K) whereafter downstream Akt is activated, resulting in platelet activation. [64-66]

In clinical settings, the $P2Y_{12}$ receptor is blocked for the prevention and treatment of cardiovascular diseases, by drugs such as clopidogrel, cangrelor, ticagrelor or prasugrel.

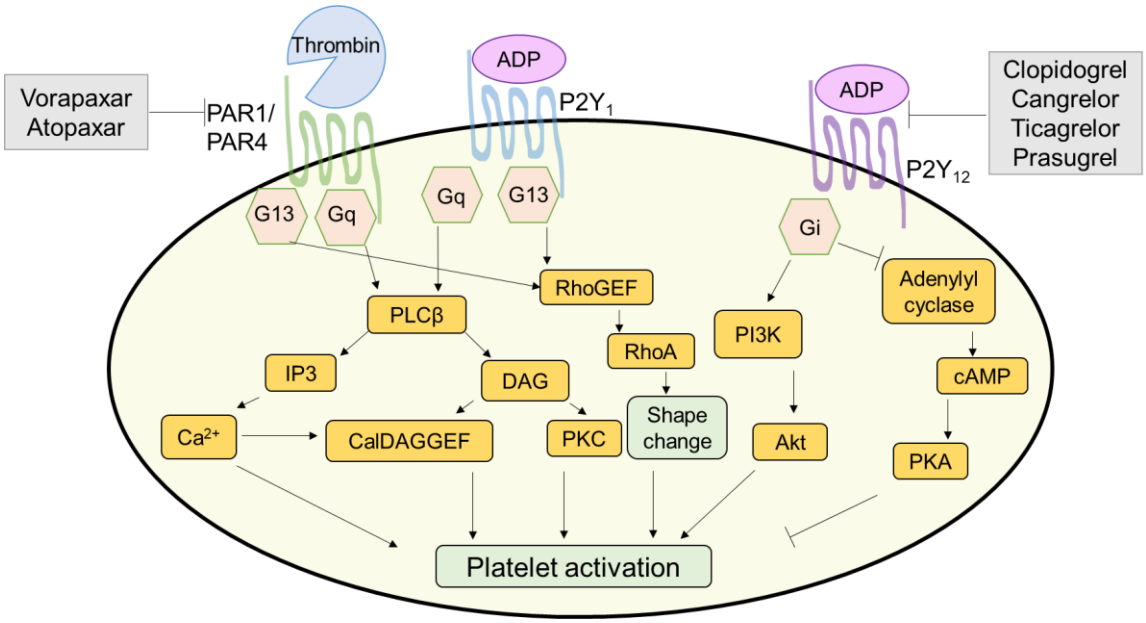

Figure 3. Platelet activation through PAR1/4 and $P2Y_{1/12}$. Thrombin mediates platelet activation through PAR1/4. When thrombin cleaves PAR1/4 or when ADP binds to $P2Y_1$, then heterotrimeric G proteins G13 and Gq are activated. G13 activation leads to reorganisation of the cytoskeleton and shape change, by activating Rho guanine nucleotide exchange factor (RhoGEF) and RhoA. Activation of Gq stimulates

phospholipase C isoform β (PLCβ), whereafter diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) are formed from phosphatidylinositol-(4,5)-bisphosphate (PIP_2). The second messenger IP3 releases Ca^{2+} from intracellular stores and DAG activates protein kinase C (PKC) and the Rap1b-exchange factor, CalDAG-GEF.[60] The other ADP receptor, $P2Y_{12}$ couples to Gi instead, which activates phosphoinositide 3-kinase (PI3-K) whereafter downstream Akt is activated. [64] On the other hand, Gi also inhibits adenylyl cyclase, thereby inhibiting the production of cAMP and PKA. [65, 66] Inhibitors for the receptors are shown in the grey boxes.

Thrombin activates platelets through PAR1 and PAR4 receptors, by cleaving the amino-terminal exodomain of PARs, generating a tethered ligand that binds to the body of the receptor to induce signalling.[67] The PAR1/4 receptors couple to G13 and Gq and signal in the same way as $P2Y_1$.[60] PAR1 (~2500 copies/platelet) has a high affinity for thrombin, while PAR4 is a low-affinity thrombin protease-activated receptor.[68] (For further details, see figure 3.) In the laboratory, the synthetic peptide TRAP6 (Thrombin receptor activating peptide 6, derived from the PAR1 tethered ligand) is used to specifically trigger PAR1. The TRA2P-TIMI 50 study, provided evidence for benefit of using the PAR-1 inhibitor vorapaxar in addition to aspirin, compared to aspirin alone, in the secondary prevention of patients with acute coronary syndromes, but at the expense of increased bleeding.[69, 70] Atopaxar is also used *in vitro* to inhibit PAR1, yet, this component never made it to phase III in clinical trials.[71, 72]

The platelet-coagulation interplay

The activation of coagulation occurs via the intrinsic (contact activation) or extrinsic pathways (Figure 4). The extrinsic pathway is triggered by tissue factor (TF) expressed on adventitial cells surrounding blood vessels, or under pathophysiological conditions, on macrophages and tumour cells.[73, 74] After initiation of the pathway, enzymatic reactions follow, in which zymogens of serine proteases are converted into activated coagulation factors.[73] On the other hand, the intrinsic pathway is initiated by factor (F)XII activation via physiological components such as RNA, DNA and polyphosphates. In vitro, kaolin or ellagic acid are commonly used as intrinsic triggers.[75] The extrinsic and

intrinsic pathway converge at FX, and this is known as the common pathway. The culmination of coagulation pathways is the generation of thrombin, which is responsible for the formation of an insoluble fibrin clot.

Platelets and the coagulation cascade interact in several ways.[4, 70, 73] Platelets support coagulation by providing a procoagulant surface and secreting (anti)coagulation factors upon activation, such as fibrinogen, FXIII, FV, prothrombin, antithrombin (AT) and tissue factor pathway inhibitor (TFPI) (Figure 4). On the other hand, thrombin can activate platelets via PAR1/4, while recently it was demonstrated that fibrin(ogen) induces platelet responses through interaction with GPVI and integrin $\alpha_{IIb}\beta_3$. So far, thrombin and fibrin are known to be the main coagulation-generated compounds that activate platelets. Interactions of other (anti)coagulation factors with platelets have been reported, such as the binding of FXa, FXIIIa and activated protein C (APC) to the PAR, integrin $\alpha_{IIb}\beta_3$ and ApoER2 receptors, respectively.[76-78] However, it is currently unclear what the importance of those interactions is in physiology.

FXa

FXa plays a role in the common pathway, making it an interesting target for therapeutics.[79] The zymogen FX circulates in blood at concentrations of 8-10 µg/ml (135-170 nM). Partial FX deficiencies lead to severe bleeding diathesis and complete deficiencies are lethal.[79] FX deficiency is rare and has a prevalence of 1 in 1 million. FXa signals through the PAR2 receptor on endothelial cells,[80] and induces protective intracellular responses (upregulation of TF, and induces expression of pro-inflammatory mediators IL-6, IL-8, MCP-1).[81-83] Rivaroxaban, a commonly used FXa inhibitor, is administered to prevent and treat venous thromboembolism and stroke. The COMPASS trial showed that in patients with stable atherosclerotic vascular disease, rivaroxaban in addition to aspirin significantly lowered the risk of major adverse cardiovascular events, in comparison to aspirin alone.[70, 84] FXa interacts with platelets via the phosphatidylserine (PS) surface of procoagulant platelets, which allows FXa to cleave

prothrombin, in a FVa dependent fashion.[4] FXa has been implicated in platelet activation, secretion and aggregation through stimulation of PAR1.[76] However, it cannot be ruled out that part if not all of these effects were due to thrombin generation.

FXIIIa

FXIII circulates in plasma as heterotetramer, FXIII-A_2B_2 or as cellular protein, FXIII-A_2.[85, 86] The heterotetramer has 2 catalytical (A) and 2 inhibitory (B) units. FXIII becomes activated by proteolytical cleavage by thrombin in the presence of Ca^{2+}, however, for cellular FXIII elevated Ca^{2+} levels are sufficient to transform the zymogen into a transglutaminase.[87, 88] The functions of FXIII are amongst others cross-linking fibrin, supporting the formation of PS positive platelets, protection against fibrinolysis and bone and matrix remodelling. Normal FXIII levels (1U/ml) are required to achieve haemostatic functioning and deficiencies are characterized by severe bleeding diathesis. However, FXIII deficiency is an extremely rare disorder, with a prevalence of 1 in 4 million.[89, 90] FXIII is found in platelets, megakaryocytes, monocytes and macrophages.[86] Upon potent receptor-mediated platelet activation, cellular FXIII is translocated to the platelet surface,[88, 91] as well as secreted in microparticles.[92] Non-receptor mediated platelet stimulation, e.g. via Ca^{2+} ionophores, does not result in FXIII translocation to the platelet surface. FXIII interacts with platelets via the integrins $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$.[78] The interaction with $\alpha_{IIb}\beta_3$ stimulates the activation of Syk.[78]

APC

The active form of the serine protease protein C (APC) has cytoprotective and anticoagulant properties and circulates in the plasma at concentrations of 70 nM.[93] APC functions as an anticoagulant factor by inhibiting FVa and FVIIIa. In addition, APC mediates cytoprotective and anti-inflammatory effects by binding to PAR1 on endothelial cells.[94, 95] APC also induces platelet spreading, via ApoEr2 and GPIbα.[77] By binding

platelets, APC on the one hand contributes to haemostasis at the site of injury, while on the other hand it limits thrombus growth by its anticoagulant properties.

The prevalence of mild PC deficiency, which is mostly asymptomatic, is 1 in 200-500, while severe PC deficiency is a rare disorder with a prevalence of 1 in 500000.[96, 97] Severe PC deficiency is associated with disseminated intravascular coagulation, purpura fulminans and vascular thromboembolic events.[97]

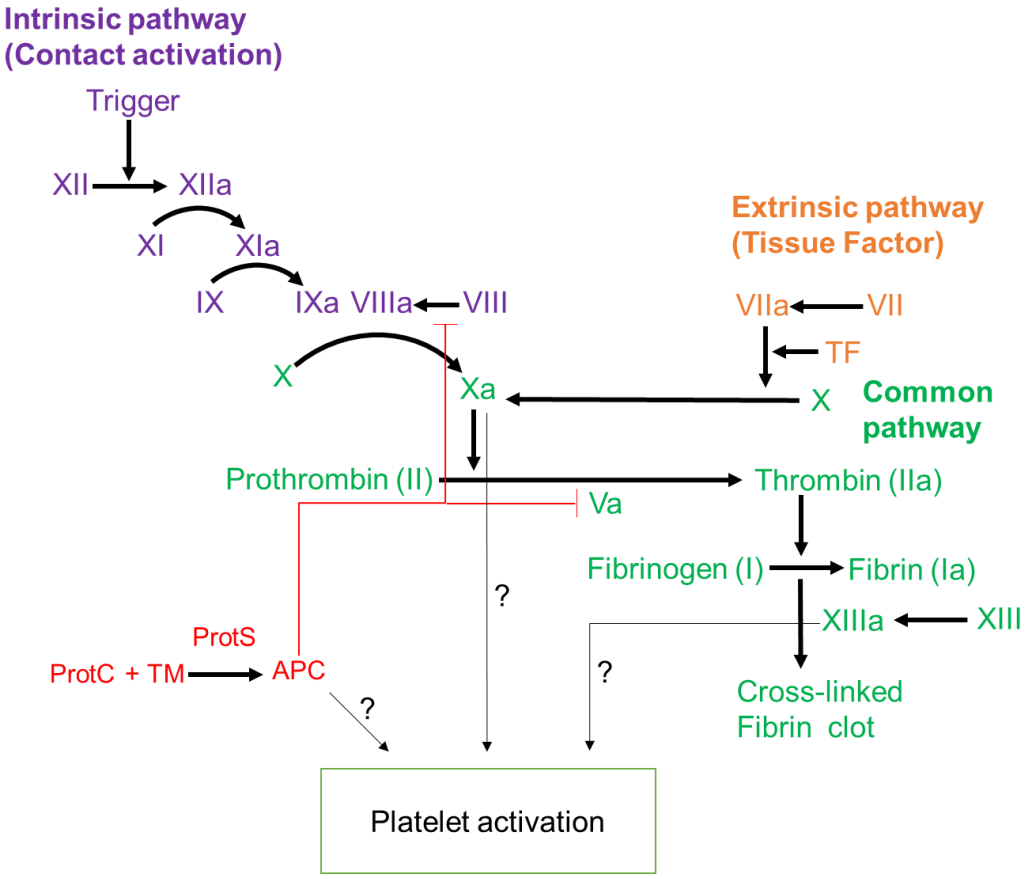

Figure 4. Interplay between platelets and coagulation. The coagulation system is activated intrinsically or extrinsically. Coagulation-generated components can function as ligands for platelets (thrombin, fibrin). Several coagulation-generated components interact with platelets; however, the relative physiological contribution of this interaction is not completely clear so far.

Inter-individual variability

Anti-platelet therapy (APT) is commonly used for the prevention and treatment of cardiovascular diseases. APT may comprise of a single drug, i.e. aspirin or clopidogrel,

or consist of a combination of aspirin with a $P2Y_{12}$ receptor inhibitor, such as clopidogrel, ticagrelor, prasugrel or cangrelor. [35] Clopidogrel is commonly used and is a prodrug which is converted into its active metabolite by the enzyme cytochrome p450 (CYP450). In patients carrying specific loss of function CYP450 mutations, such as CYP2C19 polymorphisms, clopidogrel is less effective.[98] Clopidogrel might be replaced by ticagrelor, however, at the expense of a higher bleeding risk.[99] Intravenous cangrelor is also suitable as alternative for clopidogrel but only for short term intravenous management in selected patients.[100] Genetic screening offers the possibility to determine whether clopidogrel is an optimal choice for a certain individual.[101] This genotype based prescription of clopidogrel has been successfully applied in large clinical trials in patients with CAD.[102] Another approach is to test platelet function where in addition to variation in enzyme activity, variability between patients' platelet response may also be related to age and gender. For instance, several studies reported that aging results in elevated plasma platelet factor 4 (PF4) and plasma factors, such as plasma fibrinogen.[103-105] Further, women in general have higher platelet counts than men.[106] Moreover, co-morbidities such as diabetes mellitus type II also contribute to inter-individual variation, as platelets of diabetics are found to be hyperreactive and less sensitive to aspirin and clopidogrel.[107, 108] Also in other disease states, such as CAD and cancer, platelets have been described to be pre-activated, making them less responsive to activation in vitro. As circulating platelets are continuously exposed to activating agents, they might be desensitised.[109-111] Whether platelet desensitisation relates to the drug responsiveness is to be investigated. Taken together, inter-individual variability is a multi-factorial process and there is a need for tools that allow adjustment and monitoring of antiplatelet medications, personalised for each individual.

Aims and outline of this thesis

The overall aim of this thesis is to provide more insight into acute and persistent consequences of interactions between platelet receptors and their ligands, mainly

through the GPVI and PAR receptors. Coagulation-generated ligands, as well as extracellular matrix components and peptides were used to study platelet functioning through these receptors. In **chapter 2,** the aim was to provide an overview of gene mutations involved in altered platelet traits, like platelet production and/or functionality. Here, the importance of intrinsic factors affecting platelet functioning is illustrated. The goal of **chapter 3** was to investigate the role of the GPVI-Syk pathway in thrombus formation on fibrillar collagens and collagen peptides. We hypothesised a Syk-dependent role via GPVI in thrombus formation under flow. The selective Syk inhibitor PRT060318 was used as tool, as well as a panel of surface-immobilised or soluble collagens and collagen peptides. In **chapter 4**, we aimed to gain more insight into the interplay between platelets and the coagulation cascade, as well as new coagulation-generated ligands for functional platelet receptors GPVI and PAR1. We hypothesised that coagulation factors, other than thrombin and fibrin, can affect platelet functioning through GPVI and PAR1. In **chapter 5**, the goal was to elucidate differences between short- and long-term effects of platelet activation through GPVI or GPCRs. We hypothesised that platelet stimulation through GPVI is persistent, while triggering via GPCRs is transient. Further, we hypothesised that platelets can be reactivated to a certain extent, and can still contribute to thrombus formation, after previous stimulation. The overall objective of this chapter was to investigate platelet activation, restimulation, exhaustion and recycling. In **chapter 6**, we aimed to use a flow cytometric screening, as a potential tool to tailor antiplatelet medication, based on the capacity and sensitivity of a patients' platelet to respond to agonists. In **chapter 7**, the general discussion puts the main findings of this thesis into a broader perspective.

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Chapter 2

Clonal haematopoietic mutations linked to platelet traits and the risk of thrombosis or bleeding

Platelets derive from megakaryocytes, which are differentiated from hematopoietic stem cells. Not only inherited but also somatic (acquired) mutations in hematopoietic stem cells are linked to platelet abnormalities, which could form the basis for thrombo-hemorrhagic complications. This has led to the concept of clonal hematopoiesis of indeterminate potential (CHIP), defined as somatic mutations caused by clonal expansion of mutant hematopoietic cells without evident disease. An overview of genes associated with clonal hematopoiesis and altered platelet production/and or functionality are presented in this chapter. We consider how reported CHIP genes can influence the risk of cardiovascular disease, by exploring the consequences for platelet function related to (athero)thrombosis, or the risk of bleeding. More insight into the functional consequences of the CHIP mutations may favor personalized risk assessment, not only with regard to malignancies but also in relation to thrombotic vascular disease.

We searched for CHIP mutations that are directly or indirectly linked to qualitive or quantitative platelet traits. We started from the Online Mendelian Inheritance in Man (OMIM) database complemented with recent literature and selected genes that were linked to clonal hematopoiesis as well as to the platelet traits count and function. The potential relation of CHIP mutations to both (athero)thrombotic and hemostatic disorders is presented in this Chapter. Hence, this chapter illustrates how the functional status of platelets can be altered by intrinsic platelet factors, while in the next chapters, novel mechanisms of platelet activation are investigated following specific ligand-receptor interactions.

I have been part of designing this project, as well as collecting data from the Online Mendelian Inheritance in Man (OMIM) and searching literature on PubMed. Likewise, I drafted the manuscript and addressed reviewers' comments. For these reasons, I'm co-first author of this work.

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Clonal haematopoietic mutations linked to platelet traits and the risk of thrombosis or bleeding

Alicia Veninga[1,†], Ilaria De Simone[1,†], Johan W. M. Heemskerk[1], Hugo ten Cate[1,2], Paola E. J. van der Meijden[1*]

[1]Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, Maastricht, The Netherlands, [2]Department of Internal Medicine, CARIM, Maastricht University Medical Center, Maastricht, The Netherlands

[†] Authors contributed equally to this work

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[*] Correspondence: Dr. P.E.J. van der Meijden, Dept. of Biochemistry, Maastricht University, P.O. Box 616, 6200 MD Maastricht (The Netherlands); e-mail: p.vandermeijden@maastrichtuniversity.nl

Abstract

Platelets are key elements in thrombosis, particularly in atherosclerosis-associated arterial thrombosis (atherothrombosis), and haemostasis. Megakaryocytes in the bone marrow, differentiated from haematopoietic stem cells are generally considered as a uniform source of platelets. However, recent insights into the causes of malignancies, including essential thrombocytosis, indicate that not only inherited but also somatic mutations in haematopoietic cells are linked to quantitative or qualitative platelet abnormalities. In particular cases, these form the basis of thrombo-haemorrhagic complications regularly observed in patient groups. This has led to the concept of clonal haematopoiesis of indeterminate potential (CHIP), defined as somatic mutations caused by clonal expansion of mutant haematopoietic cells without evident disease. This concept also provides clues regarding the importance of platelet function in relation to cardiovascular disease. In this summative review, we present an overview of genes associated with clonal haematopoiesis and altered platelet production and/or functionality, like mutations in *JAK2*. We consider how reported CHIP genes can influence the risk of cardiovascular disease, by exploring the consequences for platelet function related to (athero)thrombosis, or the risk of bleeding. More insight into the functional consequences of the CHIP mutations may favour personalised risk assessment, not only with regard to malignancies but also in relation to thrombotic vascular disease.

Introduction

Atherosclerotic cardiovascular disease is a chronic inflammatory condition that frequently occurs in the ageing population.[1] Current understanding is that upon rupture or erosion of an atherosclerotic plaque, a thrombus is formed of aggregated platelets and fibrin which can become vaso-occlusive.[2] Platelets furthermore contribute to

ensuing thrombo-inflammatory reactions through their multiple interactions with vascular cells, leukocytes and the coagulation system, thereby promoting disease progression.[3]

Platelets are formed from megakaryocytes in the bone marrow through a differentiation and maturation process known as megakaryopoiesis. Several transcription factors have been identified over the years that regulate megakaryopoiesis and platelet production, and the knowledge on key transcriptional regulators is still expanding. Mutations in genes encoding for these transcription factors, along with epigenetic regulators, are accompanied with quantitative and/or qualitative platelet abnormalities, causing thrombo-haemorrhagic complications.[4] Multiple growth factors control megakaryopoiesis and platelet production, of which thrombopoietin and its binding to the thrombopoietin receptor plays a primary role.[5] Megakaryocytes undergo endomitosis to become polyploid and during maturation extensive reorganisation of cytoskeletal proteins is required for proplatelet formation and the budding of platelets.[6]

A number of recent studies stipulate that the incidence of cardiovascular disease (CVD), such as coronary artery disease, heart failure and ischaemic stroke, is higher in patients with so-called somatic driver mutations in haematopoietic stem or progenitor cells, resulting in a clonal expansion of a subpopulation of blood cells.[1] This process, referred to as clonal haematopoiesis of indeterminate potential (CHIP), was proposed to define individuals with somatic clonal mutations in genes related to haematological malignancies with variant allele fractions of >2%, but without a known haematological malignancy or other clonal disorder.[7] This premalignant state is considered to be relatively frequent in the elderly population, where somatic mutations accumulate in a variety of genes controlling haematopoietic stem cell maintenance, expansion and survival. Although CHIP increases the risk of developing haematological cancer, mostly myeloid neoplasms, the absolute risk is still small. Several excellent recent reviews describe in detail the aetiology of clonal haematopoiesis and its relation with CVD.[1,8,9]

So far, attention is mainly focussed on proposed mechanisms of accelerated inflammation-driven atherosclerosis and increased thrombosis risk through altered function of innate immune cells.

In the present paper, we took a different approach. We confined to the current evidence on CHIP mutations that are directly or indirectly linked to qualitative or quantitative platelet traits. Starting from the OMIM (Online Mendelian Inheritance in Man) database complemented with recent literature, we selected and discussed genes that were linked to clonal haematopoiesis as well as to the platelet traits count and function. Since CHIP mutations appeared not to be only associated with increased platelet count and/or function, but also with decreases in these platelet traits, its potential relation to both (athero)thrombotic and haemostatic disorders is presented in this review.

Section 1: clonal mutations in genes associated with increased platelet count and/or function

For several genes encoding for transcription regulators (*ASXL1),* epigenetic regulators *(DNMT3A, IDH2)* and cell signalling proteins *(ABL1, BCR, BRAF, JAK2, SH2B3),* clonal mutations are known that enhance platelet production, which can be accompanied by enhanced platelet functionality. Related effects are described for several genes with divergent roles *(ABCB6, SF3B1)* (Table 1 and Figure 1).

ABCB6. Multiple so-called ABC transporters play a role in lipid trafficking, and thus may contribute to atherosclerosis. However, the *ABCB6* gene product (*ATP binding cassette subfamily B member 6*) facilitates the ATP-dependent import of porphyrins and haem into mitochondria.[10] Markedly, germline mutations of *ABCB6* are associated with several disease phenotypes, including dyschromatosis, microphthalmia and pseudo-hyperkalaemia.

The gene *ABCB6* is highly expressed in bone marrow megakaryocyte progenitor cells and megakaryocytes, but only moderately in platelets. Evidence regarding CVD mainly comes from animal studies. In mice, deficiency in *Abcb6* increased megakaryopoiesis and thrombopoiesis, resulting in an increased platelet count and larger platelet volume, effects that were explained by higher oxidative stress in the presence of accumulating porphyrins.[11] The platelets produced in these mice were hyperreactive and furthermore, against a high-lipid background, attracted leukocytes, thus enhancing atherosclerosis.[10,11]

In patients with acute promyelocytic or myeloid leukaemia, RNA expression levels of *ABCB6* are reduced, suggesting the occurrence of also acquired clonal mutations in this gene.[12] However, so far no strong association with CHIP has been found.[8]

ASXL1. The transcriptional regulator *Additional sex combs like 1* (ASXL1) is a chromatin-binding protein, which acts as tumour suppressor and is implicated in the maintenance of normal haematopoiesis. Somatic mutations of this gene are found in patients with a variety of myeloid malignancies, including acute myeloid leukaemia (AML), chronic myelomonocytic leukaemia (CMML), myelodysplastic syndrome (MDS), and myeloproliferative neoplasm (MPN).[13] In particular, mutations in *ASXL1* are detected in 10% of MDS patients and 40% of CMML patients.[14] Hence, this gene is considered as a leukaemia and myelodysplasia driver. The majority of (somatic) mutations provokes a truncation of the C-terminus of the protein, resulting in a loss of transcription regulation. In addition, the truncated form can interact with other proteins to modulate cell proliferation.[15] In mouse models, transgenic expression of a C-terminal truncated Asxl1 mutant resulted in age-dependent anaemia, thrombocytosis, and morphological dysplasia.[13] A similar type of thrombocytosis is seen in MDS-refractory anaemia patients, carrying *ASXL1* mutations.[13] The prevalence of acquired

haematopoietic mutations in *ASXL1* in a healthy population of 60-69 years was estimated at 1.5%, and was associated with a doubled risk of developing CVD.[16]

BCR and ABL1. The somatic gene effects of *Breakpoint cluster region protein* (BCR) and *Abelson murine leukaemia viral oncogene homolog 1* (ABL1) are highly related, if only because the two proteins share signalling pathways. The proto-oncogene *ABL1* contains an auto-inhibitory SH3 domain which, when deleted, turns it into an oncogene. During a somatic reciprocal translocation of chromosomes 22 and 9, both genes can fuse together. The encoded BCR-ABL1 fusion protein is frequently detected in patients with chronic myeloid leukaemia (CML) (90%) or ALL (30%).[17] While CML patients mostly carry the 210 amino-acid variant of BCR-ABL, in ALL patients also a shorter 185 amino-acid variant is present. Both fusion forms display constitutive protein tyrosine kinase activity.[17]

The current understanding is that aberrant roles of BCR and ABL1 in haematopoiesis are a consequence of fusion formation, although the main evidence comes from case studies. A fusion variant has been described, which is associated with an increased platelet count, although the mechanism is still unclear.[18] In the few healthy adults, carrying a BCR-ABL1 fusion mutation, haematopoietic malignancies were not detected. On the other hand, BCR-ABL1 fusions can be considered as indicators for a premalignant state, while the absolute risk of developing CVD is smaller than for the *JAK2* V617F mutation.[19]

BRAF. The serine-threonine protein kinase BRAF is an essential partner in the mitogenic RAS/RAF/MEK/ERK signalling pathway. The *BRAF* proto-oncogene is expressed in all tissues, where it controls cell proliferation, apoptosis, and differentiation. In addition, BRAF is necessary for embryonic development, as *Braf*-deficient embryos die because of disturbed blood vessel formation.[20]

Evidence on the role of BRAF in normal megakaryopoiesis comes from work mainly with immortalised human megakaryoblastic cell lines. Upon stimulation with thrombopoietin, differentiation and proliferation of the cells appeared to rely on BRAF-mediated signalling to ERK.[21] Downregulation of BRAF thus lowered the number of megakaryocytic lineage cells, a phenomenon that was confirmed *in vivo* in chimeric mice.[20]

In the Noonan, LEOPARD and cardiofaciocutaneous syndromes, patients carry germline mutations in *BRAF* in regions distinct from those of somatic cancerous mutations. However, only limited changes in BRAF signalling are reported.[22] On the other hand, somatic gain-of-function mutations in the *BRAF* gene accumulate in patients with AML, malignant lymphomas or solid cancers.[23] Next to more common point mutations, rare chromosomal translocations are described for this gene.[24]

A frequently observed gain-of-function mutation ($V^{600}E$) is the driver mutation present in different cancers, including melanomas, solid cancers and hairy cell leukaemia (HCL). Patients who suffer from HCL have low blood cell counts, likely due to bone marrow aberrations and splenomegaly.[25] Whether megakaryopoiesis is altered due to a constitutively increased MEK/ERK signalling via BRAF still needs to be confirmed.

DNMT3A. Clonal mutations of three genes (*DNMT3A, IDH2, TET2*) have been reported which, directly or indirectly, affect histone methylation and hence these can be considered as epigenetic regulators.

The gene *DNA methyltransferase 3α* (DNMT3A) encodes for a DNA methylation enzyme that regulates gene imprinting, chromosome inactivation and tumour suppression. Genetic mutations in the *DNMT3A* gene occur in the rare Tatton-Brown-Rahman syndrome which, as far as known, is not accompanied by haematopoietic aberrations.

In several acquired blood cancers, but especially in adults with AML, somatic mutations in *DNMT3A* have been reported.[26] About a quarter of all AML patients with *de novo* disease carries variant forms of this protein, most commonly with $R^{882}H$ mutation. The loss-of-protein-function in those patients resulted in chromosomal islands of hypomethylation.[27] The same mutation, albeit less frequent, can occur in patients with CMML, MDS or MPN.[26] It is considered that *DNMT3A* mutations in haematopoietic stem cells lead to a pre-leukaemic state, waiting for additional mutations to induce leukaemia. The time interval from first mutation appearance to disease is however unclear.[26]

In agreement with its relevance for clonal haematopoiesis, a recent report points to an increased incidence of acquired *DNMT3A* mutations in the elderly, with a prevalence of about 15% at 60-69 years.[1] Combined with a $JAK2V^{617}F$ mutation (see below), the mutated *DNMT3A* gene associates with essential thrombocythemia (ET) and polycythaemia vera (PV).[26] Current understanding is that first acquirement of a *DNMT3A* mutation followed by *JAK2* will result in an ET phenotype. On the other hand, first appearance of the *JAK2* mutation may result in a PV phenotype.[26] Overall, *DNMT3A* mutations in AML patients are associated with higher platelet counts, when compared to patients with WT-*DNMT3A*, however the absolute count is still low ($<150x10^9/L$).[28]

Regarding atherosclerosis development and atherothrombosis, studies report increased inflammation, linked to mutated *DNMT3A*, possibly due to a higher production of cytokines.[29] Indeed, in patients carrying an acquired mutation of *DNMT3A*, the risk of CVD appears to be doubled.[1] The higher platelet count observed in AML patients with *DNMT3A* mutations likely occurs secondarily the pro-inflammatory phenotype. So far, no mechanism is found to link *DNMT3A* mutations directly to platelet traits.

IDH2. The enzyme *isocitrate dehydrogenase* $NADP^+$ *2* (IDH2) localized in mitochondria generates NADPH from $NADP^+$, whilst catalysing the oxidative decarboxylation of

isocitrate ultimately producing D-2-hydroxy-glutarate. By producing NADPH, IDH2 regulates the mitochondrial redox balance, hence mitigating cellular oxidative damage.[30] As expected, genetic mutations in *IDH2* are described to be associated with metabolic diseases. On the other hand, somatic mutations of *IDH2* are found in several cancers, including haematologic malignancies, sarcomas and colon cancer. This is compatible with a role of *IDH2* as epigenetic regulator, although the direct evidence for epigenetic effects is still indirect.

The most frequent clonal mutations in *IDH2,* identified in patients with *de novo* AML, concern the protein arginine residues $R^{140}Q$ and $R^{172}K$. These variants cause a gain-of-function resulting in an abnormal, damaging production of D-2-hydroxyglutaric acid, leading to a hypermethylated state of DNA and histones.[30] In comparison to non-carriers, AML patients carrying the somatic *IDH2* mutations showed a higher platelet count, although the absolute platelet count was still low ($<150x10^9$/L).[31] The same trend for platelet count has also been found in MDS patients.[32] Next to this, in primary myelofibrosis, *IDH* mutations can form a risk factor for leukaemic transformation.[33] No association with thrombotic events is known for these patient groups, but bleeding was more common in mutant-carrying patients. In the elderly, the percentage of individuals with clonal haemostasis driver mutations in *IDH2* appeared to be rather low, in the order of 1%.[16] Together, this suggests only mild effects of somatic changes in this gene on clonal haematopoiesis, associated with a slight increase in platelet count by a so far unknown mechanism and an increased bleeding tendency.

JAK2. The non-receptor tyrosine kinase *Janus kinase 2* (JAK2) is one of the general regulators of cell survival and proliferation, by controlling for instance cytokine receptor signalling pathways. Also in haematopoiesis, JAK2 controls precursor cell maintenance and function.[34] Inherited mutations of *JAK2* are detected in patients with hereditary

thrombocytosis,[35] whilst somatic mutations of the gene link to various phenotypes including erythrocytosis.

A well-known acquired *JAK2* variant is the mutation $V^{617}F$, which is carried by the majority of patients with MPN, *i.e.* in nearly all PV patients and half of patients with ET or primary myelofibrosis. In general, the $V^{617}F$ mutation affects the proliferation of myeloid cells and leads to increased inflammatory responses.[34] However, this somatic mutation as such is not considered to enhance the risk of thrombotic events in patients with ET or PV.[36] Nevertheless, platelets from *JAK2* $V^{617}F$-positive patients demonstrated an enhanced activation status and procoagulant potential. In addition, the fraction of immature platelets, which can be more active than mature platelets, was higher in carriers of the JAK2 $V^{617}F$ mutation versus non-carriers.[37]

Transgenic mice have been generated, carrying the human *JAK2* $V^{617}F$ mutation in the megakaryocyte lineage.[38] The *JAK2* $V^{617}F$ megakaryocytes responded better to thrombopoietin, and displayed a greater migratory ability, proplatelet formation and increased ploidy. The produced platelets responded stronger to multiple agonists.

In ageing healthy individuals, the prevalence of the *JAK2* $V^{617}F$ variant is only 1%, but carriers have a tenfold increased risk of CVD.[16] Depending on the degree of mutation expansion, subjects may develop MPN instead of CHIP.[8] How or under which accompanying conditions the thrombocytosis is linked to somatic JAK2 mutations aggravating CVD is still a matter of debate and requires further investigation.

SF3B1. The gene *splicing factor subunit 1* (*SF3B1)* encodes for a core component of the RNA spliceosome machinery.[39] Somatic mutations in this gene, along with other genes of the spliceosome, have been identified in more than half of MDS patients.[39] Common mutations in the SF3B1 protein are those of $K^{700}E$, $K^{666}N$ and $R^{625}C$.[40] To study

the impact of the frequent $K^{300}E$ mutation, a conditional knock-in mouse model was developed, which revealed a RNA splicing defect similar as supposed in MDS patients harbouring this mutation.[39] Regarding the thrombotic risk, studies revealed that patients carrying a mutated *SF3B1* gene had higher platelet counts and were more prone to develop CVD than patients without mutation,[40] although the altered molecular players are unclear. A sequencing study furthermore identified *SF3B1* mutations in 5% of ET patients.[41] In the ageing healthy population, clonal haematopoietic mutations of *SF3B1* appear to be infrequent, ranging from 2 to 5%.[42]

SH2B3. The signalling adaptor protein *Src homology 2 B3* (SH2B3, also named LNK) acts as an interactor of JAK2, and negatively regulates thrombopoietin-induced megakaryopoiesis. An associated inherited disease is B-precursor acute lymphoblastic leukaemia. Somatic mutations in the *SH2B3* gene are found in >5% of MPN patients. These concern frameshift and missense mutations throughout the whole gene, often co-existing with mutations in driver genes, including *JAK2*, *CALR* and *MPL*.[43] The loss-of-function of SH2B3 can lead to a higher expansion of haematopoietic stem cells, acting by increased thrombopoietin signalling and megakaryopoiesis.[44] The higher platelet and leukocyte counts may worsen atherosclerosis and the thrombotic risk.[43] In *Sh2b3* knockout mice, it was found that hyperlipidaemia aggravated both atherosclerosis and thrombosis, likely due to positive platelet priming.[45] Whether this priming event due to *SH2B3* mutations also occurs in humans, is not known.

Section 2: clonal mutations in genes associated with decreased platelet count and/or function

Several mutations in genes encoding for transcription regulators (*ETV6, GATA2, GFI1B, SMAD4),* cell signalling proteins *(TP53, WAS),* and other proteins *(FANCA, FANCC)* are linked to impaired haematopoiesis, causing thrombocytopenia of varying severity with evidence for concomitant platelet function defects (Table 1 and Figure 1).

ETV6. The transcriptional repressor *E26 transformation-specific variant 6* (ETV6) serves to maintain the development of haematopoietic stem cells in the bone marrow, as a continuous survival signal. It acts by inhibiting other transcription factors, such as FLI1. However, it appears not be required for embryonic stem cell expansion.[46]

ETV6 is known as a proto-oncogene, since it can be a fusion partner with over 30 other genes, but in case of truncating mutations it acts as a tumour suppressor gene. Depending on the fusion site, the fused protein can alter the transcription levels of ETV6 target genes, which may support the development of leukaemia.[46,47] On the other hand, germline heterozygous *ETV6* mutations have been identified in some patients with dominantly inherited thrombocytopenia.[48] Such patients seem to have a predisposition to haematologic malignancies, most commonly acute lymphatic leukaemia (ALL), AML or MDS. Given that the complete loss of *ETV6* is lethal, truncating or protein-inactivating mutations are mainly found as somatic events, and rarely as germline variants.[46]

As a transcriptional repressor, ETV6 has an established role in megakaryocyte and platelet (patho)physiology. Patients with germline *ETV6* variants show a large expansion of immature megakaryocyte colony-forming units, accompanied by a reduced formation of proplatelets, thus explaining the thrombocytopaenia. The mutant platelets are of normal size, although characterised by aberrant cytoskeleton organisation, lower levels of small GTPases, and defective clot retraction.[49] Evidence is lacking to link clonal variants of *ETV6* to thrombotic phenotypes, however a related bleeding tendency has been described.

FANCA, FANCC. The proteins *Fanconi anaemia complementation group A and C* (FANCA/C) are repair factors after DNA damage or apoptosis. Inherited mutations in either gene are seen in the disorder Fanconi anaemia, where patients in 60-70% of the

cases show a mutation in *FANCA* and in 15% a mutation in *FANCC*. Such patients suffer from progressive bone marrow failure, pancytopaenia and predisposition to cancer.[50] Knockout mouse studies revealed that FANCA is needed for normal megakaryopoiesis and platelet production. Megakaryocytes in the deficient mice were found to be in a senescent state.[50]

In humans, heterozygous mutations of *FANCA* are observed in a proportion of patients with AML.[51] Yet, carriers of such mutations do not seem to have a significant risk of developing cancer.[52] On the other hand, *FANCA* deletion mutations, especially in combination with other germline mutations, might contribute to breast cancer susceptibility.[53] The phenotype coupled to somatic mutations in *FANCA* and/or *FANCC* is probably linked to genomic instability caused by defective FANC proteins.[51] How these somatic mutations contribute to CHIP-related CVD needs to be established.

GATA2. In immature haematopoietic stem cells, the transcription factor *GATA-binding factor 2* (GATA2) is earlier expressed than GATA1, and becomes downregulated upon differentiation.[54] This has also been observed in *Gata2*-knockout mice, revealing that GATA2 is required for haematopoietic stem cell and progenitor cell development.[55] In humans, congenital GATA2 deficiency is accompanied by a hypocellular and dysplastic bone marrow, resulting in low platelet counts.[55] Furthermore, germline deletion mutations in the *GATA2* gene are associated with an increased predisposition to infection, AML, CMML or MDS.

On the other hand, a somatic mutation ($L^{359}V$) in *GATA2* has been identified in about 10% of patients in the progression phase of CML.[56] This concerns a gain-of-function resulting in increased transcription factor activity, in contrast to gene deletion. Reports indicate that in about 50% of patients with any *GATA2* mutation, the megakaryocyte development is abnormal.[57] Unlike GATA1, GATA2 regulates platelet GPIIb rather than

GPIb expression.[54] Variants of *GATA2* have also been associated with increased susceptibility for coronary artery disease,[58] linking this gene to CHIP.

GFI1B. Another transcription regulator crucial for erythroid and megakaryocytic differentiation is *growth factor independent 1B transcription repressor* (GFI1B). As a DNA-binding protein, it regulates the dormancy and mobilisation of haematopoietic stem cells.[59] Next to the full-size protein of 330 amino acids implicated in megakaryopoiesis, a shorter form is expressed that may rather regulate erythroid development.[60] The longer protein modulates the expression of several proto-oncogenes and tumour suppressor genes.[59] Accordingly, a functional disturbance of GFI1B can contribute to leukaemia development. In mice, genetic deletion of *Gfi1b* resulted in early lethality, where the embryos showed failed megakaryocyte development.[61]

For human *GFI1B*, both germline and somatic mutations have been identified. These generally result in a truncated or a dysfunctional form of the protein, thereby reducing DNA binding and transcription repressor activity.[62] In the inherited disorder gray platelet syndrome, patients with a GFI1B mutation display (macro)thrombocytopenia with platelets that are reduced in α-granules.[63] The patient's platelets were also found to be reduced in GPIb and GPIIb/IIIa expression, whereas that of the haematopoietic precursor marker CD34 was markedly increased. The suggestion that, in these and other patients with a truncating mutation of *GFI1B,* megakaryocyte development is impaired was recently supported by platelet proteome analysis.[64] In mice, a megakaryocyte-specific deletion of *Gfi1b* enhanced the expansion of megakaryocytes, yet resulting in severe thrombocytopenia.[65] Here, the (tubulin) cytoskeleton appeared to be underdeveloped in the mutant megakaryocytes, explaining an inadequate proplatelet formation.

Whole-exome sequencing efforts have revealed the presence of alternative GFI1B splice variants, which is accompanied by impaired megakaryocyte differentiation and thrombopoiesis.[60] However, in heterozygous carriers, platelet counts and function were in normal ranges. With respect to clonal haematopoiesis, only limited information is available. A somatic mutation of *GFI1B* has been identified in patients with AML.[59]

SMAD4. The 'vascular' transcription factor *SMAD family member 4* (SMAD4) acts as a tumour suppressor, triggered by signalling pathways evoked by transforming growth factor-β or bone morphogenetic protein.[66] Within the cellular nucleus, SMAD4 forms a complex with other SMAD isoforms to control gene expression. In mice, SMAD4 was found to play a role especially in vascular development.[66] On the other hand, a megakaryocyte-specific deficiency of SMAD4 is described, causing mild thrombocytopenia with partially dysfunction platelets, likely as a consequence of altered promotor activities.[67]

In humans, both somatic and inherited mutations of *SMAD4* are known. Inherited mutations of the gene present with distinct phenotypes, ranging from a vascular bleeding disorder (hereditary haemorrhagic telangiectasia) to gastro-intestinal and bone marrow abnormalities.[68] Somatic mutations of the 358-515 amino-acid region are linked to pancreatic carcinoma.[69] During the screening for somatic driver mutations linked to clonal haematopoiesis, a similar mutation of *SMAD4* was found.[16] Mutations in *SMAD4* are related to a bleeding rather than thrombotic phenotype.

TP53. The tumour suppressor *tumour protein p53* (TP53 or p53) is a critical player in cell cycle progression and apoptosis. Herein, TP53 maintains the quiescent state of haematopoietic stem cells, and controls DNA damage responses upon cellular stress.[70] In megakaryocytic cells derived from *Tp53* knockout mice, cell size and polyploidisation

were increased due to higher DNA synthesis and decreased apoptosis. In human cell cultures, *TP53* knockdown affected the expression of platelet integrins, granule components and cytoskeletal proteins, which was accompanied by functional platelet defects.[71]

Regarding human disease, the *TP53* deletion occurring in multiple myeloma is accompanied by a lowering in platelet count.[72] In this context, mutant *TP53* forms are considered to drive clonal haematopoiesis via the epigenetic regulator EZH2, leading to over-methylation of histone H3. This can down-regulate several genes associated with self-renewal and differentiation of haematopoietic stem cells.[70] A common consequence is expansion of the affected haematopoietic cell clones. Markedly, the *TP53* gene is top ranking in mutated genes found in CHIP.[73] How mutated *TP53* in haematopoietic cells contributes to CHIP-associated CVD still remains to be determined. Few studies have shown that there is higher expression of pro-inflammatory cytokines in p53-deficient murine leukocytes, which may accelerate the development of CVD.[9] However, evidence directly linking platelet traits to CVD development is lacking.

WAS. The *Wiskott-Aldrich syndrome* (WAS) protein is selectively expressed in haematopoietic cells, where it regulates actin cytoskeleton rearrangements. In the classical X-linked Wiskott-Aldrich syndrome, patients suffer from thrombocytopaenia with smaller sized platelets and recurrent infections, due to an impaired functionality or availability of WAS.[74] A milder phenotype is that of X-linked thrombocytopaenia, in which patients only suffer from bleeding because of low platelet count.[74] Rare inherited mutations that instead cause constitutive WAS activation are seen in patients with X-linked neutropaenia, experiencing recurrent bacterial infections while having normal platelet count and size.[75] In addition, these patients show an increased predisposition for AML or MDS.[76]

In classical Wiskott-Aldrich syndrome patients, the prevalence of malignancy is 13-22%, mostly due to development of lymphoma, but also to lymphoblastic leukaemia, MDS or MPD.[76] The thrombocytopaenia is likely caused by increased platelet removal. In *Was*-deficient mice, platelet turnover was shortened, with proteomic evidence for alterations in proteins of metabolic and proteasomal pathways.[77] Furthermore, in both the mutant mice and patients, there is evidence for a hyperactivation status of the platelets, thus explaining the higher elimination rate. Several groups reported on alterations in integrin activation in the patient's platelets.[78,79] However, one patient study concluded for normal platelet activation properties.[80]

There is limited evidence for the presence of somatic mutations in the *WAS* gene. This mainly concerns gain-of-function mutations, associating with poor outcome in patients with juvenile myelomonocytic leukaemia.[81] This suggested a clonal role of the gene in the pre-malignant state.

Section 3: clonal mutations in other genes

For the genes *FLI1* and *GATA1* encoding for transcription regulators, the type of mutation being either gain-of-function or loss-of-function likely determines its respective effect on platelet count and/or function. Mutations in *TET2* have been associated with increased inflammation-induced atherosclerosis and thrombotic disease, although possible effects on platelets remain to be established (Table 1 and Figure 1).

FLI1. The protein *Friend leukaemia virus integration 1* (FLI1) is a member of the ETS transcription factor family, which is highly expressed in the haematopoietic lineage and endothelial cells. Due to a faulty vasculature, *Fli1* knockout mice die during embryonic development, but heterozygous mice are viable without apparent phenotype.[82] Detailed studies indicate that FLI1 plays an important role in both erythropoiesis and megakaryopoiesis by regulating the expression of multiple genes.[83] It acts together with

the transcription factor GABPA, especially in later phases of megakaryopoiesis. This is exemplified by the fact that, in *Fli1* knockout mice, megakaryocytes are specifically reduced in the expression of late-stage genes, *e.g.* genes encoding for glycoprotein (GP)Ibα, GPIX and platelet factor 4.[82]

In humans, heterozygous mutations in *FLI1* are commonly grouped together as 'Bleeding disorder platelet-type 21' (Phenotype MIM 617443). Examples are the Jacobsen syndrome and Paris-Trousseau syndrome, which are characterised by a heterozygous partial deletion of chromosome 11, encompassing the *FLI1* gene. Such patients characteristically suffer from abnormal growth and mental retardation, accompanied by thrombocytopaenia, most likely due to impaired megakaryopoiesis.[84] In the Paris-Trousseau syndrome, platelets are enlarged and contain large fused α-granules.[84] In patients with a mutated *FLI1* gene, presenting with congenital macrothrombocytopaenia, also an impaired agonist-induced platelet aggregation has been reported.[85]

In case of somatic mutations, *FLI1* can become fusion partner with the transcriptional repressing gene *EWSR1*, a condition known as Ewing sarcoma.[86] The effect on platelets is unclear. On the other hand, *in vitro* studies have indicated that in stem cells the overexpression of FLI1 enhances megakaryopoiesis, thrombopoiesis and platelet functionality.[87] Deregulated high levels of FLI1 are furthermore found in various types of cancer. In agreement with this, a predisposition to pre-T cell lymphoblastic leukaemia and lymphoma is described for transgenic mice overexpressing *Fli1* in the haematopoietic progenitor cells.[83] It remains to be established whether *FLI1* is a main contributing gene in CHIP-related CVD.

GATA1. The transcription factor *GATA-binding protein 1* (GATA1) controls the development and production of megakaryocytes, platelets and erythrocytes. In mouse studies, the loss of *Gata1* in the megakaryocyte lineage resulted in smaller size megakaryocytes and a defect in proplatelet formation. The *Gata1*-deficient platelets were larger in size, showed an excess in rough endoplasmic reticulum, and contained fewer α-granules.[88] The deficient mice furthermore were impaired in red blood cell development, and often died because of anaemia.[89] Consistent with this, *GATA1* is highly expressed in human megakaryocytes and erythroid cells. Mutations in *GATA1* can appear as germline or somatic. Inherited mutations associate with haematopoietic disorders, characterised by low blood cell counts. On the other hand, somatic mutations often result in the production of shorter GATA1 variants, for instance in cases of AMKL (acute megakaryoblastic leukaemia) or Down syndrome.[54] Here, platelets tend to be low in counts and display an atypical morphology.

A common consequence of germline *GATA1* mutations in haematopoietic disorders is the altered interaction of GATA1 with its cofactor FOG1, *i.e.* a zinc finger protein cooperating with GATA1 to regulate cell differentiation. This has been reported for patients with X-linked thrombocytopaenia, or other forms of macrothrombocytopaenia, who experience bleeding diatheses.[90] The patient's megakaryocytes are abnormal in structure and the platelets show decreased numbers of α-granules.[90] Patients with primary myelofibrosis, having upstream driver mutations resulting in low GATA1 levels in megakaryocytes, show an increased risk of both thrombosis and bleeding. Mice with Gata1^{low} mutation resemble this phenotype, demonstrating similar megakaryocyte abnormalities, such as abnormal P-selectin localization, and thrombo-haemorrhagic events. The prothrombotic state was ascribed to increased platelet-leukocyte interactions through P-selectin.[91]

In cultured megakaryocytes, GATA1 has been shown to regulate the expression of GPIIb (fibrinogen receptor) and GPIb (von Willebrand factor receptor). Markedly, in GATA1-deficient megakaryocytes, expression levels of GPIIb can be maintained by GATA2 substitution, whereas those of GPIb are decreased.[54] As expected, inherited mutations of *GATA1* are accompanied by a bleeding phenotype rather than by an increased thrombosis risk. On the other hand, high levels of GATA1 transcripts are found in patients with ET or PV.[92] Overexpression of GATA1 in mice results in a similar phenotype.[93] Regarding CHIP, somatic gain-of-function may increase the cardiovascular risk including atherothrombosis, whereas loss-of-function may be more associated with bleeding.

TET2. The protein *Tet oncogene family member 2* (TET2) has a key role in DNA methylation, explaining how it functions as a tumour suppressor, maintaining normal haematopoiesis. The *TET* gene product in particular represses the transcription of inflammatory molecules, such as interleukin-6 and -8, which are known as pro-atherogenic mediators.[1,94] This explains why somatic loss-of-function mutations in *TET2* associate with an increased inflammation tendency. Similarly, as described for *DNMT3A*, the mutations may increase the burden of atherosclerosis and arterial CVD.

Murine *Tet2*-null models are used to confirm that CHIP-like mutations lead to inflammation-driven cardiovascular pathologies,[7,95] markedly without changes in blood cell counts. In the ageing population, clonal haematopoietic mutations of *TET2* have a prevalence of 2.5 %.[16] On the other hand, such mutations are found in about 25% of patients with myeloid neoplasms, and are then associated with an increased cardiovascular risk.[7] So far, it is unclear to which extent the mutations affect megakaryopoiesis or platelet function, either directly or indirectly via enhanced inflammation.

Table 1. Relevant genes in clonal haematopoiesis with effects on platelet traits and disease. Abbreviations: ALL, Acute lymphocytic leukaemia; AMKL, Acute megakaryoblastic leukaemia; AML, Acute myeloid leukaemia; CML, Chronic myeloid leukaemia; CMML, Chronic myelomonocytic leukaemia; ET, Essential thrombocythaemia; JMML, Juvenile myelomonocytic leukaemia; MDS, Myelodysplastic syndrome; MPN, Myeloproliferative neoplasms; PV, Polycythaemia vera.

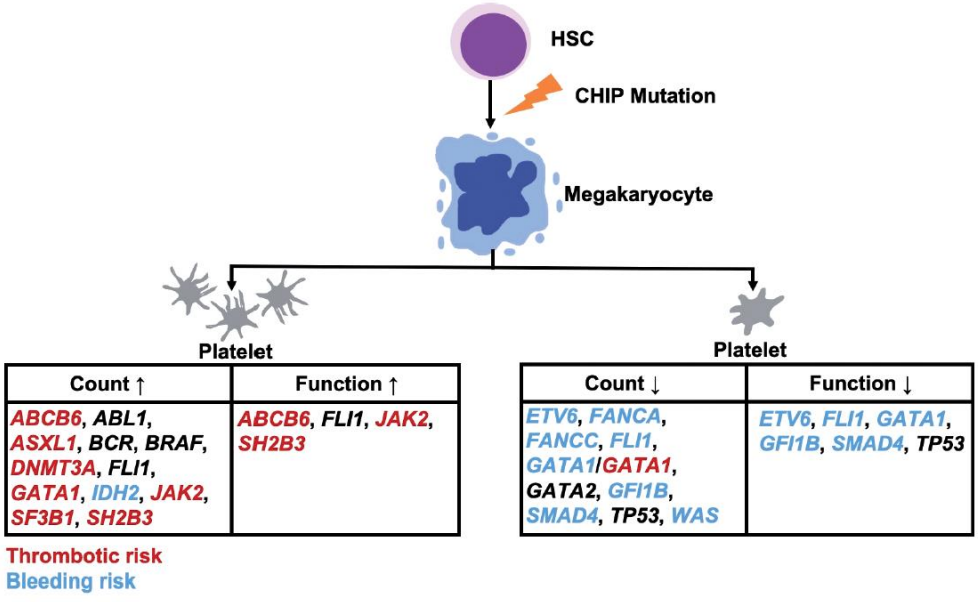
Gene name	Gene (OMIM)	Protein function	Overall role in hematopoiesis	Germline/ somatic	Inherited disease classification (OMIM, PMID)	Somatic phenotype (OMIM, PMID)	Mutation effect on protein	Mutation effect on platelet traits	Thrombosis risk	Bleeding risk	Predisposition to malignancy	Ref. platelet traits
ABCB6	605452	Mitochondrial transporter	Mitochondiral stability	G, S	Dyschromatosis universalis hereditaria 3 (615402); Microphthalmia (614497); Pseudohyperkalemia familial 2 (609153); Blood group Langereis system (111600)	Undefined	Loss-of-function	Count ↑, size ↑, function ↑ (m)	Yes	No	Undefined	11
ABL1	189980	Signaling regulator	Proliferation and survival of HSC	G, S	Congenital heart defect skeletal malformations syndrome (617602)	Leukemia Philadelphia chromosome-positive resistant to imatinib (608232)	Gain-of-function	Count ↑	n.d.	No	ALL, CML	18
ASXL1	612990	Transcription regulator	Tumor suppression; maintenance of normal hematopoiesis	G, S	Bohring-Opitz syndrome (605039)	Myelodysplastic syndrome somatic (614286)	Loss-of-function	Count ↑ (h, m)	Yes	No	Aplastic anemia, AML, CMML, MDS, MPN	13
BCR	151410	Signaling regulator	Development and survival of HSC	S	Undefined	Acute lymphocytic leukemia Philadelphia chromosome positive somatic (613065); Chronic myeloid leukemia Philadelphia chromosome positive somatic (608232)	Gain-of-function	Count ↑	n.d.	No	ALL, CML	18
BRAF	164757	Signaling protein kinase	Controlling development and proliferation of HSC	G, S	Cardiofaciocutaneous syndrome (115150); LEOPARD syndrome 3 (613707); Noonan syndrome 7 (613706)	Adenocarcinoma of lung somatic (211980); Colorectal and other cancers somatic	Gain-of-function	Count ↑/=	n.d.	n.d.	HCL, solid cancers	20
DNMT3A	602769	Epigenetic regulator	Tumor suppression	G, S	Tatton-Brown-Rahman syndrome (615879)	Acute myeloid leukemia somatic (601626)	Loss-of-function	Count ↑	Yes	No	AML, CMML, MDS, MPN (including ET, PV)	26,28
ETV6	600618	Transcription repressor	Development and survival of HSC; when fused either proto-oncogene or tumor suppressor	G, S	Thrombocytopenia 5 (616216)	Acute myeloid leukemia somatic (601262)	Loss-of-function	Count ↓, size =, function ↓	No	Yes	ALL (pre-B), AML, MDS	49

Gene name	Gene (OMIM)	Protein function	Overall role in hematopoiesis	Germline/ somatic	Inherited disease classification (OMIM, PMID)	Somatic phenotype (OMIM, PMID)	Mutation effect on protein	Mutation effect on platelet traits	Thrombosis risk	Bleeding risk	Predisposition to malignancy	Ref. platelet traits
FANCA	607139	DNA repair protein	Chromosomal stability regulating differentiation of HSC	G, S	Fanconi anemia complementation group A (227650)	Undefined	Loss-of-function	Count ↓ (h, m)	No	Yes	AML, MDS, solid cancers	50
FANCC	613899	DNA repair protein	Protection against cytotoxicity	G, S	Fanconi anemia complementation group C (227645)	Undefined	Loss-of-function	Count ↓	No	Yes	AML, MDS, solid cancers	50
FLI1	193067	Transcription regulator	Development and maintenance of HSC	G, S	Bleeding disorder platelet-type 21 (617443)	Ewing sarcoma (29977059)	Loss-of-function	Count ↓, size ↑, function ↓	No	Yes	Solid cancers	82,85
							Gain-of-function	Count ↑, function ↑	n.d.	No	Lymphoblastic leukemia, lymphoma	87
GATA1	305371	Transcription regulator	Development and differentiation of HSC and megakaryocytes	G, S	Anemia X-linked with/without platelet abnormalities (300835); Thrombocytopenia (314050, 300367)	Leukemia megakaryoblastic with/without Down syndrome somatic (190685)	Loss-of-function	Count ↓, size ↑, function ↓ (m)	Yes	Yes	AMKL (in Down syndrome)	88,91
							Gain-of-function	Count ↑	Yes	No	MPN (including ET, PV), AMKL (in non-Down syndrome)	93
GATA2	137295	Transcription regulator	Development and survival of HSC	G, S	Emberger syndrome (614038); Immunodeficiency 21 (614172)	Acute myeloid leukemia (601626); Myelodysplastic syndrome (614286); Predisposition to infection and chronic myelomonocytic leukemia (25359990)	Loss-of-function	Count ↓	n.d.	n.d.	AML, CMML, MDS	55
							Gain-of-function	n.d.	n.d.	n.d.	CML	56
GFI1B	604383	Transcription regulator	Development and mobilization of HSC and megakaryocytes	G, S	Bleeding disorder platelet-type 17, Gray platelet syndrome (187900)	Acute myeloid leukemia (26851695)	Loss-of-function	Count ↓, size ↑, function ↓	no	Yes	Various leukemias	63,65
IDH2	147650	Epigenetic regulator (indirect)	Development and differentiation of HSC	G, S	D-20 hydroxyglutaric aciduria 2 (613657)	Myeloproliferative neoplasm (20428194); Aucte myeloid leukemia (20884716)	Gain-of-function	Count ↑/=	n.d.	Yes	AML, solid cancers	31
JAK2	147796	Signaling regulator	Proliferation and survival of HSC	G, S	Thrombocythemia 3 (614521)	Erythrocytosis somatic (133100); Acute myeloid leukemia somatic (601626); Myelofibrosis somatic (254450), Polycythemia vera somatic (263300); Thrombocythemia 3 (614521); Budd-Chiari syndrome somatic; (600880)	Gain-of-function	Count ↑, size ↑/=, function ↑ (h, m)	Yes	No	MPN (including ET, PV)	36,38
SF3B1	605590	Splicing factor	Development of HSC	S	Undefined	Myelodysplastic syndrome somatic (614286)	Unclear	Count ↑ (h, m)	Yes	No	ET, MDS, MPD	40
SH2B3	605093	Signaling regulator	Development of megakaryocytes and platelet production	G, S	B-precursor acute lymphoblastic Leukemia (23908464)	Erythrocytosis somatic (133100); Myelofibrosis somatic (254450); Thrombocythemia somatic (187950)	Loss-of-function	Count ↑, function ↑ (m)	Yes	No	MPN	44,45
SMAD4	600993	Transcription regulator	Tumor suppression	G, S	Hemorrhagic telangiectasia syndrome (175050); Myhre syndrome (139210); Polyposis juvenile intestinal (174900)	Pancreatic cancer somatic (260350)	Loss-of-function	Count ↓, size =, function ↓ (m)	No	Yes	AML, solid cancers	67

Gene name	Gene (OMIM)	Protein function	Overall role in hematopoiesis	Germline/ somatic	Inherited disease classification (OMIM, PMID)	Somatic phenotype (OMIM, PMID)	Mutation effect on protein	Mutation effect on platelet traits	Thrombosis risk	Bleeding risk	Predisposition to malignancy	Ref. platelet traits
TET2	612839	Epigenetic regulator	Tumor suppression	S	Undefined	Myelodysplastic syndrome somatic (614286)	Loss-of-function	Count = (m)	Yes	No	MDS	95
TP53	191170	Transcription regulator	Quiescing of HSC; tumor suppression	G, S	Bone marrow failure syndrome 5 (618165); Li-Fraumeni syndrome (151623); Adrenocortical carcinoma pediatric (202300); Basal cell carcinoma 7 (614740); Choroid plexus papilloma (260500); Colorectal cancer (114500); Glioma susceptibility 1 (137800)	Breast cancer somatic (114480); Hepatocellular carcinoma somatic (114550); Nasopharyngeal carcinoma somatic (607107); Pancreatic cancer somatic (260350); Colorectal cancer (114500); Glioma susceptibility 1 (137800); Osteosarcoma (259500)	Loss-of-function	Count ↓ (h) = (m), size ↑, function ↓ (m)	n.d.	n.d.	Solid cancers, various leukemias	71,72
WAS	300392	Signaling regulator	Morphogenic development of HSC	G, S	Neutropenia severe congenital X-linked (300299); Thrombocytopenia X-linked (313900); Wiskott-Aldrich syndrome (301000)	Juvenile myelomonocytic leukemia (29316027); Somatic mosaicism in Wiskott-Aldrich syndrome (19129986)	Loss-of-function	Count ↓, size ↓, function unclear (h, m)	No	Yes	lymphoma, lymphoblastic leukemia, MDS, MPD	77,79
							Gain-of-function	Count ↓/=, size =	n.d.	n.d.	AML, MDS, JMML	75

Figure 1. CHIP-related genes affecting platelet traits and the risk of thrombosis or bleeding.

Mutations in genes associated with a thrombotic or bleeding risk are indicated in red and blue, respectively. For genes indicated in black, no such associations are known yet.



Conclusions and perspectives

As outlined above, somatic mutations in multiple genes affecting haematopoiesis contribute as a risk factor to the development of CVD. So far, studies have focussed on the effects of somatic and CHIP-linked mutations on blood cells, linking to increased inflammation, atherosclerotic disease and thrombosis risk. In this review, we provide evidence that many of the common CHIP genes are involved in quantitative (count) and/or qualitative (function) platelet traits, and hence in this way can influence CVD, in particular triggered by thrombo-inflammatory mechanisms. On the other hand, insight is gained in a link between mutations in CHIP genes and impairment of haematopoiesis and haemostatic function.

Reactive (secondary) thrombocytosis, which is not due to a primary haematological disorder but driven by inflammatory stimuli, trauma or acute bleeding, does not seem to increase the risk of thrombotic or haemorrhagic complications.[96] In line with this, the degree of elevation in the platelet count does not correlate with the thrombosis risk in myeloproliferative disease, where clonal (primary) thrombocytosis has been demonstrated.[97] This indicates that the platelet count as such is not the only determinant of the increased thrombosis risk in myeloproliferative disorders.[98,99] Also, several CHIP mutations (e.g. *DNMT3A* mutations) can indirectly cause a rise in platelet count by inducing increased expression of inflammatory molecules that subsequently upregulate the thrombopoietin production by the liver, However, the combination of alterations in count and function may play an essential role in CHIP mutations related to thrombosis. So far, we found evidence for seven CHIP-related genes (*ABCB6, ASXL1, DNMT3A, GATA1, JAK2, SF3B1, SH2B3*) with elevated platelet counts and an associated thrombotic risk (Figure 1). For the other genes, there is not enough evidence to make estimates of this kind. Only for *ABCB6*, *JAK2* and *SH2B3* mutations, it is known that the

elevated platelet count is accompanied by a hyperreactive platelet phenotype. Apparently, information regarding the functional status of platelets in the context of CHIP mutations is still scarce and further studies are needed to elucidate the contribution of platelets to the risk of thrombosis.

One of the most thoroughly investigated conditions, demonstrating the consequences of altered platelet traits due to somatic driver mutations, is essential thrombocythaemia. Markedly, in these patients, there appears to be no direct correlation between platelet count and thrombosis. On the other hand, the JAK2 $V^{617}F$ mutation is known to increase the thrombosis risk in ET patients, when compared to patients without the mutation.[100] The reported enhanced activation status of platelets in JAK2 $V^{617}F$-positive patients provides a strong indication that platelet function changes induced by a CHIP mutation contributes to the risk of thrombosis, thus explaining part of the risk associations of CHIP mutations with CVD. Platelet reactivity also involves interactions with leukocytes, secretion of pro-inflammatory mediators and release of extracellular vesicles that may all contribute to CVD, like atherosclerosis and -thrombosis. Given the increasing prevalence of CHIP mutations in the elderly, prone to develop CVD - along with malignancies -, more thorough investigation of platelet function linked to CHIP mutations is a worthwhile undertaking. More insight into the functional consequences of such acquired mutations may also favour personalised risk assessment, not only with regard to malignancies but also in relation to thrombotic vascular disease.

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Chapter 3

Role of platelet glycoprotein VI and tyrosine kinase Syk in thrombus formation on collagen-like surfaces

The previous chapter illustrated the importance of intrinsic factors, such as somatic mutations, in platelet functions and thrombosis. On the other hand, extrinsic factors, among which collagens, also play an important role in the pathogenesis of thrombosis. The two platelet collagen receptors, $\alpha_2\beta_1$ and GPVI mediate platelet adhesion and activation. Protein tyrosine kinase Syk is central in the GPVI-induced signalling pathway. GPVI is a target for novel antithrombotic therapy, because of its role in the pathogenesis of collagen-induced thrombosis. Therefore, direct GPVI inhibitors, as well as Syk inhibitors are currently investigated as tools to target GPVI, as explained in chapter 1. Horm-derived, collagen type I (Collagen-H) is the gold standard to investigate platelet functions in the clinical lab. Over the past years, several collagen-derived peptides have been synthesized, which bind to GPVI and/or integrin $\alpha_2\beta_1$, similarly to collagen-H. Peptides binding GPVI contain the (GPO)n motif, while peptides containing the GFOGER motif act as ligands for integrin $\alpha_2\beta_1$. Subtle changes in those sequences have been found to alter the affinity for the previously mentioned receptors. The relative importance of the Syk pathway has not been investigated thus far in platelets interacting under flow with surface-immobilized collagen peptides or fibrillar collagens.

We aimed to determine the Syk-mediated thrombogenic activity of several collagen peptides and (fibrillar) type I and III collagens. In platelet suspensions, only collagen peptides containing the consensus GPVI-activating sequence (GPO)n and Horm-type collagen evoked Syk-dependent intracellular Ca^{2+} rises. In whole blood under flow, Syk inhibition suppressed platelet activation and aggregation parameters for the collagen peptides with or without a (GPO)n sequence and for most of the fibrillar collagens.

I have been part of designing this project, as well as collecting and analysing experimental data. Likewise, I contributed to drafting the manuscript, writing (introduction, methods, results) and addressed reviewers' comments. For these reasons, I am co-first author of this paper.

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Role of platelet glycoprotein VI and tyrosine kinase Syk in thrombus formation on collagen-like surfaces

Natalie J. Jooss,[1*] Ilaria De Simone,[1*] Isabella Provenzale,[1*] Delia I. Fernandez,[1*] Sanne L. N. Brouns,[1] Richard W. Farndale,[2] Yvonne M. C. Henskens,[3] Marijke J. E. Kuijpers,[1] Hugo ten Cate,[1,5] Paola E. J. van der Meijden,[1*] Rachel Cavill,[4*] Johan W. M. Heemskerk[1†]

Departments of Biochemistry[1] and Data Science and Knowledge Engineering[4], Cardiovascular Research Institute, Maastricht University; Central Diagnostic Laboratory[3] and Department of Internal Medicine[5], Maastricht University Medical Centre, Maastricht, The Netherlands. Department of Biochemistry[2], University of Cambridge, Cambridge, United Kingdom

***** Equal contribution

† Correspondence: jwm.heemskerk@maastrichtuniversity.nl; Tel.: +31-43-3881671 (3881674) (NL)

Platelet interaction with collagens, via von Willebrand factor, is a potent trigger of shear-dependent thrombus formation mediated by subsequent engagement of the signalling collagen receptor, glycoprotein (GP)VI, enforced by integrin $\alpha_2\beta_1$. Protein tyrosine kinase Syk is central in the GPVI-induced signalling pathway leading to elevated cytosolic Ca^{2+}. We aimed to determine the Syk-mediated thrombogenic activity of several collagen peptides and (fibrillar) type I and III collagens. High-shear perfusion of blood over microspots of these substances resulted in thrombus formation, which was assessed by eight parameters, indicative of platelet adhesion, activation, aggregation and contraction, which were affected by the Syk inhibitor, PRT-060318. In platelet suspensions, only collagen peptides containing the consensus GPVI-activating sequence $(GPO)_n$ and Horm-type collagen evoked Syk-dependent Ca^{2+} rises. In whole blood under flow, Syk inhibition suppressed platelet activation and aggregation parameters for the collagen peptides with or without $(GPO)_n$ sequence and for all the collagens. Prediction models, based on regression analysis, indicated a mixed role of GPVI in thrombus formation on fibrillar collagens, which was abolished by Syk inhibition. Together, these findings indicated that GPVI-dependent signalling through Syk supports platelet activation in thrombus formation on collagen-like structures regardless of the presence of a $(GPO)_n$ sequence.

Introduction

Platelet interaction with subendothelial collagen is a crucial step in haemostasis or arterial thrombosis after vascular injury or rupture of an atherosclerotic plaque, respectively.[1,2] In blood flowing to high shear rates, the initial capture of platelets is mediated by von Willebrand factor (VWF), which avidly binds to collagens and is a ligand for the glycoprotein (GP) complex GPIb-V-IX.[3] The two platelet collagen receptors, integrin $\alpha_2\beta_1$ and GPVI, ensure stable platelet adhesion and mediate platelet activation.[4,5] For over twenty years, GPVI has been recognised as the central signalling collagen receptor on platelets.[6,7]

Studies using genetically modified mice have shown that the (patho)physiological process of arterial thrombus formation can be approximated using microfluidics devices, in which whole-blood is perfused over a collagen surface.[8] The collagen fibres immobilised in such devices, for instance applied as microspots, also bind plasma VWF, and thus promote shear-dependent adhesion, activation and aggregation of platelets.[3,9] Previous results have revealed a strong interplay of GPIb-V-IX, GPVI and integrin $\alpha_2\beta_1$ in the formation of large and stable multi-layered thrombi, in a way that the two other receptors enforce the GPVI-induced activation of platelets.[10,11] Markedly, the thrombi that were formed on collagen fibres appeared to be heterogeneous in structure with, on the one hand, patches of aggregated platelets with activated integrin $\alpha_{IIb}\beta_3$ binding fibrinogen and with CD62P expression via α-granule secretion and, on the other hand, single procoagulant platelets, exposing phosphatidylserine (PS) that is required for coagulation factor binding.[12] Particularly active in supporting thrombus formation is the standard collagen preparation 'Horm' (collagen-H), which is a fibrillar type I collagen, prepared in a proprietary process, that is commonly used for diagnostics in the clinical laboratory. Still unexplained is why other fibril-forming type I and III collagen preparations, also binding VWF, are less active in supporting thrombus formation under flow.[9,13]

In previous years, a number of synthetic collagen-derived triple-helical peptides have been identified which, similarly to collagen-H, bind to GPVI and/or integrin $\alpha_2\beta_1$, and thus induce platelet adhesion and activation *in vitro*.[10] Peptides containing the $(GPO)_n$ motif, in contrast to the supposedly inactive $(GPP)_n$ motif, bind to GPVI, whilst peptides with the GFOGER motif act as strong ligands for integrin $\alpha_2\beta_1$.[14-16] Prototypes of such triple-helical peptides are the cross-linked collagen-related peptide (CRP-XL), with a $(GPO)_{10}$ sequence, hence acting as a potent GPVI agonist; and the combined GFOGER-$(GPO)_n$ sequence, binding to platelets via both receptors. Subtle changes in the GFOGER sequence were found to alter the affinity for $\alpha_2\beta_1$. For instance, substitution of phenylalanine in GFOGER by alanine in GAOGER resulted in lower affinity $\alpha_2\beta_1$ binding, and in diminished platelet adhesion under static conditions.[17]

Platelet activation through GPVI,[18-20] but not via GPIb-V-IX,[21] relies on a potent protein tyrosine kinase cascade, culminating in activation of the tyrosine kinase Syk. This GPVI signalling pathway involves phosphorylation of the Fc receptor γ-chain via Src-family kinases, construction of a GPVI signalosome, in which Syk phosphorylates and activates phospholipase C (PLC)-γ2, causing a rise in the central second messenger, Ca^{2+}.[18,22-24] However, the relative importance of this pathway is not so far investigated in platelets interacting under flow with surface-immobilised collagen peptides or fibrillar collagens.

In the present paper, we aimed to investigate the role of the GPVI-Syk pathway in the thrombus formation on collagen-like surfaces at high shear rate. In particular, we assessed the sub-processes of platelet adhesion, aggregation and contraction as well as specific platelet activation responses. For this purpose, we used a panel of collagens, collagen peptides and collagen-H (with established GPVI dependency), and the selective Syk inhibitor PRT-060318 (Syk-IN). The latter compound has recently been used to specify Syk-dependent pathways in mouse platelets[21,25] and in human T cells[26]. As a direct readout of this signalling pathway, we also assessed the Syk-dependent rises in cytosolic Ca^{2+}.

Results

GPVI-Dependent and Syk-Dependent Platelet Activation by Collagen Peptides

As a first estimation of the potency of distinct collagen peptides to act as a ligand for platelet GPVI, we examined their ability to stimulate the PLCγ2-mediated rises in cytosolic Ca^{2+} using Fura-2-loaded platelets. As a selective inhibitor of the GPVI pathway, we used the compound PRT-060318 (Syk-IN), which has earlier been used to identify Syk-dependent activation processes in platelets mediated by GPVI[21,25] or CLEC2[27]. The Syk-IN compound was found to phenocopy the effects of Syk depletion on platelet responses in *Syk*$^{-/-}$ bone marrow chimeric mice.[28] Moreover, in human platelets, Syk-IN selectively blocked the GPVI/Syk-dependent tyrosine phosphorylation and aggregation responses induced by fibrin.[29]

To confirm the selectivity of Syk-IN as an inhibitor of GPVI-induced responses of human platelets, we monitored its effect (using 5 µM throughout) on the aggregation of platelets induced by CRP-XL, thrombin or stable ADP. This inhibitor caused complete inhibition of aggregation only with the GPVI agonist CRP-XL, whereas with thrombin or ADP it was ineffective (Figure S1A). This is in agreement with earlier studies performed with mouse platelets.[21,25] Additional control experiments with Fura-2-loaded human platelets indicated that Syk-IN did not suppress the thrombin- or ADP-induced Ca^{2+} rises (Figure S1B).

Table 1. Overview of composition of microspots (*M1-9*), platelet receptors implicated in thrombus formation. Also indicated are analysed thrombus parameters (*P1-8*) from brightfield and fluorescence microscopic images. Measured ranges and scaling for heatmap analysis were as indicated.

	Microspot	Platelet Receptors		
		GPVI	$\alpha_2\beta_1$	*GPIb*
M1	GFOGER-GPO + VWF-BP	++	++	++
M2	CRP-XL + VWF-BP	++	o	++
M3	GAOGER-GPO + VWF-BP	++	+	++
M4	GFOGER-GPP + VWF-BP	(o)*	++	++
M5	VWF-BP	o	o	++
M6	collagen-H (Horm type)	++	++	++
M7	collagen-I (human)	n.a.	n.a.	++
M8	monomeric collagen-I (human)	n.a.	n.a.	++
M9	collagen-III (human)	n.a.	n.a.	++
	Parameter	range	scaling	
	Bright-Field Images			
P1	**platelet deposition (% SAC)**	0–51.52	0–10	
P2	platelet aggregate coverage (% SAC)	0–21.09	0–10	
P3	thrombus morphological score	0–4.10	0–10	
P4	thrombus multilayer score	0–2.60	0–10	
P5	thrombus contraction score	0–2.94	0–10	
	Fluorescence Images			
P6	PS exposure (% SAC)	0–13.91	0–10	
P7	CD62P expression (% SAC)	0–46.71	0–10	
P8	fibrinogen binding (% SAC)	0–28.33	0–10	

* No GPVI-activating $(GPP)_n$ motif.

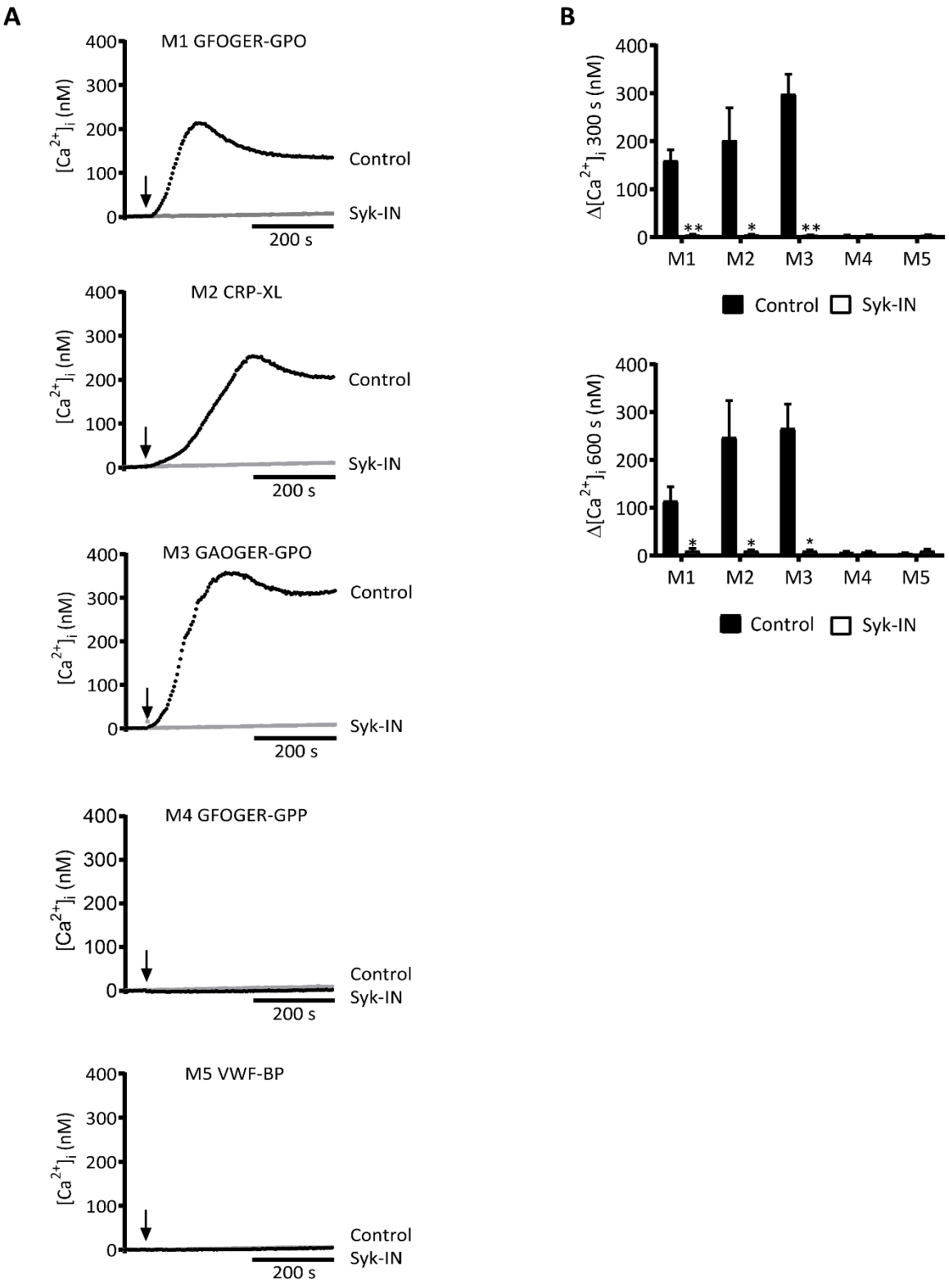

Figure 1. Syk inhibition affecting platelet Ca^{2+} rises by collagen peptides with $(GPO)_n$ motif. Fura-2-loaded platelets in 96-well plates were pre-incubated with Syk-IN (5 µM) or left untreated before stimulation with collagen peptide (*M1-5*, 10 µg/ml). Changes in $[Ca^{2+}]_i$ were recorded over time per well-plate row by ratio fluorometry using a FlexStation 3. Peptides were injected into wells at 60 s (arrow), and reached platelets in a diffusion limited way. **(A)** Calibrated $[Ca^{2+}]_i$ traces, recorded during 600 s in the

absence (black, control) or presence (grey) of Syk inhibitor. Traces are representative of 3 experiments. **(B)** Quantification for *M1-5* of increased $[Ca^{2+}]_i$ at 300 s (top graph) or 600 s (bottom graph). Means ± SEM (n=3). Paired Student t-tests; **p*<0.05, ***p*<0.01.

Using Syk-IN, we then evaluated the role of Syk in platelet Ca^{2+} fluxes, induced by several collagen peptides presumed to be GPVI-dependent or -independent. With three peptides containing the consensus GPVI-activating motif $(GPO)_n$, *i.e*. GFOGER-GPO (for convenience designated as *M1*, see Table 1), CRP-XL (*M2*) and GAOGER-GPO (*M3*), we observed a potent rise in $[Ca^{2+}]_i$, which was fully abolished in the presence of Syk-IN (Figure 1). Close examination of the Ca^{2+} traces showed some differences between *M1-3* in onset and maximum value. The reason for these differences is unclear, but it may be linked to variations in peptide conformation or in GPVI clustering capacity of the triple-helical peptide in question. On the other hand, two other collagen peptides, containing a $(GPP)_n$ motif instead of $(GPO)_n$, were unable to induce $[Ca^{2+}]_i$ rises; these were GFOGER-GPP (*M4*) and the VWF-binding peptide (*M5*). These Ca^{2+} traces were not influenced by the presence of Syk-IN. Overall, these results indicated a high Syk-dependency of the platelet $[Ca^{2+}]_i$ rises that were induced by the $(GPO)_n$-containing collagen peptides, containing an established GPVI-activating sequence.

GPVI- and Syk-Dependent Parameters of Thrombus Formation on Collagen Peptides

To assess how the five collagen peptides supported whole-blood thrombus formation, we applied these as microspots (*M1-5*) in a microfluidic device, as described before.[9] The microspots were supplemented with VWF-BP (binding plasma VWF) to allow GPIb-V-IX-mediated trapping of platelets. Whole-blood perfusion was performed at a wall-shear rate of 1000 s^{-1}. By end-stage (3.5 min) multicolour microscopic imaging, it was possible to analyse up to eight thrombus and platelet characteristics: overall platelet deposition (parameter *P1*, see Table 1); platelet aggregation (*P2*); thrombus signature, *i.e.* morphology, multilayer and contraction (*P3-5*); platelet procoagulant activity,

measured as PS exposure (*P6*); and the platelet activation parameters CD62P expression (*P7*), and fibrinogen binding to activated integrin $\alpha_{IIb}\beta_3$ (*P8*).

Typically, the collagen peptides containing $(GPO)_n$ (*M1-3*) produced large thrombi with aggregated platelets with high levels of activation markers, *i.e.* PS exposure, CD62P expression and integrin activation (Figure 2). In contrast, the non-GPVI-stimulating $(GPP)_n$ peptide, GFOGER-GPP (*M4*) caused formation of smaller thrombi, with residual CD62P expression and integrin activation, but essentially no PS exposure. Quantification of the raw image data confirmed high parameter values for all $(GPO)_n$ microspots *M1-3*, indicating a strong ability to support thrombus formation (Figure S2). Interestingly, when comparing the two GFOGER peptides with or without the GPVI-binding motif (*M1* or *M4*), the latter still induced residual platelet activation, in spite of lower thrombus signature scores (*P4-5*) and limited PS exposure (*P6*). Furthermore, *M1* (GFOGER) with a supposedly higher affinity $\alpha_2\beta_1$ binding motif than *M3* (GAOGER), was less effective in promoting almost all thrombus parameters (*P1-2,3-5,7,8*). The differences between microspots were visualised in a univariate scaled heatmap of all parameters (Figure 3A). Together, the data suggested that the earlier distinction made between high- and low-affinity $\alpha_2\beta_1$-binding sites established under static conditions,[11,15] becomes confused in part when immobilised collagen peptides are exposed to platelets in flowed whole-blood. On the other hand, the apparent lack of both GPVI- and $\alpha_2\beta_1$-binding sites, as in *M5*, resulted in almost no stable platelet adhesion and activation.

Parallel flow runs on all microspots *M1-5* were performed with blood samples pre-treated with Syk-IN (max. effective dose of 20 µM) or DMSO vehicle. This resulted in marked reductions in the majority of thrombus parameters (Figure 3A). A subtraction heatmap, pinpointing only relevant changes (p<0.05), indicated that for *M1-4* essentially all parameters except for *P1* (platelet deposition) were reduced by Syk inhibition (Figure 3B). Most drastic complete reductions were seen for PS exposure (*P6*) on the 'active' $(GPO)_n$ surfaces *M1-3*. Surprisingly, Syk inhibition also affected platelet activation at the

supposedly non-GPVI $(GPP)_n$ surface *M4*. The other microspot *M5* was inactive in the absence of Syk-IN.

A summative plot was made indicating how individual (scaled) parameters were changed by Syk inhibition across all microspots (Figure 3C). This revealed a complete reduction in *P6* (PS exposure), along with strong reductions in *P2* (aggregate coverage), *P4* (thrombus multilayer), *P5* (thrombus contraction) and *P8* (fibrinogen binding). Less affected were *P3* (thrombus morphology) and *P7* (CD62P expression).

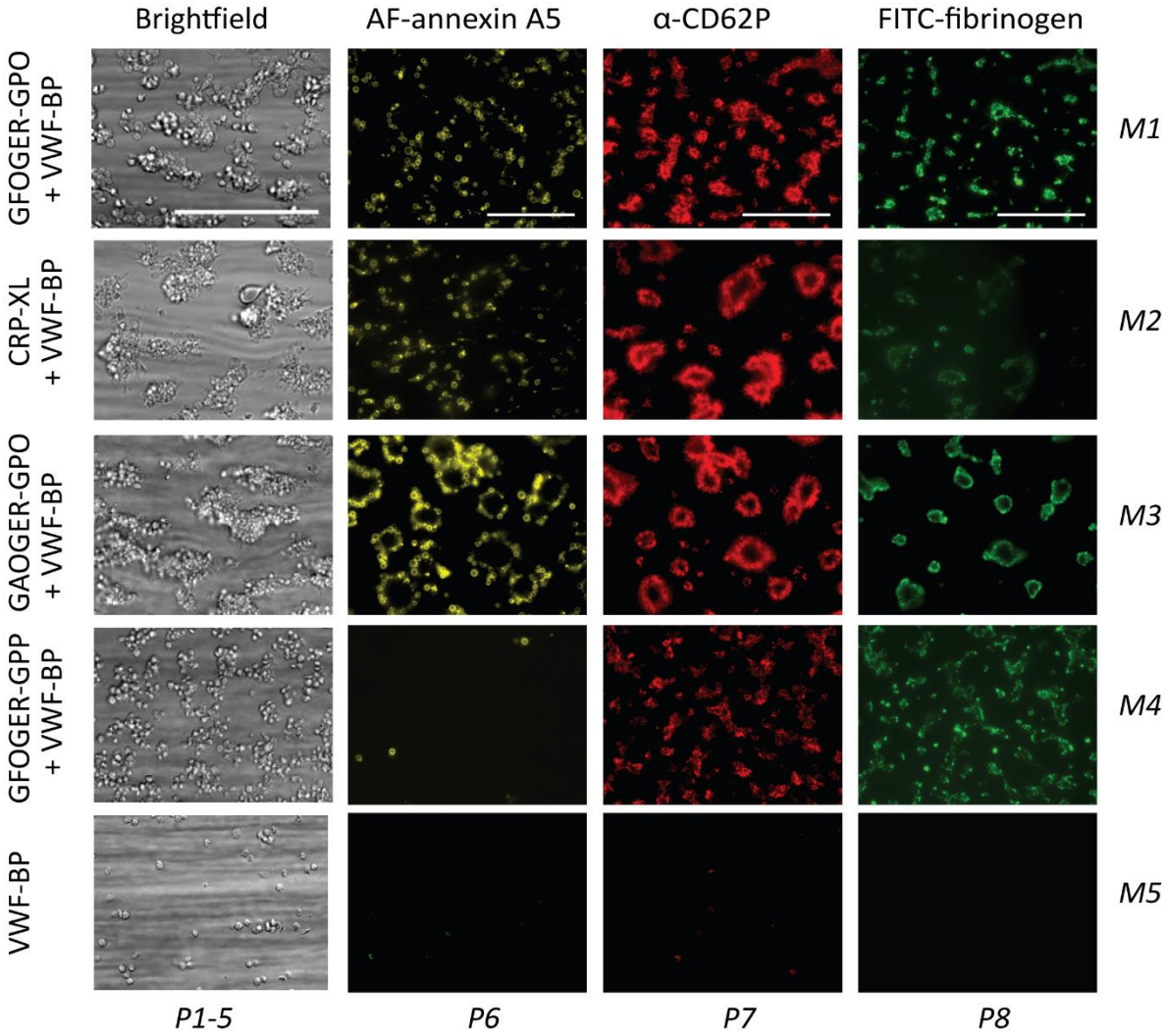

Figure 2. Thrombus formation on immobilised collagen peptides with or without $(GPO)_n$ motif. Whole-blood was perfused over microspots *M1* (GFOGER-GPO + VWF-BP), *M2* (CRP-XL + VWF-BP), *M3* (GAOGER-GPO + VWF-BP), *M4* (GFOGER-GPP + VWF-BP), and *M5* (VWF-BP), with assumed platelet adhesion via GPIb, GPVI and/or integrin $\alpha_2\beta_1$, as in Table 1. Wall-shear rate was 1000 s^{-1} at perfusion time of 3.5 min. Shown are representative brightfield microscopic images at end-stage, for analysis of platelet deposition (parameter *P1*) and thrombus characteristics (*P2-5*). In addition, end-

stage 3-colour fluorescence images for analysis of PS exposure (AF568 annexin A5, *P6*), CD62P expression (AF647 α-CD62P, *P7*), and fibrinogen binding (FITC, *P8*). Scale bars represent 50 µm.

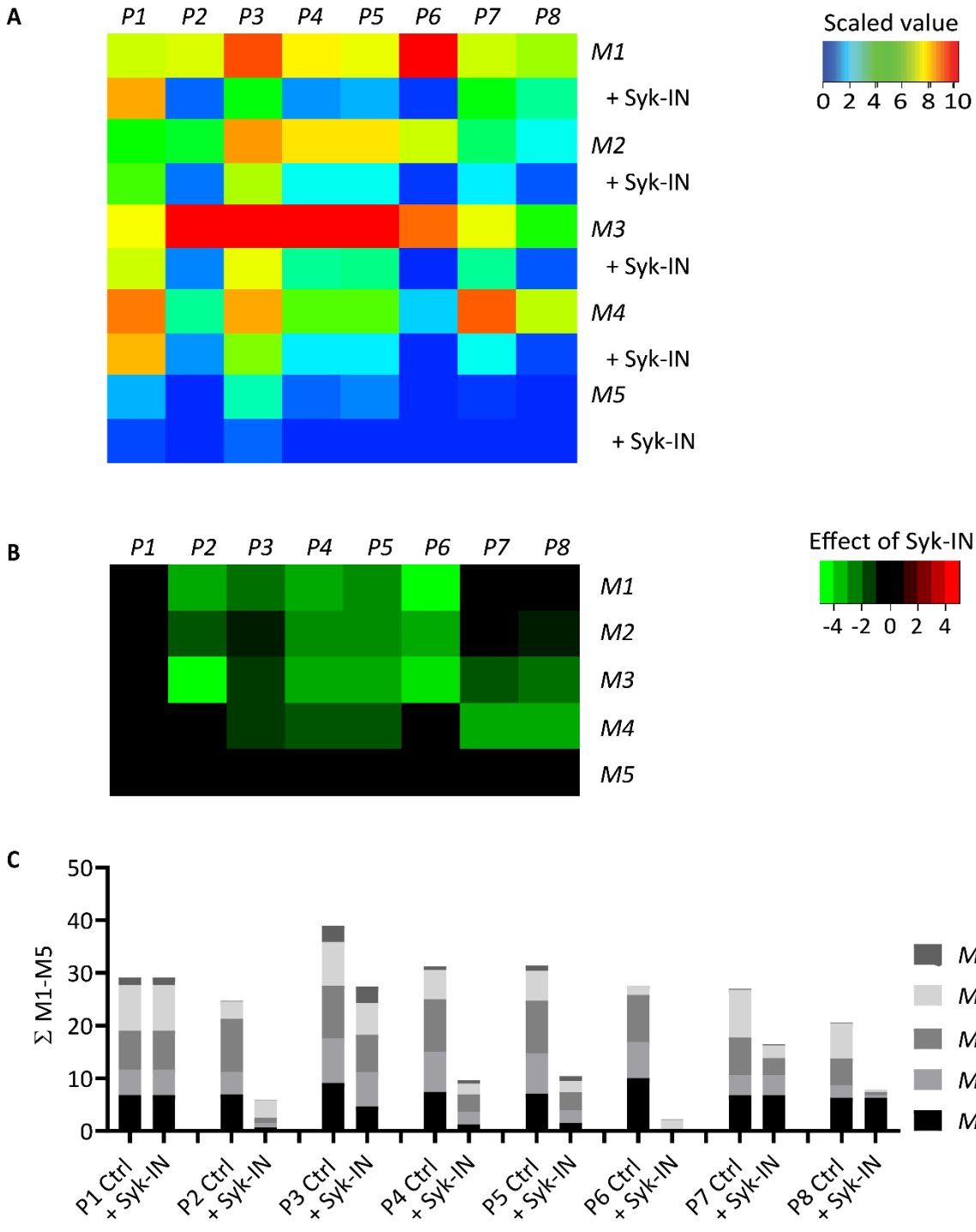

Figure 3. Effect of Syk inhibition on parameters of thrombus formation on immobilised collagen peptides. Blood samples pre-incubated with vehicle (Ctrl) or Syk-IN (20 µM) were flowed over microspots *M1-5,* and thrombi formed were imaged to obtain parameters *P1-8*, as in Figure 2. Effects of Syk-IN were assessed per blood sample, surface and parameter. Mean values from individual blood samples (n=5-7)

were univariate scaled to 0-10 per parameter across all surfaces *M1-9*. **(A)** Heatmap of scaled parameters, demonstrating mean effects of Syk-IN. Rainbow colour code indicates scaled values between 0 (blue) and 10 (red). **(B)** Subtraction heatmap, representing scaled effects of Syk-IN, filtered for relevant changes (p<0.05, paired Student t-tests per surface and parameter). Colour code represents decreases (green) or increases (red) in comparison to control runs. **(C)** Cumulative inhibitory effect per parameter over all microspots, indicating relevant changes.

GPVI-Induced and Syk-Dependent Platelet Activation by Different Collagens

Subendothelial collagen types I and III are considered to be the major platelet-activating collagens in the vessel wall, acting via GPVI and $\alpha_2\beta_1$.[30] The equine standard collagen (collagen-H), likely a modified type I collagen, is the most commonly used collagen to study GPVI-induced platelet activation. This prompted us to compare four collagen preparations on their ability to support the GPVI-PLCγ2-Ca^{2+} activation pathway: *i.e.,* the fibrous collagen-H (*M6*), human fibrillar collagen-I (*M7*), a degraded collagen-I (*M8*), and human fibrillar collagen-III (*M9*). While realising that the very high-molecular mass of collagens results in a heterogeneous interaction with platelets in suspension, we evaluated the $[Ca^{2+}]_i$ rises induced by these collagens. Markedly, the four collagens (*M6-9*) evoked a biphasic rise in $[Ca^{2+}]_i$, with an initial plateau level and a later second phase, which was highest for *M7* and *M9* (Figure 4A, B). In absolute levels, the rises in $[Ca^{2+}]_i$ obtained with *M6,7,9*, at the late time point of 600 s, were 2-3 fold lower than those seen with the $(GPO)_n$-containing collagen peptides (Figure 4 *vs.* Figure 1). This difference is likely due to the high-molecular mass of the fibrillar-type collagens, slowing down the rate and extent of diffusion-limited interactions with platelets, but also the higher density of activation motif within the peptides. In addition, it appeared that Syk inhibition completely suppressed the $[Ca^{2+}]_i$ transients induced by the standard collagen-H (*M6*), but it did not alter the transients by other collagens (Figure 4). In the presence of indomethacin (10 µM, thromboxane A_2 pathway inhibitor), AR-C69931MX (10 µM, $P2Y_{12}$ receptor inhibitor) or MRS2179 (100 µM, $P2Y_1$ receptor inhibitor), the rises in $[Ca^{2+}]_i$ with

collagen I-III were suppressed by 15-28%, 31-32% or 17-31%, respectively, in a non-redundant way (data not shown). Taken together, this suggests the presence of a Syk-independent pathway of Ca^{2+} mobilisation of suspended natural collagens, which in part comes from autocrine activation mechanisms.

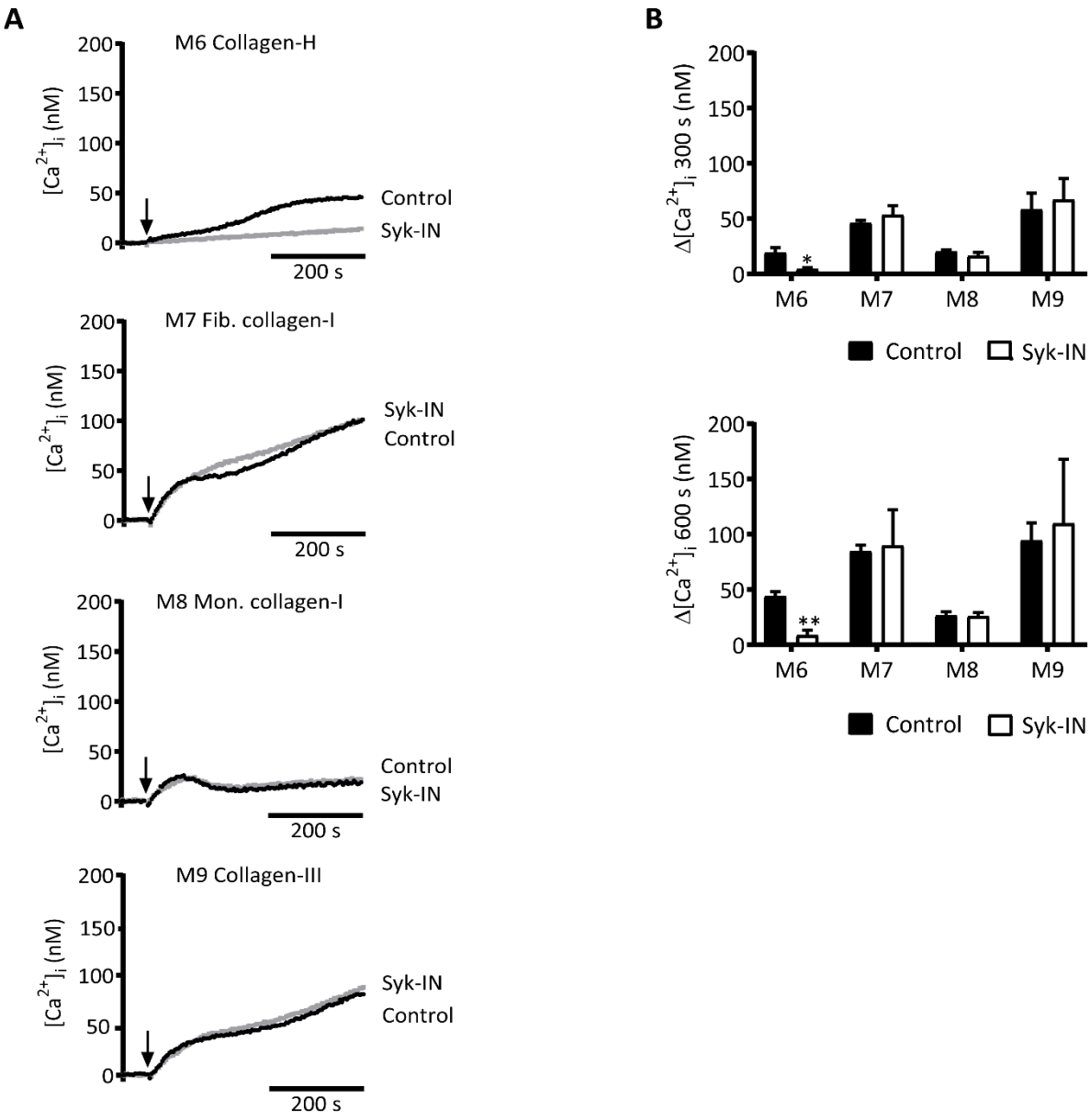

Figure 4. Syk inhibition differently affecting platelet Ca^{2+} rises by collagens. Fura-2-loaded platelets in 96-well plates were pre-incubated with Syk-IN (5 µM) or left untreated before stimulation with different collagens (*M6-9*, 10 µg/ml). Changes in $[Ca^{2+}]_i$ were continuously monitored per well-plate row by ratio fluorometry using a FlexStation 3. Collagens were injected at 60 s (arrow), and reached platelets in a diffusion limited way. **(A)** Calibrated $[Ca^{2+}]_i$ traces, recorded for 600 s in the absence (black, control) or

presence (grey) of Syk inhibitor. Traces are representative of 3 experiments. **(B)** Quantification of $[Ca^{2+}]_i$ rises after 300 s (top graph) and 600 s (bottom graph) for *M1-5*. Means ± SEM (n=3). Paired Student t-tests; **p*<0.05, ***p*<0.01.

GPVI- and Syk-Dependent Platelet Responses in Thrombus Formation on Collagens

The same collagen preparations (*M6-9*) were also applied as microspots to test their ability to support thrombus formation under flow. As indicated in Figure 5, collagen-H (*M6*) was most potent in provoking the formation of large aggregates of platelets with high PS exposure, granule secretion and fibrinogen binding, in agreement with the known high GPVI- and $\alpha_2\beta_1$-activating potency of this collagen when immobilised.[9,11,12] In comparison, the fibrillar-type I (*M7*) and III (*M9*) collagens formed only small aggregates of platelets with remaining secretion and fibrinogen binding, with only *M9* causing residual PS exposure (Figure 5). The degraded collagen-I (*M8*) caused mostly single platelet adhesion with incidental small-sized aggregates. The same information was obtained from the raw mean values of individual parameters with these surfaces (Figure S3).

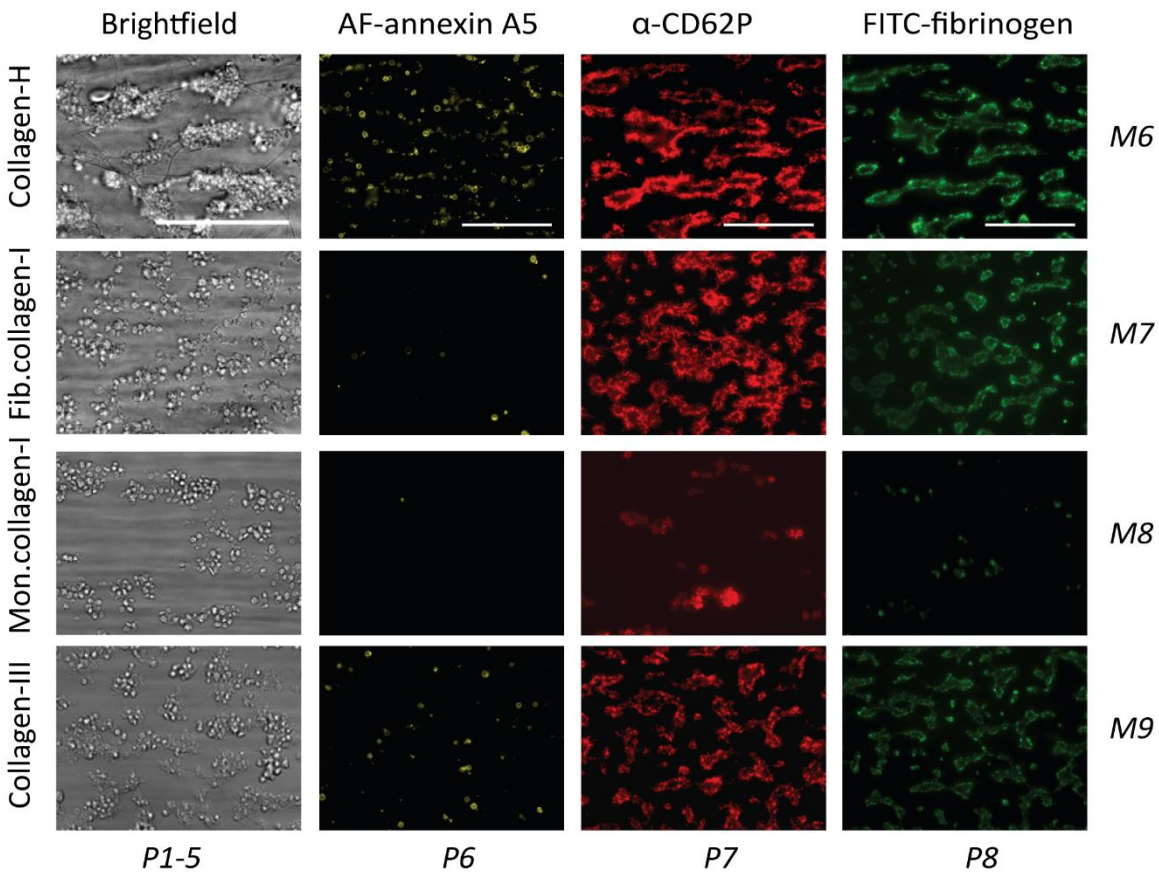

Figure 5. Thrombus formation on immobilised collagens. Whole-blood was perfused over microspots *M6* (collagen-H), *M7* (fibrillar collagen-I), *M8* (degraded collagen-I), *M9* (collagen-III). Wall-shear rate was 1000 s^{-1} and perfusion time 3.5 min. Shown are representative brightfield microscopic images at end-stage for analysis of platelet deposition (parameter *P1*) and thrombus characteristics (*P2-5*). In addition, end-stage 3-colour fluorescence images for analysis of PS exposure (AF568 annexin A5, *P6*), CD62P expression (AF647 α-CD62P, *P7*), and fibrinogen binding (FITC, *P8*). Scale bars represent 50 µm.

Heatmapping of the eight scaled parameter values confirmed that the overall microspot thrombogenicity decreased in the order of *M6* > *M7,9* > *M8* (Figure 6A). Treatment of the blood with Syk-IN left platelet deposition (*P1*) unchanged, but it decreased the thrombus signature and the platelet activation parameters (*P2-5, P7-8*) for several collagens. A subtraction heatmap was built with a filter for relevant changes (p<0.05). This showed for collagen-H (*M6*) as well as for fibrillar collagen-I and III (*M7,9*) a reduction in almost all parameters, except for *P1*, in the presence of Syk-IN (Figure 6B). Most reduced were the parameters of platelet aggregation and contraction (*P2,4,5*), and of platelet activation (*P6* for *M6*, and *P7-8* for *M7,9*).

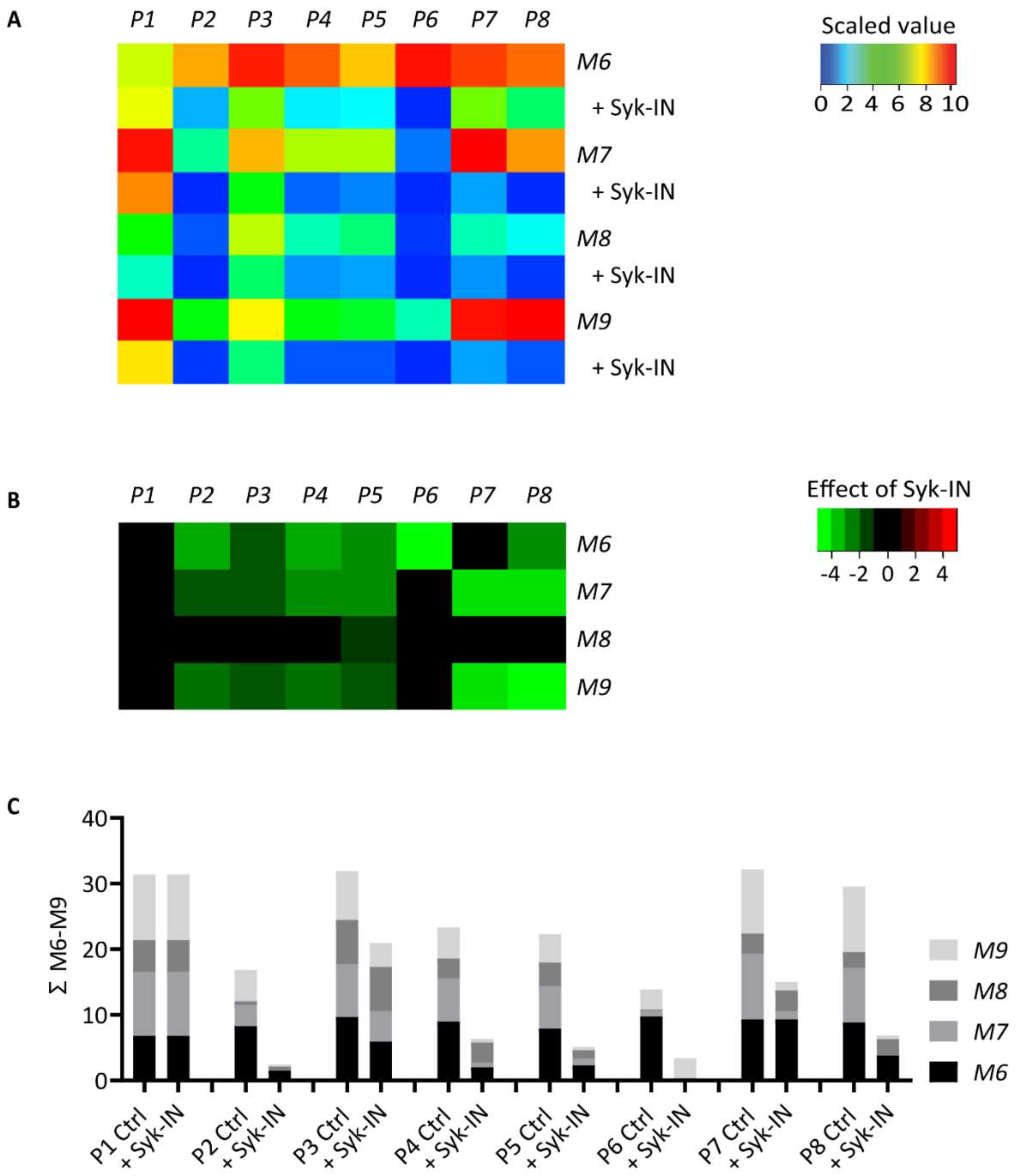

Figure 6. Effect of Syk inhibition on parameters of thrombus formation on immobilised collagen. Whole blood pre-incubated with vehicle (Ctrl) or Syk-IN (20 µM) was perfused over microspots *M6-9,* and thrombus formation was imaged to obtain parameters *P1-8*, as in Figure 5. Effects of Syk-IN were calculated per blood sample, surface and parameter. Mean values for all blood samples (n=5-7) were univariate scaled to 0-10 per parameter across all surfaces *M1-9*. **(A)** Heatmap of scaled parameters, showing mean effects of Syk-IN. Rainbow colour code gives scaled values between 0 (blue) and 10 (red). **(B)** Subtraction heatmap representing scaled effects of Syk-IN, filtered for relevant changes (p<0.05, paired Student t-tests per surface and parameter). Colour code represents decreases (green) or increases (red) in comparison

to control runs. **(C)** Cumulative inhibitory effect over all microspots per parameter, indicating relevant changes from control runs.

To obtain an overall insight into the effect of Syk inhibition, a summative plot was again constructed for each scaled parameter across all collagen microspots (Figure 6C). Importantly, this revealed a highly similar effect of Syk inhibition, as previously seen for the collagen peptides. Summing up the values for *M6-9*, we noticed a near-complete reduction in *P6* (*M6*, PS exposure), along with strong reductions in *P2* (platelet aggregate coverage), *P4* (thrombus multilayer), *P5* (thrombus contraction) and *P7* (CD62P expression), *P8* (fibrinogen binding), as compared to vehicle-treated blood. Less affected by Syk inhibition was *P3* (thrombus morphology), while platelet adhesion (*P1*) was unchanged.

In view of a possible role of GPVI also for platelet interaction with collagen type IV, additional whole-blood flow runs (n=3 donors) were performed with collagen IV microspots. Under control conditions, we noticed a similar pattern of thrombus formation as for collagen-I (*M7*) or collagen-III (*M9*). In the presence of Syk-IN, all parameters on collagen-IV were significantly reduced ($p<0.01$), with the exception of *P2* and *P6*. Across all parameters, the median inhibitory effect of Syk-IN for collagen-I, -III and -IV was 87.8%, 88.0% and 85.7%, respectively (data not shown). Hence, we observed a similar extent of thrombus inhibition by Syk-IN for all these fibrillar collagens

Modelling of Role of GPVI in Thrombus Formation on Various Collagens

We then applied regression analysis to provide a systematic examination of the generated data (*M1-9*), consisting of 416 data points (52 mean control flow runs of 9 surfaces, 8 parameters), to reveal the GPVI dependency of each surface. First, a partial least square (PLS) regression model was generated for collagen peptides *M1-3,* with an assumed high GPVI dependency, and for *M4-5* with supposedly no role of GPVI, after which the data from *M6* (collagen-H) were entered into the model. This analysis resulted in relevant components 1 and 2, explaining 68 and 15% of the variation, respectively

(Figure S4). The principal component plot indicated a tight cluster of flow runs with *M1-3,6*. Data of *M5* (negative component 1) and data of *M4* (negative component 2) lay further out in the model. This agrees with the large observed differences in (parameters of) thrombus formation on *M4* and *M5*. The calculated beta matrix indicated that *P2-6* to a similar extent contributed to the modelled results.

Because of the separation of *M4-5* parameters, the 1-component model was used for further analysis. Prediction testing of the model showed near complete prediction accuracy for all surfaces, except for *M4* (because no component 2) (Table 2). The model was further used to predict the role of GPVI on the remaining collagen surfaces *M7-9*. For both fibrillar collagens (*M7,9*), the prediction for GPVI dependency was mixed, while it was negative for the degraded collagen-I (*M8*). Subsequently, we integrated into the model the second set of 416 data points of Syk-inhibited blood samples (52 mean flow runs with Syk-IN for 9 surfaces, 8 parameters) to predict the absence of GPVI activity. Markedly, across all surfaces, 51 out of all 52 samples predicted a negative GPVI dependency, wherein the only incorrectly predicted sample was just above the conventional 0.5 threshold value for right prediction. Taken together, the constructed PLS model indicated, in addition to complete GPVI-independency of all Syk-inhibited samples, no role of GPVI for surfaces *M5* and *M8*.

Table 2. Modelled PLS analysis (based on 1 component PCA) of range-scaled data for collagen peptides (*M1-5*) plus collagen-H (*M6*), with assumed GPVI dependency. Shown are means and ranges of prediction values. Predicted accuracy is given by numbers of mean flow runs per donor (control and Syk-IN); by default, a correct prediction was set at >0.5. In addition, back-prediction of GPVI dependency of mean flow runs per donor for *M7-9.* Prediction outcomes are given here in italic. Contribution of parameters to the prediction model was in the order of *P2-6 >> P1,7,8*.

Microspot	GPVI Dependency	Correctly Predicted		
		Range	Ctrl	Syk-IN
M1	positive	0.41–1.06	5/6	6/6
M2	positive	0.27–0.76	4/5	5/5
M3	positive	0.86–1.07	5/5	5/5
M4	negative	0.57–0.97	0/6	6/6
M5	negative	0.21–0.34	5/5	5/5
M6	positive	0.68–1.11	7/7	6/7
M7	mixed	0.44–0.85	5/7	7/7
M8	negative	0.13–0.41	0/5	5/5
M9	mixed	0.49–0.67	3/5	5/5

Discussion

Collagen Peptides and GPVI-Dependent Platelet Activation

The data obtained indicate a clear separation between effects of triple-helical collagen peptides that contain the established GPVI recognition motif, $(GPO)_n$,[15] and peptides that have a $(GPP)_n$ backbone instead. We found that the $(GPO)_n$-containing collagen peptides (*M1-3*): *(i)* induced high platelet Ca^{2+} rises under stasis; *(ii)* accomplished a fast build-up of thrombi with aggregated and activated platelets under flow; and *(iii)* evoked platelet responses both under flow and static conditions that were highly sensitive to inhibition of Syk. Accordingly, these peptides provided strong proof-of-principle evidence for potent stimulation of the GPVI-Syk-PLCγ2-Ca^{2+} pathway of platelet activation.

Immobilised, the $(GPO)_n$ peptide CRP-XL (*M2*), lacking an $\alpha_2\beta_1$ interaction motif, produced smaller size thrombi (low parameter values *P2-6*) than the other collagen peptides, which is in agreement with the known synergy between GPVI, integrin $\alpha_2\beta_1$ and GPIb-V-IX receptors in thrombus formation at high shear rate.[9-11] Synergy of GPVI and $\alpha_2\beta_1$ can also explain why peptides containing the integrin-binding motif G(F/A)OGER evoked a faster Ca^{2+} signal, when compared to CRP-XL. Seemingly in contrast with its lower binding affinity to platelets under stasis,[17] we observed higher

parameters of thrombus formation with GAOGER-GPO (*M3*) than with GFOGER-GPO (*M1*). The explanation for this higher activity remains unclear.

In contrast, the $(GPP)_n$-containing peptides GFOGER-GPP (*M4*) and VWF-BP (*M5*) did not evoke detectable Ca^{2+} rises in platelets under stasis. Yet, when immobilised under flow, the integrin-binding peptide GFOGER-GPP evoked low-parameter thrombus formation in terms of platelet activation and aggregation; and this activity was again suppressed by Syk inhibition. This may reflect a weak interaction of the $(GPP)_n$ motif with GPVI, reinforced by strong integrin binding activity.

Jointly these results pointed to a Syk-dependent role via GPVI in the support of thrombus formation. This conclusion was supported by a reanalysis of earlier experiments, where effects of the single-chain variable fragment antibody 10B12 were studied for the surfaces *M1*, *M5* and *M6.*[16] Markedly, image reanalysis, providing the parameters *P1,3-6,* indicated a similar effect pattern of 10B12 as presently seen with Syk-IN (not shown).

Collagens and GPVI-Dependent Platelet Activation

Fibrillar type I and type III collagens are among the vessel wall components that most strongly activate platelets.[7,30] Due to the structural complexity of multiple adjacent triple helices in these collagens, little is known about how platelet receptors bind to the fibrils, although there is evidence that the co-presence of multiple binding sites in a collagen fibre enforces platelet adhesion and activation.[31,32] Recent high-resolution microscopy indicates that multiple copies of GPVI dimerise and cluster along the fibres of such collagens, a process that is considered to enforce GPVI-dependent platelet activation.[33,34] Previous sequence analysis has shown that both type I and III collagens are made for up to 10% of GPO triplets, with $\alpha_2\beta_1$-binding sequences present in both cases, *e.g.* GFOGER in collagen-I and GAOGER in collagen-III.[35]

Here we compared the effects of preparations of human fibrillar collagen-I and collagen-III with the standard collagen-H, *i.e.* a commercial equine type I-enriched preparation with less defined supramolecular characteristics.[36] Markedly, added to suspended platelets, collagen-H (*M6*) was the only collagen that induced Syk-dependent $[Ca^{2+}]_i$ rises, whereas the other collagens (*M7,9*) induced low $[Ca^{2+}]_i$ rises that were insensitive to Syk inhibition. When microspotted, collagen-H triggered the formation of large-size thrombi, with high parameters of platelet aggregation and activation, *i.e.* responses that are known to be strongly GPVI-dependent,[9] and which in the present setting were consistently affected by Syk inhibition.

In addition, we tested a protease-treated, monomeric collagen-I preparation (*M8*), which appeared to be inactive in supporting thrombus formation with no appreciable effect of Syk inhibition. This finding supports the notion that the fibrillar structure of immobilised collagens helps to expose receptor (GPVI) binding sites upon stretching under flow conditions.

In comparison to collagen-H, the immobilised type-I (*M7*) and type-III *(M9*) collagens triggered formation of smaller thrombi with lower platelet activation parameters. Yet, for the fibrillar collagens, the summed suppressive effects of Syk inhibition were remarkably similar to those seen for collagen-H and the $(GPO)_n$-containing collagen peptides. Given the similar abundance of GPO triplets in both collagen-I and -III,[15] these findings point to a limited role of GPVI-induced activation under flow conditions. In agreement with such a role for GPVI, others have shown that immobilized collagens can induce GPVI dimer clustering in adhered and spreading platelets.[34] In this setting, immobilized collagen-III was found to be more effective in cluster formation than collagen-H or CRP-XL. Furthermore, inhibition of Syk did not abrogate the GPVI clustering. These findings suggest that there is not a direct link between GPVI cluster formation and strength of the GPVI-Syk-PLCγ2 signal. However, under flow conditions, the additional involvement of

VWF/GPIb-V-IX and integrin $\alpha_2\beta_1$ interactions[13,16] might enforce the GPVI clustering pattern, but this still needs to be demonstrated.

Comparative Roles of GPVI and Syk in Platelet Activation

A remarkable finding was that Syk inhibition also affected parameters of thrombus formation on surfaces that were considered to act independently of GPVI (*i.e.*, GFOGER-GPP, *M4*) or with a low GPVI dependency (collagen-I, *M7*; collagen-III, *M9*). As another approach to examine this, a PLS model was constructed and used for principal component analysis. The PCA plots indicated a narrow cluster for all high GPVI-activating surfaces (*M1-3,6*), with data of *M4,5* partly centring out. Prediction of the role of GPVI for other surfaces gave a mixed outcome for the fibrillar collagens (*M7,9*), whereas this was negative for the degraded collagen-I (*M8*). Importantly, the prediction model revealed a consistent GPVI independence for the Syk-inhibited samples, regardless of the type of microspot composition. Accordingly, this analysis supported the indication of low level GPVI and Syk activity at these weakly thrombogenic surfaces.

In recent years, evidence has been accumulated for a role of GPVI signalling in platelets also contacting non-collagen surfaces. For instance, GPVI dependency has been discovered for platelets interacting with laminin,[37] fibrin,[29,38] or fibrinogen[39,40]. In this context, it is also likely that also for the $(GPP)_n$ containing surfaces the Syk-dependent platelet responses can be traced back to residual GPVI activity. On the other hand, based on early studies, it cannot yet be excluded that (part of) the Syk-dependent platelet responses in thrombus formation at 'weaker' surfaces are mediated by signalling via integrin $\alpha_{IIb}\beta_3$,[41-43] hence bypassing GPVI. This will need to be studied by using specific GPVI-inhibitory tools.

Conclusion

The present data reveal typical differences of preparations of collagens or collagen peptides if used in suspension with platelets, or when immobilised as microspots and subjected to whole-blood flow (Figure 7). Especially for the 'weaker' fibrillar collagens, immobilisation appeared to enhance the signalling capability of GPVI, thus stimulating Syk-dependent platelet activation processes in thrombus formation. Apart from changes in the (immobilised) collagen structure, other factors that may contribute to the enhanced signalling capacity are the shear-dependent interaction of GPIb-V-IX with collagen-bound VWF and the priming of platelet activation via integrin $\alpha_2\beta_1$. These and perhaps also other receptor interactions with collagen fibres may ensure increased activation of the GPVI-PLCγ2-Ca^{2+} pathway.

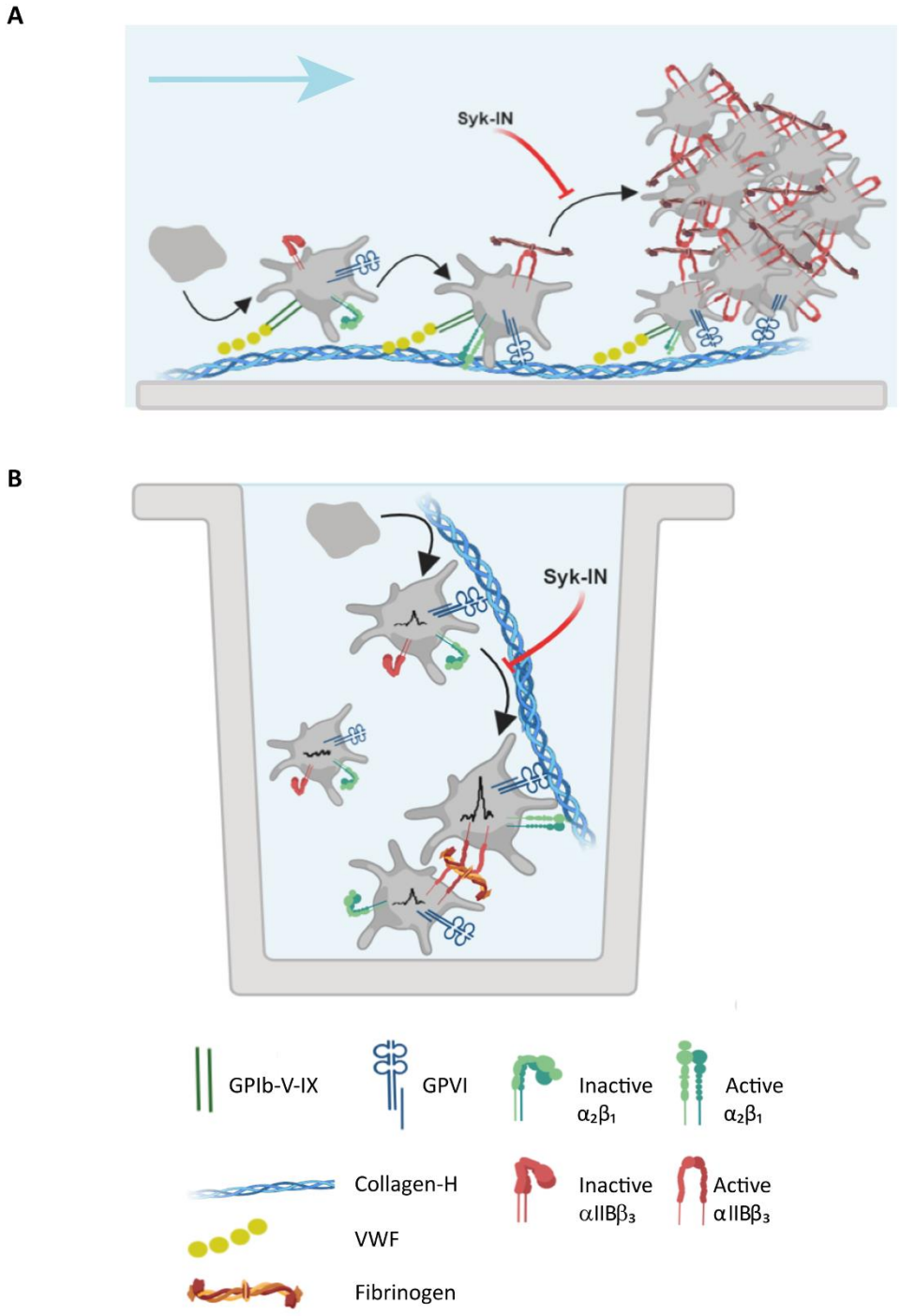

Figure 7. Schematic platelet adhesion and activation by collagen under flow or in suspension. (A) Under flow conditions, immobilised collagen-H interacts with VWF to capture platelets via GPIb-V-IX, and activate platelets via GPVI and integrin $\alpha_2\beta_1$. Thrombi build up by recruitment of flowing platelets interacting with collagen/VWF-adhered platelets. Syk inhibition suppresses initial platelet activation and platelet aggregate formation. (B) Collagen-H added to a suspension of platelets transiently interacts with GPVI, resulting in Syk-dependent Ca^{2+} rises. Autocrine agonists will stimulate non-adhered platelets, responding by Syk-independent signals.

Materials and Methods

Materials

Collagen-related triple-helical peptides were synthesised as C-terminal amides and purified by reverse phase high performance liquid chromatography[44,45]: H-GPC(GPO)$_3$GFO GER(GPO)$_3$GPC-NH_2 (GFOGER-GPO); H-GPC(GPP)$_5$GFOGER(GPP)$_5$GPC-NH_2 (GFOGER-GPP); cross-linked collagen-related (GPO)$_n$ peptide (CRP-XL); GPC(GPO)$_3$GAOGER(GPO)$_3$GPC-NH_2 (GAOGER-GPO); collagen type-III derived VWF-binding peptide VWF-III (VWF-BP), H-GPC(GPP)$_5$GPRGQOGVMGFO(GPP)$_5$GPC-NH_2.[46] Collagen-I Horm derived from equine tendon (collagen-H) was obtained from Nycomed (Hoofddorp, The Netherlands); Human placenta-derived collagen-III (C4407), collagen-IV (C7521), and fibrillar collagen-I (C7774) came from Sigma-Aldrich (Zwijndrecht, The Netherlands). The latter was used to prepare monomeric collagen-I by pepsin treatment, as described.[47] The selective spleen tyrosine kinase (Syk) inhibitor PRT-060318, 2-((1*R*,2*S*)-2-aminocyclohexylamino)-4-(*m*-tolylamino)pyrimidine-5-carboxamide (Syk-IN) came from Bio-Connect (Huissen, The Netherlands). Used for fluorescence staining were: Alexa Fluor (AF)647-labelled anti-human CD62P mAb (304918, Biolegend, London, UK), FITC-labelled fibrinogen (F0111, Dako, Amstelveen, The Netherlands), and AF568-labelled annexin A5 (A13202, ThermoFisher, Eindhoven, The Netherlands). Fura-2 acetoxymethyl ester and pluronic were from Invitrogen (Carlsbad CA, USA). Human α-thrombin was from Kordia (Leiden, The Netherlands); stable ADP (Me-S-ADP) and MRS-2179 were from Sigma-Aldrich. Other materials were from sources described before.[48]

Blood Isolation

Blood was obtained by venepuncture from healthy volunteers, who had not received antiplatelet medication for at least two weeks. All subjects gave full informed consent

according to the declaration of Helsinki. Studies were approved by the local Medical Ethics Committee. Blood samples were collected into 3.2% trisodium citrate (Vacuette tubes, Greiner Bio-One, Alphen a/d Rijn, The Netherlands). All subjects had platelet counts within the reference range, as measured with a Sysmex XN-9000 analyser (Sysmex, Cho-ku, Kobe, Japan).

Platelet Isolation and Loading with Fura-2

Platelet-rich plasma (PRP) was obtained from citrated blood by centrifugation at 870 g for 10 min. After addition of 1:10 vol./vol. acid citrate dextrose (ACD; 80 mM trisodium citrate, 183 mM glucose, 52 mM citric acid), the isolated PRP was centrifuged at 2,360 g for 2 min. Platelet pellets were resuspended into Hepes buffer pH 6.6 (10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 5.5 mM glucose and 0.1 % bovine serum albumin). After addition of apyrase (1 U/ml) and 1:15 vol./vol. ACD, another centrifugation step was performed to obtain washed platelets.[48] The final pellet was resuspended in Hepes buffer pH 7.45.

Light Transmission Aggregometry

Aggregation of washed platelets was measured by light transmission aggregometry, as described[48] using an automated Chronolog aggregometer (Havertown PA, USA). Platelet aggregation rate was determined from maximal curve slopes (% transmission change per min).

Whole-Blood Microfluidic Perfusion over Microspots

Selected collagen-like peptides and collagens were microspotted on glass coverslips, essentially as described before.[9] Coding of nine microspots (*M1-9)* is displayed in Table 1. In brief, washed coverslips were coated with three different microspots, each containing a collagen (100 µg/ml) or a combination of collagen-like peptide (250 µg/ml) with VWF-BP (100 µg/ml). Coating doses were chosen to obtain maximal platelet

adhesion in flow assays.[9] The most active microspots were always located downstream, thus preventing cross-activation of platelets between microspots.[9] The coated coverslips were incubated overnight in a humid chamber at 4 °C, and then blocked with Hepes buffer pH 7.45 containing 1% bovine serum albumin for 30 min, before mounting into the Maastricht microfluidic chambers.

For flow perfusion, 500 µl of citrated whole blood was preincubated for 10 min with either vehicle (0.5% DMSO and 0.4 µg/ml pluronic, f.c.) or inhibitor PRT-060318 (Syk-IN, 20 µM in vehicle solution, f.c.). After the addition of 40 µM PPACK and recalcification (3.75 mM $MgCl_2$ and 7.5 mM $CaCl_2$), blood samples were perfused through a microspot-containing flow chambers for 3.5 min at a wall shear rate of 1000 s^{-1}. After 2 min of staining for PS exposure (AF568-annexin A5), CD62P expression (AF647 anti-CD62P mAb) and integrin $\alpha_{IIb}\beta_3$ activation (FITC fibrinogen), residual label was removed by post-perfusion with Hepes buffer pH 7.45, containing 2 mM $CaCl_2$ and 1 U/ml Heparin. Vehicle controls were performed in duplicate, while samples containing Syk-IN were repeated in triplicate, using blood obtained from >5 different healthy donors.

Brightfield and Fluorescence Microscopy

From each microspot, two brightfield images (during labelling) and three 3-colour fluorescence images (after removing label) were taken using an EVOS-FL microscope (Life Technologies, Bleiswijk, The Netherlands), equipped with Cy5, RFP and GFP LEDs, an Olympus UPLSAPO 60x oil-immersion objective, and a sensitive 1360 x 1024 pixel CCD camera.[49] Standardised image analysis was performed using semi-automated scripts operated in Fiji (ImageJ), as described before.[49] Parameters extracted from brightfield images (*P1-5*), including thrombus signature scores (*P3-5*), and parameters from fluorescence images (*P6-8*) are specified in Table 1.

Cytosolic Ca^{2+} Measurements

Washed human platelets (2 x 10^8/ml) were loaded with Fura-2 acetoxymethyl ester (3 µM) and pluronic (0.4 µg/ml) by a 40-min incubation at room temperature. After another wash step and resuspension of the platelets at the same concentration, changes in cytosolic $[Ca^{2+}]_i$ were measured in 96-well plates using a FlexStation 3 (Molecular Devices, San Jose, CA, USA). In brief, 200 µl of platelet suspension were pre-treated with Syk-IN (5 µM) for 10 min or were left untreated. After addition of 1 mM $CaCl_2$, the Fura-2-loaded cells were stimulated by automated pipetting with one of the following agonists (10 µg/ml), for convenience indicated as *M1-9* (see Table 1): GFOGER-GPO (*M1*), CRP-XL (*M2*), GAOGER-GPO (*M3*), GFOGER-GPP (*M4*), VWF-BP (*M5*), collagen-H (*M6*), fibrillar collagen-I (*M7*), monomeric collagen-I (*M8*) or collagen-III (*M9*).

Changes in Fura-2 fluorescence were measured over time at 37 °C by ratiometric fluorometry, at dual excitation wavelengths of 340 and 380 nm, and an emission wavelength of 510 nm. Agonist injection speed was set at 125 µl/s, resulting in a complete, diffusion-limited mixing. Separate wells contained Fura-2-loaded platelets that were lysed with 0.1% Triton-X-100 in the presence of either 1 mM $CaCl_2$ or 9 mM EGTA/Tris, for the determination of R_{max} and R_{min} values, respectively.[50] After correction for background fluorescence, $[Ca^{2+}]_i$ (as nM) was calculated from ratio values.[51] Measurements were performed in duplicate wells and completed within 2-3 h of preparation of the cells.

Data Handling and Statistics

GraphPad Prism 8 was employed for statistical analysis. Heatmaps were generated with the program R. For heatmap representation, all parameter values were univariate normalised at a scale of 0-10.[49] Thrombus values of duplicate or triplicate flow runs from the same blood donor were averaged to obtain one parameter set (vehicle or Syk-inhibited) per microspot and donor. Mean values of control and inhibitor runs were then

compared per blood sample, using paired Student t-tests. *P*-values below 0.05 were considered to be significant. For subtraction heatmaps, a standard filter of $p<0.05$ was set to visualise relevant effects.

Modelling to Predict GPVI Activity

Complete datasets (8 parameters, 9 surfaces) for flow runs of ≥5 donors were used to construct a partial-least square (PLS) model, to predict back for GPVI dependency. First, range-scaled data for the collagen peptide surfaces *(M1-5*) with known GPVI dependency were used to generate a PLS model, after which collagen-H (*M6*) then used to test the reliability of the model. Principal component analysis (PCA) in 1- and 2-component mode was then applied, the predictions of which were supported by cross-validated analysis of Q^2, defined as 1 - (PRESS/TSS).[52] Subsequently, parameter sets of *M7-9* were predicted for GPVI-dependency from the PLS model, as were parameters of *M1-9* in the presence of Syk-IN. By default, prediction values of >0.5 were considered as being positive for GPVI dependency.

Supplementary Materials: See separate file

Author Contributions: Conceptualization, JWMH, PEJvdM and MJEK; Methodology, NJJ, IDS, IP and DIF; Formal Analysis, NJJ, IDS, IP DIF, SLNB and RC; Investigation, NJJ, IDS, IP and DIF; Resources, RWF and JWMH; Data Curation, NJJ; Writing – Original Draft Preparation, NJJ, IDS, IP and DIF; Writing – Review & Editing, JWMH, RWF; Visualisation, NJJ, IDS, IP and DIF; Supervision, JWMH, YMCH, PEJvdM, MJEK and HtC; Funding Acquisition, JWMH, PEJvdM, MJEK and HtC.

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Supplementary Materials

Role of platelet glycoprotein VI and tyrosine kinase Syk in thrombus formation on collagen-like surfaces

Natalie J. Jooss,[1*] Ilaria De Simone,[1*] Isabella Provenzale,[1*] Delia I. Fernandez,[1*] Sanne L. N. Brouns,[1] Richard W. Farndale,[2] Yvonne M. C. Henskens,[3] Marijke J. E. Kuijpers,[1] Hugo ten Cate,[1,5] Paola E. J. van der Meijden,[1*] Rachel Cavill,[4*] Johan W. M. Heemskerk[1]†

Departments of Biochemistry[1] and Data Science and Knowledge Engineering[4], Cardiovascular Research Institute, Maastricht University; Central Diagnostic Laboratory[3] and Department of Internal Medicine[5], Maastricht University Medical Centre, Maastricht, The Netherlands. Department of Biochemistry[2], University of Cambridge, Cambridge, United Kingdom

***** Equal contribution

† Correspondence: jwm.heemskerk@maastrichtuniversity.nl; Tel.: +31-43-3881671 (3881674) (NL)

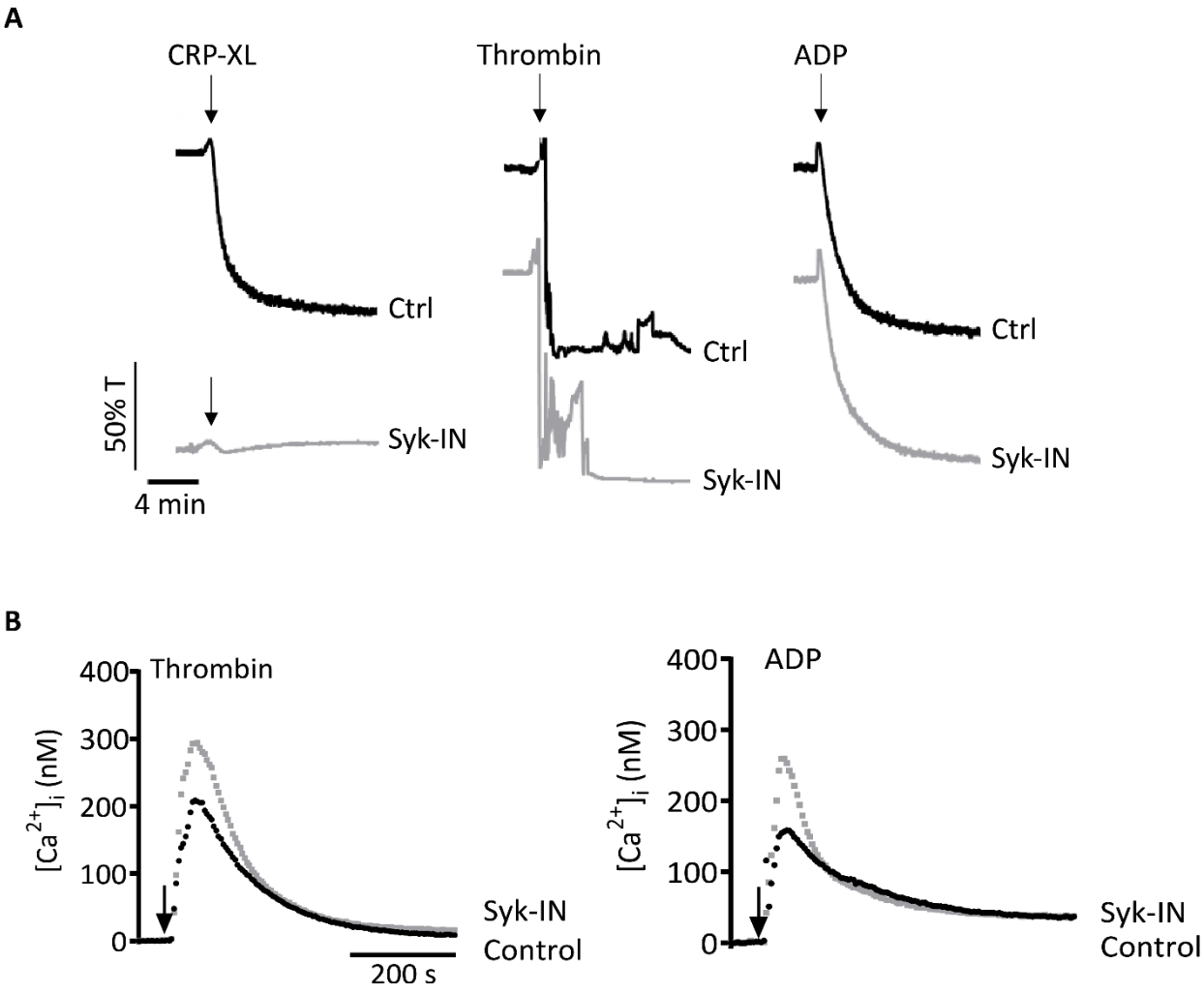

Figure S1. Effect of Syk inhibitor PRT-060318 (Syk-IN) on agonist-induced platelet responses. (A) Platelets in plasma (2.5 x 10^8/ml) were pre-incubated with vehicle (DMSO) or Syk-IN (5 µM) for 10 min, and then activated with CRP-XL (10 µg/ml), thrombin (8 nM) or stable ADP (5 µM), as indicated. Shown are representative traces from light transmission aggregometry. **(B)** Fura-2-loaded platelets in 96-well plates were pre-incubated with Syk-IN (5 µM) or left untreated before injection of thrombin (4 nM) or stable ADP (5 µM), as in Figure 1. Shown are representative traces of changes in $[Ca_{2+}]_i$ of control (black) and Syk-IN (grey) incubations. Arrows indicate addition indicated of agonists.

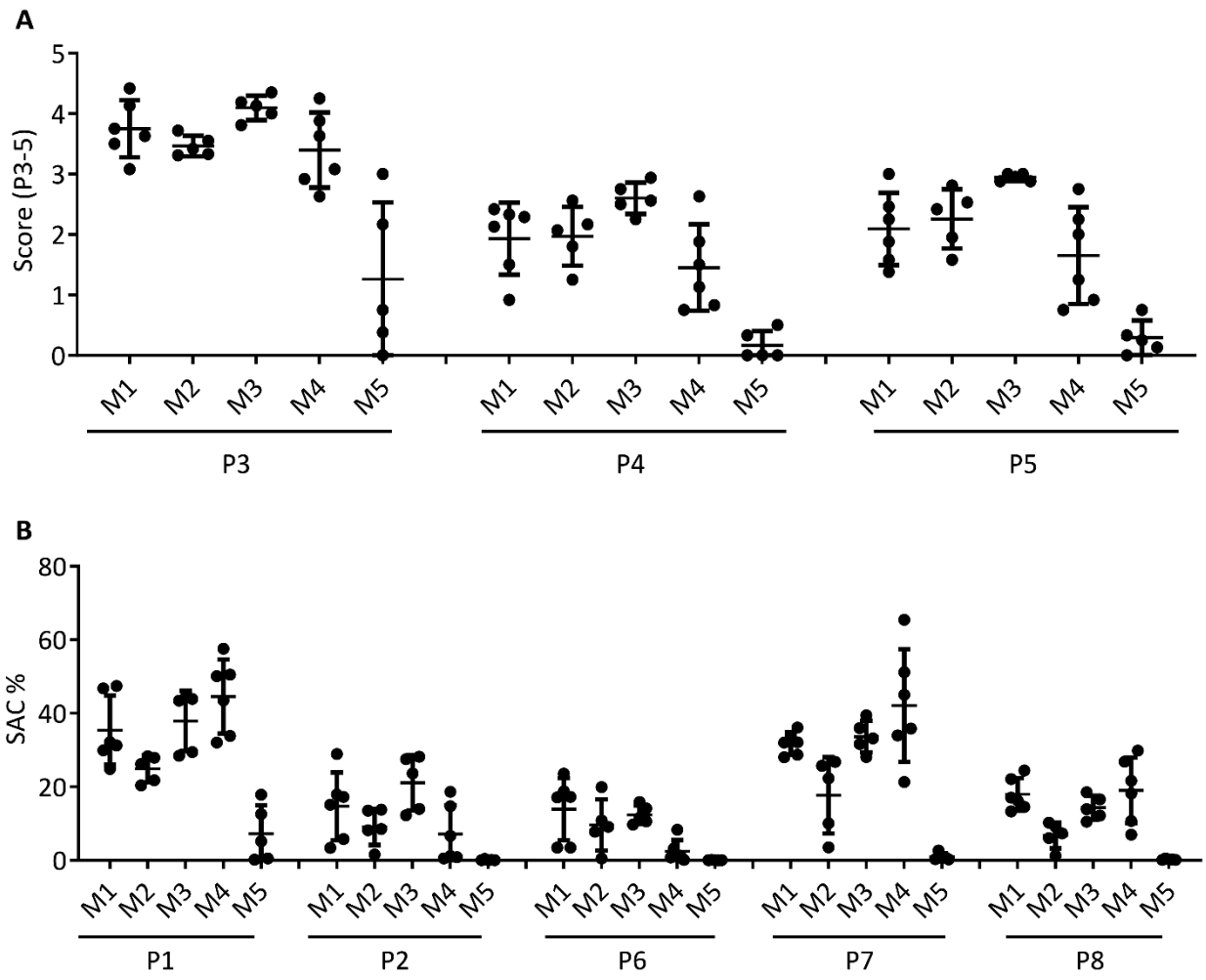

Figure S2. Parameters of thrombus formation on immobilised collagen peptides: raw data. Whole-blood was perfused over microspots *M1* (GFOGER-GPO + VWF-BP), *M2* (CRP-XL + VWF-BP), *M3* (GAOGER-GPO + VWF-BP), *M4* (GFOGER-GPP + VWF-BP), and *M5* (VWF-BP). Microscopic images were analysed for parameters *P1-8*, as for Figure 2. Shown are raw mean outcome values from individual blood donors. **(A)** Parameters providing surface area coverage (SAC%) information: *P1*, platelet deposition; *P2*, platelet aggregate coverage; *P6*, PS exposure; *P7*, CD62P expression; *P8*, fibrinogen binding. **(B)** Score parameters: *P3*, thrombus morphological score (range 0-5); *P4*, thrombus multilayer score (range 0-3); *P5*, thrombus contraction score (range 0-3). Means ± SD (n=5-7 donors).

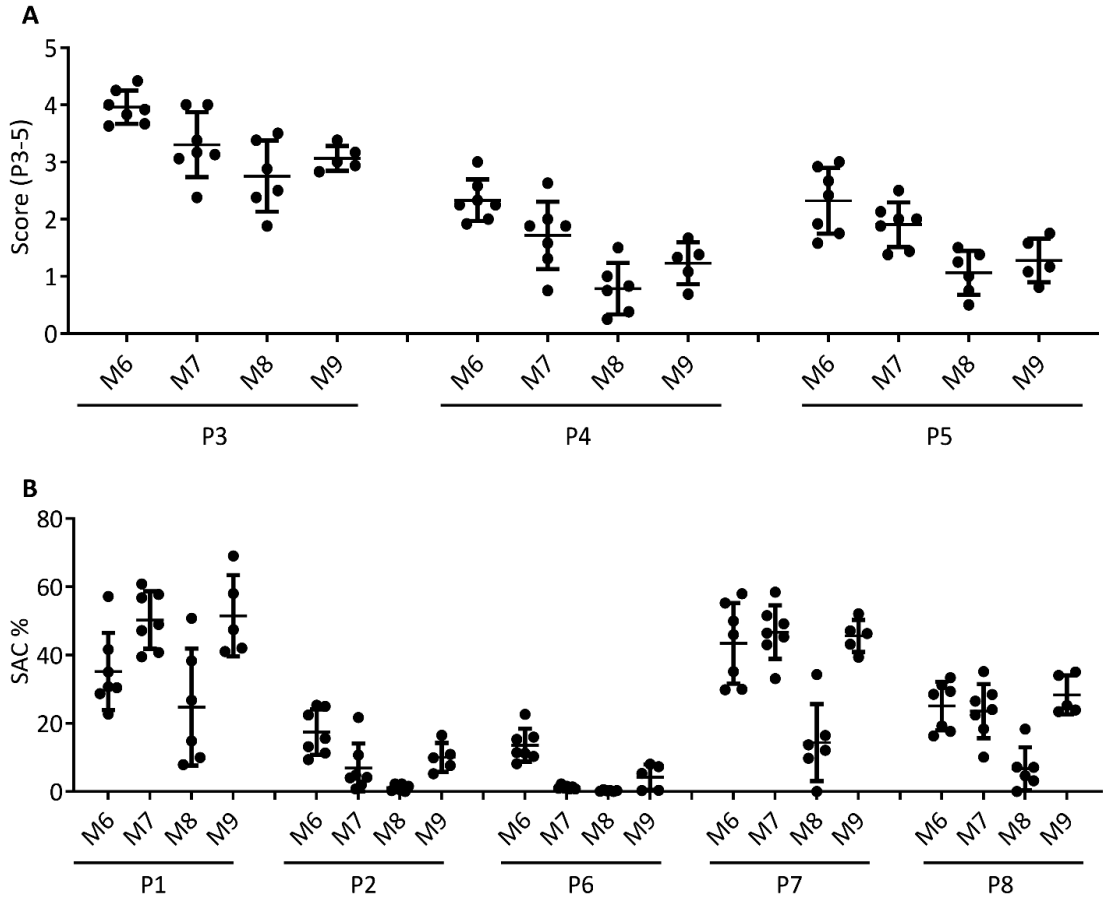

Figure S3. Parameters of thrombus formation on immobilised collagens: raw data. Whole-blood was perfused over microspots *M6* (collagen-H), *M7* (fibrillar collagen-I), *M8* (monomeric collagen-I), *M9* (collagen-III). Microscopic images were captured and analysed for parameters P1-8, as for Figure 5. Shown are raw mean outcome values from individual blood donors. **(A)** Parameters providing surface area coverage (SAC%) information. **(B)** Score parameters. See further Suppl. Figure S2. Means ± SD (n=5-7 donors).

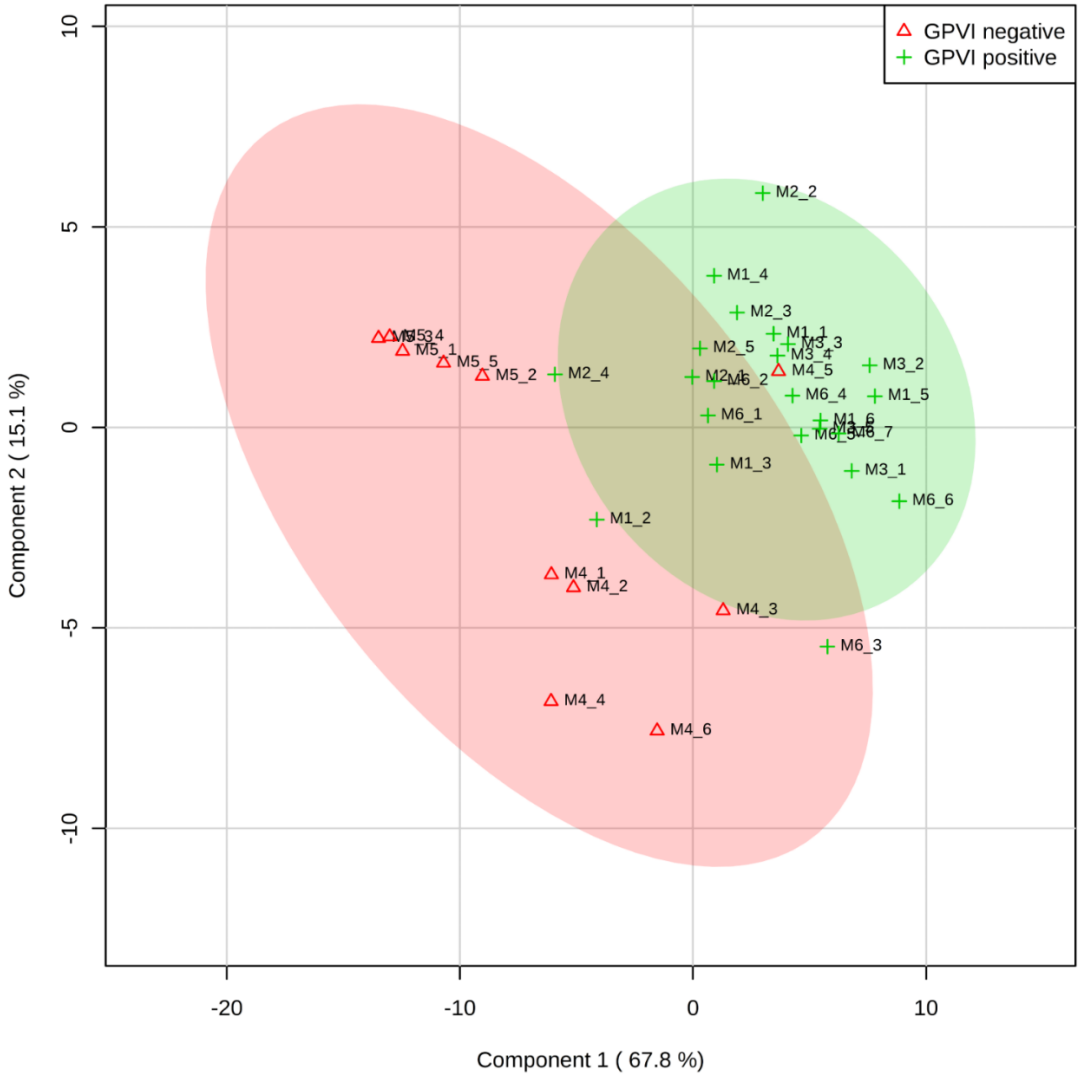

Figure S4. Partial Least Squares with components 1 and 2, indicating distribution of thrombus formation parameters at microspots *M1-6* for 5-7 individual blood samples per microspot. Note, the clustering (green area) of flow runs over $(GPO)_n$ containing surfaces *M1-3* and *M6*, whereas flow runs with other surfaces M4 and M5 out-clustered with more negative contributions to component 2 or 1, respectively (red area). Red triangles indicate assumed negative GPVI contribution, green plusses indicate positive contribution.

Table S1. Scaled subtracted parameter values of thrombus formation (means), indicating effects of Syk-IN, for microspots *M1-9* and parameters *P1-8*.

	P1	*P2*	*P3*	*P4*	*P5*	*P6*	*P7*	*P8*
M1	1.18±0.46	0.10±0.14	0.51±0.17	0.15±0.16	0.21±0.20	0.03±0.05	0.71±0.49	0.62±0.66
M2	1.15±0.24	0.16±0.13	0.77±0.15	0.30±0.20	0.30±0.15	0.04±0.06	0.69±0.39	0.31±0.28
M3	0.94±0.24	0.10±0.07	0.71±0.17	0.37±0.23	0.34±0.12	0.01±0.00	0.47±0.27	0.11±0.08
M4	0.93±0.29	0.17±0.29	0.73±0.09	0.34±0.34	0.37±0.40	0.13±0.16	0.25±0.18	0.04±0.03
M5	0.97±1.18	1.27±2.13	0.13±0.12	0.00±0.00	0.00±0.00	0.63±0.79	1.40±2.65	0.36±0.43
M6	1.12±0.33	0.19±0.10	0.61±0.15	0.22±0.17	0.28±0.18	0.02±0.02	0.65±0.35	0.42±0.44
M7	0.88±0.21	0.03±0.06	0.60±0.20	0.10±0.11	0.17±0.12	0.03±0.02	0.13±0.08	0.02±0.02
M8	0.87±0.56	2.91±6.23	0.61±0.35	0.64±0.74	0.42±0.28	0.16±0.15	0.25±0.16	0.12±0.17
M9	0.79±0.20	0.07±0.07	0.49±0.06	0.13±0.23	0.11±0.13	0.03±0.03	0.14±0.12	0.05±0.05

Chapter 4

Coagulation factor XIIIa and activated protein C activate platelets via GPVI and PAR1

In chapter 3, we investigated collagen(-like peptides), and whether their thrombogenicity was dependent on the protein tyrosine kinase Syk and GPVI. In the next chapter, we aimed to expand the knowledge on the ligands for the platelet receptor GPVI, but also for PAR1. Since it is well recognized that platelet and coagulation activation are highly reciprocal processes, we aimed to investigate the contribution of coagulation-generated ligands to platelet activation. So far, the coagulation-generated ligands for GPVI and PAR1 were fibrin and thrombin, respectively. We found that coagulation factor XIIIa also induced platelet activation through Syk and GPVI, while the anticoagulation factor APC induced platelet responses through PAR1. We also found that combining the coagulation factors, increased their potency to enhance platelet functions in solution, suggesting that combinations of several activated coagulation factors could prime the platelets and tip the balance to the site of thrombosis. Also, effects evoked by the factors were more pronounced when the factors were immobilized on a surface, indicating those factors are more effective when recruited to the site of injury.

I designed this project, collected and analysed experimental data. Likewise, I drafted the manuscript, writing (introduction, methods, results, discussion, conclusion and supplement) and prepared the paper for submission. For these reasons, I'm first author of this work.

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Coagulation factor XIIIa and activated protein C activate platelets via GPVI and PAR1

Ilaria De Simone[1,2], Constance C.F.M.J. Baaten[1,3], Martine Jandrot-Perrus[4], Jonathan M. Gibbins[2], Hugo ten Cate[1,5], Johan W.M. Heemskerk[1,6], Chris I. Jones[2*], Paola E.J. van der Meijden[1,5*]

[1]Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, Maastricht, The Netherlands; [2]Institute for Metabolic and Cardiovascular Research, School of Biological Sciences, University of Reading, Reading, UK; [3]Institute for Molecular Cardiovascular Research, University Hospital Aachen, RWTH Aachen University, Aachen, Germany; [4]UMR_S1148, Laboratory for Vascular Translational Science, INSERM, University Paris Cité, France; [5]Thrombosis Expertise Center, Heart and Vascular Center, Maastricht University Medical Center, Maastricht, The Netherlands; [6]Synapse Research Institute, Maastricht, The Netherlands

*equal contribution

Running title: Coagulation and anticoagulation factors affecting platelets

Correspondence:

Chris I. Jones, School of Biological Sciences, Institute for Cardiovascular and Metabolic Research, University of Reading, Reading, UK. Tel: +44 (0) 118 378 7047. E-mail: c.i.jones@reading.ac.uk

Paola E. J. van der Meijden, Department of Biochemistry Maastricht University P.O. Box 616, 6200 MD, Maastricht, The Netherlands Tel.: +31 43 3881684, E-mail: p.vandermeijden@maastrichtuniversity.nl

Abstract

Platelet and coagulation activation are highly reciprocal processes driven by multi-molecular interactions. Activated platelets secrete several coagulation factors and expose phosphatidylserine, which supports the activation of coagulation factor proteins. On the other hand, the coagulation cascade generates known ligands for platelet receptors, such as thrombin and fibrin. Coagulation factor (F)Xa, (F)XIIIa and activated protein C (APC) can also bind to platelets, but the functional consequences are unclear. Here, we investigated the effects of the activated (anti)coagulation factors on platelets, other than thrombin. Multicolor flow cytometry and aggregation experiments revealed that the 'supernatant of (hirudin-treated) coagulated plasma' (SCP) enhanced CRP-XL-induced platelet responses, i.e., integrin $\alpha_{IIb}\beta_3$ activation, P-selectin exposure and aggregate formation. We demonstrated that FXIIIa in combination with APC enhanced platelet activation in solution, and separately immobilized FXIIIa and APC resulted in platelet spreading. Platelet activation by FXIIIa was inhibited by molecular blockade of glycoprotein VI (GPVI) or Syk kinase. In contrast, platelet spreading on immobilized APC was inhibited by PAR1 blockade. Immobilized, but not soluble, FXIIIa and APC also enhanced in vitro adhesion and aggregation under flow. In conclusion, in coagulation, factors other than thrombin or fibrin can induce platelet activation via GPVI and PAR receptors.

Introduction

Blood platelets and the coagulation system both contribute to hemostasis and thrombosis in a highly interactive manner.[1, 2] Well-characterized coagulation products that activate platelets are thrombin and fibrin. Thrombin induces platelet responses via the G-protein coupled receptors, protease-activated receptor (PAR)1 and 4.[3] Fibrin can stimulate platelet activation, jointly via molecular actions of the integrin $\alpha_{IIb}\beta_3$ and glycoprotein VI (GPVI),[4, 5] leading to thrombus growth and stabilization.[6, 7] GPVI is also known to be the central signaling collagen receptor on platelets.[8, 9]

Several other activated factors induced by the coagulation process have been described to bind and activate platelets. Factor Xa (FXa) was reported to cleave PAR1 at the thrombin-cleavage site and to evoke platelet responses which were inhibitable by PAR1 inhibitors.[10, 11] The formation of FXa occurs at the surface of phosphatidylserine (PS)-exposing platelets by the tenase complex, after which FXa cleaves prothrombin into thrombin in a factor FVa-dependent way.[12] The transglutaminase factor XIIIa (FXIIIa), which crosslinks fibrin fibers, supports platelet spreading and filipodia formation via the activation of Tyr-kinases.[13] In addition, activated protein C (APC, an anticoagulation factor) was found to stimulate platelets via the receptors ApoER2 and GPIb-V-IX.[1, 14] Additionally, other factors, such as FV, FIX and FXI, are known to bind to platelets for instance via GPIb-V-IX and integrin $\alpha_{IIb}\beta_3$.[15-17]

Targeting molecular interaction processes of both platelet and coagulation has been shown to be beneficial in terms of cardiovascular risk reduction. Both, the ATLAS-ACS 2 TIMI 51 study, where the FXa inhibitor rivaroxaban was combined with dual antiplatelet therapy and the COMPASS trial, where a low-dose rivaroxaban administered in addition to aspirin, provided proof that combining platelet and coagulation inhibitors resulted in a lower rate of cardiovascular events, compared to platelet inhibition alone.[2, 18, 19] In addition, combined antiplatelet and anticoagulation therapy might be promising in other patient populations as well, for example in patients with myocardial injury after non-cardiac surgery (MINS),[20] in which the MANAGE trial recently showed that dabigatran reduced the risk for major vascular complications.[21]

In the literature, we encountered a gap in detailed knowledge regarding the relative contribution of key coagulation and anticoagulation factors—other than thrombin and fibrin—in platelet recruitment, platelet activation and thrombus formation. In the present paper, we aimed to close this gap by investigating, on a molecular and signaling level, how the interactions of FXa, FXIIIa and APC with platelets influenced the activation processes of platelets. We hypothesize that all these factors support these processes

and hence may act together. We selected FXa, FXIIIa and APC for further investigation because of their central role in the initiation of thrombin generation, fibrin crosslinking and anticoagulation, respectively. In addition, these (anti)coagulation factors are clinically used or are of current high interest for novel therapies to control thrombosis and hemostasis.

Materials and Methods

Blood collection and platelet isolation

Human blood was obtained by venipuncture from healthy volunteers, free from antithrombotic medication after written informed consent in accordance with the Declaration of Helsinki. Protocols were reviewed by the local ethics committee. Blood samples were collected into 3.2% trisodium citrate (Vacuette tubes, Greiner Bio-One, Alphen a/d Rijn, The Netherlands). The first 2 mL of blood was discarded to avoid contact activation effects. All subjects had platelet counts within the reference range (150–450 × 10^9/L), as determined with a Sysmex XP-300 thrombocounter (Sysmex, Cho-ku, Kobe, Japan). The platelets were isolated, washed and resuspended in Hepes buffer pH 7.45 (10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 0.1% glucose and 0.1% BSA), as described earlier.[22] For further details, see supplementary data.

Preparation of Supernatant of Hirudin-Treated Coagulated Plasma (SCP)

Citrate anticoagulated platelet-poor plasma was obtained from blood samples by a double centrifugation at 2200× g for 10 min (22 °C, acc. 9, brake 3; Rotina 380 R, Hettich Benelux B.V., Geldermalsen, The Netherlands). As described earlier,[23] the collected plasma was recalcified with 16.6 mM $CaCl_2$ and activated with 10 pM tissue factor at 37 °C for one hour, resulting in the extrinsic activation of the coagulation cascade. Fibrin clots were manually removed and the fluid remnant was centrifuged at 22,500× g for 5 min (22 °C; Hettich EBA 12, Hettich Benelux B.V., Geldermalsen, The Netherlands) to remove remaining fibrin fibers and cell debris. The collected supernatant was post-

treated with 10 U/mL hirudin, at room temperature for 10 min, to completely block residual thrombin activity. No additional inhibitors were added. Treated clot supernatants from four healthy donors were pooled and frozen for later experimentation. See supplementary Data.

Statistical Analysis

GraphPad Prism 8 software (La Jolla, CA, USA) was used for statistical analysis. Data are presented as mean ± SD. Mean values were compared using an ordinary one- or two-way ANOVA. The Shapiro–Wilk test was used to test for normal distribution of the data. P-values below 0.05 were considered statistically significant in that: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Results

Supernatant of Hirudin-Treated Coagulated Plasma Enhances Platelet Activation

To determine whether activated factors generated during the coagulation process, other than thrombin, affect platelet activation processes, washed platelets were isolated and exposed to supernatant of hirudin-treated coagulated plasma (SCP). SCP was used to mimic the (patho)physiological situation upon injury and activation of extrinsic coagulation. Platelets exposed to SCP were stimulated with varying concentrations of CRP-XL, whereafter platelet activation markers were assessed by flow cytometric analysis (Figure 1). SCP did not induce platelet activation by itself, but significantly enhanced the CRP-XL-induced integrin $\alpha_{IIb}\beta_3$ activation (PAC-1 labeling) and P-selectin exposure (anti-P-selectin mAb labeling) by 30–50% over a range of submaximal concentrations (Figure 1A). In addition, the effect of SCP on platelet aggregation in response to CRP-XL, TRAP6 or ADP was determined using a plate-based aggregation method. Again, SCP significantly increased the percentage of platelet aggregation upon stimulation with submaximal concentrations of CRP-XL, TRAP6 or ADP, compared to

noncoagulated (control) plasma (Figure 1B). These results indicated that components generated during coagulation, other than thrombin, support platelet responses.

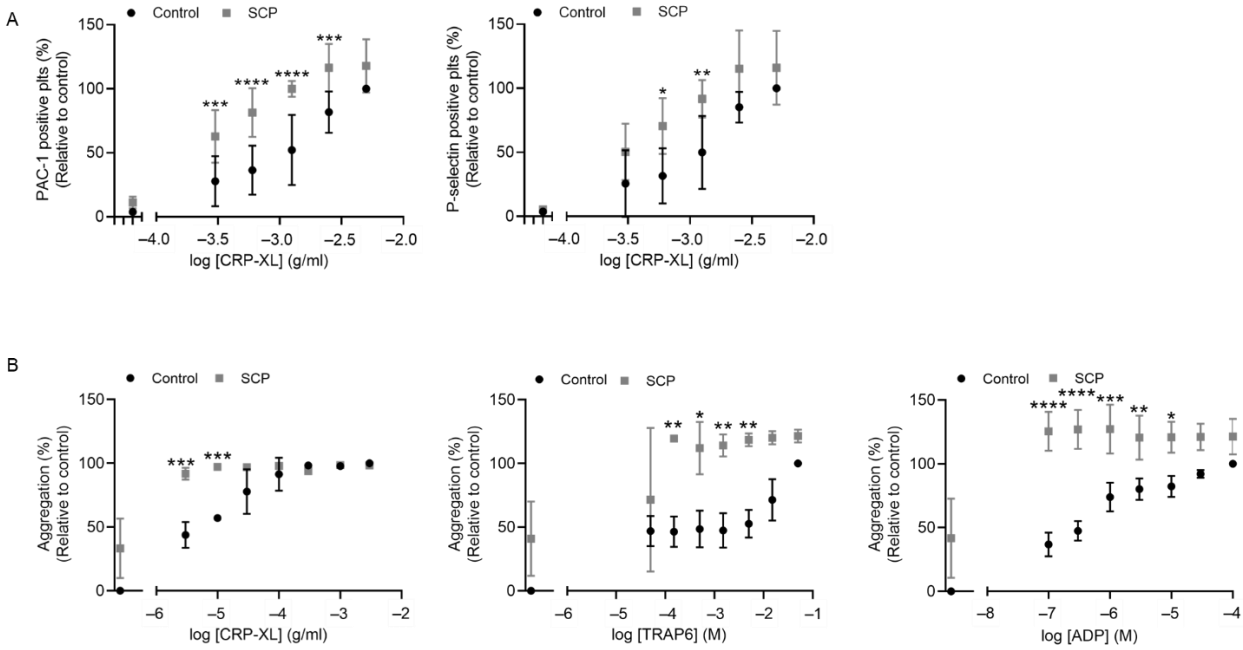


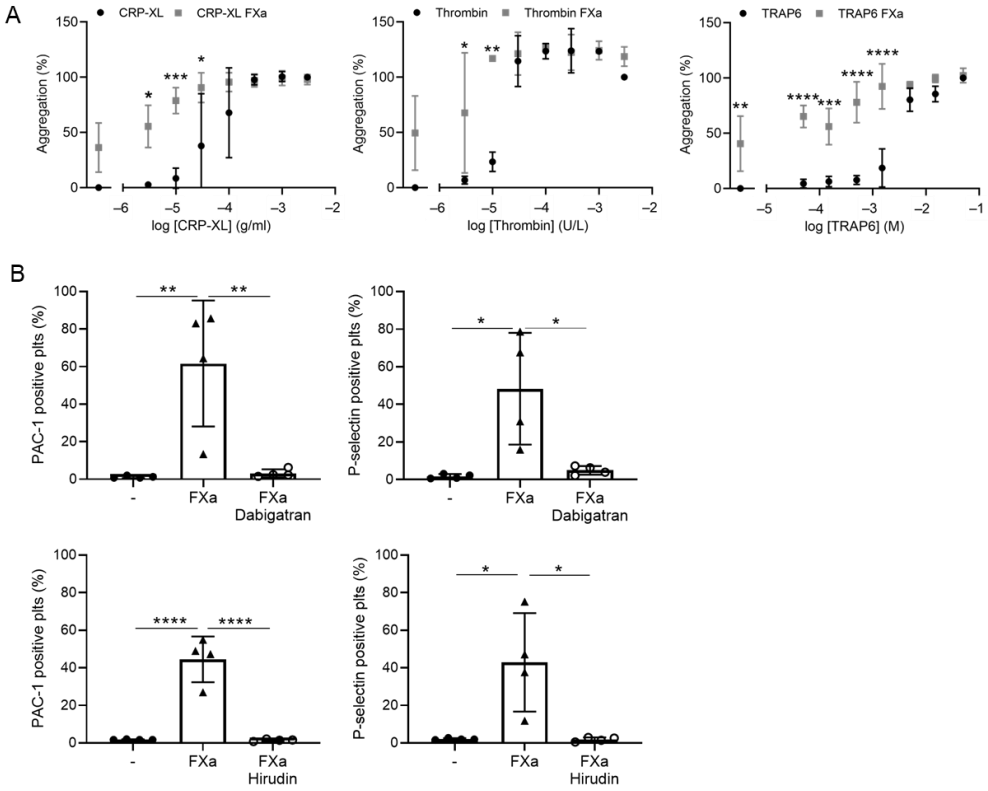
Figure 1. Coagulation-dependent activation of platelets independent of thrombin. (A) Washed human platelets in the presence or absence of supernatant of hirudin-treated, coagulated plasma (SCP) were stimulated with a range of CRP-XL concentrations. Activation of integrin $\alpha_{IIb}\beta_3$ and P-selectin expression were assessed by flow cytometric analysis, using FITC labeled PAC-1 mAb and Alexa Fluor (AF) 647-labeled anti-human CD62P mAb, respectively. Mean ± SD, n = 4. All data were scaled relative to aggregation obtained upon highest CRP-XL concentration in control condition (100%). Ordinary two-way ANOVA, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. (B) Aggregation of washed platelets in control or SCP induced by CRP-XL, TRAP6 or ADP, as assessed by well plate-based aggregation method. Mean ± SD, n = 3. All data were scaled relative to aggregation obtained upon highest agonist concentration. in control condition (100%). Ordinary two-way ANOVA, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Effect of Individual Coagulation Factors on Platelet Activation

Since previous literature described that FXa, FXIIIa and APC interact with platelets,[10, 13, 14] we investigated whether these factors contribute to the enhancing effects of SCP on platelet activation. Washed platelets were primed with FXa (10 µg/mL), APC (10 nM),

FXIIIa (10 U/mL) and activated with varying concentrations of CRP-XL, thrombin or TRAP6.

The addition of FXa significantly enhanced the platelet aggregation response triggered by submaximal doses of CRP-XL, TRAP6 or ADP (Figure 2A). Flow cytometric analysis showed that FXa increased CRP-XL-induced integrin $\alpha_{IIb}\beta_3$ activation and P-selectin exposure (Figure 2C). FXa alone also triggered platelet activation (Figure 2B). Strikingly, all effects evoked by FXa were abolished by the thrombin inhibitors dabigatran and hirudin (Figure 2B–D). Consistent with this, increased cytosolic Ca^{2+} levels measured in FXa-treated fura-2-loaded platelets were also inhibited by dabigatran (Figure 2D). This suggests that the FXa-dependent platelet responses are due to the in situ formation of thrombin traces.



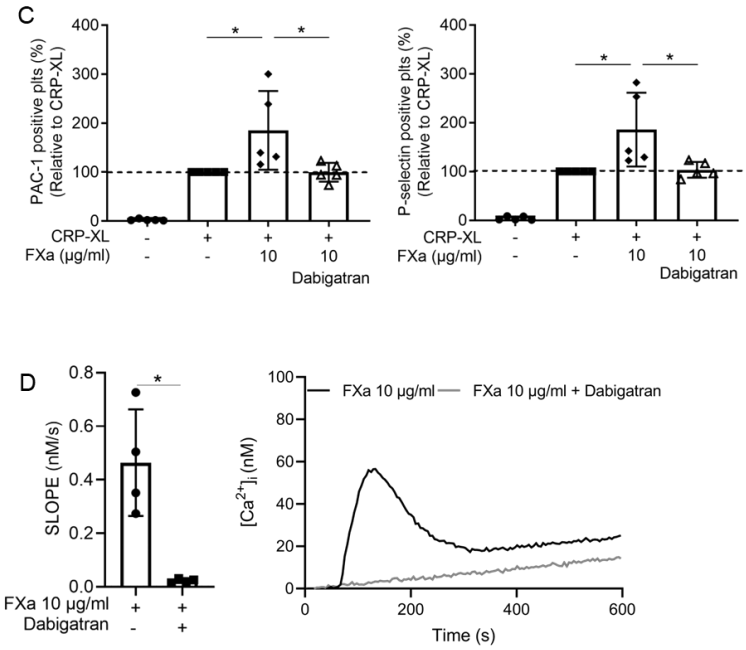


Figure 2. Factor Xa induces platelet activation and enhances agonist-induced platelet effects, in a thrombin-dependent way. (A) Factor Xa enhances agonist-induced platelet aggregation. Washed platelets preincubated with vehicle or FXa (10 µg/mL) were stimulated with CRP-XL (0.003–3 µg/mL), thrombin (0.003–3 U/mL) or TRAP6 (0.05–50 µM). Platelet aggregation was assessed by well plate-based light transmission changes. All data were scaled relative to aggregation obtained upon highest agonist concentration in the presence of vehicle (100%). Mean ± SD, n = 3; two-way ANOVA, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. (B) FXa-induced platelet activation is inhibitable by dabigatran or hirudin. Flow cytometry, washed platelets. Active integrin and P-selectin expression are shown. Percentage positive platelets. One-way ANOVA, multiple comparisons, mean ± SD, n = 4–5, one-way ANOVA, * $p < 0.05$ and ** $p < 0.01$, **** $p < 0.0001$. (C) Enhancement of CRP-XL-induced platelet activation by FXa is abolished by dabigatran. One-way ANOVA, multiple comparisons, data are compared to CRP-XL, * $p < 0.05$ and ** $p < 0.01$, mean ± SD, n = 4–5. (D) FXa-induced cytosolic Ca^{2+} release is inhibitable by dabigatran. Washed platelets, loaded with Fura-2 acetoxymethyl ester (3 µM). Changes in cytosolic $[Ca^{2+}]_i$ were measured using FlexStation 3. Outcome was assessed from slope of initial Ca^{2+} rises and representative time traces. Unpaired t-test, * $p < 0.05$, mean ± SD, n = 3–4.

In contrast, neither FXIIIa nor APC alone enhanced agonist-induced aggregation (Figure 3A,B). Additionally, platelet activation markers following CRP-XL stimulation were not altered by APC or FXIIIa (Supplementary Figure S1). However, in line with the effects of SCP, combining APC and FXIIIa significantly enhanced CRP-XL-induced integrin $\alpha_{IIb}\beta_3$ activation by approximately 20% (Figure 3C).

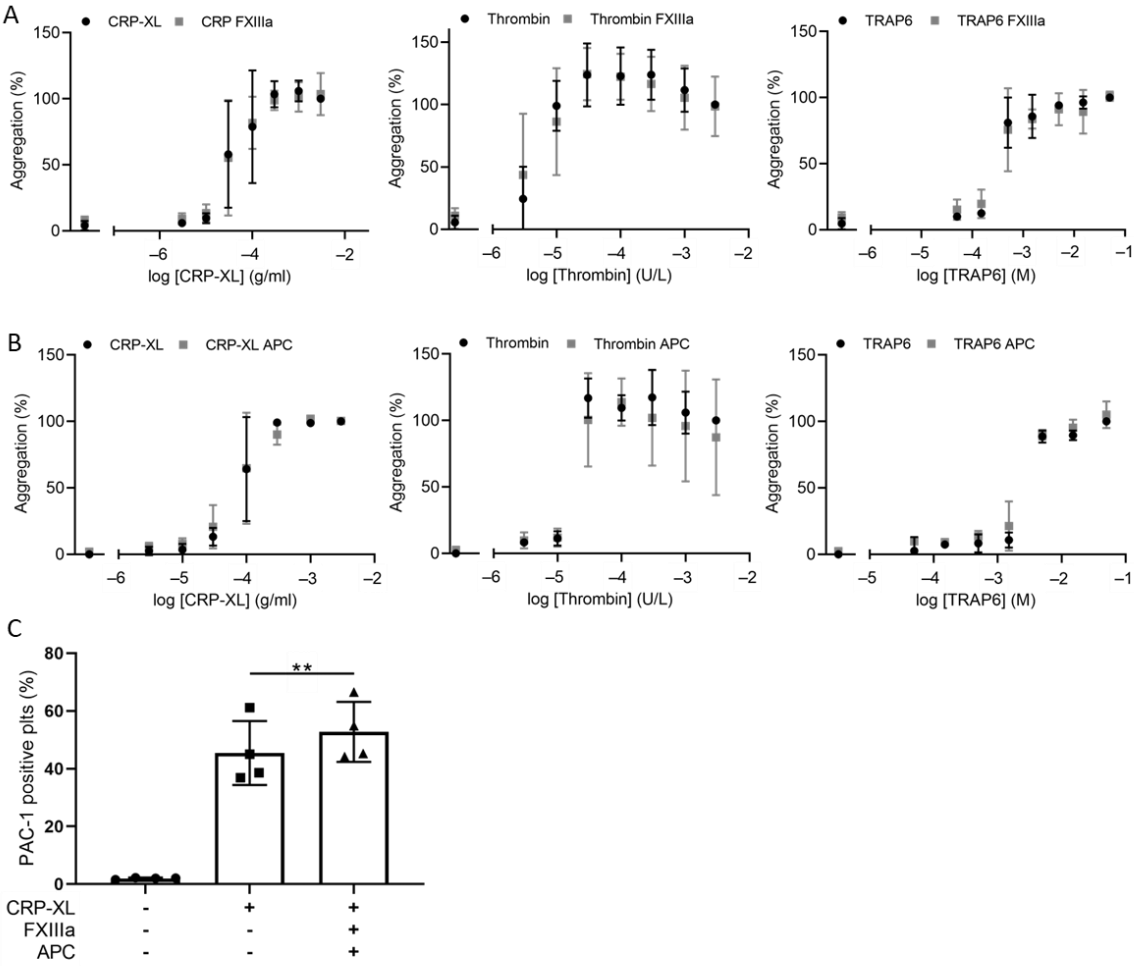


Figure 3. Combinations of coagulation factors (F)XIIIa and anticoagulation factor APC enhance CRP-XL induced platelet activation. (A,B) Washed platelets preincubated with vehicle, FXIIIa (10 U/mL) or APC (10 nM) were stimulated with CRP-XL (0.003–3 µg/mL), thrombin (0.003–3 U/mL) or TRAP6 (0.05–50 µM). Platelet aggregation was assessed by well plate-based light transmission changes. All data were scaled relative to aggregation obtained upon highest agonist concentration in the presence of vehicle (100%). Mean ± SD, n = 3; two-way ANOVA., * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. (C) Washed platelets were preincubated with vehicle or FXIIIa and APC and activated with a submaximal CRP-XL concentration (0.03–0.5 µg/mL). Flow cytometry was used to measure activated integrin $\alpha_{IIb}\beta_3$ using FITC labelled PAC-1 mAb. Paired t-test, ** $p < 0.015$, mean ± SD, n = 4.

Immobilization of APC and FXIIIa Favors Their Activating Effect on Platelets

Since the effects induced by soluble APC and FXIIIa were variable, we examined whether immobilizing APC and FXIIIa could favor their interaction with platelets using platelet spreading assays. FXa was not further investigated, as we observed that effects evoked by FXa were entirely thrombin dependent.

Immobilized APC

Surface-immobilized APC triggered the adhesion and spreading of unstimulated platelets (Figure 4A). Of all adhered platelets, 18.29 ± 13.33% did not undergo shape change, while only a small percentage of platelets protruded filopodia 14.68 ± 9.223% and all others formed lamellipodia 67.03 ± 18.9%. Similar results were obtained for plasma-derived APC (not shown). Since the binding of APC to the EPCR receptor on endothelial cells results in N-terminal PAR1 cleavage,[24] we studied a possible role of PAR1 in APC-induced platelet spreading. Therefore, washed platelets were pretreated with the PAR1 inhibitor Atopaxar before spreading on APC-coated surfaces. Atopaxar substantially reduced platelet adhesion by 59.96 ± 15.04% ($p = 0.0131$) to the APC-coated surfaces and abolished the formation of lamellipodia ($p < 0.001$), demonstrating the role of PAR1 in APC-induced platelet spreading (Figure 4A).

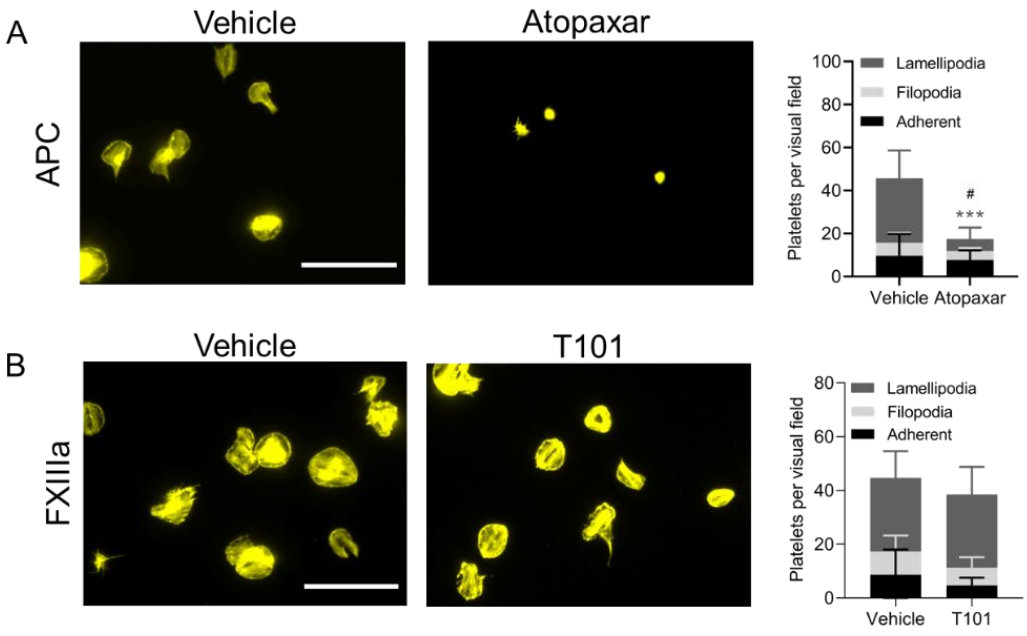


Figure 4. Immobilized APC and FXIIIa induce platelet adhesion and spreading. (A) Role of PAR1 in APC-induced platelet spreading. Washed platelets (20×10^9 platelets/L) were incubated with vehicle (DMSO) or Atopaxar (5 µM) and were allowed to spread for 45 min under static conditions, on a surface coated with APC. The platelets were then fixed, permeabilized and stained with CF543-phalloidin. Spreading was assessed with fluorescence microscopy (bars, 20 µm). Two-way ANOVA, *** $p < 0.001$ compared between lamellipodia, mean ± SD, n = 4. Unpaired t-test, # $p < 0.05$ comparison of

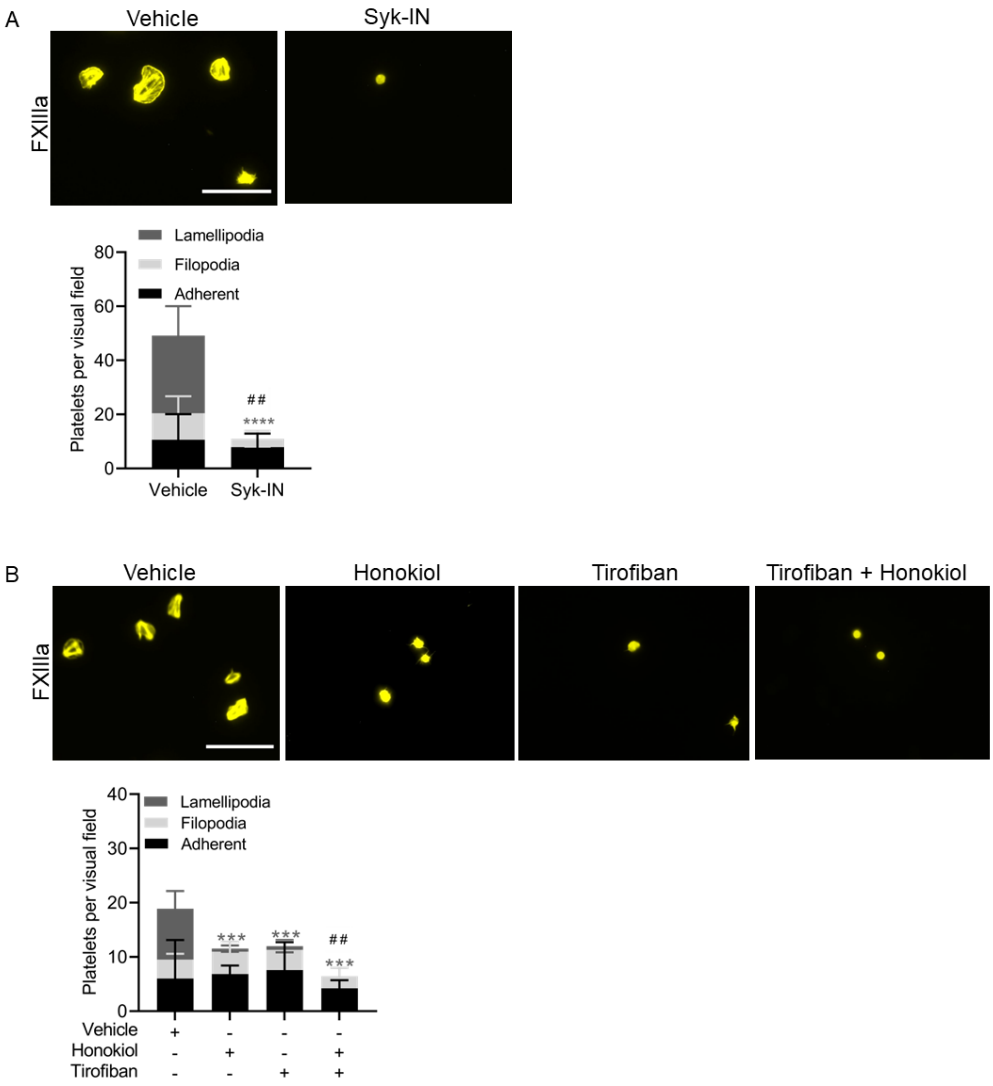
platelets per visual field, mean ± SD, n = 4. (B) FXIIIa-induced platelet spreading is independent of transglutaminase activity. Washed platelets (20 × 109 platelets/L) were treated with vehicle or transglutaminase inhibitor T101 (20 μM) and were allowed to spread for 45 min under static conditions, on surfaces coated with FXIIIa. Samples were fixed, permeabilized and stained with CF543-phalloidin. Spreading was assessed by fluorescence microscopy (bars, 20 μm). Two-way ANOVA, not significant, mean ± SD, n = 3–4. Unpaired t-test, not significant, mean ± SD, n = 3–4.

Immobilized FXIIIa

Immobilized FXIIIa also induced platelet adhesion and spreading (Figure 4B). The majority of platelets 69.41 ± 15.04% formed lamellipodia on FXIIIa surfaces. To assess the contribution of the transglutaminase activity of FXIIIa in platelet adhesion and spreading, platelets were pretreated with the transglutaminase inhibitor T101. There was no significant decrease in platelet adhesion and in the formation of filopodia and lamellipodia when transglutaminase activity was blocked (Figure 4B).

Since previous studies have demonstrated that the mechanism of FXIIIa-induced platelet spreading and filopodia formation was dependent on integrin $\alpha_{IIb}\beta_3$ and tyrosine-kinase activity,[13, 25, 26] we preincubated the platelets with an inhibitor of the kinase Syk, PRT060318 (Syk-IN). Platelet adhesion on surface-immobilized FXIIIa was reduced ($p = 0.001$) and lamellipodia formation was abolished ($p < 0.0001$) after treatment with Syk-IN (Figure 5A). In platelets, the binding of Syk to the β3 cytoplasmic domain of $\alpha_{IIb}\beta_3$ integrin is known to be important in lamellipodia formation.[27] However, Syk is also a major signaling molecule downstream of GPVI. Therefore, we reasoned that this may indicate the stimulation of GPVI signaling by FXIIIa. Platelets were therefore treated with the small-molecule GPVI inhibitor honokiol[28] or the blocking anti-GPVI Fab 9O12[29] with or without the integrin $\alpha_{IIb}\beta_3$ inhibitor tirofiban. Treatment with either GPVI inhibitors or tirofiban significantly reduced platelet lamellipodia formation on FXIIIa ($p < 0.01$), but not adhesion (Figure 5B,C). Combination of either one of the GPVI inhibitors with the integrin $\alpha_{IIb}\beta_3$ receptor inhibitor tirofiban, resulted in a significant further decreased platelet

adhesion and inhibited lamellipodia formation (Figure 5B,C). These data indicate the importance of GPVI in platelet activation by FXIIIa, and the synergistic roles of integrin $\alpha_{IIb}\beta_3$ and GPVI in the binding and spreading of platelets to immobilized FXIIIa.



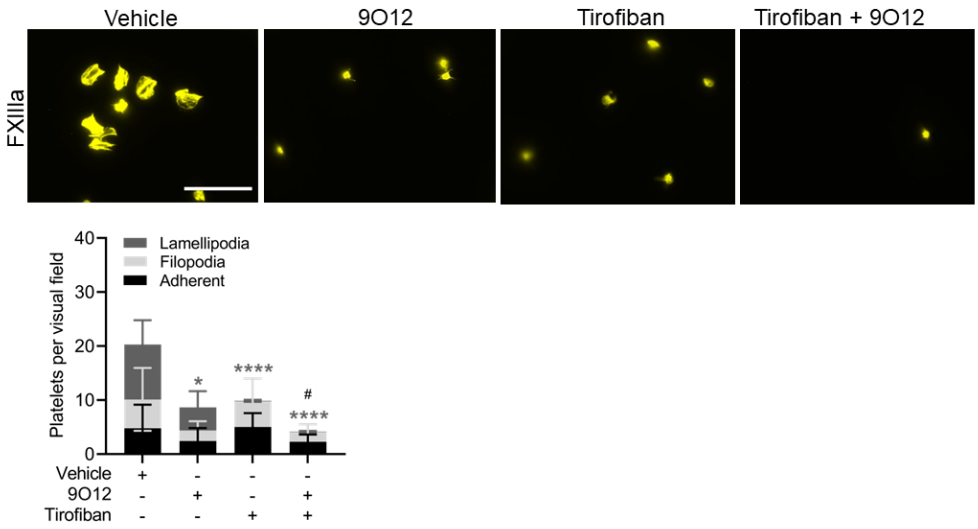


Figure 5. The role of Syk, integrin $\alpha_{IIb}\beta_3$ and GPVI in platelet spreading on FXIIIa. (A) Spreading on FXIIIa is Syk-dependent. Washed platelets (20 × 10^9 platelets/L) were treated with vehicle or Syk inhibitor PRT-060318 (20 µM) and added to the FXIIIa-coated surface. Samples were fixed, permeabilized and stained with CF543-phalloidin. Spreading was assessed by fluorescence microscopy (bars, 20 µm). Two-way ANOVA, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001, comparison between lamellipodia, mean ± SD, n = 4. Unpaired t-test, # p < 0.05, ## p < 0.01, comparison of platelets per visual field, mean ± SD, n = 4. (B) The role of GPVI in spreading on FXIIIa. Washed platelets (20 × 109 platelets/L) were treated with honokiol (50 µM) or 9O12 (50 µg/mL) ± tirofiban (1 µg/mL) as indicated and added to the FXIIIa-coated surface. Samples were fixed, permeabilized and stained with CF543-phalloidin. Spreading was assessed by fluorescence microscopy (bars, 20 µm). Two-way ANOVA, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001, comparison between lamellipodia, mean ± SD, n = 3–5. Unpaired t-test, # p < 0.05, ## p < 0.01, comparison of platelets per visual field, mean ± SD, n = 3–5.

Immobilized FXIIIa and APC Enhance Platelet Adhesion under Flow

To obtain more insight into the effect of FXIIIa and APC on thrombus formation, whole blood samples were used for the assessment of thrombus formation under flow using the Maastricht flow chamber.[30] Coagulation factors in physiological conditions can be found soluble in the plasma or immobilized by other ligands or vascular cells. Therefore, APC and FXIIIa were added either directly to the blood or coated on a surface. Surfaces were coated with VWF, since this subendothelial matrix protein only induces weak

platelet responses. We reasoned that additional platelet effects evoked by APC or FXIIIa would be rather detected on VWF than on more potent surfaces, such as collagens. To investigate whether soluble FXIIIa and APC enhance thrombus formation on a VWF surface, blood was incubated for 5 min with vehicle or FXIIIa or APC. For experimentation with FXIIIa, citrated blood was used, which was recalcified in the presence of PPACK. For the investigation of APC, blood was taken on hirudin, to avoid any inhibitory effects of PPACK on APC.[31] Blood was perfused over a VWF-coated surface, at an arterial wall shear rate of 1000 s−1 or venous wall shear rate of 300 s−1. There was no difference in overall platelet deposition or microaggregate formation when blood was incubated with vehicle, soluble FXIIIa (sFXIIIa) or soluble APC (sAPC) (Figures 6A and 7A). Interestingly, at an arterial wall shear (1000 s−1), co-coating FXIIIa or APC with VWF significantly enhanced VWF-induced platelet adhesion ($p = 0.026$ and 0.043) (Figures 6B and 7B). Additionally, microaggregate formation was significantly increased upon FXIIIa co-coating ($p = 0.043$) (Figure 6B). Moreover, co-coating FXIIIa and APC together with VWF increased platelet adhesion to the surface with 32% or 15% compared to co-coating APC or FXIIIa with VWF alone, respectively (not shown).

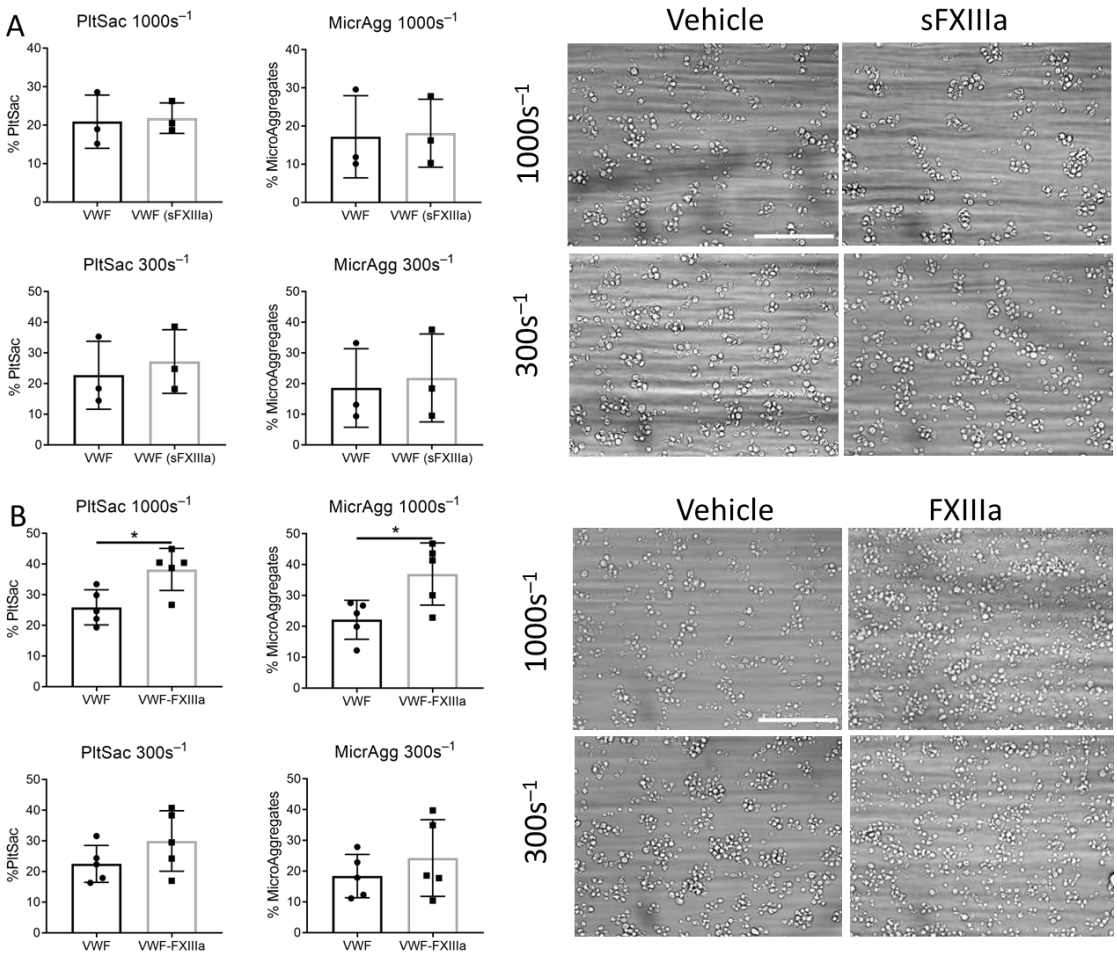


Figure 6. Immobilized FXIIIa enhances platelet adhesion and aggregate formation on VWF. (A) Blood samples were preincubated for 5 min with FXIIIa (10 U/mL) and perfused over a surface coated with VWF. (B) Blood samples were perfused over surfaces coated with VWF ± FXIIIa. Wall shear rate was 300 s−1 or 1000 s−1. Representative brightfield images (bars, 40 µm). Platelet adhesion (expressed as percentage surface area coverage, % PltSac). Platelets forming (micro)aggregates (expressed as percentage microaggregates, %MicrAgg). Unpaired t-test, * $p < 0.05$, mean ± SD, n = 3–5.

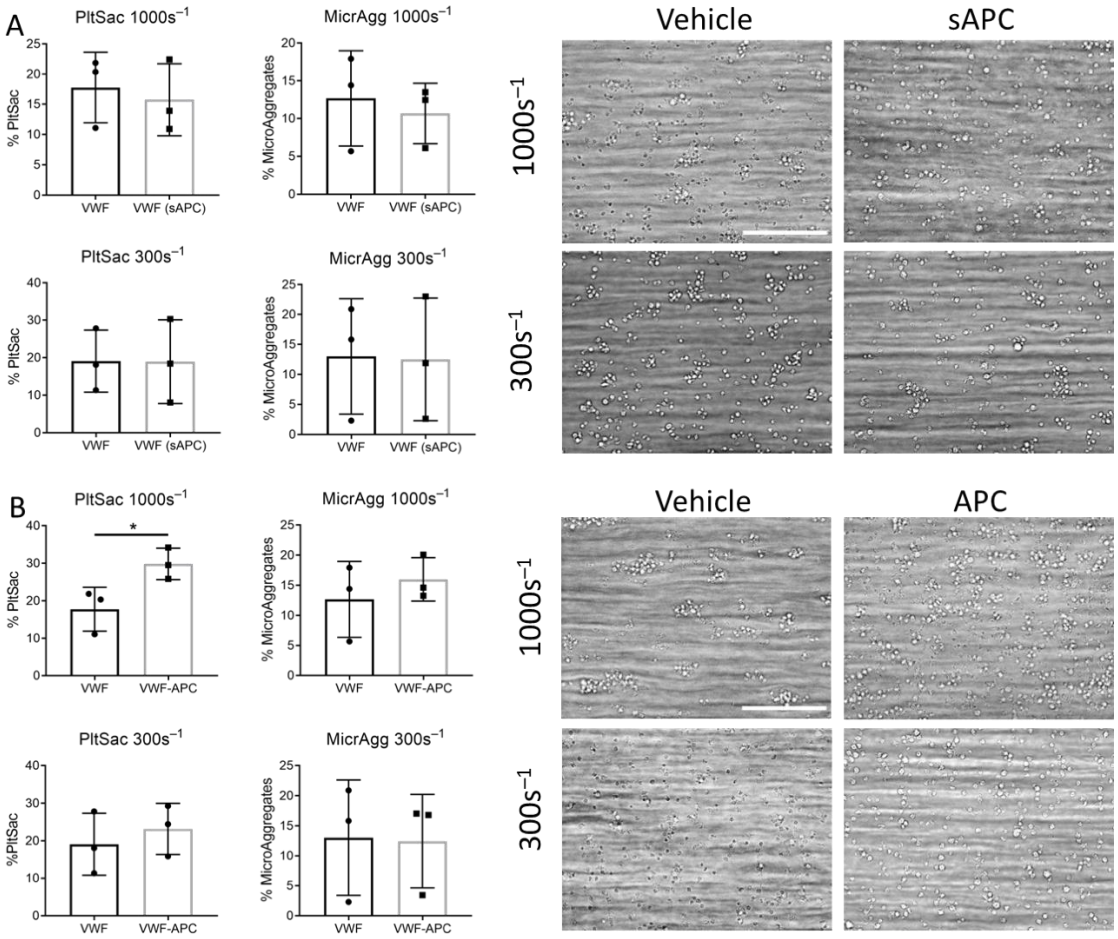


Figure 7. Immobilized APC enhances platelet adhesion on VWF. (A) Blood samples were preincubated for 5 min with APC (10 nM) and perfused over a surface coated with VWF. (B) Blood samples were perfused over surfaces coated with VWF ± APC. Wall shear rate was 300 s−1 or 1000 s−1. Representative brightfield images (bars, 40 µm). Platelet adhesion (expressed as percentage surface area coverage, %PltSac). Platelets forming (micro)aggregates (expressed as percentage microaggregates, %MicrAgg). Unpaired t-test, * $p < 0.05$, mean ± SD, n = 3.

Discussion

Platelet and coagulation activation occur contemporarily, but are often studied separately, while both mechanisms sustain thrombus formation and impact thrombosis. Our data show that coagulation factors, factors other than thrombin or fibrin, can induce platelet activation. FXIIIa induced platelet spreading via GPVI, and APC induced platelet spreading via PAR1. As GPVI and PAR1 are interesting targets for novel antiplatelet therapy, it is important to identify the ligands for those receptors. Previously, the only

coagulation-derived product which was described to activate GPVI was fibrin(ogen), and the activation of PAR1 by APC was only described for endothelial cells. We observed that the effects of individual (anti-)coagulation factors FXIIIa and APC on platelets were bigger when immobilized than when soluble, and that the combined effects of soluble FXIIIa and APC were capable of enhancing platelet activation. In pathological conditions, i.e., at sites of vascular injury or atherothrombosis, the activated factors FXIIIa and APC, both of which are key in regulating the extent of clot formation, are likely to act in a balanced way to prevent, allow and/or restrict the formation of a thrombus.

Given that during acute thrombotic events platelets are exposed to high levels of multiple activated (anti-)coagulation factors, we investigated the effect of the supernatant of hirudin-treated coagulated plasma (SCP) on platelets. Hereby, we have shown that (anti-)coagulation factors formed upon the activation of the extrinsic pathway jointly enhanced agonist-induced platelet activation and aggregation in a thrombin-independent manner. Although the formed fibrin clot was removed from the SCP and a thrombin inhibitor was added to exclude thrombin and fibrin effects, the residual presence of fibrin(ogen) in SCP and thus its influence on platelet activity cannot be completely excluded. To further explore which elements within SCP could be involved in the enhancement of platelet activation, the effects of individual factors of the coagulation cascade on platelets were investigated, by themselves and in combination.

We observed that the addition of FXa caused platelet integrin $\alpha_{IIb}\beta_3$ activation, secretion, aggregation and the release of cytosolic Ca^{2+}, confirming the effects reported by others. A report by Al-Tamimi and colleagues[11] concluded that platelet activating effects of FXa were mediated via PAR1 as effects were abolished in the presence of the PAR1-inhibitors SCH79797. Accordingly, Petzold and colleagues reported that FXa-mediated effects on platelets were abolished by the FXa inhibitor Rivaroxaban or the PAR1 inhibitor Vorapaxar.[10] However, our data showed that all effects evoked by FXa were abolished upon the addition of thrombin inhibitors, suggesting that the effects of FXa rely

on the in situ formation of low levels of thrombin, activating platelets via PAR1. Since platelets' alpha granules and open canalicular system contain several coagulation factors and co-factors, the generation of thrombin could possibly be explained by the release of traces of prothrombin and factor V/Va by platelets.[32]

APC has previously been shown to mediate cytoprotective effects in endothelial cells via PAR1 signaling,[14, 33, 34] which are inhibited by the orthosteric PAR1 inhibitors Vorapaxar and Atopaxar.[35] We revealed that APC-induced platelet spreading is also dependent on PAR1 and spreading could be inhibited by Atopaxar. Platelet adhesion, however, was not completely abolished upon PAR1 inhibition, suggesting a complementary role of other receptors, for example ApoER2 and GPIbα, which have also been shown to be involved in APC-induced platelet spreading.[14] Compatible with earlier findings by White et al.,[14] we could detect an additive effect of APC on platelet adhesion under flow.

The endothelial cell protein C receptor (EPCR) captures and immobilizes APC on the endothelium. Whether this can influence platelet responses in vivo and contribute to platelet adhesion warrants further investigation. The action of APC in this context is uncertain because as well as facilitating platelet adhesion, the binding of APC to platelets could possibly also limit thrombus growth, by localizing the anticoagulant property of the protein C system on the thrombus. To what extent platelet activating and anticoagulant properties of APC influence thrombus formation and growth remains to be established.

FXIIIa has previously been shown to support platelet adhesion and spreading through Syk and integrin $\alpha_{IIb}\beta_3$.[13, 26] For the first time, we show that GPVI also has a role in FXIIIa-induced platelet spreading. We observed that the combined inhibition of GPVI and integrin $\alpha_{IIb}\beta_3$ almost completely abolished platelet adhesion and spreading, pointing towards synergistic roles of integrin $\alpha_{IIb}\beta_3$ and GPVI in the effects of FXIIIa on platelets. Fibrin(ogen) is, to our knowledge, the only coagulation product previously described that induces platelet responses via the platelet receptor GPVI. Complementary to our findings, Moroi M. et al. reported proof that GPVI-dimer selectively binds to FXIII A-

subunit.[36] Our study demonstrates the functional consequences of the interaction between FXIIIa and GPVI.

Our results highlight the importance of the immobilization of FXIIIa, suggesting that FXIIIa may need to be captured before eliciting a platelet response, which may be due to a change in FXIIIa conformation,[25] or due to the importance of clustering GPVI.[37, 38] Under physiological conditions, FXIIIa is captured by fibrin(ogen), and by platelet surface receptors in a growing thrombus.[39] FXIIIa is also exposed on the surface of activated platelets, suggesting that there are ample sources of immobilized FXIIIa in a forming thrombus.[40]

In conclusion, our data provide novel evidence that: (i) coagulation products generated upon the activation of the extrinsic pathway support platelet activation, independently of thrombin; (ii) coagulation factor (F)Xa-induced platelet responses rely solely on the in situ formation of thrombin; (iii) immobilized anticoagulation factor APC induces platelet adhesion and spreading, with a role for PAR1; and (iv) immobilized coagulation factor (F)XIIIa induces platelet adhesion and spreading, through GPVI and integrin $\alpha_{IIb}\beta_3$. This provides new insights into the molecular processes that drive the interactions between coagulation-generated factors and platelets, and the roles of the platelet receptors PAR1 and GPVI herein. GPVI is a promising target for novel antiplatelet therapy, given its involvement in the collagen-induced pathogenesis of thrombosis, but its minor role in hemostasis.[41] There is, therefore, a need to elucidate the ligands for the GPVI receptor. Similarly, it is clear that the interplay between platelet and coagulation activation needs to be considered if we are to understand the propagation of thrombus, the formation of pathological thrombosis and the efficacy of novel anti-thrombotic therapies.

Author Contributions: Conceptualization, P.E.J.v.d.M., C.I.J., J.W.M.H., H.t.C. and J.M.G.; Methodology and Investigation, I.D.S. and C.C.F.M.J.B.; Analysis and Data Curation, I.D.S.; Resources, M.J.P., P.E.J.v.d.M., C.I.J., H.t.C., J.M.G. and J.W.M.H.; Writing—Original Draft Preparation, I.D.S.; Review and Editing, P.E.J.v.d.M., C.I.J., C.C.F.M.J.B., J.W.M.H., M.J.P., H.t.C. and J.M.G.; Visualization, I.D.S. and C.C.F.M.J.B.; Funding Acquisition, P.E.J.v.d.M., C.I.J., J.W.M.H., J.M.G. and H.t.C. All authors have read and agreed to the published version of the manuscript.

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Supplementary materials

Coagulation factor XIIIa and activated protein C activate platelets via GPVI and PAR1

Ilaria De Simone[1,2], Constance C.F.M.J. Baaten[1,3], Martine Jandrot-Perrus[4], Jonathan M. Gibbins[2], Hugo ten Cate[1,5], Johan W.M. Heemskerk[1,6], Chris I. Jones[2*], Paola E.J. van der Meijden[1,5*]

[1]Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, Maastricht, The Netherlands; [2]Institute for Metabolic and Cardiovascular Research, School of Biological Sciences, University of Reading, Reading, UK; [3]Institute for Molecular Cardiovascular Research, University Hospital Aachen, RWTH Aachen University, Aachen, Germany; [4]UMR_S1148, Laboratory for Vascular Translational Science, INSERM, University Paris Cité, France; [5]Thrombosis Expertise Center, Heart and Vascular Center, Maastricht University Medical Center, Maastricht, The Netherlands; [6]Synapse Research Institute, Maastricht, The Netherlands

*equal contribution

Running title: Coagulation and anticoagulation factors affecting platelets

Correspondence:

Chris I. Jones, School of Biological Sciences, Institute for Cardiovascular and Metabolic Research, University of Reading, Reading, UK. Tel: +44 (0) 118 378 7047. E-mail: c.i.jones@reading.ac.uk

Paola E. J. van der Meijden, Department of Biochemistry Maastricht University P.O. Box 616, 6200 MD, Maastricht, The Netherlands Tel.: +31 43 3881684, E-mail: p.vandermeijden@maastrichtuniversity.nl

Materials

Collagen-related peptide cross-linked (CRP-XL) was purchased from Prof. Richard Farndale (University of Cambridge, UK). Human α−thrombin came from Kordia (Leiden, The Netherlands. PAR1-activating peptide TRAP6 (SFLLRN) was from Bio Connect (Toronto, Canada); ADP and honokiol were from Sigma (Zwijndrecht, The Netherlands). Collagen-I Horm derived from equine tendon was from Nycomed (Hoofddorp, The Netherlands). Von Willebrandt factor (VWF) was from Invitrogen (Waltham, Massachusetts, United states). Human FXa was from ERL (Enzyme Research Laboratories, Swansea, UK). Human FXIIIa (A subunit, recombinantly produced in insect cells) and the transglutaminase inhibitor T101 were from Zedira (Darmstadt, Germany). The human recombinant activated protein C (APC) was produced, as described elsewhere.[1] Dabigatran etexilate was purchased from Alsachim (Illkirch Graffenstaden, France). Atopaxar was purchased from Axon Medchem (Groningen, the Netherlands). The selective spleen tyrosine kinase (Syk) inhibitor PRT 060318, 2-((1R,2S)-2-aminocyclohexylamino)-4-(m-tolylamino)pyrimidine-5-carboxamide (Syk-IN), came from Bio-Connect (Huissen, the Netherlands). Tirofiban (aggrastat) from Merck Sharp & Dohme. Anti-GPVI Fab 9O12 was a kind gift from Dr. M. Jandrot-Perrus (INSERM, University Paris Diderot, Paris, F).[2] (FITC)-conjugated PAC-1 antibody against active integrin αIIbβ3 was from Becton-Dickinson Bioscience (Franklin Lakes, NJ, USA). Alexa Fluor (AF)647-labelled anti-human CD62P mAb was from Biolegend (San Diego, California, United States).

Preparation of washed platelets

Blood was first centrifuged for 15 min at 258 g (22°C, acc. 9, brake 0; Rotina 380R, Hettich Benelux B.V., Geldermalsen, The Netherlands) to obtain platelet-rich plasma (PRP). PRP was collected and 1:10 Acid Citrate Dextrose (ACD; 80 mM Tri-sodium citrate (.2H2O), 52 mM Citric acid (.H2O), 183 mM D-(+)-glucose) was added, whereafter PRP was centrifuged at 2200 g for 2 min (22°C; Hettich EBA 12, Hettich

Benelux B.V., Geldermalsen, The Netherlands). The platelet pellet was resuspended in Hepes buffer pH 6.6 (10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 0.1% glucose and 0.1 % BSA), whereafter Apyrase (0.1 U/ml) and ACD (1:15) were added. After another centrifugation step (2200 g, 2 min, 22°C; Hettich EBA 12, Hettich Benelux B.V., Geldermalsen, The Netherlands), platelets were resuspended in Hepes buffer pH 7.45 (10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 0.1% glucose and 0.1 % BSA) as described earlier.[3]

Cytosolic $[Ca^{2+}]i$ measurements

Washed human platelets (200×10^9/L) were incubated with Fura-2 acetoxymethyl ester (3 µM) and pluronic (0.4 µg/ml) for 40 minutes at room temperature. After washing away the residual probe, the Fura-2 loaded platelets were resuspended in Hepes buffer pH 7.45 and changes in cytosolic $[Ca^{2+}]i$ were measured in 96-well plates using a FlexStation 3 (Molecular Devices, San Jose, CA, USA). In brief, after adding 1 mM $CaCl_2$, the platelets were stimulated by automated pipetting 10 µg/ml FXa. Changes in Fura-2 fluorescence were continuously measured over time at 37 °C by ratiometric fluorometry at excitation wavelengths of 340 and 380 nm, and an emission wavelength of 510 nm. After correction for background fluorescence, ratio values were used to calculate $[Ca^{2+}]i$ (nM).

Flow cytometric analysis

Integrin αIIbβ3 activation was measured via FITC-conjugated PAC-1 chimeric Ab (1.25 µg/ml), which binds the active integrins. Platelet granular secretion was measured by P-selectin expression, detected using Alexa Fluor (AF)647-labelled antihuman CD62P mAb (2.5 µg/ml).

For the experiment assessing the effect of SCP on platelet activation, washed platelets (500x109 platelets/L) were resuspended in SCP or Hepes buffer (pH 7.45) at a 1:5 ratio and were then stimulated with a range of CRP-XL concentrations (0.003-3 µg/ml) for 10

minutes, in the presence of 2 mM $CaCl_2$. Thereafter, platelets were fixed for at least 10 minutes, by addition of formyl saline (0.2% formaldehyde in 0.15 M NaCl).

For all other flow cytometry experiments, washed platelets ($50x10^9$ platelets/L) were stimulated with a range of CRP-XL (0.03-0.5 µg/mL) concentrations in the presence of PAC-1 to determine a suboptimal agonist concentration per donor (i.e. the agonist concentration generating 40-60% PAC-1 positive platelets). Platelets were then stimulated with a suboptimal agonist concentration combined with FXIIIa (10 U/ml), APC (10 nM) and/or FXa (10 µg/ml), in the presence of 2mM $CaCl_2$. Coagulation factor concentrations were as used previously,[4-6] presumed that physiologically at least 1/10 of the plasma zymogen concentration is activated. Platelets were stimulated for 10 minutes, whereafter they were fixed for at least 10 minutes, by addition of formyl saline (0.2% formaldehyde in 0.15 M NaCl). 5000 platelets per sample were measured using a BD Accuri C6 flow cytometer and analysed with CFlow Plus software (BD Bioscience, Franklin Lakes, New Jersey, United States).

Plate-based aggregation

Measurements of aggregation were performed using 96-well plates (Greiner) containing the following platelet agonists at a range of concentrations: ADP, CRP-XL, TRAP6, Thrombin. No additional fibrinogen was added. Washed platelets resuspended in SCP and control plasma, or washed platelets in Hepes buffer ($400x10^9$ platelets/L) isolated from the citrated blood of healthy donors was loaded onto plates and shaken at 1200 rpm for 5 minutes at 37°C using a plate shaker (Quantifoil Instruments), as described earlier;[7] absorption of 405 nm light was measured using a FlexStation 3 (Molecular Devices, San Jose, CA, USA).

Platelet spreading

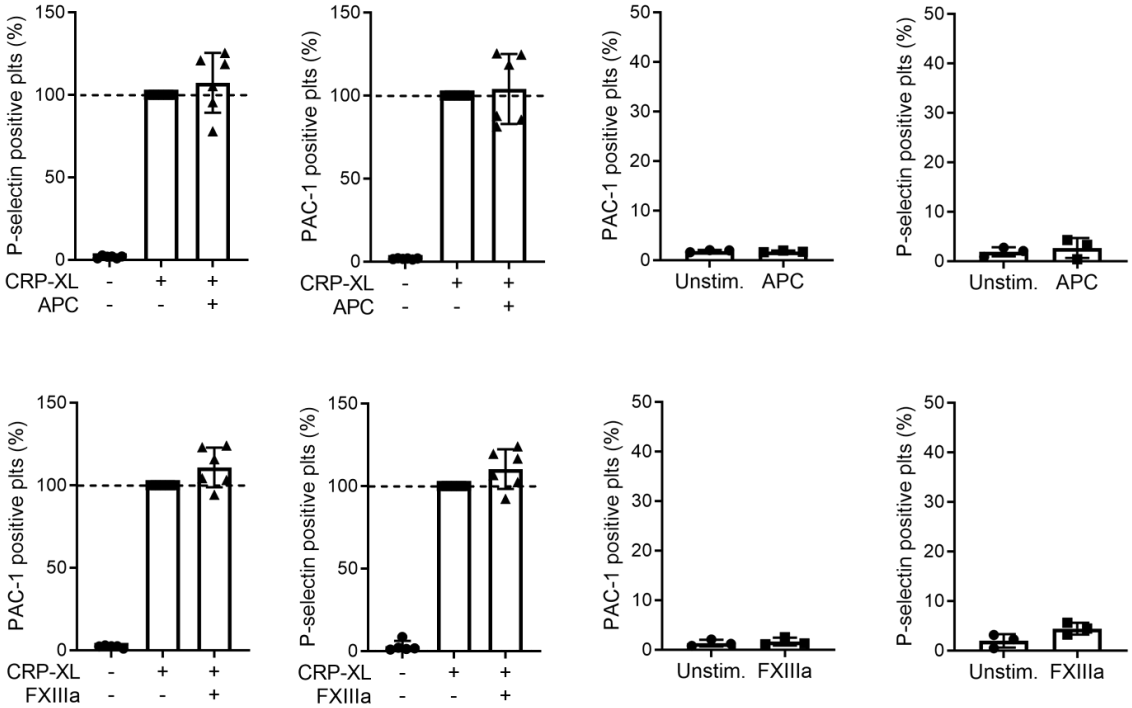
Washed glass coverslips were coated with coagulation factors at concentrations as used previously,[4-6] FXIIIa (10 U/ml), recombinant APC (10 nM) and blocked with 1% BSA.

Washed platelets ($20x10^9$ platelets/L) were allowed to adhere and spread for 45 minutes at 37°C. Non-adherent platelets were removed by gently washing the slides three times with Hepes buffer, pH 7.45. Then, remaining adherent platelets were fixed for 10 minutes, using 1% paraformaldehyde solution. After washing, fixated platelets were permeabilized with 0.005% SDS in phosphate-buffered saline (PBS) for 10 minutes and subsequently blocked with 5% BSA in PBS during 20 minutes. F-actin was stained using phalloidin (1U/mL CF543-phalloidin in 1% BSA in PBS) for 1 hour at room temperature, after which fluorescence images were captured using an EVOS-FL microscope (Life Technologies, Bleiswijk, the Netherlands), equipped with Cy5, RFP, and GFP LEDs; an Olympus UPLSAPO 60× oil immersion objective; and a sensitive 1360×1024 pixel CCD camera. The platelets were scored as being adherent, extending filopodia and fully spread (formation of lamellipodia).

Whole blood perfusion assays

Platelet adhesion and aggregation under flow was studied using the Maastricht parallel-plate flow chamber.[8] Washed coverslips were coated with 0.5 µl microspots, containing VWF (12.5 µg/ml) with or without FXIIIa (200 µg/ml) or recombinant APC (20 nM). The coated coverslips were incubated in a humid chamber for 1 hour, then washed with saline and blocked for 30 minutes with blocking buffer (1% BSA in HEPES buffer, pH 7.45). When indicated, citrated blood was incubated with FXIIIa (10 U/ml), while blood taken on hirudin was incubated with APC (10 nM), for 5 minutes. Subsequently, the citrated blood was recalcified with 3.75 mM $MgCl_2$ and 7.5 mM $CaCl_2$ in the presence of 40 µM PPACK, whereafter it was perfused over a coverslip for 3.5 min or 5 min at a wall-shear rate of 1000 s−1 or 300s-1, respectively. Brightfield images were taken per microspot, using the EVOS-FL microscope (Life Technologies, Bleiswijk, the Netherlands); an Olympus UPLSAPO 60× oil immersion objective; and a sensitive 1360 × 1024 pixel CCD camera. Image analysis was performed using the program Fiji.[9] Parameters extracted from brightfield images were ‘platelet surface area coverage’ (%

PltSac), which is the percentage of area covered with adhered platelets, and 'Microaggregates' (% MicrAgg), which is the percentage of area covered by microaggregates.



Supplementary Figure 1. Individual, soluble (anti-)coagulation factors APC and FXIIIa do not affect CRP-XL induced platelet activation. Washed platelets were preincubated with vehicle or FXIIIa and APC and activated with a submaximal CRP-XL concentration (0.03-0.5 µg/mL). Data scaled relative to CRP-XL. Flow cytometry was used to measure activated integrin αIIbβ3 using FITC labelled PAC-1 mAb and P-selectin expression using Alexa Fluor (AF)647-labelled antihuman CD62P mAb. One sample t-test with false discovery rate correction, mean ± SD, n = 4.

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Chapter 5

Repeated platelet activation and the potential of previously activated platelets to contribute to thrombus formation

While in Chapter 4 the aim was to increase insights on physiological ligands for GPVI and PAR1, in this Chapter we questioned short- and long-term effects of GPVI and PAR1.

This Chapter therefore describes short-and long-term effects of GPVI and GPCR stimulation on platelet activation and aggregation potential which were evaluated via flow cytometry (PAC-1 binding and P-selectin expression) and via plate-based aggregation. Using fluorescence and electron microscopy, platelet morphology and content (granules and fibrinogen uptake) were assessed, as well as thrombus formation. We showed that reversal of platelet activation and re-activation by a second stimulus are possible when platelets are exposed to G-protein coupled receptor (GPCR) agonists (ADP, TRAP6), but to a lesser extent in GPVI-stimulated (CRP-XL) platelets. The main findings of this chapter are: i) Integrin $\alpha_{IIb}\beta_3$ activation and aggregation decreased over time especially in TRAP6- or ADP-activated platelets; ii) Upon reversal of integrin $\alpha_{IIb}\beta_3$ activation, platelets started to return from a filopodia-forming to a smooth, disc-shaped morphology; iii) Previously activated platelets could be re-activated again with specific agonists, depending on the initial trigger, resulting in the reformation of filopodia and secretion of residual granule content; iv) Platelets previously activated with a GPCR agonist regained their potential to contribute to thrombus formation under flow. In contrast, prior platelet triggering with a GPVI agonist was accompanied by more prolonged platelet activity, leading to a decreased secondary platelet adhesion under flow.

I designed this project, collected and analysed all of the data presented here. In addition, I have drafted the manuscript and prepared the paper for submission. For these reasons, I'm first author of this work. The paper is currently under major revision at Journal of Thrombosis and Haemostasis.

Repeated platelet activation and the potential of previously activated platelets to contribute to thrombus formation

Ilaria De Simone [1,2], Constance C.F.M.J. Baaten[1,3], Jonathan M. Gibbins[2], Hugo ten Cate [1,4], Johan W.M. Heemskerk[1,5], Chris I. Jones[2]* and Paola E.J. van der Meijden[1,4]*

[1]Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, The Netherlands. [2]Institute for Cardiovascular and Metabolic Research, School of Biological Sciences, University of Reading, Reading, UK. [3]Institute for Molecular Cardiovascular Research, University Hospital Aachen, RWTH Aachen University, Aachen, Germany. [4]Thrombosis Expertise Center, Heart and Vascular Center, Maastricht University Medical Center, Maastricht, The Netherlands. [5]Synapse Research Institute, Maastricht, The Netherlands. *equal contribution

Running title: Previously activated platelets in thrombus formation

Correspondence:

Ilaria De Simone, Department of Biochemistry, Maastricht University, Maastricht, The Netherlands Tel.: +32 485208280, E-mail: i.desimone@maastrichtuniversity.nl

Abstract

Background: Especially in disease conditions, platelets can encounter activating agents in the circulation.

Objectives: To investigate to which extent previously activated platelets can be reactivated and whether in- and reactivation also applies to different aspects of platelet activation and thrombus formation.

Methods: Short-and long-term effects of GPVI and GPCR stimulation on platelet activation and aggregation potential were compared via flow cytometry and plate-based aggregation. Using fluorescence and electron microscopy, we assessed platelet morphology and content, as well as thrombus formation.

Results: After 30 min of PAR1 or $P2Y_{1/12}$ receptor stimulation, platelets were secondarily decreased in PAC-1 binding and ability to aggregate. Reactivation was possible via another receptor. In contrast, GPVI stimulation evoked persistent effects in $\alpha_{IIb}\beta_3$ activation and platelet aggregation. However, after 60 min of GPVI stimulation, when $\alpha_{IIb}\beta_3$ activation slightly decreased, restimulation with ADP increased integrin activation again. After longer GPCR stimulation, the platelet morphology returned from filopodia-forming to discoid. Interestingly, reactivation of reversed platelets again resulted in the reformation of filopodia and if not fully degranulated, additional secretion. Moreover, platelets previously activated with a GPCR agonist regained their potential to contribute to thrombus formation under flow, similarly to previously unstimulated platelets. Contrary, prior platelet triggering with a GPVI agonist was accompanied by prolonged platelet activity, leading to a decreased secondary platelet adhesion under flow.

Conclusions: This work emphasizes that prior platelet activation can be reversed, whereafter platelets can be reactivated through a different receptor, and previously activated platelets returning to a resting state can contribute to thrombus formation.

Introduction

Blood platelets express a broad range of receptors, that support the roles of platelets in thrombus formation, such as shape change, granule release and aggregation,[1,2] among which the integrin $\alpha_{IIb}\beta_3$ receptor is one of the most highly expressed receptors on platelets, with an abundance of 80000 surface copies.[3] In resting platelets, the affinity of integrin $\alpha_{IIb}\beta_3$ for its ligands is low.[4] Upon platelet activation, shape change occurs, integrins undergo an activating conformational change and the granular content is secreted. During shape change, discoid platelets change into spheres which protrude filopodia.[5] The integrins are activated by extending or 'opening' the extracellular regions of both integrin chains, which allows them to bind ligands, like fibrinogen, with a higher affinity.[6] However, upon strong stimulation, integrin closure can occur when platelets reach a procoagulant state.[7] This process is unidirectional as the integrin β3 subunit is cleaved by calpain.[7-9] Alternatively, integrin inactivation can occur when continuous agonist-induced signalling is abrogated by inhibitors, resulting in the disassembly of thrombi.[10, 11] It is still unclear when and to which extent reversal of platelet activation processes occurs. Further, it is unknown whether platelets take up plasma components after secretion, to form granules again, as a mechanism to recycle. Wencel-Drake et al. showed that TRAP6-induced binding of fibrinogen to platelets decreases over time, thereby demonstrating increased integrin $\alpha_{IIb}\beta_3$ internalisation and reduced aggregability.[12] Interestingly, these platelets were still able to respond to ADP. The ability to reverse platelet activation by different agonists and its consequences for thrombus formation remains to be investigated.

Within a thrombus, platelets in contact with adhesive ligands such as the GPVI ligand collagen, form the thrombus core, which is tightly packed and sustained by high concentrations of thrombin. This core is surrounded by the thrombus shell, in which the platelets bind loosely and activation here is sustained by soluble agonists such as the

GPCR ligands ADP and thrombin (at a lower concentration compared to in the core). As the variability in potency of stimuli, by type and concentration of agonist is crucial for thrombus organisation,[13] we set out to investigate to which extent platelet inactivation occurs upon agonist stimulation by comparing GPVI *vs.* GPCR stimulation. Given that platelets can encounter activating agents during their lifetime (8-10 days), in (patho)physiological conditions,[14] after flowing over an incipient thrombus or after being loosely incorporated in a thrombus shell, we aimed to determine whether previously activated platelets recycle and reset their capacity to respond to agonists. In addition, we studied the thrombogenic potential of previously activated platelets.

Methods

Materials

Collagen-related peptide cross-linked (CRP-XL) was obtained from Prof. Richard Farndale (University of Cambridge, UK). TRAP6 was from Bio Connect (Toronto, Ontario). Adenosine Diphosphate (ADP) and fibrinogen were purchased from Sigma-Aldrich (Dorset, UK)**,** Horm type I collagen was obtained from Nycomed Pharma (Munich, Germany). Fluorescein isothiocyanate (FITC)-conjugated PAC-1 antibody against active integrin $\alpha_{IIb}\beta_3$ and FITC-conjugated anti-CD61 antibody against β_3 were from Becton Dickinson Bioscience (Franklin Lakes, New Jersey, USA). Alexa Fluor (AF)647-conjugated human fibrinogen and Annexin A5 AF647-conjugated were from Invitrogen (Bleiswijk, The Netherlands). AF647-labelled anti-human CD62P mAb was from Biolegend (San Diego, California, United States). AF488-conjugated anti-human CD42b (anti-GPIb) was from R&D systems (Minneapolis, Minnesota, USA).

Blood collection

Blood was obtained by venepuncture from healthy volunteers, who had not received antiplatelet medication for at least two weeks. All volunteers gave full informed consent according to the declaration of Helsinki. Studies were approved by the local Medical

Ethics Committee (MET2017-0285). After discarding the first 2 mL of blood, to avoid contact activation, blood samples were collected into 3.2% trisodium citrate (Vacuette tubes, Greiner Bio-One, Alphen a/d Rijn, The Netherlands). Platelet counts were within the reference range (150-450x10^9 platelets/L), as measured with a Sysmex XP-300 analyser (Sysmex, Cho-ku, Kobe, Japan).

Preparation of washed platelets and plasma

Platelet isolation was performed as described before.[15] In short, platelet-rich plasma (PRP) was obtained by centrifugation of citrated whole blood at 258 g for 15 min. To isolate platelets from the PRP, PRP was centrifuged at 2200 g for 2 min, after addition of 1:10 Acid Citrate Dextrose (ACD; 80 mM Tri-sodium citrate (.2H2O), 52 mM Citric acid, 183 mM D-(+)-glucose). Hereafter, the platelet pellet was resuspended in Hepes buffer pH 6.6 (10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 0.1% glucose and 0.1 % BSA). After addition of Apyrase (0.1 U/mL) and ACD (1:15), another centrifugation step (2200 g, 2 min) followed. Eventually, platelets were resuspended in Hepes buffer pH 7.45 (10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 0.1% glucose and 0.1 % BSA). Platelet-poor plasma (PPP) was obtained from citrated blood, by double centrifugation at 2500 g for 10 min.

Plate-based aggregation

Measurements of aggregation were performed using 96-well plates (Greiner). PRP isolated from the citrated blood of healthy donors was incubated with 30 µM ADP, 3 µg/mL CRP-XL or 15 µM TRAP6. Immediately after stimulation, after 30 and 60 min of incubation with the agonist on 37°C, subsamples were loaded into plates and shaken at 1200 rpm for 5 min at 37°C using a plate shaker (Quantifoil Instruments). Platelet-poor plasma (PPP) was set as control for maximal aggregation. Absorption at 405 nm was measured using a FlexStation 3 (Molecular Devices, San Jose, CA, USA).

Flow cytometric analysis

Washed platelets ($50x10^9$ plts/L) were incubated with low or high dose of agonist (5 and 50 µM ADP; 7.5 and 15 µM TRAP6; 0.5 and 5 µg/mL CRP-XL) in the presence of 2 mM $CaCl_2$, at 37 °C. Integrin activation and P-selectin exposure were measured in subsamples after 10, 30 or 60 min of stimulation using FITC PAC-1 (1.25 µg/mL) or AF647 anti-CD62P (2.5 µg/mL). PAC-1 and anti-CD62P antibodies were added during the last 10 min of agonist incubation. After 60 min of agonist incubation, samples were restimulated with the highest concentrations of ADP (50 µM), TRAP6 (15 µM) or CRP-XL (5 µg/mL) for 10 min and again integrin activation and P-selectin were measured. Flow cytometry was performed using an Accuri C6 flow cytometer and software (Becton-Dickinson Bioscience).

Scanning and Transmission Electron Microscopy

Washed platelets in the presence of 2 mM $CaCl_2$, unstimulated or stimulated with 15 µM TRAP6, 50 µM ADP or 5 µg/mL CRP-XL were subsampled after 10 and 60 min and fixed with 1.5% glutaraldehyde in 0.1 M phosphate buffered to pH 7.4. Subsamples were also taken after 60 min for restimulation with 50 µM ADP or 5 µg/mL CRP-XL, whereafter fixation followed.

For scanning electron microscopy (SEM), fixed samples were washed with 0.1 M cacodylate (pH 7.4), followed by incubation in 1% osmium tetroxide and 1.5% $K_4Fe(CN)_6$ in 0.1 M sodium cacodylate (pH 7.4) for 1 hour at 4 ^oC. Platelets were rinsed with MQ and dehydrated at room temperature in a graded ethanol series (from 70 up to 100%), whereafter they were washed twice in hexamethyldisilazane for 10 min. When dried, SEM samples were ready for imaging using a Scanning Electron Microscope Jeol JSM-IT200 InTouchScope (Jeol, Japan)

For transmission electron microscopy (TEM), fixed samples were washed with 0.1 M cacodylate (pH 7.4) followed by incubation in 1% osmium tetroxide and 1.5%

$K_4Fe(CN)_6$ in 0.1 M sodium cacodylate (pH 7.4) for 1 hour at 4 °C. Platelets were rinsed with MQ, dehydrated at room temperature in a graded ethanol series (from 70 up to 100%) and embedded in Epon. Epon was then polymerized for 48h at 60 °C. 50 nm sections were cut using a diamond knife (Diatome) on a Leica UC7 ultramicrotome and were transferred onto 50 Mesh copper grids covered with a formvar and carbon film, whereafter they were post-stained with uranyl acetate and lead citrate. TEM samples were imaged using FEI Tecnai T12 microscopes (Thermo Fisher Scientific, The Netherlands) at 120kV using an Eagle CCD camera.

Thrombus formation with pre-activated platelets

The ability of prior stimulated platelets to contribute to thrombus formation was investigated using the Maastricht flow chamber.[16] Therefore, washed platelets ($500x10^9$ plts/L) in the presence of 2 mM $CaCl_2$ were either left unstimulated (control) or were stimulated with ADP (50 µM), TRAP6 (15 µM) or CRP-XL (5 µg/ml) at 37°C. A subsample of the (un)stimulated platelets was taken directly or after 30 min and was added ($250x10^9$ plts/L) to washed red blood cells (40%) and fibrinogen (80 µg/mL). $DiOC_6$ (0.5 µg/ml, f.c.) was added to the 'reconstituted blood' to visualise adhered platelets. Coverslips were coated with microspots of 100 µg/mL collagen type I or 100 µg/mL human fibrinogen + 12.5 µg/mL VWF-BP, as described elsewhere.[16]

After a 1-minute plasma perfusion, platelets in the reconstituted environment were perfused through the Maastricht flow chamber, at a wall-shear rate of 1000 s^{-1}. After 3 min of perfusion, tile scans and confocal fluorescence images were made of the whole microspot, using a Zeiss LSM7 Microscope equipped with a 63x oil-immersion objective (Carl Zeiss, Oberkochen, Germany).

Fibrinogen uptake and secretion

Washed platelets ($100x10^9$/L), incubated with 30 µg/mL AF647-fibrinogen in the presence of 2 mM $CaCl_2$, were left unstimulated (control) or were stimulated with 15 µM TRAP6. Subsamples were stained with an anti-GPIbα AF488 conjugated antibody (1.3 µg/mL), 10 min prior to fixation. After 10 and 60 min of stimulation, subsamples were fixed with paraformaldehyde (1% f.c.) for 15 min. Hereafter, fixed samples were centrifuged at 2200 g for 2 min. The platelet pellet was resuspended in PBS and samples were subsequently spun down at 250 g for 10 min on poly-L-lysine coated coverslips (0.01%). Z-stacks were recorded, using a Leica DMI 4000 microscope (Leica Microsystems, Wetzlar, Germany) to investigate cellular localisation of AF647 fibrinogen in platelets using Fiji.[17]

Statistical analysis

Data are presented as mean ± SD. Mean values were compared using an ordinary one-way ANOVA. Differences with p values below 0.05 were considered statistically significant in that: *p<0.05, **p<0.01 and ***p<0.001. GraphPad Prism 8 software (La Jolla CA, USA) was used for statistical analysis.

Results

Platelet activation and aggregation potential decreases over time following agonist stimulation

To investigate the capacity of platelets to aggregate after previous exposure to an agonist, PRP was incubated with CRP-XL (3 µg/mL), TRAP6 (15 µM) or ADP (30 µM) for different time intervals (5, 30, 60 min) (Figure1A). The aggregation response appeared to be highest immediately after the stimulation. After 30 min of incubation with TRAP6, aggregation significantly decreased with 32±10%. After 60 min of incubation, aggregation significantly reduced with all three agonists, although the reduction was higher after TRAP6 (48±8%) or ADP (35±18%), than after CRP-XL (15±6%).

We hypothesised that there may be a different reversibility of platelet activation in washed platelets compared to platelets in plasma. To study the intrinsic potential of platelets, experiments were therefore carried out using washed platelets. Agonist-induced integrin $\alpha_{IIb}\beta_3$ activation (FITC-PAC1 mAb), secretion (P-selectin expression, AF647-CD62P mAb) and PS exposure (AF647-Annexin A5) were thus measured by performing two-color flow cytometric analysis using washed platelets (Figure 1B-C; Supplementary Figure 1). Platelets were activated with low or high concentrations of CRP-XL, TRAP6 or ADP for 10, 30 or 60 min.

After 10 min of agonist stimulation, there was a significant increase in PAC-1 positive platelets, compared to the unstimulated condition for all agonists (5 µg/mL CRP-XL, 15 µM TRAP6 and 5 or 50 µM ADP) (Figure 1B). After 30 and 60 min of stimulation with TRAP6 or ADP, PAC-1 binding decreased and did not significantly differ from the unstimulated condition anymore (Figure 1B). On the other hand, PAC1-binding after 30 and 60 min stimulation with CRP-XL was still significantly higher than PAC-1 binding of the unstimulated (control) platelets (Figure 1B). P-selectin expression after high dose CRP-XL or TRAP6 stimulation remained high over time (80-90%) (Figure 1C). Platelet activation by the agonists did not result in significant phosphatidylserine exposure (Supplementary figure 1A). Since PAC-1 binding following agonist stimulation decreased over time, we checked whether this was due to β3 cleavage or internalisation by measuring CD61 expression (Supplementary figure 1B). No alterations in levels of CD61 could be detected over time. Together, this suggests that at least part of the activated integrins on activated platelets can transform into a ‘low affinity’ state.

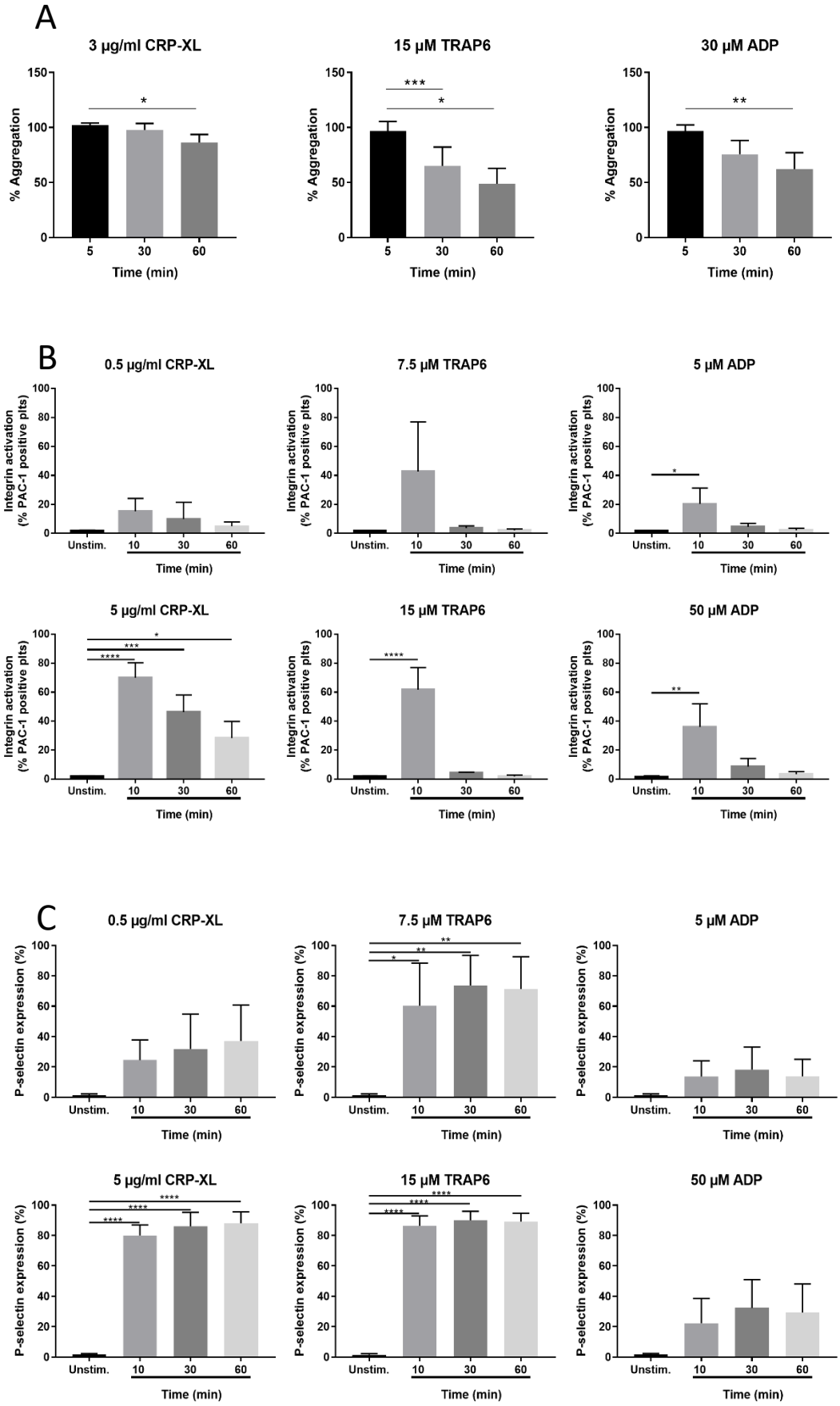

A
3 µg/ml CRP-XL
15 µM TRAP6
30 µM ADP
% Aggregation
Time (min)
B
0.5 µg/ml CRP-XL
7.5 µM TRAP6
5 µM ADP
5 µg/ml CRP-XL
15 µM TRAP6
50 µM ADP
Integrin activation (% PAC-1 positive plts)
Unstim.
Time (min)
C
0.5 µg/ml CRP-XL
7.5 µM TRAP6
5 µM ADP
5 µg/ml CRP-XL
15 µM TRAP6
50 µM ADP
P-selectin expression (%)

Figure 1. Agonist-induced platelet integrin $\alpha_{IIb}\beta_3$ activation and aggregation decrease over time. A. Platelet-rich plasma pre-incubated with 3 µg/mL CRP-XL, 15 µM TRAP6 and 30 µM ADP, for 5, 30 or 60 min. Platelet aggregation was assessed by well plate-based light transmission changes. **B-C**. Washed platelets were activated with low and high concentrations of agonist (0.5 and 5 µg/mL CRP-XL; 7.5 and 15 µM TRAP6; 5 and 50 µM ADP), in the presence of 2 mM $CaCl_2$. After 10, 30 and 60 min of activation, integrin $\alpha_{IIb}\beta_3$ activation **(B)** and P-selectin expression **(C)** were measured by flow cytometry. Mean ±SD, n=3-4; one-way ANOVA, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

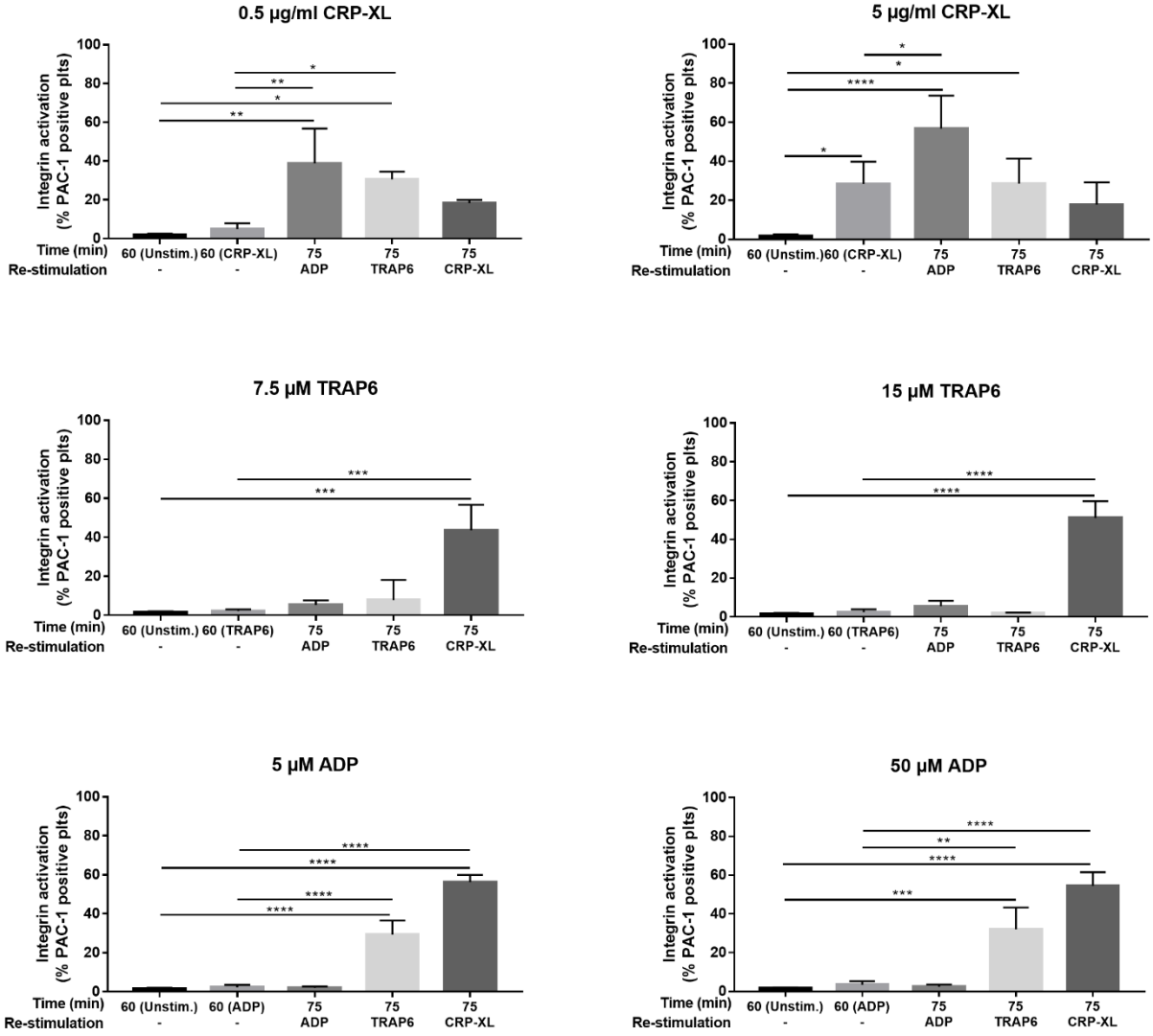
Previously activated platelets can be reactivated by agonists

Next, we investigated whether previously stimulated platelets can be reactivated by a second stimulus, once integrin activation was reversed (60 min after stimulation). To this end, previously stimulated platelets were re-activated with a high agonist dose (5 µg/mL CRP-XL, 15 µM TRAP6 and 50 µM ADP). Interestingly, integrin activation of platelets previously incubated with CRP-XL, only increased significantly after restimulation with ADP (from 5±3% to 39±18% for low CRP-XL and from 29±11% to 57±17% for high CRP-XL); or after restimulation with TRAP6 if previously stimulated with low CRP-XL (from 5±3% to 31±4%). Restimulation with a second dose of CRP-XL did not increase again integrin activation (Figure 2A). Further, platelets that were prior activated with TRAP6, only showed elevated integrin activation after restimulation with CRP-XL, up to 44±13% if previously stimulated with low TRAP6, or up to 51±9% after high TRAP6 stimulation (Figure 2A). Markedly, platelets previously stimulated with ADP, showed reactivation of the integrins induced by either TRAP6 or CRP-XL (Figure 2A). TRAP6 increased the fraction of PAC1-positive platelets from 2.3±1.3% to 29±7.3% (low dose ADP) and 32±11% (high dose ADP), while CRP-XL resulted in 56±3.7% (low dose ADP) and 55±7.0% (high dose ADP) PAC-1 positive platelets (Figure 2A).

Under conditions where upon the initial activation P-selectin expression was maximal, as observed with high TRAP6 or CRP-XL (Figure 2B), P-selectin expression remained maximal over time, regardless of restimulation. However, as expected ADP induced only

limited P-selectin expression, which then significantly increased upon restimulation with TRAP6 or CRP-XL (Figure 2B). Taken together, in platelets, there is a time-dependent pattern of granular secretion, integrin inactivation and reactivation through heterologous receptors.

A



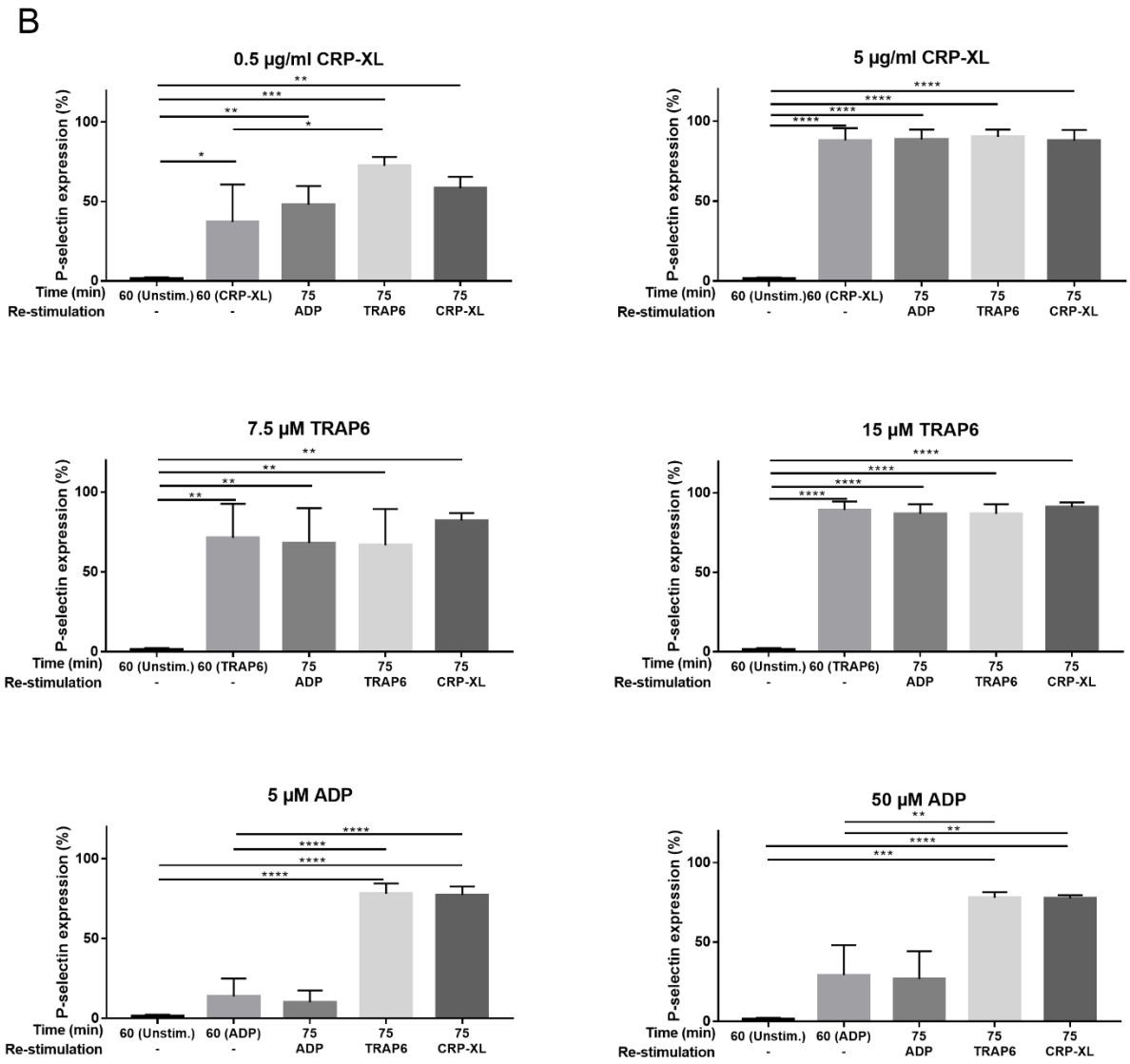
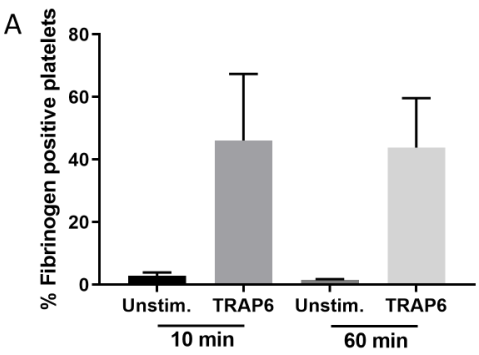


Figure 2. When platelet integrin $\alpha_{IIb}\beta_3$ reverses, platelets can be reactivated again. Washed platelets were activated with low and high concentrations of agonist (0.5 and 5 µg/mL CRP-XL; 7.5 and 15 µM TRAP6; 5 and 50 µM ADP), in the presence of 2 mM $CaCl_2$. After 60 min of activation, platelets were restimulated with high agonist concentrations (50 µM ADP, 7.5 µM TRAP6 and 5 µg/mL CRP-XL), integrin $\alpha_{IIb}\beta_3$ activation **(A)** and P-selectin expression **(B)** were measured by flow cytometry, using PAC-1 and anti-P-selectin antibody, respectively. Mean ±SD, n=4; one-way ANOVA, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Platelet fibrinogen uptake and secretion

We questioned whether reversal of platelet activation also entails the re-uptake of secreted proteins or plasma proteins, such as fibrinogen, which can be secreted again upon activation by a second stimulus. Therefore, washed unstimulated or TRAP6-stimulated platelets were exposed to AF647-labelled fibrinogen and after 10 and 60 min incubation, fibrinogen binding and uptake was assessed by flow cytometry and confocal microscopy. Flow cytometric analysis revealed that TRAP6-stimulated platelets were fibrinogen-positive after 10 min, but also after 60 min (Figure 3A). Since the location of AF647-fibrinogen, on the top or in the middle of the platelets, could not be distinguished by flow cytometry, we applied confocal microscopic imaging. As observed from z-stack images, after incubation in the absence of an agonist, AF647-fibrinogen was not bound to platelets or taken up (Figure 3B, C upper panels). After 10 min of stimulation with TRAP6 instead, AF647-fibrinogen was localised in the middle and on the top of the platelets, while after 60 min of incubation with TRAP6, AF647 fibrinogen was completely in the middle (Figure 3B, C lower panels), as no fibrinogen signal could be detected at the plasma membrane. In case fibrinogen was observed in the middle the platelets, it was unclear whether fibrinogen was partly or completely internalised, or bound to platelets in the open canalicular system. Eventually, upon (re)stimulation of the platelets with CRP-XL, AF647-fibrinogen was again bound to the middle and the top of the platelets (Figure 3D), as fibrinogen was observed in z-stack images from the surface and the middle of the platelets.



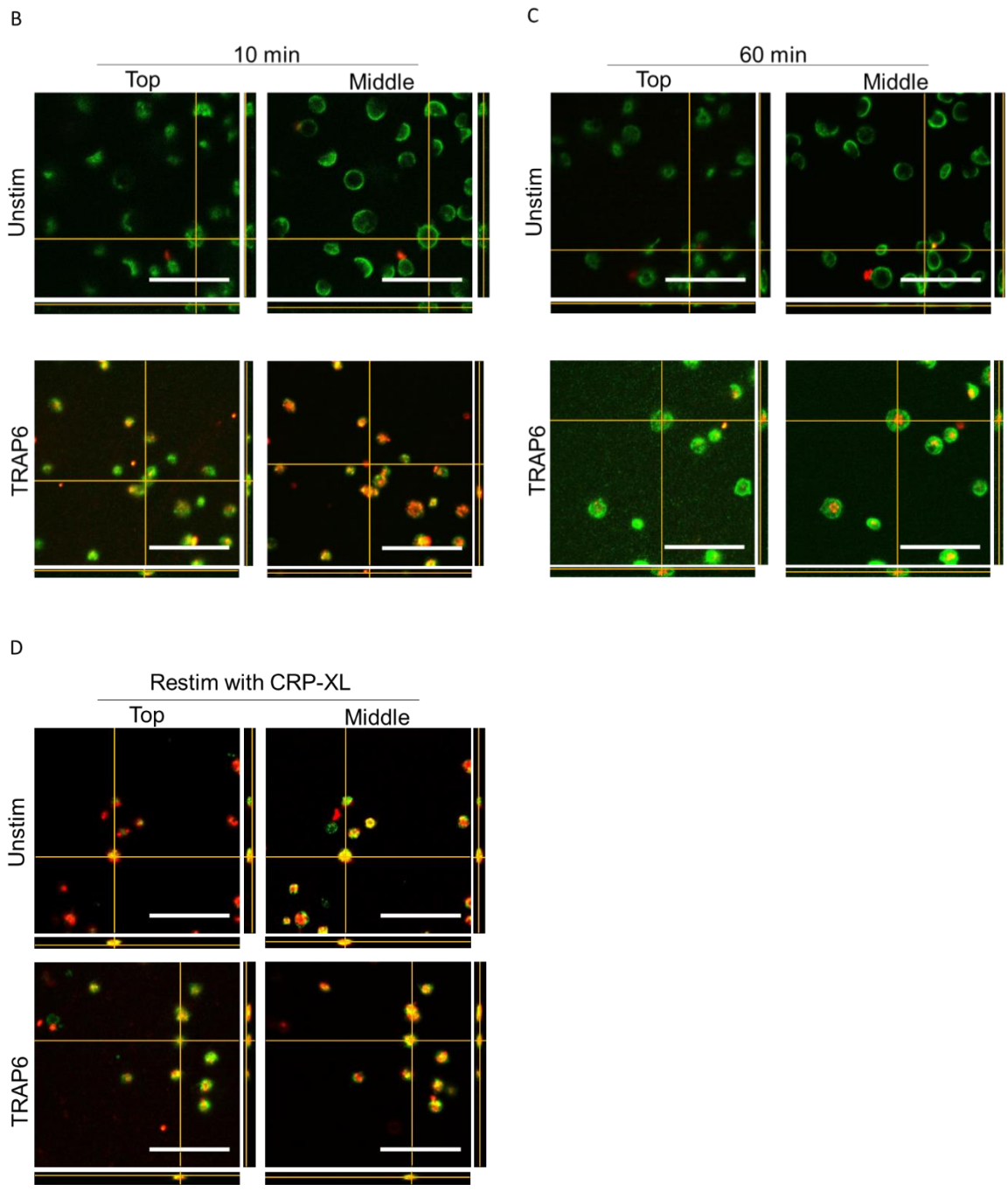


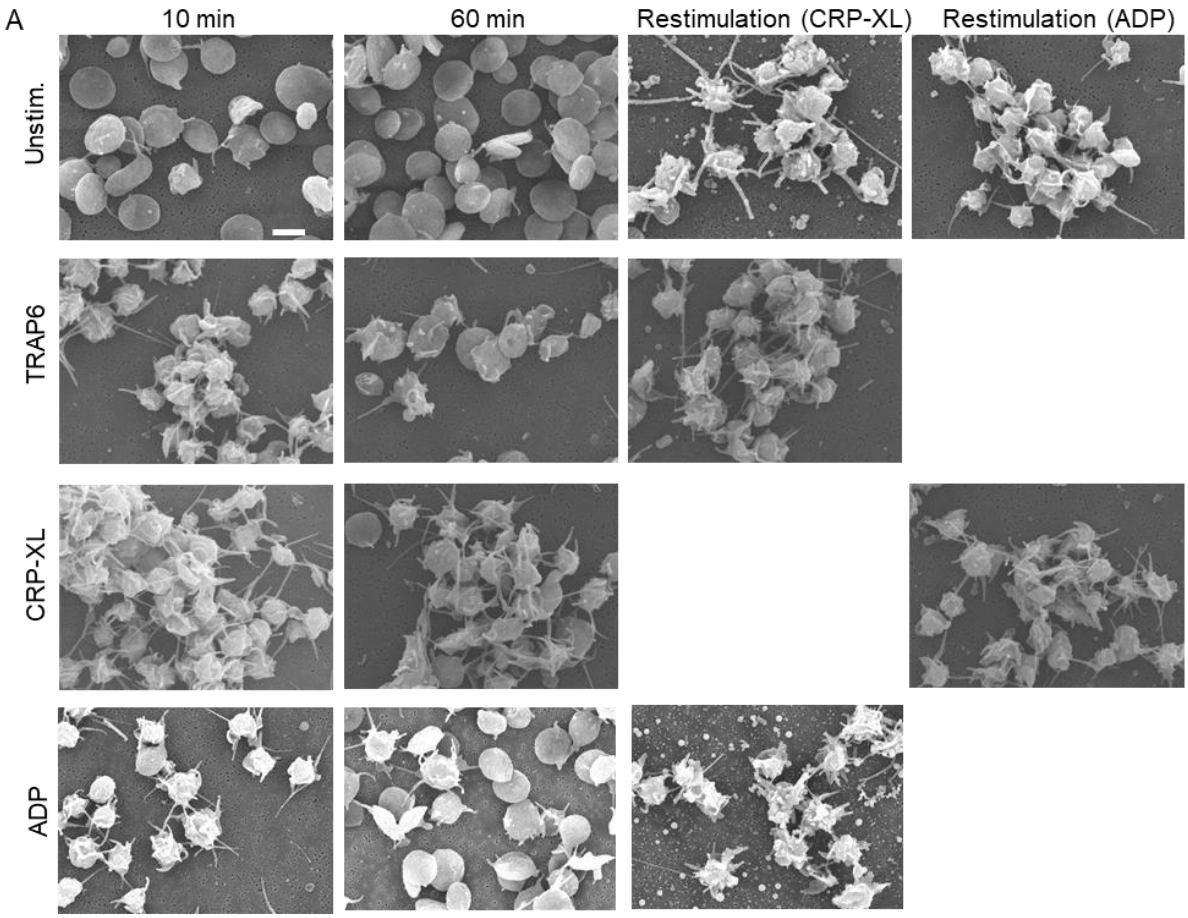
Figure 3. Activated platelets internalise fibrinogen. Washed platelets in the presence of AF647 Fibrinogen (red) and 2 mM $CaCl_2$, unstimulated or stimulated with 15 µM TRAP6 for 10 min or 60 min. **A.** Fibrinogen binding was measured by flow cytometry. **B-D.** Washed platelets in the presence of AF647 Fibrinogen and 2 mM $CaCl_2$, unstimulated or stimulated with 15 µM TRAP6 for 10 min **(B)** or 60 min **(C)**, and restimulated with CRP-XL **(D)**, were fixed and stained for GPIb (anti-GPIba AF488 conjugated antibody, green). Shown are representative microscopy overlays, extracted from Z-stacks. Scale bar=40 µm. n=3

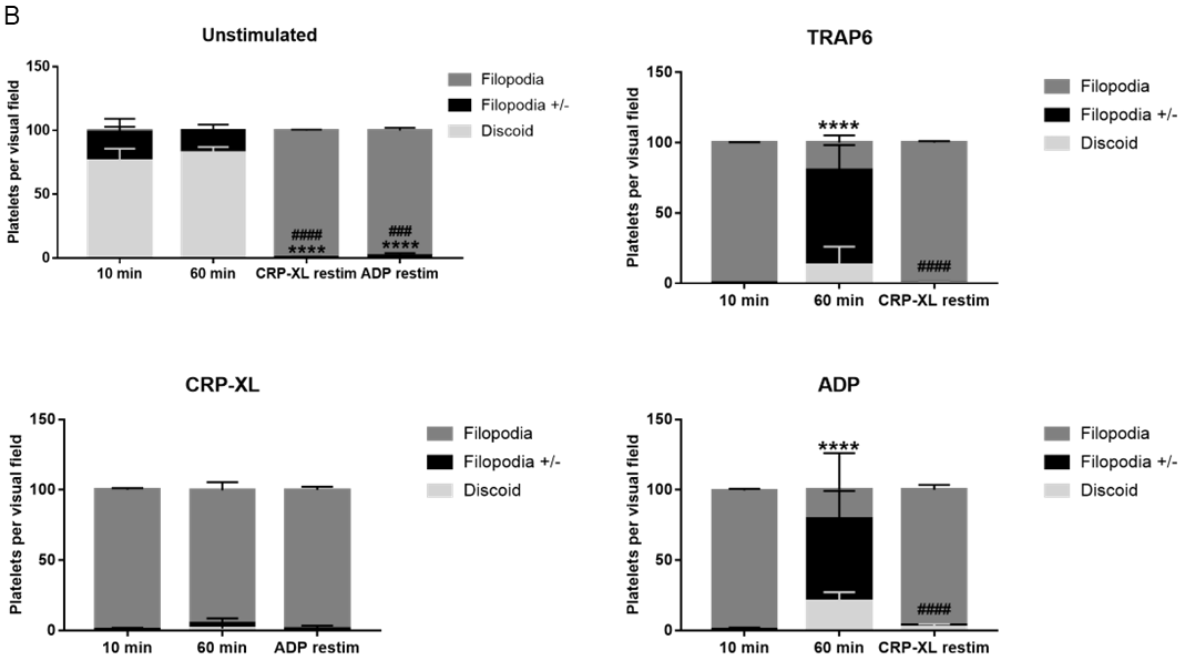
Platelet shape and content change upon activation and inactivation

To assess whether platelets that underwent shape change and formed filopodia can return to the smooth, discoid morphology, which is typical for resting platelets, we performed scanning electron microscopy on platelets stimulated with 15 µM TRAP6, 5 µg/mL CRP-XL or 50 µM ADP for 10 or 60 min. Platelets stimulated with TRAP6, CRP-XL or ADP protruded filopodia, while the unstimulated platelets retained their smooth and discoid shape (Figure 4A,B). Interestingly, after 60 min, TRAP6- or ADP-stimulated platelets almost completely returned to resting morphology (designated as 'Filopodia+/-' phenotype), with a major reduction in the number and length of protruding filopodia, indicating that not only integrin activation is reversible, but the morphological change as well (Figure 4A,B). Under conditions where integrin activation was more persistent, as upon GPVI activation, the activated platelet morphology remained unchanged. In addition, no major change could be observed when CRP-XL-stimulated platelets were restimulated with ADP. Remarkably, when TRAP6- or ADP-stimulated platelets were restimulated with CRP-XL, platelets were able to form filopodia again.

Further, we investigated the platelet content in resting and activated conditions using Transmission Electron Microscopy (TEM) (Figure 4C). As expected, resting washed platelets were filled with granules, both after 10 and 60 min. The open canalicular system (OCS), which is a network of intracellular membrane channels where granules accumulate before secretion,[18] was visible in resting platelets, but upon activation with TRAP6 it expanded, as observed after 10 and 60 minutes incubation. On the other hand, the OCS seemed to become less pronounced after stimulation with CRP-XL, possibly because of fusion with the platelet plasma membrane and fragmentation of the platelets. Since the stimulation with TRAP6 or CRP-XL for 10 and 60 min resulted in secretion, almost no alpha or dense granules could be observed. Although SEM images showed that TRAP6-stimulated platelets reversed after 60 minutes, such reversal was not detectable in the TEM images of TRAP6-stimulated platelets over time. No differences

were noticed intracellularly upon restimulation after 60 minutes of TRAP6- or CRP-XL-stimulated conditions. Stimulation with the weak agonist ADP did not result in granule-depleted platelets, as α-granules were still visible after stimulation. The α-granules, however, started to accumulate in the middle of the platelet, after ADP stimulation, as described before.[19] After restimulation with CRP-XL, previously ADP-stimulated platelets further secreted their granules. The outside morphology of ADP-stimulated platelets showed filopodia, as observed from the SEM samples. Overall, the SEM images showed that platelet morphology upon natural inactivation is reversible from filopodia-forming platelets to disc-shaped platelets, while the TEM images revealed that initial signs of intracellular reversibility were not detectable yet.




B
Unstimulated
TRAP6
CRP-XL
ADP
Platelets per visual field
150
100
50
0
Filopodia
Filopodia +/-
Discoid
10 min
60 min
CRP-XL restim
ADP restim
####
###
****

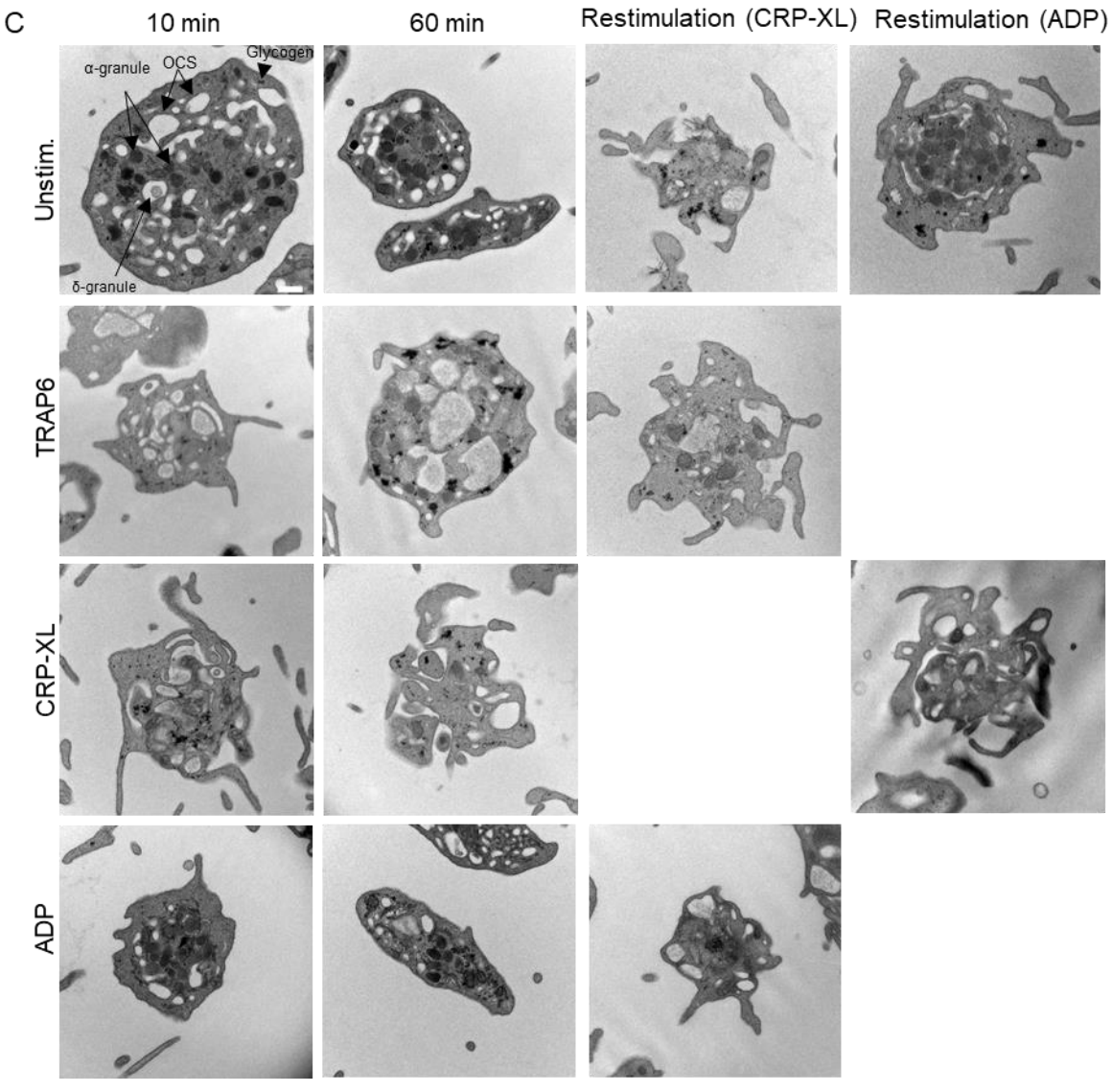
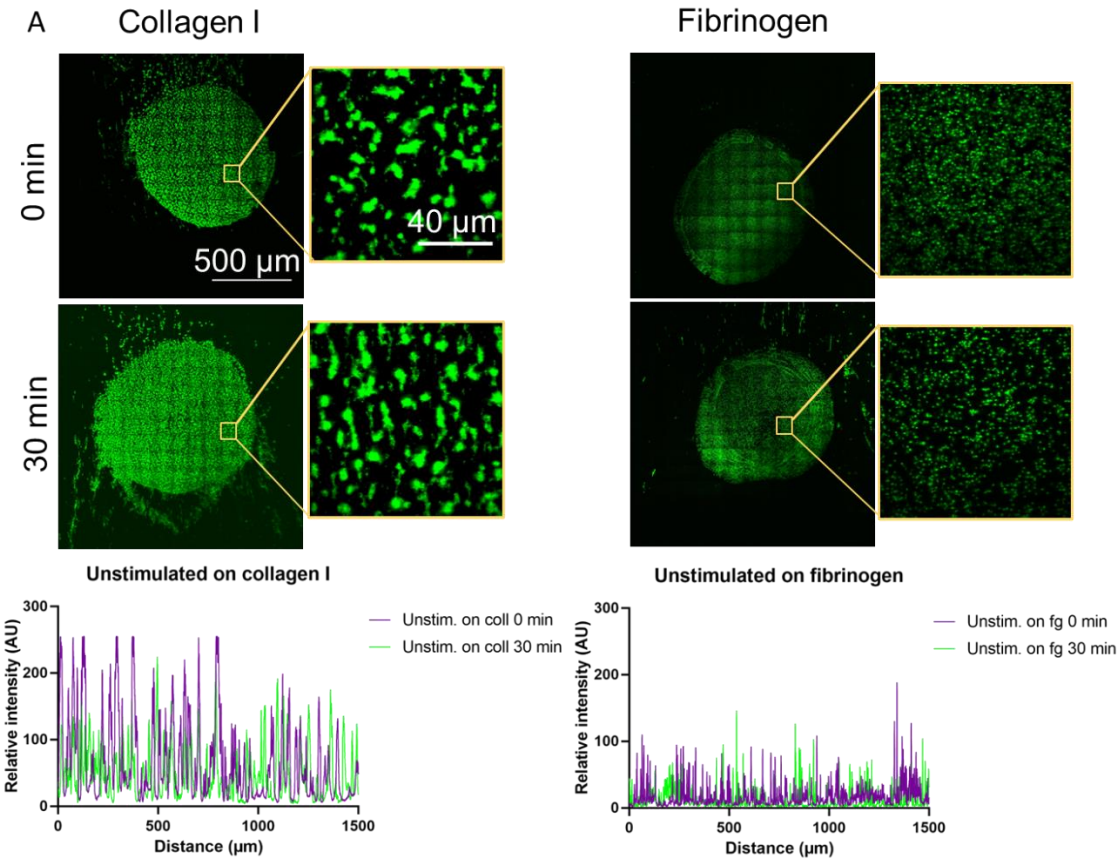

C
10 min
60 min
Restimulation (CRP-XL)
Restimulation (ADP)
Unstim.
TRAP6
CRP-XL
ADP
α-granule
OCS
Glycogen
δ-granule

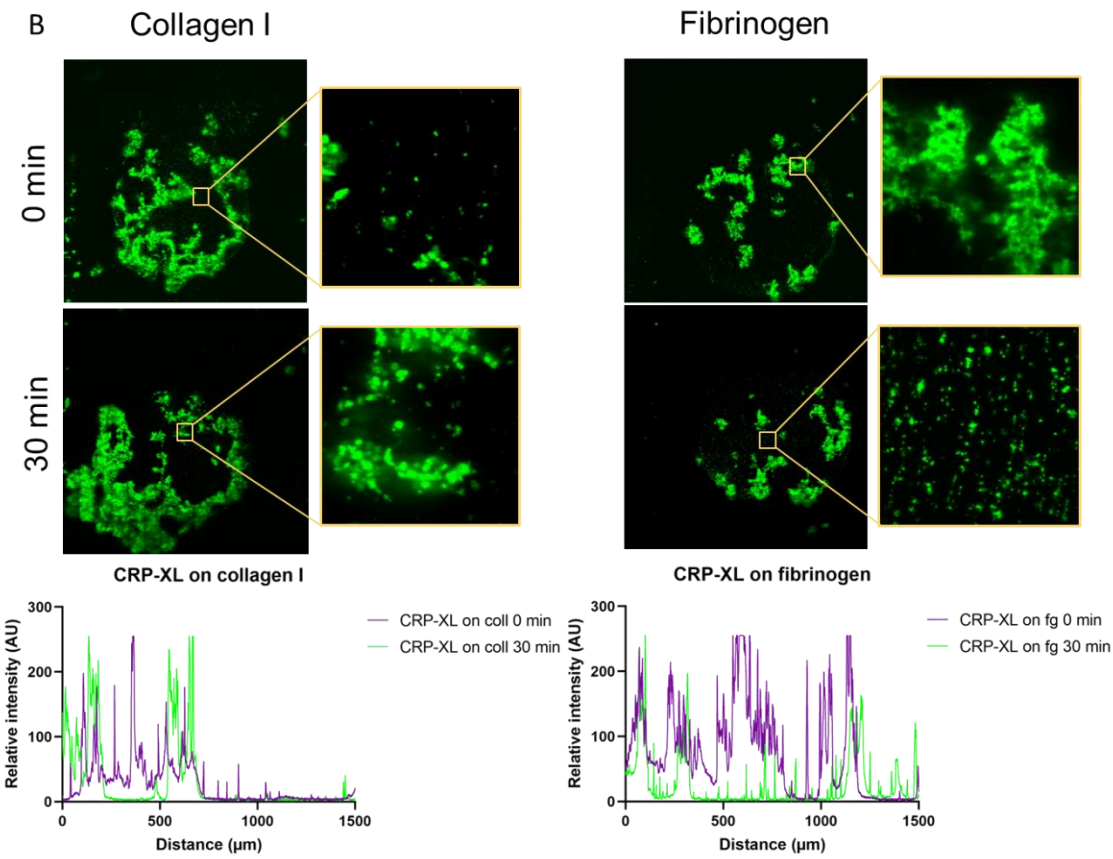
Figure 4. Upon reversal of platelet activation, platelets return to their initial discoid morphology, but intracellular reversibility was not detected. Washed platelets were unstimulated or stimulated with 15 µM TRAP6, 5 µg/mL CRP-XL or 50 µM ADP, for 10 or 60 min, in the presence of 2 mM $CaCl_2$. After 10 or 60 min of stimulation, or after restimulation, platelet morphology was imaged using scanning electron microscopy, scale bar=2µm **(A)**. Quantification of platelets being disc-shaped ('Discoid'), having an intermediate phenotype ('Filopodia +/-) or forming filopodia ('Filopodia') **(B)**. Mean ±SD, n=3; two-way ANOVA, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, compared to 10 min and #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001, compared to 60 min. The platelet content was imaged using transmission electron microscopy, scale bar=500nm **(C)**.

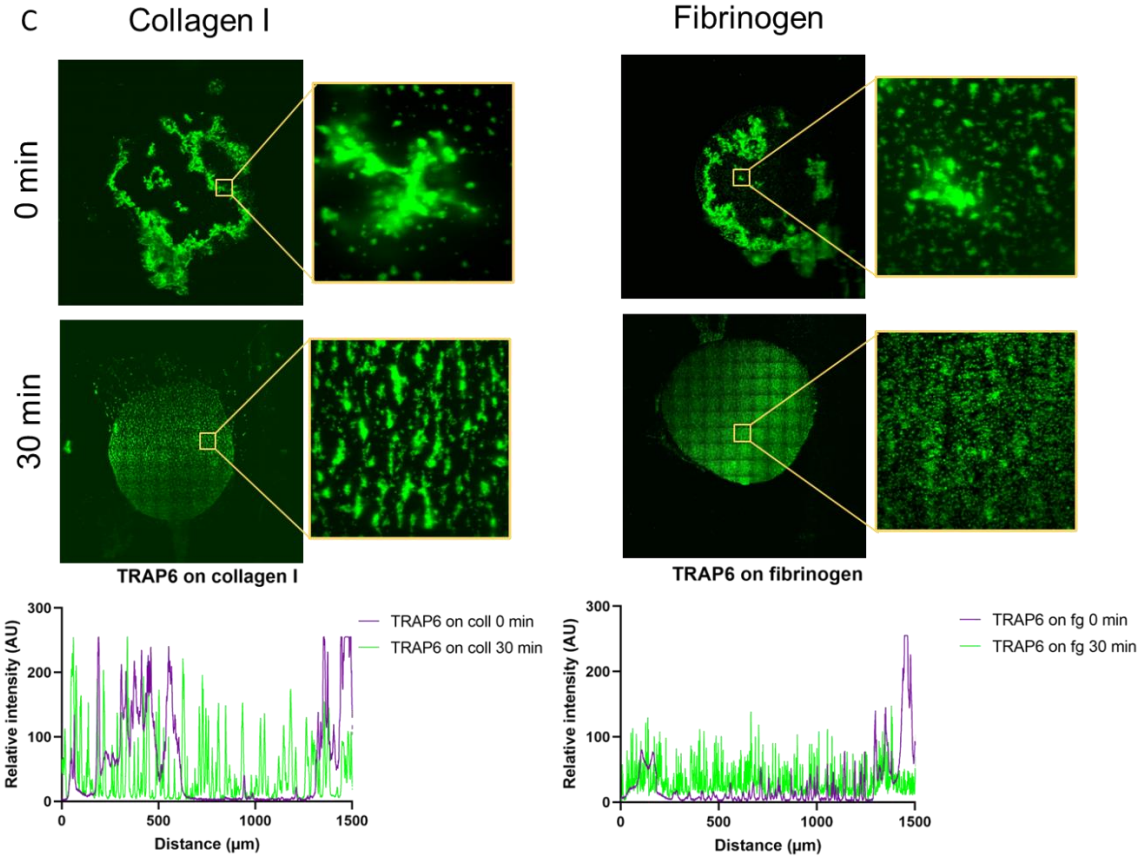
Thrombus formation of previously activated platelets on collagen and fibrinogen surfaces

Since we observed that platelets could be reactivated after prior stimulation, we hypothesised that previously stimulated platelets could still contribute to thrombus formation in a blood-like environment under flow. Therefore, washed platelets were incubated with CRP-XL, TRAP6 or ADP and reconstituted with fibrinogen and red blood cells, immediately or after long-term incubation of 30 min. Then, the reconstituted blood was perfused over microspots coated with collagen or fibrinogen, which were previously rinsed with plasma for VWF binding. Unstimulated (control) platelets, reconstituted with fibrinogen and red blood cells, formed thrombi homogeneously spread over the collagen and fibrinogen surfaces (Figure 5A), with a similar coverage of the area per microspot after 0 or 30 min (Figure 5E). Platelets pre-stimulated with CRP-XL formed large thrombi, which were heterogeneously distributed over the microspots resulting in an overall decrease in surface coverage, both when the platelets were added directly after stimulation (0 min) and after 30 min of stimulation (Figure 5B, E). When platelets were pre-treated with ADP or TRAP6, again large thrombi were formed shortly after stimulation, however after 30 min stimulation the thrombi formed on the collagen and fibrinogen surfaces, again resembled those formed by unstimulated platelets which homogeneously covered the spot (Figure 5C, D). The percentage of surface area coverage on collagen I spots was significantly lower for platelets immediately after

stimulation with ADP or TRAP6, compared to after 30 min of stimulation, in which case the thrombi resembled those of unstimulated (control) platelets (Figure 5E). Taken together, after 30 minutes, the effects induced by stimulation through GPCR receptors were abolished, whereafter platelets previously stimulated with GPCR agonists could again contribute to thrombus formation, with a potential comparable to unstimulated platelets. On the other hand, effects of stimulation through GPVI were sustained, leading to a decreased potential of GPVI-stimulated platelets to contribute to secondary thrombus formation under flow.




B
Collagen I
Fibrinogen
0 min
30 min
CRP-XL on collagen I
CRP-XL on fibrinogen
Relative intensity (AU)
Distance (µm)
CRP-XL on coll 0 min
CRP-XL on coll 30 min
CRP-XL on fg 0 min
CRP-XL on fg 30 min


C
Collagen I
Fibrinogen
0 min
30 min
TRAP6 on collagen I
TRAP6 on fibrinogen
Relative intensity (AU)
Distance (µm)
TRAP6 on coll 0 min
TRAP6 on coll 30 min
TRAP6 on fg 0 min
TRAP6 on fg 30 min

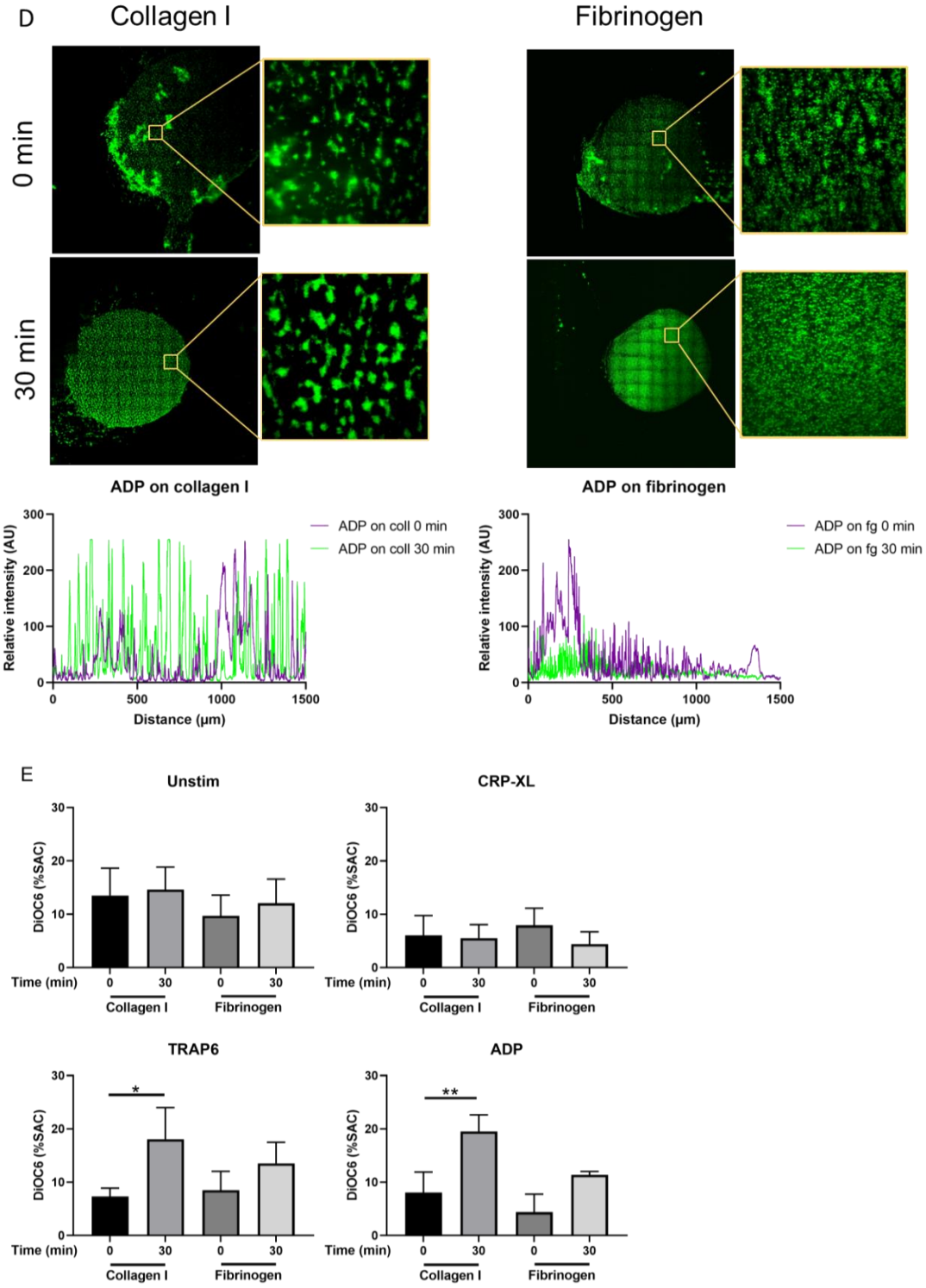


Figure 5. GPCR-stimulated platelets regain their aggregation potential under flow, while platelet stimulation through GPVI is accompanied by more prolonged platelet activity, leading to a decreased secondary platelet adhesion under flow. Washed platelets, in the presence of 2 mM $CaCl_2$, unstimulated **(A)**, stimulated with 5 µM CRP-XL **(B)**, 15 µM TRAP6 **(C)** or 50 µM ADP **(D)**, stained with DiOC6, reconstituted in a 'blood-like' environment, perfused over the microspots collagen I and fibrinogen, at a wall-shear rate of 1000 s^{-1}. After 3 min of perfusion, tile scans were made of the whole microspot. Representative images and cross-sectional intensity profile plots of surfaces

are shown. **E.** Percentages of fluorescence surface-area-coverage (%SAC). Mean ±SD, n=3-5; one-way ANOVA, *p<0.05, **p<0.01.

Discussion

In the present paper, we show that reversal of platelet activation and re-activation by a second stimulus occur when platelets are exposed to G-protein coupled receptor (GPCR) agonists (ADP, TRAP6), but to a lesser extent in GPVI-stimulated (CRP-XL) platelets. Integrin $\alpha_{IIb}\beta_3$ activation and aggregation decreased over time especially in TRAP6- or ADP-activated platelets. Remarkably, upon reversal of integrin $\alpha_{IIb}\beta_3$ activation, platelets started to return to their initial smooth, discoid morphology. These platelets could be re-activated again with specific agonists, depending on the initial trigger, resulting in the reformation of filopodia and secretion of residual granule content. Interestingly, we showed for the first time that platelets previously activated with a GPCR agonist regained their potential to contribute to thrombus formation under flow. In contrast, prior platelet triggering with a GPVI agonist was accompanied by more prolonged platelet activity, leading to a decreased secondary platelet adhesion under flow. The observation that platelets previously stimulated through GPCRs regained their potential to contribute to thrombus formation under flow supports previous reports which described that GPCRs recycle after desensitisation.[20, 21] The finding that integrin $\alpha_{IIb}\beta_3$ activation after GPCR stimulation is transient, and rather persistent upon GPVI stimulation, is in agreement with the recent findings by Zou et al. showing that there is a maintained Ca^{2+} signal with CRP-XL, in comparison to TRAP6, which points to a longer-term activation state of the platelets.[11] With the agonist concentrations used in our assay, we showed that platelets did not increase in phosphatidylserine exposure, thus that the observed decreased integrin activation is not due to a procoagulant state. In other reports, reversibility of platelet integrin activation was studied,[10, 11] however, the reversibility was evoked with a $P2Y_{12}$ receptor inhibitor. In this paper, we investigated natural integrin inactivation over time, thus without use of inhibitors. Whether inhibitors enhance the process of natural integrin inactivation is so far unclear. Also, to which

extent plasma affects natural integrin inactivation and contributes to intrinsic platelet reversal, should be further investigated in the future.

We observed that upon GPCR stimulation followed by natural integrin inactivation, re-activation of platelets was possible through other receptors than through which the activation was initially evoked, indicating that the platelets were desensitised for previously stimulated receptors, but not completely exhausted. After long-term CRP-XL stimulation, platelets also showed slightly less integrin activation compared to immediately after stimulation. A striking observation was that integrin activation in prior CRP-XL-stimulated platelets was induced by a second stimulation with ADP, however the underlying mechanism for this remains unclear. The present data hence point to a regulated mechanism of receptor switch off. We speculate that our observations of integrin inactivation upon GPCR stimulation over time are due to GPCR receptor desensitisation, a mechanism which is dependent on the type of receptors, they are internalised and can later be degraded or recycled.[20, 22-25] It is probable that integrin inactivation is observed to a lower extent following long-term GPVI stimulation, because the GPVI receptor is not desensitized. However, ADAM-mediated shedding of the GPVI receptor after ligand exposure,[26] might explain the unresponsiveness to restimulation of these platelets.

Our flow cytometry data showed that when platelets were stimulated with weak agonists (such as ADP) or low concentration of agonist (eg. low CRP-XL), further secretion was possible upon restimulation. It is likely that upon weak stimulation only part of the individual granules fuse with the platelet surface, while upon potent triggers, there is first granule-to-granule fusion followed by full degranulation.[19] Our data suggest that further secretion is only possible if platelets did not fully secrete. We found that TRAP6-stimulated platelets were able to take up fibrinogen from the environment, but whether the fibrinogen is actually internalised or accumulated in the OCS, is still unclear. The available literature suggests that fibrinogen after uptake concentrates in the OCS,[27] and

that the major determinant of platelet fibrinogen uptake is integrin $\alpha_{IIb}\beta_3$, since platelet fibrinogen was reduced in samples of Glanzmann patients,[28] and that fibrinogen internalization modulates the return of stimulated platelets to a resting state.[12]

Typically, we found that reversed platelets after GPCR stimulation, in contrast to GPVI stimulation, could fully participate again in thrombus formation on coated surfaces. Translated to physiology, the difference between GPVI and GPCR (PAR1 or $P2Y_{1/12}$) receptor stimulation indicates that activation of platelets by extracellular matrix ligands is more persistent, while activation of circulating platelets by soluble agonists is more transient. In vivo, platelets are exposed to a mixture of agonists, but depending on the location of a platelet in a thrombus the influence of certain agonists will be more prominent.[13] The formation of fibrinogen bridges is described to be a multistep process, in which initial contact between platelets and fibrinogen is reversible, followed by irreversible binding, whereafter it is probably not possible for platelets to disaggregate again.[29, 30] It thus remains to be shown whether platelets passing by an incipient thrombus without being incorporated but being exposed to localised high concentrations of several soluble agonists, as well as platelets upon initial reversible contact with fibrinogen, can return to their resting state before being removed from the circulation and as such can still contribute to haemostasis. Future studies are needed to unravel whether platelets can be recycled after exposure to multiple agonists, as this will more likely be the case *in vivo*, during thrombotic events. On another note, based on our data showing that platelets can be reactivated, it is questioned whether exhausted platelets, characterised by desensitised receptors, inactive integrins, and previous secretion,[31] are truly exhausted and if this definition needs to be reconsidered.

In conclusion, our data show that platelet triggering through GPVI induces prolonged responses on platelets, while stimulation through the GPCR receptors PAR1 or $P2Y_{1/12}$ induces rather transient platelet effects, whereafter platelets return to a state in which they can be restimulated and might still contribute to vascular repair.

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Authorships and disclosures:

Authorship: Conceptualization, PEJvdM, CIJ, JWMH, HtC, JMG; Methodology & Investigation, IDS and CCFMJB; Analysis & Data Curation, IDS; Resources, PEJvdM, CIJ, HtC, JMG and JWMH; Writing – Original Draft Preparation, IDS; Review & Editing, PEJvdM, CIJ, CCFMJB, JWMH, HtC, JMG; Visualisation, IDS and CCFMJB; Funding Acquisition, PEJvdM, CIJ, JWMH, JMG, HtC.

Conflicts of interest: The authors declare no relevant conflict of interest

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Supplementary material and methods

Materials

Collagen-related peptide cross-linked (CRP-XL) was obtained from Prof. Richard Farndale (University of Cambridge, UK). TRAP6 was from Bio Connect (Toronto, Ontario). Adenosine Diphosphate (ADP) was purchased from Sigma-Aldrich (Dorset, UK)**,** FITC-conjugated anti-CD61 antibody against β_3 was from Becton Dickinson Bioscience (Franklin Lakes, New Jersey, USA). Annexin A5 AF647-conjugated was from Invitrogen (Bleiswijk, The Netherlands).

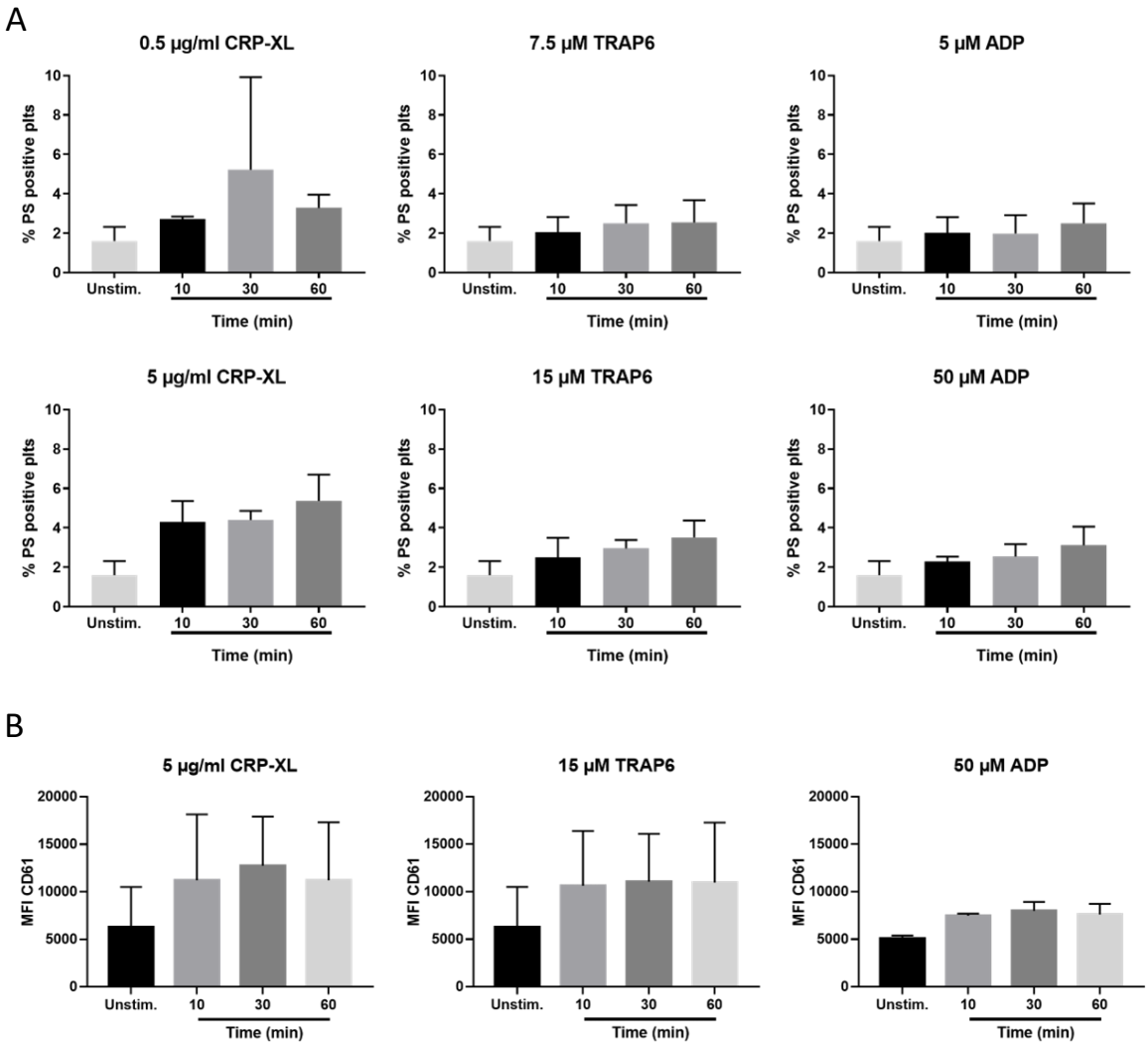
For washed platelet preparation, see section '*Preparation of washed platelets and plasma'*.

Flow cytometric analysis

Washed platelets ($50x10^9$ plts/L) were incubated with low or high dose of agonist (5 and 50 µM ADP; 7.5 and 15 µM TRAP6; 0.5 and 5 µg/mL CRP-XL) in the presence of 2 mM $CaCl_2$, at 37 °C. To measure phosphatidylserine exposure, annexin buffer (Hepes 7.45, 5 µg/mL AF647 Annexin A5, 2 mM $CaCl_2$) was added during the last 2 min of incubation.

To measure AF647-fibrinogen binding, and β3 internalisation (anti-CD61 antibody), washed platelets ($50x10^9$ plts/L) were incubated at 37°C with 5 µg/mL CRP-XL, 15 µM TRAP6 and 50 µM ADP for 10, 30, or 60 min. Fluorescent labels (150 µg/mL AF647-fibrinogen and 1.25 µg/mL FITC-conjugated anti-CD61 antibody) were added during the last 10 min of the incubation. Flow cytometry was performed using an Accuri C6 flow cytometer and software (Becton-Dickinson Bioscience).

Supplementary data



Supplementary Figure 1. Agonist-induced platelet phosphatidylserine exposure and CD61 expression levels are unaltered over time. A. Washed platelets were activated with low and high concentrations of agonist (0.5 and 5 µg/mL CRP-XL; 7.5 and 15 µM TRAP6; 5 and 50 µM ADP), in the presence of 2 mM $CaCl_2$. After 10, 30 and 60 min of activation, Annexin AF647 binding was measured by flow cytometry. **B.** Washed platelets were activated with high concentrations of agonist (5 µg/mL CRP-XL, 15 µM TRAP6 and 50 µM ADP), in the presence of 2 mM $CaCl_2$. After 10, 30 and 60 min of activation, CD61 expression levels were analysed by flow cytometry. Mean ±SD, n=3-4; one-way ANOVA, ns.

Chapter 6

Inter-individual variation in platelet function and its impact on the effectiveness of antiplatelet medication

As patients often experience bleeding complications or drug ineffectiveness due to inter-individual differences, there is still an unmet clinical need for tools which allow a more personalised antiplatelet therapy. In the next Chapter, we therefore used a flow cytometric assay to assess platelet functioning and drug responsiveness in patients with CAD with or without T2DM, which we compared to demographically matched patient controls and healthy controls. In this assay, platelet capacity and sensitivity to the GPVI and PAR1 agonists, CRP-XL and TRAP6, respectively, were investigated and were thereafter correlated with reduction in response due to Cangrelor treatment. We showed a positive correlation between initial capacity to respond to an agonist, and reduction upon Cangrelor treatment, indicating that drug efficacy could be predicted by assessment of initial platelet responses. Overall, this chapter describes how a flow cytometric assay could possibly be used to predict antiplatelet drug effectiveness and bleeding risks.

I have contributed to collection of the data presented in this work. Further, I have drafted the manuscript. For these reasons, I'm second author of this work. The paper is currently in preparation.

Inter-individual variation in platelet function and its impact on the effectiveness of antiplatelet medication

Yasmin Mohammed[1], Ilaria De Simone[1,2], Joanne Dunster[1], Neline Kriek[1], Tanya Sage[1], Mark Brunton[3], Sharon Mark[3], Abigail Whyte[3], Neil Ruparelia[3], Charlie McKenna[3], Chris I Jones[1] and Jonathan M.Gibbins

[1]Institute for Cardiovascular and Metabolic Research, School of Biological Sciences, University of Reading, Reading, UK

[2]Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, The Netherlands

[3]University Department of Cardiology, Royal Berkshire Hospital, UK

Running title: Platelet Functional Variability in Health and Disease

Correspondence: j.m.gibbins@reading.ac.uk

Abstract

Platelet activation and thrombus formation are the primary processes behind coronary artery disease and stroke. Physicians widely use antiplatelet medications for the prevention and treatment of those diseases, however, variation in response to anti-platelet medication is well documented. To investigate if the efficacy of antiplatelet medication is related to patients' clinical status and/or their platelet responsiveness before therapy, we assessed the sensitivity and maximum capacity of platelet response in 79 individuals, among which 23 were healthy controls, 13 patient controls, 31 patients with coronary artery disease and 12 patients with coronary artery disease and type II diabetes. Platelet-rich plasma was pre-treated with Cangrelor and stimulated with a concentration range of CRP-XL and TRAP-6 using 96 well freeze-dried plates and fibrinogen binding to integrin $\alpha_{IIb}\beta_3$ measured by flow cytometry. In all participants combined, the effect of Cangrelor was significantly but modestly correlated with the initial response to CRP-XL and TRAP-6 (r^2=0.14, p=0.02 and r^2=0.37, p<0.0001 respectively) and the reduction in platelet response due to Cangrelor significantly correlated with the initial sensitivity to CRP-XL and TRAP-6 (r^2=0.12, p<0.01 and r^2=0.32, p≤0.0001). The correlations observed in the healthy and patient control groups were lower than those in the CAD and CAD+T2DM groups. Cangrelor exerted significant inhibition of platelet activation with a wide variation among the patients group, with a greater reduction obtained in the patient groups than in the healthy controls. Greater response to platelet agonists was associated with greater degree of inhibition by cangrelor, and vice versa. The variation in responses observed, particularly notable with patients' platelets, may have impact on the efficacy of antiplatelet medications, and therefore capacity and sensitivity assessment of patients' platelets could help in applying a tailored approach for the use of antiplatelet medications.

Introduction

Platelets have a major role in haemostasis and thrombosis,[1] and their response to stimulating and inhibitory factors varies widely within the population.[2-6] Inappropriate platelet activation leads to pathological thrombus formation resulting in heart muscle ischemia, or myocardial infarction, which can be fatal.[7] Antiplatelet medications are widely used in the prevention and treatment of cardiovascular diseases and successfully reduce the recurrence of myocardial infarction and stroke.[8-11] One of the major side effects of these drugs, however, is bleeding complications.[10] The precise reasons why many patients benefit from antiplatelet drugs, yet others experience side effects remains unclear.

The current standard antiplatelet therapy consists of a combination of aspirin with a $P2Y_{12}$ receptor inhibitor, such as Clopidogrel, Ticagrelor, Prasugrel, or Cangrelor.[12] Clopidogrel, the most used $P2Y_{12}$ receptor inhibitor, is a prodrug that is converted to its active metabolite by the liver enzyme cytochrome p450 (CYP450),[13] and is therefore less effective in patients with CYP450 mutations.[14] Ticagrelor is a more potent $P2Y_{12}$ receptor inhibitor, that unlike Clopidogrel does not require metabolic conversion to interact with the ADP receptor.[13] Its increased potency, however, comes at the expense of increased bleeding risk.[15] The $P2Y_{12}$ receptor inhibitor Cangrelor is administered intravenously and achieves a faster and more potent inhibition than oral $P2Y_{12}$ inhibitors.[16]

Apart from inter-individual differences in drug metabolism based on enzyme activity (eg CYP450 mutations), variability between patients' platelet responses to antiplatelet medication may be related to age and sex,[17, 18] however, the mechanisms underlying inter-individual variability are largely unknown. Several disease states can also alter platelet responsiveness. Patients with type 2 diabetes mellitus (T2DM) have a two-to-four-fold higher risk of recurrent atherothrombotic events and vascular complications as compared to non-T2DM patients.[19] Platelets play a major role in the pathogenesis of

thrombo-ischaemic complications in diabetic patients and altered platelet morphology and function, causing platelet hyper-reactivity, have been reported in patients with diabetes.[20]

Current antiplatelet medications are thus often effective, however, resistance to medication, recurrent thrombotic events, and side effects such as bleeding, indicate the need for novel antiplatelet strategies and a more tailored approach. It is important to consider that disease processes are multifactorial and complex interacting forces should be looked at collectively. Studies on platelets in CVD or diabetics should consider the complex factors including inflammatory, immune and genetic factors. In this paper, we employ the Platelet Phenomic Analysis (PPAnalysis) [21] to measure concentration-dependent responses to agonists and antiplatelet therapies, in order to investigate if there are characteristic differences in platelet function and whether this is related to response to the $P2Y_{12}$ antagonist Cangrelor in patients with established coronary artery disease (CAD) with or without type 2 Diabetes Mellitus (T2DM) compared to healthy controls and demographically matched controls.

Methods

Materials

Collagen-related peptide (CRP-XL) was obtained from Professor Richard Farndale (University of Cambridge, Cambridge, UK). Thrombin-receptor activated peptide (TRAP-6) was purchased from Bachem (Switzerland). Cangrelor was a gift from the Medicines Company. Fluorescein isothiocyanate (FITC) conjugated polyclonal rabbit anti-human fibrinogen antibody was from Dako (Glostrup, Denmark; catalog no. F0111).

Patient recruitment: inclusion and exclusion criteria

Peripheral blood from 23 fasted, healthy, aspirin free donors between the ages of 30-65 (Healthy Control) was collected with informed consent and with approval by the University of Reading Research Ethics Committee.

Patients were investigated for stable CAD as a part of their clinical work-up which includes coronary angiography at the Royal Berkshire Hospital in Reading, UK, and were classified into 3 different groups; those with no CAD following coronary angiography and no Type II Diabetes Mellitus (T2DM) (Patient Control; n=13); with CAD and no T2DM (CAD; n=31); with CAD and T2DM (CAD+T2DM; n=12). Exclusion criteria were: acute coronary syndrome (ACS) in the past 12 months, taking $P2Y_{12}$ receptor inhibitors, anti-coagulants, other metabolic dysfunctions, active or recent malignancy (<2 years), pregnancy, evidence of alcohol or drug misuse, any underlying haematological pathologies. All samples were collected with informed consent and with approval by the University of Reading Research Ethics Committee and NHS health Research Authority (Ref:19/LO/0572).

Blood collection and platelet-rich plasma preparation

Blood samples were collected into vacutainers containing 3.8% (w/v) sodium citrate (Greiner Bio-one LTD, UK); the first 3 mL was discarded. Whole blood was centrifuged at 102 g for 20 minutes at 20°C to obtain platelet-rich plasma (PRP).

Platelet functional assay using flow cytometry

Freeze-dried agonist plates were prepared as follows: half area 96 well plates were incubated for 4 hours with gelatin solution (0.75% w/v gelatin and 0.05% Tween-20 in 20mM phosphate buffer, pH 7.4), then washed twice with 0.01% (v/v) Triton X-100 in distilled water and three times with distilled water, whereafter they were air-dried. TRAP-6 was diluted in 0.1% ascorbic acid/ ddH2O, while CRP-XL was diluted in 0.01% Human albumin/ddH2O. 5 µl CRP-XL (0.03 - 3 µM) or TRAP-6 (0.15 - 15 µM) was added to the gelatine coated plates. Plates were frozen (-80 °C) and freeze-dried using the VirTis advantage Plus freeze-dryer (Biopharma, Winchester, UK), vacuum-sealed, and stored at room temperature in the dark for later use. Immediately before use, freeze-dried agonist plates were rehydrated using Hepes Buffered Saline (HBS; 10mM Hepes, 150 mM NaCl, 1mM $MgSO_4$, 5mM KCl, pH 7.4) and, if indicated, with the addition Cangrelor

(0.003 - 0.3 µM final) for 10 minutes. PRP was incubated with 0.14 mg/mL FITC-conjugated anti-fibrinogen antibody and, where indicated, treated with Cangrelor (0.003 - 0.3 µM) for 10 minutes at 37 °C. PRP was subsequently added to the freeze-dried agonist plate, followed by incubation in the dark for 20 minutes at room temperature. Samples were fixed by adding 0.2% formyl saline (FS; 140 mM NaCl, 0.2% formaldehyde), before flow cytometric analysis using an Accuri C6 flow cytometer (BD, Winnersh, UK). 5000 platelet events were recorded.

For characterisation of platelet function, data were analysed by plotting dose response curves, as described before [21] and as illustrated in figure 1. The x-axes represent log agonist concentration and y-axes median fluorescence intensity (MFI). Curves were fitted by performing nonlinear regression using parameter analysis, log agonist stimulation, where sensitivity (EC50, the agonist concentration that gives half maximal responses) and platelet response capacity (reflects an individual's capacity to respond to an agonist) were determined.

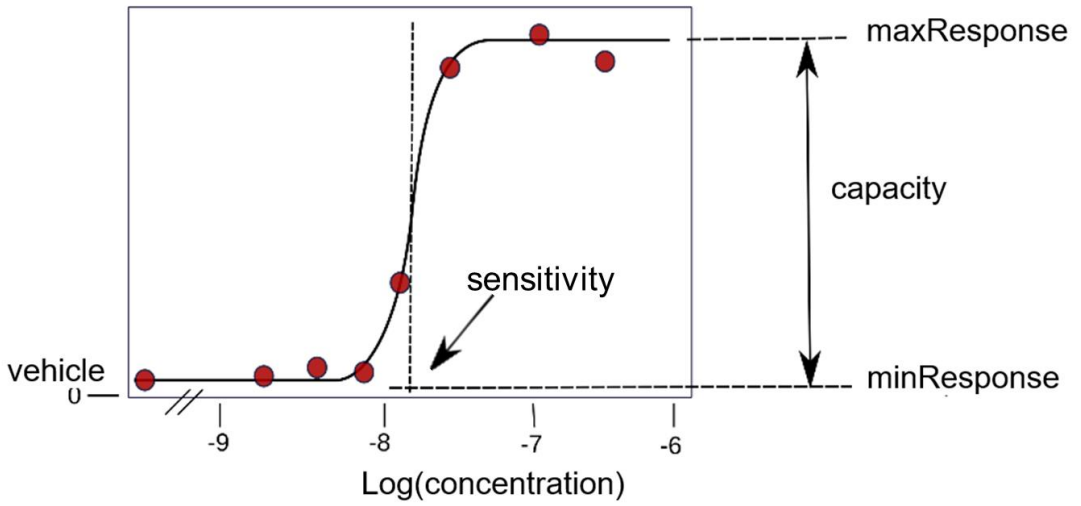


Figure 1. Determination of platelet capacity and sensitivity to assess platelet functioning. Platelet response to multiple concentrations of an agonist. Scripts coded in R were used to analyse the data on R studio (as described previously)[21] and further analysed using GraphPad prism 9. Output results as dose-response curves to provide two distinct estimates of platelet reactivity for each agonist: sensitivity and capacity.

Statistical analysis

GraphPad Prism 9 software (La Jolla CA, USA) was used for statistical analysis. Data are presented as median values. Mean values were compared using a one-way ANOVA with Dunnett's multiple comparisons or a paired Student's t-test. Categorical values were compared using the Fisher's exact test. Analysis of platelet response capacity and sensitivity to agonist was performed in R studio as previously described. [21] Nonlinear regression analysis was performed to plot dose-response curves to test responses to antiplatelet medications.

Results

Demographics of healthy volunteers and patients

Patients with coronary artery disease (CAD) with or without type 2 Diabetes Mellitus (T2DM), were recruited and compared to control groups (demographically matched patients and healthy volunteers), to analyse platelet responses in the different patient groups (Table 1). Compared to the healthy controls, CAD and CAD+T2DM patients were older, and weighed more. CAD and CAD+T2DM patients were also predominantly male, had high blood pressure and were more likely to be taking aspirin and/or statins than the healthy or patient controls (Table 1).

	Healthy Control (n=23)	Patient Control (n=13)	CAD (n=31)	CAD + T2DM (n= 12)
Age (years)	48.96 ± 9.64	56.38 ± 11.76	62.71 ± 8.22*	64.08 ± 5.95*
Men, %	26.09 (6/23)	38.46 (5/13)	87.10 (27/31)$^{\#,+}$	91.67 (11/12)$^{\#,+}$
Height (m)	1.68 ± 0.09	1.69 ± 0.09	1.73 ± 0.10	1.71 ± 0.05
Weight (Kg)	72.94 ± 14.85	74.79 ± 12.47	86.06 ± 15.47*	87.38 ± 12.62*
BMI	25.64 ± 4.36	25.9 ± 4.08	28.85 ± 5.95	28.92 ± 3.03
High blood pressure, %	0	23.07 (3/13)$^{\#}$	54.84 (17/31)$^{\#,+}$	100 (12/12)$^{\#,+}$
Smoker, %	0	0	9.68 (3/31)	8.33 (1/12)
Type of T2DM medication, %	0	Metformin, 7.69% (1/13) Empagliflozin, 7.69% (1/13)	0	Metformin, 75% (9/12) Empagliflozin, 41.67% (5/12) Gliclazide 8.33% (1/12) Humulin 8.33% (1/12) Insulin, 8.33% (1/12)
Aspirin, %	0	15.38 (2/13)	51.61 (16/31)$^{\#,+}$	66.67 (8/12)$^{\#,+}$
Statins, %	0	23.08 (3/13)$^{\#}$	58.06 (18/31)$^{\#,+}$	83.33 (10/12)$^{\#,+}$
Haemoglobin (g/L)	133.09 ± 12.10	146.13 ± 11.75	146.17 ± 11.23	147.54 ± 17.13
Platelet count (x10^9 plts/L)	243 ± 50.33	271.54 ± 75.92	262.69 ± 50.73	242.18 ± 39.52

Table 1. Clinical and demographic characteristics of the participants.
*p<0.05 compared to healthy control, one-way ANOVA, multiple comparisons
$^{\#}$p<0.05 compared to healthy control, contingency, Fisher's exact test
$^{+}$p<0.05 compared to patient control, contingency, Fisher's exact test
Continuous data are presented as mean ± SD and categorical data as percentage (number/ total number).

Cangrelor reduced platelet responses in all participant groups

In order to validate the effect of the $P2Y_{12}$ receptor inhibitor Cangrelor on platelet activation, and to select a concentration for further analysis, platelet-rich plasma from patients was treated with a range of Cangrelor concentrations (0.0003-30 μM) and stimulated with CRP-XL or TRAP6 (Figure 2). Flow cytometric analysis was performed to study integrin $\alpha_{IIb}\beta_3$ activation by measuring fibrinogen binding. This analysis revealed that the lowest concentration of Cangrelor (0.0003 μM), did not affect the capacity of

platelets to respond to CRP-XL or TRAP-6, but did result in a significant reduction in sensitivity. All higher concentrations of Cangrelor significantly decreased platelet response capacity to the two agonists CRP-XL or TRAP6. Furthermore, all concentrations of Cangrelor significantly affected the sensitivity of the platelets to CRP-XL and TRAP-6. Consistent with previous reports,[22, 23] Cangrelor significantly inhibited fibrinogen binding on the platelet surface in healthy controls (Figure 2). We observed, however, a relatively wide variation in response within each group. Because of the level of inhibition reached upon 3nM Cangrelor, this concentration was selected for further experimentation.

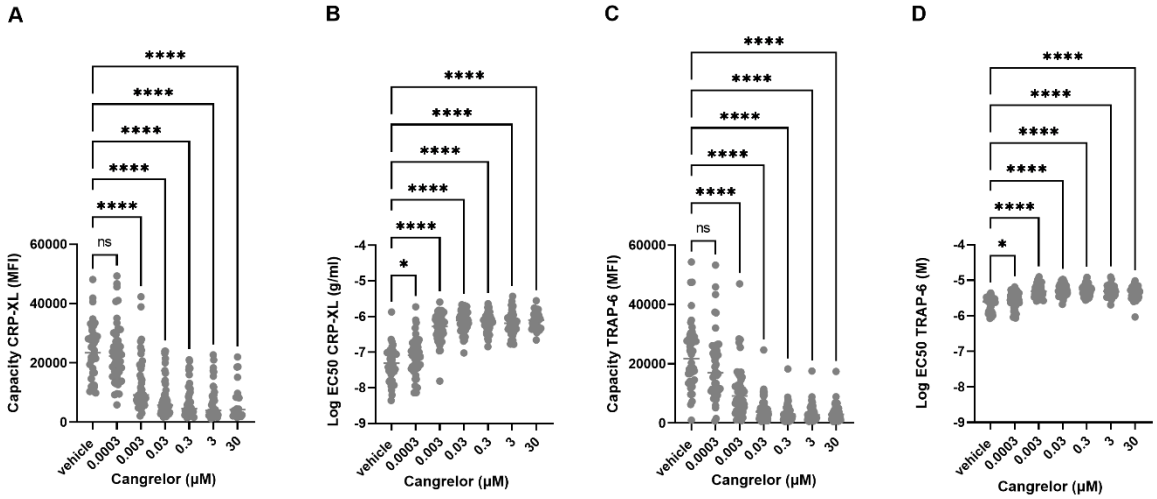


Figure 2. Cangrelor dose-dependently inhibited fibrinogen binding on the platelet surface. Platelets were pre-incubated with Cangrelor (0.0003 - 30μM) for 10 min, and then stimulated with CRP-XL **(A,B)**, TRAP-6 **(C,D)** for 20 minutes and fixed afterwards with 0.2 % formylsaline. Platelets were rested for 10 minutes before platelet function was measured as fibrinogen binding to integrin $\alpha_{IIb}\beta_3$, using flow-cytometry. Scripts coded in R were used to analyse the data on R studio and were further analysed using graph GraphPad prism 9. Output shown as dose-response curves which provided two distinct estimates of platelet reactivity for each agonist: capacity and sensitivity. Reponses to CRP-XL and TRAP-6 expressed as capacity (MFI; **A,C**) or sensitivity (EC50; **B,D**). Statistical significance was estimated by one-way ANOVA for repeated measurements against vehicle control with Dunnett's post-test. Graph represents median values, significance was taken at *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001. Each dot represents one donor (n=24-48)**.**

The response of platelets to CRP-XL or TRAP6, after treatment with 3nM Cangrelor, was hereafter studied among the healthy controls, the patient controls, the patients with

CAD and the patients with CAD+T2DM (Figure 3). In all the groups, there was a significant difference in the capacity to bind fibrinogen upon TRAP6 or CRP-XL stimulation, as well as in the sensitivity of the platelets to respond to CRP-XL or TRAP6, after Cangrelor treatment (Figure 3A,B).

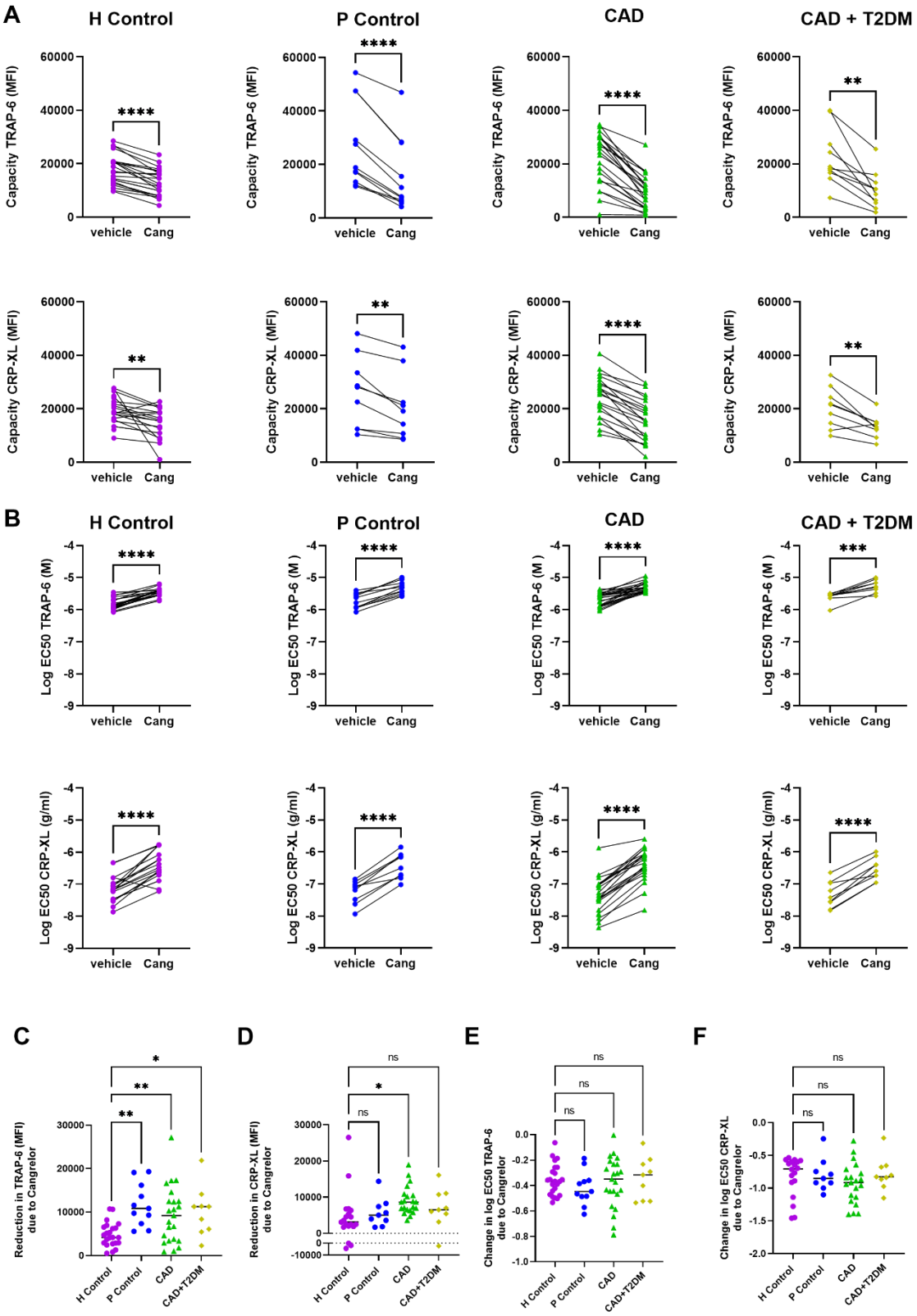


Figure 3. 3 nM Cangrelor significantly inhibited the activation of platelets from patients and healthy controls. Platelets were pre-incubated with 3nM Cangrelor for 10 min, stimulated with CRP-XL (0.03 - 3 µM) or TRAP-6 (0.15 - 15 µM) for 20 minutes and fixed with 0.2 % formylsaline afterwards. **A-B.** Platelet function was measured as fibrinogen binding to integrin $\alpha_{IIb}\beta_3$ in healthy (H) controls (n=23), patient (P) controls (n=13), patients with CAD (n=31) and CAD+T2DM (n=12) by flow-cytometry, in the

presence and absence of 3 nM cangrelor. Responses to CRP-XL or to TRAP6 were expressed as capacity (MFI) **(A)** or sensitivity (log EC50) **(B)** and plotted with each dot representing one patient. Median values were obtained after vehicle or cangrelor treatment were compared within the participant groups. Statistical significance was estimated using paired t-test for repeated measurements against vehicle for each group (stimulated platelets with no drug). Lines at median values, significance was taken at *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001. **C-F.** Reduction in capacity **(C, D)** and change in sensitivity **(E, F)** due to cangrelor treatment was assessed and compared between the different participant groups. One-way ANOVA with *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001.

Remarkably, there was a higher reduction in capacity to respond to TRAP6 after treatment with Cangrelor in all the patient groups compared to the healthy control group (Figure 3C). Further, there was a significant difference in capacity to respond to CRP-XL, in the presence of Cangrelor, between the healthy control group and the group with CAD (Figure 3D). The reduction in sensitivity to TRAP6 and CRP-XL after Cangrelor treatment, however, showed no significant difference between any of the groups (Figure 3E,F). Taken together, these results indicate that Cangrelor significantly reduced platelet activation (fibrinogen binding) within each of the groups, regardless of the condition. Again, a wide variation in responses within each group was be observed.

The effect of Cangrelor correlates with the platelet capacity and sensitivity to agonists TRAP-6 and CRP-XL

Given the inhibitory effects of Cangrelor on the platelets of all participant groups and the large variation in response that existed within each group, we assessed whether an individual's initial platelet capacity and sensitivity to agonists could determine the level of reduction in response after treatment with 3nM Cangrelor (Figure 4). Correlations between the initial platelet responses upon CRP-XL and TRAP6 indicated that the reduction of capacity to respond to agonists in the presence of 3nM Cangrelor (Figure 4A,B), correlated positively with the platelet capacity to bind fibrinogen upon stimulation with CRP-XL (r^2=0.14, p=0.02) or TRAP6 (r^2=0.37, p<0.0001), when all participants were combined. Also, to assess whether initial platelet sensitivity to the agonists CRP-XL and TRAP6 is associated with changes in sensitivity upon treatment with 3nM Cangrelor, correlations were made (Figure 4B,C). There was a positive correlation between sensitivity to CRP-XL (r^2=0.12, p=0.01) or TRAP6 (r^2=0.38, p<0.0001) and the

change in sensitivity due to 3nM Cangrelor. We further assessed the correlation between the capacity of platelets to respond to CRP-XL or TRAP6 and the reduction in response after Cangrelor treatment but separated for each of the 4 participant groups (Figure 4). In the healthy and patient control group, the lowest r^2 values were observed. In the patient group with CAD, only TRAP6 stimulation resulted in a significantly positive correlation (r^2=0.55, p<0.0001) between initial capacity of the platelet to respond to the agonist (Figure 4B). There also was a correlation between the initial sensitivity to Cangrelor and the change in sensitivity due to cangrelor, however, this correlation was only significant upon TRAP6 stimulation. (CRP-XL, r^2=0.17, p=0.07, Figure 4C and TRAP6, r^2=0.53, p<0.0001, Figure 4D). In the patient group of CAD+T2DM, the capacity of the platelet to respond to CRP-XL, but not TRAP6 significantly correlated positively with the reduction in MFI obtained with Cangrelor treatment (CRP-XL: r^2=0.71, p=0.0044 and TRAP6: r^2=0.36 and p=0.09). Taken together, these data suggest that the higher the initial capacity of the platelet response to an agonist, the greater the reduction evoked by Cangrelor treatment. Similarly, the higher the concentration needed to reach the EC50, the higher the change in sensitivity due to Cangrelor. The data we obtained hence suggest that the capacity of platelets to respond to agonists correlated with the response to Cangrelor. Thus, measurement of platelet capacity could be a potential tool to estimate effectiveness of the drug.

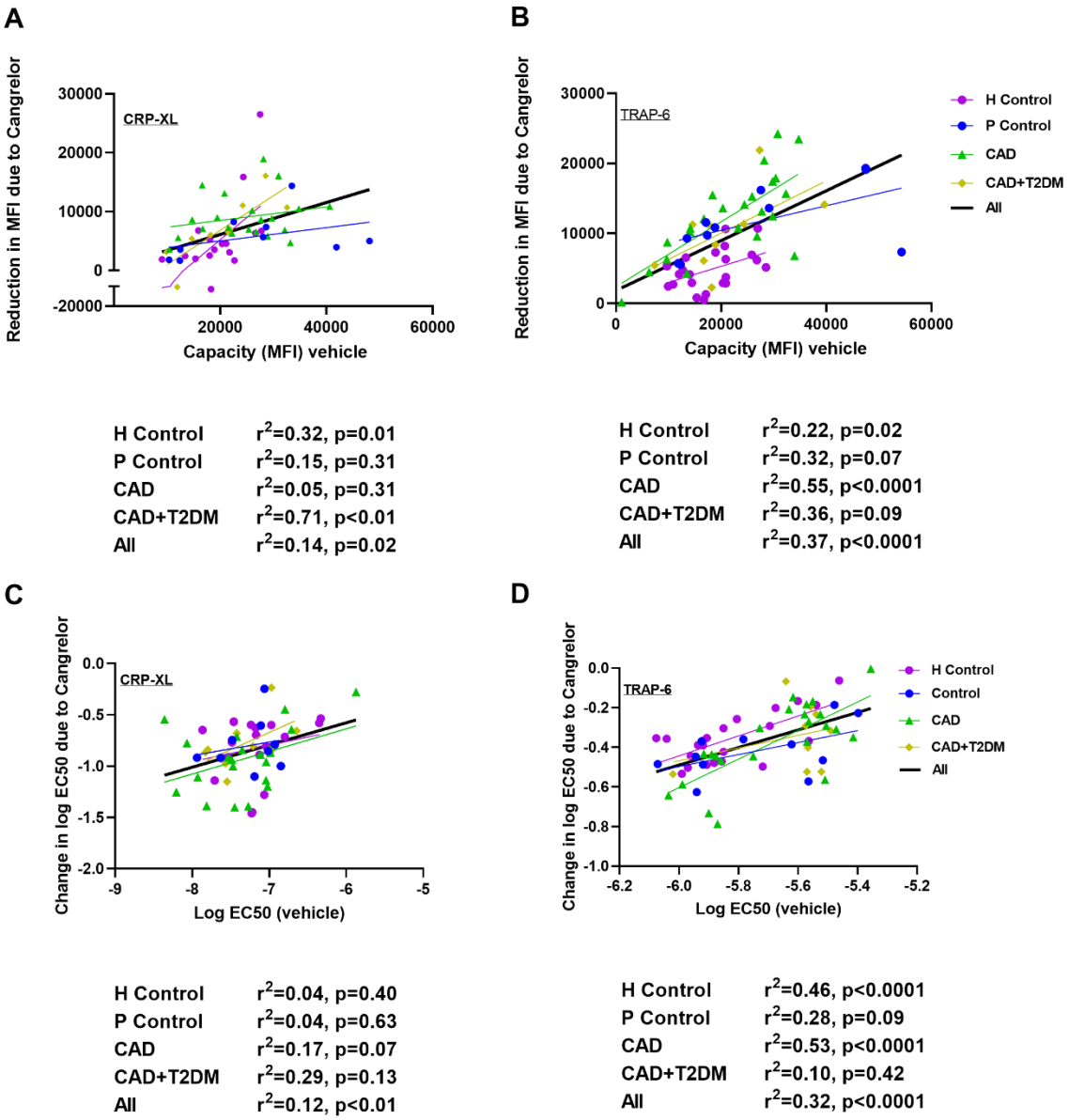


Figure 4. The effect of Cangrelor is modestly but significantly correlated with the initial response or sensitivity to CRP-XL and TRAP-6. Platelets were stimulated with CRP-XL (0.03 - 3 µM) and TRAP-6 (0.15 - 15µM) for 20 minutes and fixed afterwards. Platelet function was measured as fibrinogen binding to integrin $\alpha_{IIb}\beta_3$ using flow cytometry. **A-B:** Correlation graphs were made between the initial capacity (expressed as MFI) to agonists CRP-XL **(A)** and TRAP6 **(B)** and the reduction in capacity to respond to agonists after cangrelor treatment. The reduction in capacity was measured by calculating the absolute shift (subtracting the platelet response in the presence of Cangrelor 3nM from the responses with vehicle). **C-D:** Correlation between the initial sensitivity (expressed as log EC50) to agonists CRP-XL **(C)** and TRAP-6 **(D)** and the change in sensitivity to agonists after Cangrelor treatment. The change in sensitivity was measured by calculating the absolute shift (subtraction of the responses with Cangrelor 3nM from the initial responses). Correlations within control groups (Healthy (H) and Patients controls (P)) and patients were made as indicated. Overall correlations of all groups combined are displayed in black ('All'). Student's t-test, paired analysis was performed to check the significance of the correlations on graph pad prism 9. Significance was taken as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Capacity to respond to CRP-XL as potential tool to predict haemostatic function of platelets

The distribution of the capacity of platelets to CRP-XL was plotted (Table 2), and this revealed that patient platelets particularly were found in the higher quartile of response (Figure 5A, Table 2). Based on the capacity of the platelets, a cut-off value (9048) was determined (Figure 5B, Table 3). This cut-off value was the lowest capacity (MFI) of platelets to respond to CRP-XL, among the participants, in the presence of vehicle. After treatment with Cangrelor, 15.79% of the healthy controls and 20.51% of the patients fell below the cut-off value. A question is whether those individuals are at increased bleeding risk after receiving Cangrelor. For these participants, this raises the possibility that the capacity of the platelets to respond to CRP-XL, may be used as an indication of the bleeding risk after treatment with Cangrelor. Further work with patients pre-and post-administration of Cangrelor will be required to assess the predictive potential of this metric for personalised antiplatelet therapy.

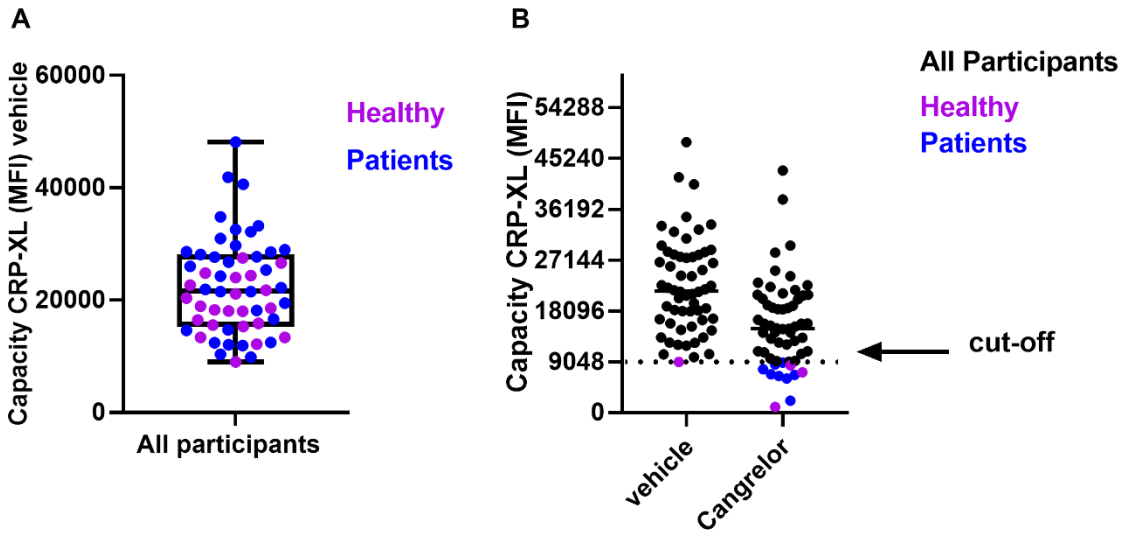


Figure 5. Distribution of healthy and patient platelets' capacity to respond to CRP-XL and determination of cut-off value. Platelets were stimulated with CRP-XL (0.03 - 3µM) and platelet activation was measured by flow cytometry, where platelet capacity to CRP-XL was obtained and expressed as median fluorescence intensity (MFI). Capacity to CRP-XL (MFI values) for all the participant groups were allocated in one group. Statistical analysis was performed using GraphPad prism 9. A. Descriptive statistics were applied to identify quartiles and further analysis was performed using contingency tables Chi-squared test (See table 2). *P value < 0.05 B. The lowest platelet capacity to

CRP-XL was taken as cut-off value, to stratify participants which fall below and above. MFI=9048 as cut-off capacity to CRP-XL upon stimulation (see Table 3).

Quartiles	H	P	Total (H+P)	% Of H
1	6 (26.08%)	9 (23.07%)	15	40
2	9 (39.13%)	7 (17.94%)	16	56.25
3	7 (30.43%)	8 (20.51%)	15	46.66667
4	1 (4.34%)	15 (38.46%)	16	6.25
Total	23	39	62	

Table 2. Contingency table of distribution of healthy and patient platelets' capacity to respond to CRP-XL. Analysis was performed using contingency tables Chi-squared test. *P value < 0.05. For graph and further details, see figure 5.

Cut-off capacity to CRP-XL (9048)	Healthy	Patients	Total
Below	**3**	**8**	**11**
Above	**16**	**31**	**47**
Total	**19**	**39**	**58**
Percentage of row total	**Healthy**	**patients**	
Below	**27.27%**	**72.73%**	
Above	**34.04%**	**65.96%**	
Percentage of column total	**Healthy**	**patients**	
Below	**15.79%**	**20.51%**	
Above	**84.21%**	**79.49%**	

Table 3. Contingency table of platelet capacity of healthy and patient groups. MFI=9048 as cut-off capacity to stimulation upon CRP-XL. For graph and further details, see figure 6.

Discussion

In the present study, we questioned whether we could identify differences in platelet function and drug responsiveness among patients with CAD, with or without T2DM, and in healthy controls and demographically matched patient controls. For this purpose, we used a flow cytometric assay to determine capacity of platelet responses and platelet sensitivity to agonists. Surprisingly, we did not detect any differences in platelet function between the participant groups even though previous studies have reported platelet hyperreactivity in diabetics.[24, 25] Within the different participant groups, considerable variability in the platelet response was, however, observed. Inter-individual variability is caused by several factors, such as sex, age, medication and co-morbidities.[17-19] Regardless of the disease state and inter-individual variability, Cangrelor treatment was effective in all participant groups.

In clinical settings, Cangrelor is used in patients with CAD undergoing percutaneous coronary intervention (PCI), and is proven to be more effective in reducing thrombotic complications compared to Clopidogrel.[26, 27] The variability in the effectiveness of Clopidogrel, has been widely reported.[14] Part of this variation comes from the metabolism of Clopidogrel by liver enzyme cytochrome CYP450, and variants (eg. CYP2C19), which prevent the conversion of Clopidogrel to an active metabolite.[28] Although inter-individual variation is lower with Cangrelor than Clopidogrel,[29] because enzymatic conversion is not necessary, inter-individual differences are still present upon Cangrelor treatment, and this is consistent with our data.

We questioned whether platelets from different clinical groups were differentially able to be inhibited by Cangrelor. Although platelets from all different groups were inhibited by Cangrelor, when we compared among the groups, the greatest effect of Cangrelor was obtained with platelets from the patient groups. We observed inter-individual variability in the capacity and sensitivity of platelet response to both agonists and to Cangrelor, regardless of the participant group. Inter-individual variability may be caused by several

factors, such as sex and age. Whether individuals are fasted might as well make a difference. In this study, patients were not fasted, which might thus contribute to inter-individual differences. The CAD and CAD+T2DM patients were significantly older than the healthy controls, and contained more men compared to healthy and patient controls. Another cause of variability is co-morbidity, such as Diabetes mellitus, which is described to alter platelet functions in response to agonists.[17-19] Angiolillo D. et al, reported that platelets from diabetic subjects exhibit increased levels of platelet activation and aggregation, however, we did not observe this in our assays.[24] T2DM has previously been associated with a higher rate of ischaemic events and increased atherothrombotic risk than non-T2DM individuals, due to risk factors such as hypertension, dyslipidaemia, insulin resistance and endothelial dysfunction.[24, 30] Beside those risk factors, previous papers reported that diabetics are less sensitive to aspirin and clopidogrel treatment.[31, 32] We did, however, not observe any differences between CAD and CAD+T2DM patients. Our findings could possibly be explained by the use and type of medication administered to the patients in our cohort, leading to controlled diabetes or absence of hyperglycaemia. In addition, previously a correlation was found between weight gain and the prevalence of CAD in recently diagnosed T2DM patients,[33] thus the fact that BMI and weight observed in our patient groups were similar in CAD and CAD+T2DM may have obscured this relationship.

Inter-individual variability in platelet response is a multifactorial process. Regardless of clinical phenotype, patients' platelet responsiveness and their response to Cangrelor is variable and is difficult to predict based on the risk factors alone. We therefore assessed whether platelet response before antiplatelet therapy may be considered to predicted platelet inhibition following therapy. We showed a positive correlation between pre-drug platelet function and reduction in platelet function due to a drug, which illustrates that responsiveness to a drug, to a certain extent, is predictable, *in vitro*. For this reason, the development of platelet function tests which could be used to predict and monitor the

response of individuals to antiplatelet therapy could be useful in clinical settings, however, this will need to be explored in a future clinical intervention study.

As the capacity of platelets to respond to agonists gives an estimation of the responsiveness of platelets within an individual, we reasoned that a very low capacity may indicate an increased bleeding risk. Therefore, measuring capacity before and after drug treatment could help in the assessment of effectiveness of the drug but also in the assessment of the bleeding risk. We observed that before Cangrelor treatment, the highest platelet responsiveness was observed in patients. After Cangrelor treatment, however, patients were overrepresented in the sub-population of individuals with the very lowest platelet function. This may reflect a greater risk of bleeding complications in these patients, upon Cangrelor treatment. Given the large variability in individuals, testing platelet capacity before and after drug administration would allow stratification of patients to increase antiplatelet medication effectiveness, limit bleeding and monitor the efficacy of therapy. Clinical intervention trials are needed to explore the potential for incorporation into stratified or precision medicine approaches to improve efficacy of thrombosis treatment and prevention while minimising unwanted side effects.

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Chapter 7

General discussion

Platelets play crucial roles in thrombosis and haemostasis.[1] In these processes, several platelet receptors are key and have therefore been studied for decades.[2] Although the knowledge on platelet receptors has expanded throughout the years, it is important to gain new insights in platelet functioning and receptors as they will offer new ways to target platelets in disease states, while minimising adverse effects. Therefore, the overall goal of this thesis was to increase understanding of acute and persistent effects of platelet activation mediated through the glycoprotein VI (GPVI) receptor and the protease-activated receptor 1 (PAR1). GPVI and PAR1 are platelet receptors for the coagulation-generated ligands fibrin(ogen) and thrombin, they are thus involved in both platelet activation, and the coagulation system.[3, 4] GPVI also is the major receptor for collagen on platelets.[5] Targeting GPVI is a potential novel antithrombotic strategy, since this receptor has a main role in the collagen-induced pathogenesis of thrombosis, but has only minimal roles in haemostasis.[6-8] A broad aim of this thesis was to investigate the working mechanisms and ligands of the receptors which are currently interesting as targets for novel antithrombotic strategies, such as GPVI and PAR1. The ultimate goal is to develop antiplatelet drugs which prevent or treat thrombosis, without increasing the bleeding risk, and to offer the patient a more tailored therapy, in which both the bleeding risk and chance of recurrent events are minimal.

In Chapter 2, we focussed on how the functional status of platelets can be altered by somatic mutations in hematopoietic stem or progenitor cells and the potential consequences for the risk of thrombosis or bleeding. We reviewed genes related to clonal haematopoiesis and changes in platelet traits, such as platelet count and function. We found that somatic mutations in the genes ABCB6, ASXL1, DNMT3A, GATA1, JAK2, SF3B1 and SH2B3 are related to an increased platelet count and/or function, making individuals more prone to develop thrombosis. Instead, somatic mutations in ETV6, FANCA, FANCC, FLI1, GATA1, GFI1B, SMAD4 and WAS are linked to impaired platelet function or production, possibly increasing the bleeding risk.

In the next chapters, we investigated the effects of environmental (extrinsic) factors, such as interactions with agonists, on platelet responses. We first studied the role of GPVI and Syk in thrombus formation on collagen and collagen-like peptides in **Chapter 3**. For this purpose, we used the small molecule Syk inhibitor PRT060318.[9] Collagens and collagen-related peptides with differences in GPVI and integrin $\alpha_2\beta_1$ dependency were investigated, in solution and coated on a surface. Peptides containing a GPVI-activating GPO sequence or with a GFOGER sequence with high affinity for integrin $\alpha_2\beta_1$ were used, as well as combinations of these. Overall, GPVI-dependent signalling through Syk supports thrombus formation induced by collagens and collagen-like peptides, independently of the GPVI-binding motif GPO. The platelet activating effects of weaker collagens were more pronounced when immobilised on a surface, compared to when in solution.

In **chapter 4**, we further investigated the roles of GPVI beyond being a receptor for collagen and fibrin. Using the fab fragment 9O12,[10] we showed that FXIIIa is a physiological coagulation-generated ligand for GPVI (Figure 1). Recently, our findings were confirmed by the paper of Moroi M. et al., where FXIII was identified as a new binding partner for GPVI dimers in binding assays.[11] While in the study by Moroi M. et al an in vitro system of FXIII and GPVI dimers was used, we confirmed the physiological importance of the GPVI-FXIIIa interaction in a system with isolated platelets. The second aim of **Chapter 4** was to explore other ligands for PAR1, beyond thrombin. In earlier work it was described that the anticoagulant factor activated protein C (APC) signals through PAR1 on endothelial cells.[12] Yet, to our knowledge, this was never investigated for platelets. We thus questioned whether platelet PAR1 is involved in APC-induced platelet functions, and we found that APC induces platelet spreading through PAR1 (Figure 1). Overall, we observed that effects induced by FXIIIa or APC were more pronounced when the proteins were immobilised on a surface, compared to in solution,

which indicates that these (anti)coagulation proteins are more effective when recruited and immobilised at the site of injury, compared to when flowing in the circulation.

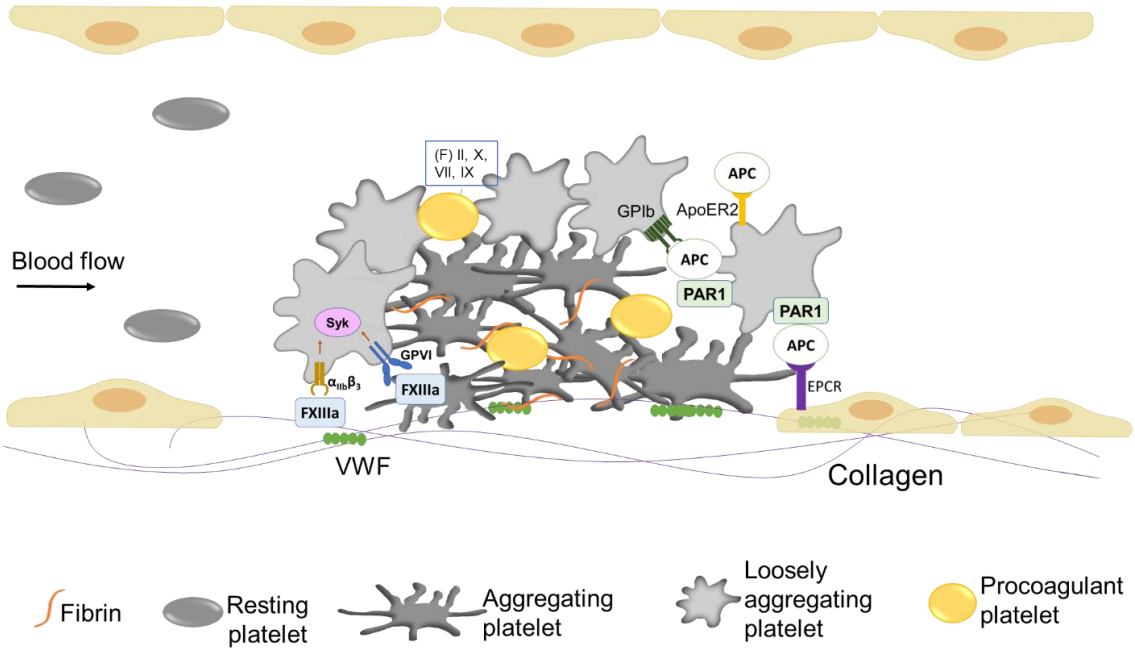

Figure 1: Novel interactions between platelets and coagulation and proposed roles in thrombus formation. The coagulation cascade generates ligands for platelets, such as thrombin and fibrin. Coagulation factor XIIIa evokes activation of platelets in a Syk-, integrin $\alpha_{IIb}\beta_3$ and GPVI-dependent fashion, and in this way FXIIIa supports recruitment of platelets to the thrombus. Also, the anticoagulation factor APC supports platelet adhesion via PAR1. On the one hand, this supports platelet recruitment to the site of injury. On the other hand, this limits thrombus growth because of anticoagulant activity of APC. Upon platelet activation, platelets secrete coagulation factors and possibly become procoagulant, thereby forming a docking site for coagulation factors and promoting the formation of thrombin.

In **Chapter 5**, we questioned whether previously activated platelets can be reactivated and still contribute to thrombus formation. We found that platelet activation through GPVI is more persistent, while responses evoked by PAR1 or $P2Y_{1/12}$ stimulation are rather transient. Platelets exposed to PAR1 or $P2Y_{1/12}$ receptor ligands, were desensitised for the receptor through which they were originally stimulated, but could be reactivated through another receptor, and then still contribute to thrombus formation under flow. This concept of receptor desensitisation is in agreement with earlier publications.[13-16] We showed that preactivated platelets, which started to return to a resting state (integrin inactivation, morphological change), but still express P-selectin on their surface, could

be activated again upon restimulation (Figure 2). The difference between GPVI and PAR1 or $P2Y_{1/12}$ receptor stimulation indicates that activation of platelets by extracellular matrix ligands is more persistent, to minimize blood loss and support haemostasis, while activation of circulating platelets by diffusible agonists is rather transient.

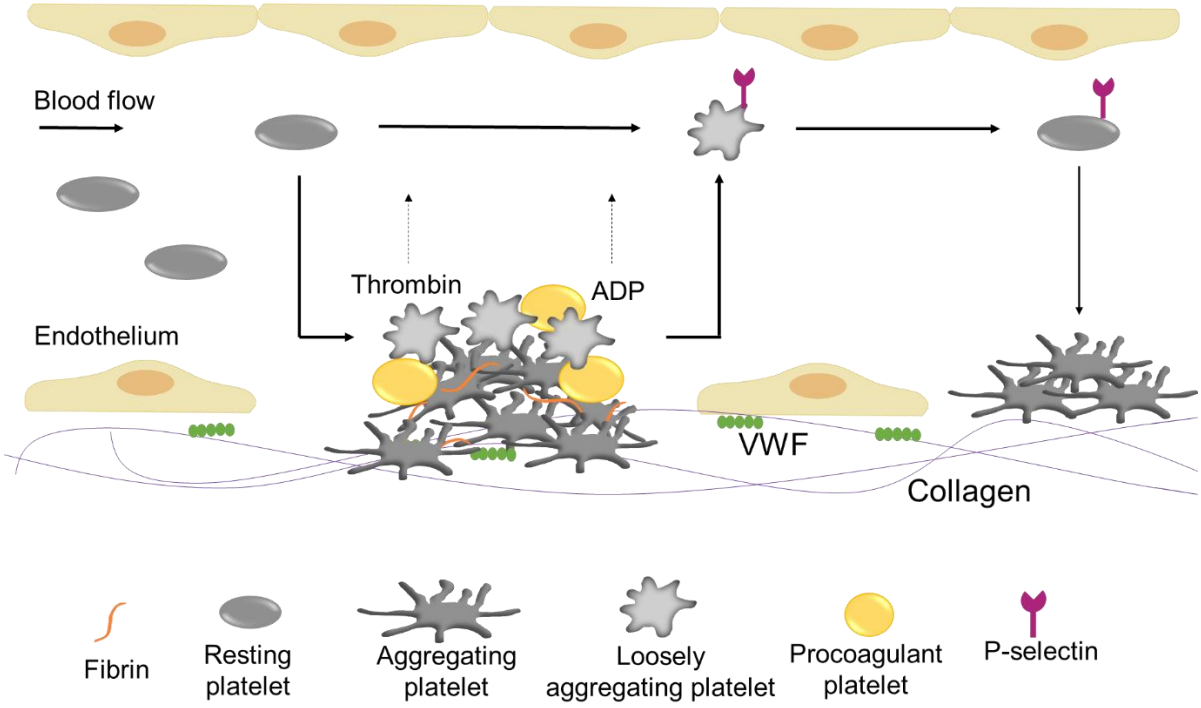

Figure 2: Proposed contribution of previously activated platelets to thrombus formation. Circulating platelets may adhere to incipient thrombi and transform into loosely aggregating platelets, while platelets in the vicinity of thrombi, which are not captured, might still be exposed to soluble ligands, such as low levels of thrombin or ADP. Exposure of the platelets to GPCR ligands leads to platelet activation, including platelet shape change, formation of filopodia and expression of P-selectin. Loosely aggregating, GPCR-stimulated platelets return to a more discoid shape, typical for resting platelets, while P-selectin remains expressed on the surface. Overall, these 'reversed' platelets are desensitised for the receptor that was activated, but re-activation can occur through different receptors, allowing them to again contribute to vascular integrity.

In **Chapter 6**, we investigated platelet responsiveness in patients with coronary artery disease (CAD), with or without diabetes. Platelet function in these patients was determined by applying a flow cytometric assay, that evaluates the platelet capacity to respond to agonists, as well as the effect of inhibition by the antiplatelet drug cangrelor. No differences were observed in the responsiveness of platelets to agonists, regardless

of the presence of cangrelor, between patients and healthy controls. Cangrelor was effective and inhibited platelet function in all participant groups. However, high inter-individual variability in platelet responsiveness was detected in the patient and control groups. Therefore, our study provides justification to further explore the relationship between platelet function and drug responsiveness in individuals, as well as the risk of adverse effects of drugs. The ultimate goal is to offer a more tailored therapy by making the flow cytometric assay applicable for clinical testing in order to maximise drug effectiveness and minimise the bleeding risk. Based on previous studies, the correlation between platelet function tests and bleeding risk is considered poor and only a few associations were found.[17] So far, the VerifyNow $P2Y_{12}$ and light transmission aggregometry (LTA) with ADP assays were the most appropriate to distinguish between patients at high and low bleeding risks.[18, 19] Our flow cytometric setup is promising, as we applied more detailed analytical approaches to measure platelet function and differences between individuals.

Overall, based on the main findings presented in this thesis, we can conclude that GPVI and PAR1 play crucial roles in platelet activation and that understanding the working mechanisms of those receptors may contribute to the development of more precise medicine approaches to improve efficacy while minimising adverse effects.

The different studies performed in the chapters of my thesis are related through several connections, which will be further explored in this chapter.

Extracellular matrix components and coagulation-generated ligands mediating platelet activation through GPVI and tyrosine kinase Syk

One overarching topic in the chapters presented in this thesis is platelet activation through GPVI and tyrosine kinase Syk. We found that effects evoked through Syk by weaker collagens, such as collagen type III, were more pronounced after immobilisation

on a surface **(Chapter 3)**. Similarly, this was also the case for the newly identified GPVI ligand, FXIIIa **(Chapter 4)**. In solution, FXIIIa did not induce platelet activation by itself, however, when immobilised FXIIIa triggered platelet spreading, which was reduced by Syk and GPVI inhibitors. In normal physiology, FXIIIa immobilisation during vascular injury can occur when FXIII-A is exposed on the activated platelet surface,[20] as well as when bound to (the γ and αC chains of) fibrin or to collagen.[21, 22] The finding that immobilised ligands evoked more potent platelet activation through GPVI, was in agreement with an earlier report of Jung SM. et al., where binding of GPVI dimers to immobilised $(GPO)_{10}$ was described, probably to multiple peptide helices, which must be in close proximity to the GPVI dimers.[23] Previous studies demonstrated that GPVI dimers (29% of all GPVI on resting platelet), not monomers, bind to ligands.[23, 24] The level of GPVI dimers is increased upon platelet activation.[23]

Next to immobilisation of ligands, also synergy of GPVI with other receptors thus determines the magnitude of the response. For instance, our work in **chapter 3** showed that CRP-XL, which only has affinity for GPVI and not for integrin $\alpha_2\beta_1$ produced smaller thrombi than peptides containing the $\alpha_2\beta_1$ interaction motif, which was in agreement with the known synergy between GPVI and integrin $\alpha_2\beta_1$ in thrombus formation.[25] Other studies also provided evidence for the synergy of GPVI with integrin $\alpha_{IIb}\beta_3$, since the tyrosine kinase Syk is activated downstream of both receptors.[26] Because of this synergy, perfusion of blood from GPVI deficient patients over fibrin spots resulted in abolished thrombus formation, although platelet adhesion was observed.[26]

Previously, Magwenzi S. et al., provided evidence that Syk and integrin $\alpha_{IIb}\beta_3$ are involved in platelet spreading evoked by FXIIIa,[27] which we also confirmed in **Chapter 4**. Furthermore, since in **Chapter 4** there was a stronger effect of combined inhibition of GPVI and integrin $\alpha_{IIb}\beta_3$, our study suggested that FXIIIa mediates platelet activation by enforcing synergy of the GPVI and integrin $\alpha_{IIb}\beta_3$ receptors. In a similar fashion, the interaction of fibrin with GPVI relies on $\alpha_{IIb}\beta_3$, as described previously by Perrella G. et

al. [26] Syk also has a critical role in thrombus stability, which is dependent on integrin $\alpha_{IIb}\beta_3$ interactions with fibrin and FXIII, and on fibrin-induced GPVI activation.[26, 28, 29]

In **Chapter 5**, we showed that platelet activation is persistent after GPVI (CRP-XL) stimulation, while upon GPCR stimulation platelet activation is rather transient. This fits with earlier cytosolic Ca^{2+} mobilisation assays, where PAR1 mediated activation evoked a platelet signalling response of shorter duration than GPVI.[30] Linking all findings to thrombus architecture, our data suggest that immobilised collagens at the site of injury evoke sustained activation of platelets in the thrombus core, whereas soluble ligands (such as ADP) present in the thrombus shell allow platelets to interact loosely. Fibrin was described to be especially relevant for the continued growth and consolidation of a thrombus,[3, 31] which is most likely enforced by FXIIIa, because of interactions with, and crosslinking by FXIIIa. A question that arises is whether the presence of FXIIIa can cause shedding of the GPVI receptor, as observed with collagens and fibrin.[32-34] According to Moroi et al., the binding of GPVI to FXIIIa may be competitive with the crosslinking activity of FXIIIa and thus affect clot stability,[11] however, this should be further investigated in the future. The same authors also reported that FXIIIa and fibrin bind GPVI independently and suggested that there is a different binding site for either ligand, however, the observed competition, could be due to steric hinderance. Based on previous reports and the findings obtained in **chapter 4**, we thus speculate that FXIIIa enforces initial adhesion of platelets to the site of injury, where collagen and VWF are exposed, after immobilisation. Thereafter, the growing thrombus is stabilised by fibrin, whose γ- and α-chains are crosslinked by FXIIIa. The stabilisation by fibrin is mediated through integrin $\alpha_{IIb}\beta_3$ and GPVI. GPVI binds with higher affinity to crosslinked fibrin than to non-crosslinked fibrin, highlighting the role of FXIII in the interaction between fibrin and GPVI.[24]

Coagulation-generated ligands mediating platelet activation through PAR1

Next to thrombin as ligand for the platelet receptors PAR1/4, a newly identified PAR1 ligand found in **Chapter 4** of this thesis is the anticoagulant factor APC (Figure 1). APC is known to mediate cytoprotective effects in endothelial cells via PAR1 signalling.[12, 35] Notably, an interaction between APC and platelets, mediated by the ApoER2 receptor and GPIb, was illustrated by White TC et al.,[36] however, a possible role of APC in PAR1 activation on platelets was not investigated before. In **Chapter 4**, we found that APC induced platelet spreading and supported platelet activation, predominantly when immobilised on a surface. APC is captured and immobilised on the endothelium by the endothelial cell protein C receptor (EPCR).[35] According to Fager A. et al, platelets also express the EPCR receptor,[37] which in theory could tether APC. Lastly, APC is captured on procoagulant platelets via its Gla domain, with protein S as co-factor.[31] Whether the above-mentioned mechanisms influence platelet responses *in vivo*, remains to be established. Overall, our findings suggest that APC in physiological conditions enhances the recruitment of platelets to the injured site, via several platelet receptors. On the other hand, the binding of APC to the thrombus may also limit thrombus growth as a feedback mechanism.[36, 38] To which extent platelet adhesion and thrombus growth are influenced by APC, warrants further investigation. Our data in **Chapter 5** showed that when platelets are previously activated through PAR1, there is a relatively fast reversal to a resting state, because of PAR1 desensitisation. For this reason, platelet effects induced by APC may be transient, whereafter platelets can return to a resting state. It is possible that APC and thrombin mediate distinct biological effects, since R41 is the thrombin cleavage site in PAR1, while R46 is the APC cleavage site.[39] Therefore, no competitive binding is expected between APC and thrombin, although steric hindrance might be possible.

As described in **Chapter 4**, we observed that FXa induced platelet activation, confirming previous reports.[40, 41] Others concluded that platelet activating effects were mediated via

PAR1. We found, however, that all effects evoked by FXa were abolished upon the addition of thrombin inhibitors. Hence, we conclude that FXa induces PAR1-dependent platelet activation, through the generation of thrombin.

Activated platelets in health and disease

Continuous platelet activation has been reported in several diseases, including CAD and cancer.[42-44] This constant activation is prevented and inhibited by antiplatelet drugs.

In patients with CAD with or without diabetes mellitus (**Chapter 6**), we did not detect hyperreactivity of platelets using flow cytometry, which can be explained by the treatment with antiplatelet drugs or diabetes medication, leading to controlled diabetes. Another study reported no hyperreactivity in platelet reactivity between healthy individuals and patients with acute ischaemic stroke.[43] Instead, platelets from those patients rather showed decreased reactivity *in vitro*, resembling a previously activated platelet phenotype, in which circulating platelets exposed P-selectin. This finding is in agreement with our work in **Chapter 5**, where previously activated, 'reversed' platelets remain P-selectin positive, and can be activated again, depending on the previous trigger. Also, another study reported that in patients with CAD, P-selectin exposure was elevated on circulating platelets.[45] Remarkably, the authors also reported that the percentage of dimeric GPVI was higher on circulating platelets of those patients. Since dimeric GPVI binds collagen and other ligands with higher affinity, this indicates that circulating platelets in CAD patients have a higher capacity to bind GPVI ligands, because of previous exposure to soluble ligands. This makes the use of GPVI inhibition in CAD patients extremely interesting.

In **Chapter 5**, we already showed that platelets desensitised for one receptor could be reactivated through another receptor. However, the limitation is that the work was

performed *in vitro*, while in patients, platelets are likely exposed to multiple activating agents simultaneously. Whether reactivation then is possible, or whether platelets are completely exhausted and multiple pathways are desensitised, remains to be determined. Another question is whether continuously activated platelets from patients are still responsive to antiplatelet medication. In **Chapter 6**, we showed a positive correlation between the initial capacity to respond to an agonist and the reduction in responsiveness by the antiplatelet drug clopidogrel. The goal is to develop this method as a clinical tool to predict the efficacy of antiplatelet drugs in patients with potentially activated platelets. Overall, the assessment of the capacity of platelets before administering drugs might be useful since platelet responses to pharmaceutical agents vary between patients. Although the inter-individual differences are largely unexplained, variability could be explained partly by age, sex and conditions such as diabetes.[46-48] As described in **Chapter 2**, with ageing, somatic mutations in haematopoietic stem cells can accumulate, resulting in clonal expansion of a mutated haematopoietic stem cell, which might increase the risk for cardiovascular diseases.[49]

The need for new drugs and more advanced clinical diagnostic testing

An overall limitation of our studies was the lack of tools to inhibit GPVI. As described in the introduction, there are several tools to target GPVI, such as GPVI mimetics,[50] fab fragments,[10] small molecules,[51] and pathway inhibitors. However, those inhibitors are not commercially available yet, making it challenging to investigate GPVI. In **Chapter 3**, we therefore used PRT060318, a Syk inhibitor. Syk is a central player under the pathway of GPVI.[9] In clinical settings, Syk inhibitors are used already. Fostamatinib for instance is administered to patients suffering from refractory immune thrombocytopenia (ITP), without increasing the bleeding risk.[52] In **Chapter 4** instead, we received permission to use the 9O12 Fab fragment, which specifically inhibits GPVI by targeting the fibrin and collagen binding sites. Currently, the safety and efficacy of 9O12 is being investigated in

phase 2 clinical trials in patients with acute ischaemic stroke.[53, 54] The aim is to add 9O12 to standard therapy (thrombolysis with recombinant tPA and thrombectomy). Furthermore, the GPVI mimetic revacept was investigated as additional treatment to aspirin in patients undergoing PCI for stable CAD. Revacept did not reduce myocardial injury in patients with stable ischemic heart disease undergoing percutaneous coronary intervention.[55] However, revacept might be a promising treatment option for patients with acute ischaemic stroke due to symptomatic carotid artery stenoses. Phase II clinical trials are finalized, and future phase III studies in patients with underlying ruptured plaque embolization, are to be started.[56] As discussed in **Chapter 6**, there is a need for improved antiplatelet medication and diagnostic tools in patients with CAD. Inhibition of GPVI as the standard treatment for patients with CAD would be beneficial, as GPVI inhibitors are probably associated with a low risk of bleeding compared to the current $P2Y_{12}$ receptor inhibitors. Overall, the data in this thesis suggest that inhibiting GPVI in patients with cardiovascular diseases might be a good strategy. Not only has GPVI minor roles in haemostasis, but it is also the receptor for multiple ligands, such as collagen, fibrin and FXIIIa.

Since there is a lot of inter-individual variation in platelet responses within the population, platelet testing should be improved, so that the effectiveness of drugs is maximised, and the side effects, such as bleeding, are limited. The assays used in **Chapter 6** are promising and warrant further investigation. In addition, as explained in **Chapter 2**, genetic screening for acquired mutations that are associated with an increased risk for cardiovascular diseases, may favour personalised risk assessment. The issue is that so far, the methods used to detect clonal mutations have a low sensitivity and specificity.

Concluding Remarks and future work

Overall, this thesis provides several new findings concerning the two platelet receptors GPVI and PAR1. We found that coagulation factors, other than thrombin and fibrin, can affect platelet functioning through GPVI and PAR1. First, we identified a role for GPVI and tyrosine kinase Syk in FXIIIa-induced platelet activation, as well as for PAR1 in APC-induced platelet functions. Then, we showed that platelet effects evoked through GPCR activation are rather transient, while effects through GPVI are persistent. Furthermore, we found that reversed platelets can be reactivated to a certain extent, and can still contribute to thrombus formation, after previous stimulation. The research described in this thesis thus expands the knowledge on the GPVI and PAR1 receptors, which are currently important novel targets for antithrombotic therapy, and illustrates the importance of more tailored antiplatelet therapy, in which bleeding risks are kept minimal. Future work should further elucidate the (patho)physiological importance of the interaction between FXIIIa and GPVI. As such, the effect of this interaction on clustering of the GPVI receptor and possible competition with fibrin crosslinking should be further investigated, as well as to which extent the recently described FXIIIa-GPVI and APC-PAR1 interactions support or limit thrombus growth. Furthermore, it is interesting to explore whether preactivated platelets can be re-used and are able to contribute to vascular repair in *in vivo* models. Also, the exposure of platelets to multiple ligands should be further studied, to mimic the (patho)physological environment in patients. Eventually, optimisation and automatization of the flow cytometric assays used in this thesis, can support patient stratification and personalised treatment.

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