Platelet glycoprotein VI in the regulation of thrombus growth

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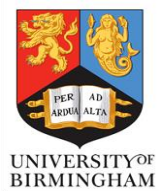
Gina Perrella



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Platelet Glycoprotein VI in the Regulation of Thrombus Growth

DISSERTATION

To obtain the degree of Doctor at Maastricht University and Doctor of Philosophy at the University of Birmingham, on the authority of the Rector Magnificus, Prof. dr. Pamela Habibović, in accordance with the decision of the Board of Deans, to be defended in public
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Abstract

Platelet glycoprotein VI (GPVI) is the principal signalling receptor for collagen, which is exposed upon damage to the extracellular matrix and at site of rupture of an atherosclerotic plaque. GPVI has recently been shown to be also a receptor for fibrinogen and fibrin, which mediate platelet aggregation and clot formation. In mice, GPVI deficiency protects form arterial thrombosis without causing excessive bleeding. Individuals with an inherited deficiency in GPVI have a mild bleeding diathesis. Together, these observations suggest a more important role of GPVI in thrombosis over haemostasis.

The research described in this thesis investigates the relative contribution of GPVI in adhesion and platelet aggregation under flow on collagen and fibrin(ogen). As overarching hypothesis, I propose that GVI is a relevant signalling receptor for fibrin and fibrinogen in supporting thrombus propagation and stability. The first part is a critical assessment of the evidence that fibrin and fibrinogen bind to monomeric or dimeric GPVI in view of the earlier existing controversies. This is followed by the exploration of the role of GPVI in platelets adhesion to collagen using blood from Chilean patients with a homozygous insertion mutation in the *GP6* gene which causes lack of GPVI expression on the platelet surface. The results show a critical role for GPVI in supporting platelet aggregation and phosphatidylserine exposure on collagen and non-collagen surfaces, but not adhesion which, on collagen, is mediated by the integrin α2β1. Further, it is estimated that in Chile there are over 4,000 individuals GPVI-deficient, of whom only a handful are known to have a bleeding diathesis. The next study investigates the contribution of GPVI to platelet adhesion and thrombus growth on fibrin, fibrinogen, using the Syk inhibitor, PRT-060318, and an anti-GPVI Fab, 9O12. The results show that GPVI contributes to platelet activation in response to fibrin and fibrinogen, that induces platelet secretion but low level of Ca^{2+} rises and that it is not required for platelet adhesion. Further, they show that in response to fibrin, GPVI acts in concert with the integrin

αIIbβ3 in a non-redundant way. Successively, the action of two small molecule inhibitors of GPVI, losartan and honokiol, on platelet activation is investigated. The results show that both compounds are not selective GPVI antagonists. The following study explores the contribution of GPVI and integrin αIIbβ3 to the stability of a preformed thrombus under flow conditions. A comparison is made between inhibitors of Syk, Src, Btk and those of secondary mediators, ADP and TxA_2. The results demonstrate a critical role for Syk in supporting aggregate stability, likely through fibrinogen-induced activation of both GPVI and integrin αIIbβ3. In addition, Syk appears to act in synergy with ADP and TxA_2 and to be independent of collagen-induced GVPI activation, as a blocking nanobody has minor effect.

Overall, the research in this thesis provides evidence that the role of GPVI extends beyond the onset of thrombus formation. GPVI activation in response to fibrin(ogen) contributes to platelet aggregation and it is key to thrombus stability. This conclusion further supports the argument that blocking GPVI may effectively prevent arterial thrombosis. Furthermore, this research highlights the importance of the integrin αIIbβ3 in supporting GPVI activation, suggesting that blocking signalling downstream both receptors, can provide an alternative therapeutic strategy for the treatment of arterial thrombosis.

Contents

Chapter 1

General Introduction

1. Platelet in haemostasis and thrombosis

Platelets are critical for haemostasis, a vital process to prevent excessive blood loss following vessel injury. The endothelium lining the blood vessels prevents platelet activation by presenting a non-thrombotic surface and by creating a barrier that physically separates platelets from the thrombogenic factors present in the subendothelium. The intact endothelium constitutively releases nitric oxide and prostacyclin, two potent platelet inhibitors, and expresses the ecto-nucleotidase CD39, which metabolises the platelet-activating nucleotides, adenosine triphosphate (ATP) and adenosine diphosphate (ADP), into platelet-inhibiting adenosine.[1] Following endothelial damage, the collagen and tissue factor in the subendothelium become exposed, and platelet activation is initiated by the combined actions of collagen and thrombin generated by tissue factor, together with ATP and ADP released from damaged endothelial cells.[2] This leads to integrin activation and stable platelet adhesion to proteins in the subendothelium, including collagen that binds to integrin α2β1 and von Willebrand factor (VWF) which binds to integrin αIIbβ3. Platelet adhesion is followed by platelet aggregation, which is driven by the release of ADP, thromboxane A_2 (TxA_2) and thrombin generation.

Arterial thrombosis is the counterpart of haemostasis and it occurs when platelets are activated in diseased vessels, mostly at the site of rupture or denudation of an arterial atherosclerotic plaque.[3] Plaques are fatty deposits rich in collagen that, in atherosclerosis, a chronic inflammatory disease, grow into the artery causing it to narrow and harden.[3] The local rupture or de-endothelialisation of a plaque exposes its components to the blood stream. This leads to platelet and coagulation activation and culminates with the formation of a thrombus, which can become occlusive or instable and detaches (embolise).[3] Depending on the site of plaque disruption and the downstream vessels where emboli are trapped, this can lead to myocardial infarction, stroke or pulmonary embolism. According to the World Health Organization, in 2019 an estimated 17.9 million (32% of all deaths) people

died from cardiovascular diseases, of these 85% were accounted for by myocardial infarction or stroke.[4]

Currently used anti-platelet agents, in particular aspirin and ADP receptor ($P2Y_{12}$) antagonists, are widely prescribed for the primary and secondary prevention of cardiovascular diseases.[5] However, long-term treatment with these medications carries the risk of minor or major bleeding events, which can be life-threatening.[5] Therefore, new forms of anti-platelet agents that more selectively target arterial thrombosis over haemostasis are needed.

2. Platelet formation and characteristics

Platelets were identified by Max Schultze in 1864,[6] but it was the Italian physician Bizzozero, who in 1881 first reported their function in haemostasis.[7] Mammalian platelets are anucleated cell fragments derived from the cytoplasm of matured megakaryocytes, which can generate up to 4000 platelets per cell.[8] During their maturation process, megakaryocytes undergo a process called endomitosis, a prematurely terminated mitosis with several DNA replications in the absence of cell division. This results into the formation of a single lobed nucleus with multiple chromosome copies.[8,9] The number of endomitotic cycles can range from two to six, but most of the megakaryocytes undergo three cycles resulting in a 16*N* polyploid state.[10] This process is essential for the production of platelets, as the gene amplification results in an increased protein synthesis.[11]

Followed by the endomitosis, the maturation process of polyploid megakaryocytes continues through the formation of an extensive demarcation membrane, by which the cytoplasm becomes concentrated at the outer cell surface, and through the formation of pseudopods, which elongate into cytoskeleton-containing tubules with a diameter of 2-4 µm. The latter then develop organelle-containing densities along their length, and through a branching process, the nascent proplatelets will assemble at the tubular ends of the branches.[12-14] This transformation unfolds over 4-10 hours, after which the formed proplatelets are released into the blood

stream.[12-14] The shear stress of the flowing blood will then convert the proplatelets into preplatelets, which then divide into platelets by the rupture of the cytoplasmic bridges between platelet-size segments.

Platelets are the smallest cells in the blood with a diameter of 2-5 µm, a thickness of 0.5 µm and a mean cell volume of 6-10 fL.[15,16] Human platelets have a lifetime of 8 to 10 days, and their count ranges from 150 to 450 million/mL.[17] The exterior surface of platelets is covered by a wide range of glycoprotein receptors and also contains a glycocalyx that rests on the plasma membrane.[18]

Platelets have a discoid shape that is maintained by a circumferential coil of microtubules and is supported by an extensive actomyosin filament system. In resting platelets, this cytoskeleton provides spatial organisation to keep the organelles suspended and separated from each other.[19] Next to mitochondria and an extensive endoplasmic reticulum, platelets contain three major types of secretory granules: α-granules, δ-granules (dense bodies) and lysosomes. The α-granules, typically 200–500 nm in diameter, originate from the trans-Golgi network and are most abundant in number, ranging from 40 to 80 per platelet. These granules contain up to 300 membrane and secretory proteins including P-selectin, fibronectin, fibrinogen and thrombospondin.[19] The δ-granules are 150 nm in diameter and number from 2 to 7 per platelet. They contain the autocrine feedback mediators ADP and ATP, as well as Ca^{2+}, polyphosphate and serotonin. Lysosomes have a diameter of 300 nm and contain enzymes involved in the degradation of the vessel wall matrix components.[20]

3. Mechanism of haemostasis

Haemostasis is a dynamic process that, from the perspective of platelet and coagulation activation, can be divided in several stages (Figure 1).

Platelet rolling, tethering and adhesion. Upon vascular injury, the exposure of the subendothelium triggers platelets adhesion by a multitude of events. At first, collagen captures the circulating VWF, which tethers the circulating

platelets. VWF is a large multimeric glycoprotein produced by endothelial cells and stored in the form Weibel–Palade bodies and secreted by exocytosis.[21] Upon secretion, the protease ADAMTS13 cleaves VWF multimers into smaller inactive proteins which are unable to bind to platelets. However, collagen exposure prevents VWF cleavage making it available for binding to the glycoprotein Ib-V-IX (GPIb-V-IX). This interaction favours platelet adhesion by facilitating integrin binding to the extracellular matrix proteins, such as integrin α2β1 to collagen, integrin α5β1 to fibronectin, integrin α6β1 to laminin and integrin αIIbβ3 binding to VWF and fibrinogen.[22]

Platelet activation and firm adhesion. Following the initial adhesion, platelet activation is started by binding of signalling receptors, including glycoprotein VI (GPVI), to ligands present in the extracellular matrix. This results in integrin inside-out signalling, by which platelet integrin assume an active conformation and more avidly bind to their ligands,[23] stabilising platelet adhesion.

Platelet shape change and spreading. Receptor-induced intracellular signals lead to a reorganisation of the platelet microtubular and actin cytoskeleton.[24] Platelets flatten their surface and assume a dendritic shape with filopodia and lamellipodia, which facilitate the adhesion to the subendothelium.[25]

Platelet secretion. The adhered and activated platelets release their cytoplasmic granules content, including the secondary mediator ADP, and generate the other secondary mediator TxA_2. The latter is produced by activation of phospholipase A_2 and the conversion of arachidonic acid into prostaglandin H_2 and then into TxA_2. Both ADP and TxA_2 are important autocrine agents that provide a positive feedback mechanism leading to recruitment of more platelets and enhanced platelet activation.[26]

Platelet aggregation. Activated platelets are brought together by activated αIIbβ3, which binds to fibrinogen and VWF.[23] This results in platelet aggregation forming a primary platelet plug.

Thrombin generation. The initial generation of thrombin, and the coagulation process in general, is triggered by the tissue factor (TF) expressed by activated endothelial, subendothelial cells and monocytes.[27] TF forms a complex with factor VII(a) and factor X(a) generating the first traces of thrombin (extrinsic coagulation pathway).[28-30] In addition, platelet activation via GPVI leads to a prolonged rise in cytosolic Ca^{2+} levels, leading to exposure of phosphatidylserine (PS) on platelet surface. The exposed PS allows the assembly of the tenase and prothrombinase complexes, which enhance the cleavage of factor X into factor Xa and the cleavage of prothrombin into thrombin, thus accelerating the coagulation.[31] The generated thrombin further activates platelets.[26]

Fibrin formation. Thrombin catalyses the cleavage of fibrinogen into fibrin. Successively, the activated factor XIII (FXIIIa) crosslinks fibrin fibrils conferring protection against fibrinolysis.[32]

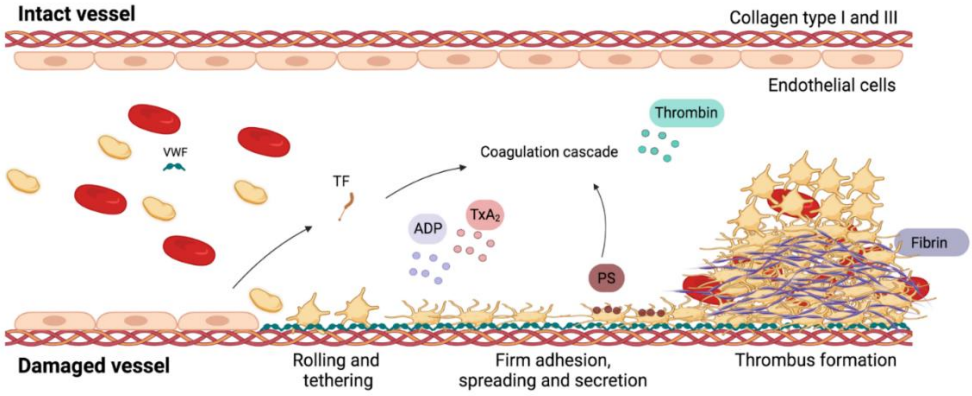

Figure 1. Schematic representation of the mechanism of haemostasis. *<u>Platelet rolling and tethering:</u> the binding of VWF to collagen tethers the circulating platelets and facilitates integrin-mediated initial adhesion of platelets to the extracellular*

matrix (ECM). Platelet firm adhesion, spreading and secretion: platelet receptors bind to ligands in the ECM, the ensuing inside-out signalling activate the integrins conferring platelet stable adhesion. The adhered platelets undergo a conformational change from a resting to a dendritic form and release their cytoplasmic granule content. ADP and TxA_2 provide a positive feedback mechanism enhancing platelet activation. Thrombus formation: the prolonged increased intracellular Ca^{2+} leads to phosphatidylserine (PS) exposure, which contributes to thrombin generation, alongside with the tissue factor generated on activated subendothelial cells. Thrombin catalyses fibrinogen cleavage into fibrin that stabilizes the clot. Figure created with BioRender.com.

4. Platelet receptors

Platelets have a wide range of signalling receptors. In this paragraph the most relevant receptor classes for this thesis are described.

4.1 Relevant G-protein coupled receptors

G-protein coupled receptors (GPCRs) include those for thrombin, ADP and TXA_2. GPCRs consist of seven transmembrane α-helices connected by three extracellular loops and three intracellular loops. The extracellular loop contains the amino terminus and the ligand (agonist) binding site; while the intracellular loop contains the carboxyl-terminal domain, which is associated to guanine nucleotide binding proteins (G proteins). The G proteins coupled to these receptors are αβγ heterotrimers and are divided into the G_i, G_q, $G_{12/13}$ and G_s families. In all families, the Gα subunit contains a guanine nucleotide-binding site, which in the inactive state is occupied by guanosine 5'-diphosphate (GDP). The Gβγ subunits are non-covalently associated with the Gα subunit. Upon activation of the concerning GPCR by its agonist, the GDP in the Gα subunit is replaced by guanosine 5'-triphosphate (GTP) causing a conformational change that leads to the dissociation of Gα from Gβγ. This dissociation makes them available for interactions with downstream effectors.[34,35]

The two thrombin receptors on human platelets are the protease-activated receptors (PAR) 1 and 4.[29] Both PAR1 and PAR4 couple to G_q and $G_{12/13}$

protein families. Thrombin-induced platelet stimulation via G_q activates phospholipase C isoforms of the β-class (PLCβ), whereas $G_{12/13}$ activates Rho-family small GTP-binding proteins leading to platelet shape change.[36] The activated PLCβ cleaves the phosphatidylinositol 4,5-bisphosphate (PIP_2), forming inositol 1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG). The IP_3 opens Ca^{2+} channels in the platelet dense tubular system (endoplasmic reticulum), raising the cytosolic Ca^{2+} concentration and subsequently triggering Ca^{2+} influx through ion channels in the plasma membrane. DAG activates protein kinase C (PKC) isoforms supporting granule secretion and aggregation. The receptor for TxA_2, named thromboxane-prostanoid (TP) receptor, belongs to the prostaglandin family of receptors and is expressed on both platelet and endothelial cells. In platelets, TP couples to the G_q and $G_{12/13}$ proteins.[37] The two ADP receptors on platelets are $P2Y_1$ and $P2Y_{12}$. The first $P2Y_1$ is G_q-coupled, while $P2Y_{12}$ is G_i-coupled.[36,38] The binding of ADP to $P2Y_{12}$, inhibits adenylyl cyclase and activates as effector enzymes phosphoinositide 3-kinases (PI3K).[39] It is considered that the concomitant activation of G_q and G_i pathways is necessary to elicit full platelet aggregation with ADP.[38]

The receptors for the three discussed agonists (thrombin, TxA_2, ADP) are targeted with current antiplatelet drugs. Vorapaxar is an oral thrombin receptor antagonist, which selectively antagonises PAR1 and it is used to prevent cardiovascular events in patients with history of myocardial infarction or stroke.[40] The cyclooxygenase (COX)-1 inhibitor, aspirin, which irreversibly blocks arachidonic acid conversion into prostaglandin H_2, is abundantly used in patients at risk of arterial thrombosis, often in combination with a $P2Y_{12}$ antagonists such as clopidogrel, prasugrel or ticagrelor,[41] which are also orally available. Ticagrelor provides faster platelet inhibition as it directly binds to the $P2Y_{12}$ receptors, whereas the other two are prodrugs requiring metabolization in the liver.[42,43] A major side-effect of all these inhibitors is undesired bleeding (1-2%).[5]

4.2 Glycoprotein VI and other tyrosine kinase-linked receptors

Platelet tyrosine kinase-linked signalling receptors contain an intracellular conserved sequence, known as the immunoreceptor tyrosine-based activation motif (ITAM). Human platelets express three ITAM-linked receptors: GPVI, FcγRIIA and C-type lectin-like receptor 2 (CLEC-2).[44] An ITAM contains two YXXL/I sequences, separated by 6-12 amino acids, in which the two Y (tyrosine) residues can be phosphorylated and X denotes any amino acid.[45] Upon activation, Src-family kinases (SFK) phosphorylate both these tyrosines, providing a docking site for Syk tandem Src homology 2 domains (SH2). After SFK-induced phosphorylation of Syk, this undergoes autophosphorylation on tyrosines 525 and 526.[46] The activated SFKs and Syk drive the assembly of a 'LAT signalosome', which includes the proteins: linker of activated T-cells (LAT), Slp76, Grb2/Gads and the Tec family kinase Bruton tyrosine kinase (Btk). The latter phosphorylates the effector enzyme phospholipase Cγ2 (PLCγ2).[47] The assembly of the LAT signalosome leads to activation of PI3K isoforms and PLCγ2. The PI3K converts PIP_2 into the phospholipid phosphatidylinositol 3,4,5-trisphosphate (PIP_3), which binds to PLCγ2 favouring its recruitment to the plasma membrane.[48] The activated PLCγ2 cleaves PIP_2 into IP_3 and DAG. As result, cytosolic Ca^{2+} increases and PKC isoforms are activated, supporting granule secretion and platelet aggregation (Figure 2). As most relevant for this thesis, I will only describe the receptor GPVI.

The immunoglobulin receptor GPVI is expressed exclusively on platelets and megakaryocytes. Human platelets contain 3-4,000 copies of the receptor, which consists of 309 amino acids. The *GP6* gene is located on chromosome 19 in the so-called leukocyte receptor cluster.[49] GPVI has two extracellular Ig domains, D1 and D2, of which D1 contains the collagen binding domain and D2 is involved in GPVI dimerization.[50] In addition it consists of a mucin-rich stalk, which is heavily O-glycosylated; a transmembrane region and a proline-rich cytosolic tail (Figure 3). In the platelet membrane, GPVI is constitutively

associated with the Fc receptor-γ (FcR-γ) chain through a salt bridge between an aspartate and an arginine.[51]

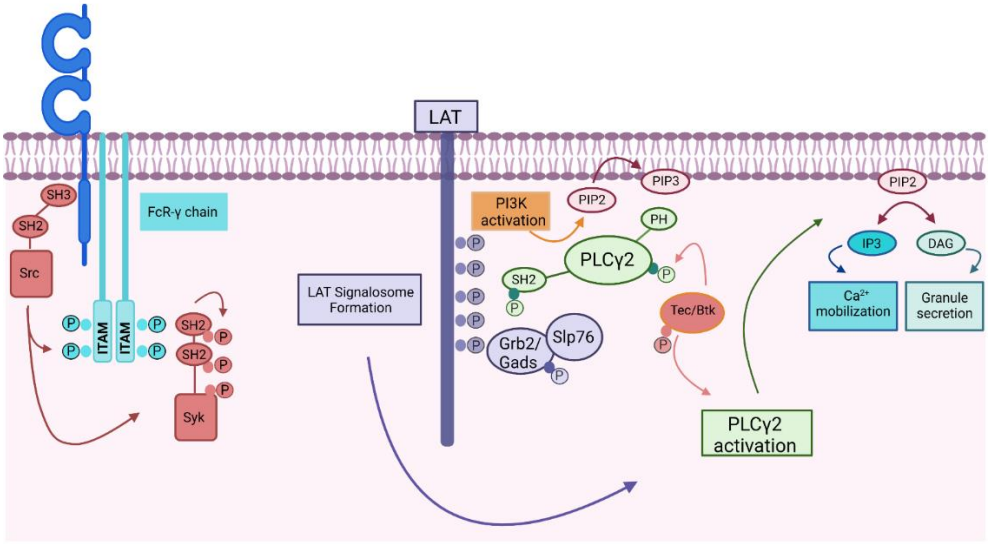

Figure 2. Schematic representation of ITAM-linked receptor signalling in platelets. *Ligand binding to ITAM-linked receptors activates the Src family kinases, Lyn and Fyn, which phosphorylate the ITAM present in the FcR-γ chain providing a docking site for the SH2 domains of the kinase Syk. The (phosphorylation) activation of Syk, together with Src proteins, results in the assembly of the LAT signalosome. This activates PI3K isoforms and PLCγ2. PI3K activation brings to the formation of PIP_3 that binds to PLCγ2 favouring its recruitment into the membrane. PLCγ2 activation induces Ca^{2+} mobilisation and granule secretion. Figure created with BioRender.com.*

The FcR-γ chain is present as a homodimer with disulphide bridges connecting its extracellular portions and it is critical for the signalling.[52]. In the proline-rich region of the cytoplasmic tail, GPVI associates with the SH3 domains of two SFK, namely Lyn and Fyn.[48,53] It has been shown that double-deficient Lyn/Fyn and Lyn/Src mouse platelets display a delayed collagen-induced platelet aggregation, indicating that Src as well as Lyn and Fyn mediates GPVI signalling.[54] The SFK have partly distinct and partly

overlapping roles, with Lyn and Fyn deficiency showing a delay and a reduction in the onset of aggregation, respectively.[55]

Next to collagen, collagen-related peptides and the snake venom convulxin, GPVI also binds to plasma-derived proteins including fibrin and fibrinogen, and a variety of other charged ligands.[56,57] In 2002, Jung and Moroi reported that dimeric but not monomeric GPVI bound to fibrillar collagen with a K_d of 576 nM and a rapid dissociation rate, explaining the importance of the other collagen receptor, integrin α2β1, for stabilising platelet adhesion.[58] Using antibodies specific to the dimeric conformation, it was concluded that in resting platelets GPVI is expressed predominantly as a monomer, with the fraction of dimeric GPVI ranging from 2% to 29% and increasing upon platelet activation.[59-61] The explanation for the differences in the binding of the dimer-specific antibodies between resting and activated platelets, and the mechanism of regulation of dimerisation is not known. These aspects are examined in Chapter 3.

In vivo mouse studies indicated that pharmacological blockage and genetic deficiency of GPVI suppresses arterial thrombosis without significantly affecting bleeding.[62] So far, only a handful of GPVI-deficient individuals have been identified worldwide. The first cases presented with an autoimmune thrombocytopenic purpura caused by antibodies to GPVI.[63] These patients had a marked bleeding diathesis which was attributed to both the loss of GPVI and the low platelet count. In these patients, the presence of thrombocytopenia made it difficult to assess the role of GPVI in haemostasis. The sequencing of *GP6* gene later permitted the identification of two individuals with compound heterozygous mutations, which associated with an altered GPVI function.[64] But it was through the generation of receptor-deficient mice[65] and, many years later, through the identification of patients with a mild bleeding diathesis, attributed solely to mutations in GPVI,[66] that the receptor was found to have a relatively minor role in haemostasis. To date, nine individuals, from eight unrelated families, with a homozygous

adenine insertion (c.711_712insA) in exon 6 of the *GP6* gene have been identified.[66,67]

This insertion generates a premature stop codon, which gives rise to a truncated protein of ~50 kDa that is retained in the platelet cytosol, as shown using an antibody against the extracellular region of GPVI.[66] Individuals with

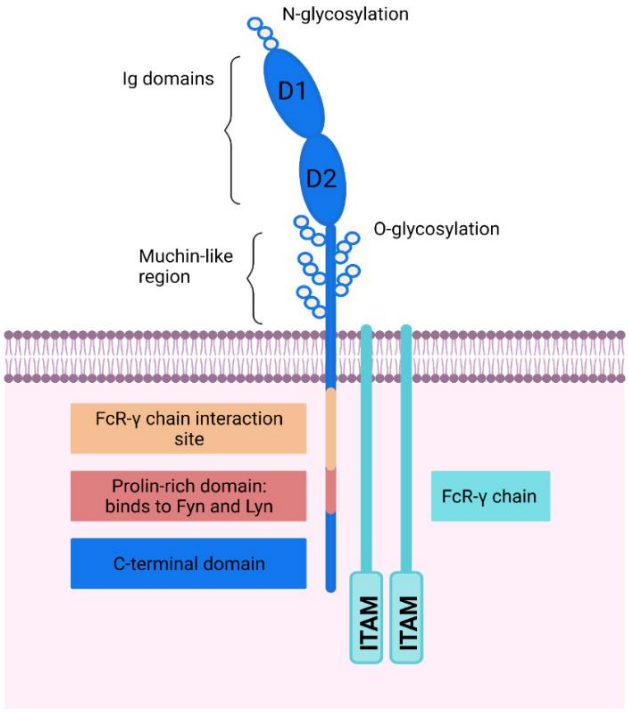

Figure 3. Schematic representation of GPVI. *GPVI is an ITAM containing receptor, it is constitutively associated with the FcR-γ chain that contains two ITAM domains. Three different domains characterise GPVI structure: 1) the extracellular domain formed by two Ig-like domains, D1 and D2, linked to a mucin-like stalk region; 2) the transmembrane domain; 3) the cytosolic tail including the binding site for the SH3 domain of Scr familty kinases. Figure created with BioRender.com.*

this insertion mutation have only been found in Chile. Recently two further individuals, again from unrelated families, have been identified in the same country (Diego Mezzano, personal communication).

The platelets from these GPVI-deficient individuals do not aggregate in response to collagen or other GPVI agonists but aggregate normally to arachidonic acid and ADP.[66] To which extent the process of thrombus formation is impaired in individuals with a GPVI deficiency is not well understood. This question is raised in Chapter 3.

The only mild bleeding diathesis of GPVI-deficient individuals suggests that GPVI is a promising therapeutic target in arterial thrombosis with limited effect on haemostasis. Over the years, several pharmaceutical companies have searched for small molecule inhibitors of GPVI and among these there were losartan and honokiol, whose action is re-examined in *Chapter 5*. However, these inhibitors were not successful probably because the glycoprotein receptor has a restricted binding to collagen.[68] In contrast, a GPVI-blocking Fab fragment (9O12 Fab), which has since been humanised (glenzocimab, ACT-017),[69] was shown to inhibit collagen-induced platelet aggregation *ex vivo* in macaques, without inducing thrombocytopenia or bleeding side effects.[69] In addition, a GPVI-FcR fusion protein, revacept, which functions by shielding collagen at the local site of vascular injury without directly blocking GPVI, was also developed.[70] Revacept was shown to have high affinity for fibrillar collagen, but not for fibrin or fibrinogen[58] and to be well-tolerated, with dose-related inhibition of platelet aggregation, in healthy volunteers.[70] However, the group of Siess later showed that inhibition of collagen- and plaque-induced platelet aggregation under static and flow conditions at low and high shear rates, was less with revacept than antibodies recognizing platelet monomeric and dimeric GPVI.[71]

Glenzocimab is currently undergoing a phase II trial in patients with acute ischemic stroke (NCT03803007). In the previous phase I clinical trial, it was shown to be well tolerated, with inhibition lasting up to 24 h for the highest dose of 2,000 mg.[72] Revacept has been tested in clinical trials in patient with

carotid artery stenosis (NCT01645306) or with stable coronary artery disease and a percutaneous coronary intervention (NCT03312855). In these studies, revacept appeared to be well tolerated, but it did not significantly reduce the incidence of myocardial infarction when compared to placebo.[73,74]

4.3 Integrin αIIbβ3

Integrins are heterodimeric transmembrane glycoproteins formed by two noncovalently bound subunits, indicated as α and β chains.[75] Each integrin chain consists of a N-terminal extracellular ectodomain, a single transmembrane-spanning helix and a C-terminal cytoplasmic domain. The latter provides docking sites for cytosolic proteins supporting integrin inside-out and outside-in signalling.[76,77] Platelets express up to 80-100,000 copies of integrin αIIbβ3 on their surface, and an additional 40,000 copies in their α-granules, making it the most highly expressed platelet integrin.[78]

On resting platelets, the ectodomain of the integrin chains assumes a bent, low-affinity conformation.[79,80] On activated platelets, the ectodomain unfolds in response to agonist-dependent intracellular signals (inside-out signaling) which stimulate the interaction of the cytoskeletal proteins, talin-1 and kindlin-3, with the integrin β3 C-terminal tail. This leads to a conformational change in the receptor extracellular domain which acquires high affinity for adhesive ligands such as fibrinogen, VWF, vitronectin and fibronectin.[81-83] The binding of the ligands then promotes integrin clustering resulting in outside-in signalling.

Binding of SFKs, especially Src and Lyn, to the β3 cytoplasmic tail mediates integrin outside-in signalling.[46] SFKs phosphorylate Syk, which binds to the integrin β3 chain-tail.[84] This binding is independent of tyrosine phosphorylation of the β3-tail and leads to Syk activation independently of an ITAM sequence.[85] However, there is also evidence for another signalling route, that involves the ITAM present in the IgG receptor FcγRIIa.[86] Integrin αIIbβ3 outside-in signalling, involving Src and Syk, leads to a activation of PLCγ2 that catalyse the generation of IP_3 and DAG.[84] Integrin αIIbβ3 elicits

weak platelet activation but it is key to clot retraction. Its absence causes a moderate to severe bleeding disorder, as seen in patients with Glanzmann thrombasthenia, a rare autosomal recessive disorder due to a dysfunction mutation in the genes encoding for the αIIb or β3 chains.[78,87] Platelets from αIIbβ3-deficient Glanzmann thrombasthaenia patients are unable to bind to fibrinogen and have impaired binding to VWF, fibronectin and vitronectin.[88] The inadequate platelet adhesion leads to an impaired inside-out signalling and reduced fibrinogen-dependent platelet aggregation, and a defect in outside-in signalling.

There are several intravenous inhibitors of integrin αIIbβ3 currently approved for clinical use, such as abciximab (reopro, a recombinant monoclonal antibody construct); eptifibatide (integrilin, based on the snake venom disintegrin, barbourin) and tirofiban (aggrastat, a small molecule compound).[89-91] However, their use is restricted to acute treatment of patients undergoing stenting, as they are not suitable for long-term therapy due to the risk of life-threatening bleeding.[92,93] Orally available inhibitors of αIIbβ3 were developed by numerous companies over 20 years ago but they either demonstrated excess mortality or lack of improvement over existing therapies,[94,95] possibly because they induced integrin activation by acting as ligand-mimetic.[94] The resulting priming for fibrinogen binding and the outside-in signalling caused a paradoxical platelet activation.[96]

5. Structure of protein based GPVI ligands

A number of protein ligands for GPVI have been identified,[56,57] in this paragraph the most relevant for this thesis will be described.

5.1 Collagen

Collagen is the major structural components in the vascular subendothelium. It consists of triple-helix polypeptide chains,[97] which self-assemble into thicker fibres,[98] forming a quaternary structure with multiple binding sites for platelet receptors.[99] GPVI was first proposed as a collagen receptor in the

later 1980s due to the identification of patients with an immune thrombocytopenia who were deficient in the glycoprotein.[60] However, it was not until dissection of its signalling pathway in the mid 1990s, showing that it signalled through a tyrosine kinase cascade, and cloning of the receptor in 1999 that it was established as a collagen receptor.[100] The glycine-proline-hydroxyproline (GPO) repeats in the collagen triple helices have been shown to be GPVI binding sites. Among the nine types of collagen present in the extracellular matrix, the fibrillar type I and III have the highest affinity for GPVI.[101]

5.2 Fibrinogen and fibrin

Fibrinogen is a glycoprotein of 340 kDa, which is present in the blood plasma at a concentration of 2 to 5 mg/mL.[102] Fibrinogen is a bivalent molecule, composed of two repeats of three polypeptides, namely Aα, Bβ and γ chains, which are linked together through disulphide bridges. These polypeptide chains are organised in a dimeric structure with two lateral D sites and a central E site. The C-terminal and N-terminal domains of the Aα, Bβ, and γ chains are located in the D and E sites (Figure 4). When coagulation is triggered, thrombin cleaves fibrinogen into fibrin. At first, the N-terminal fibrinopeptide sequence in the Aα- (FPA) and Bβ-chain (FPB) are cleaved, resulting in the exposure of two polymerisation sites, Ea and Eb.[32] The cleavage also causes a conformational change of the formed fibrin monomers. This allows the monomers to organise into fibrin protofibrils through association of the Ea/Eb sites with Da/Db sites in the the γ-chain of adjacent molecules. The polymerised fibrin is strengthened by the activated factor XIII (FXIIIa) which catalyses the formation of covalent cross-linkages in D-domain (α-α and γ-γ peptides), conferring protection against fibrinolysis.[32] Fibrin clots are degraded by plasmin, leading to release of the fibrin degradation product, D-dimer (Figure 4).[103]

Fibrin and fibrinogen have only recently been recognised as GPVI ligands through two independent lines of research. The group of Martine Jandrot-Perrus observed an impaired thrombin generation in response to collagen or

TF in platelet-rich plasma (PRP) from several GPVI-deficient patients,[104] whereas the group of Watson observed platelet activation by fibrin as part of a study examining the ability of charged ligands to activate the immunoglobulin receptor.[105] These results, together with the observation that thrombus occlusion was delayed in GPVI-deficient mice, while the onset

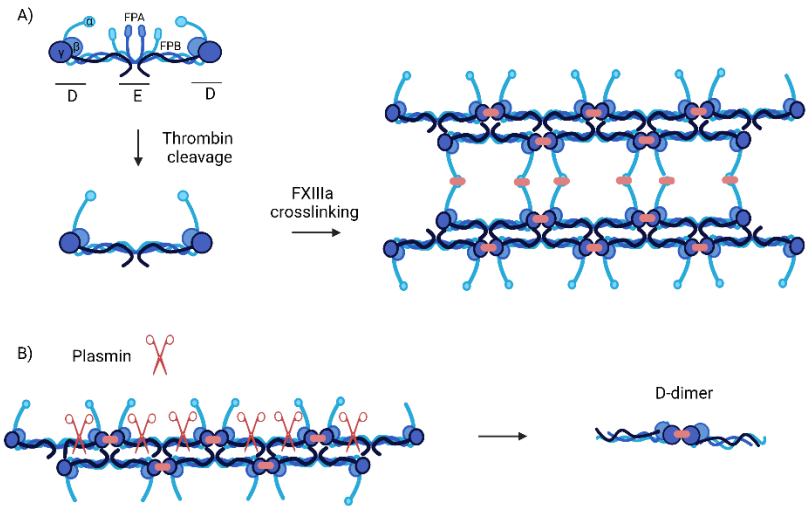

***Figure 4. From fibrinogen to fibrin**. **A)** Thrombin cleaves fibrinopeptides A and B (FPA and FPB) from the fibrinogen molecule resulting in the formation of fibrin monomers. The monomers polymerise and generate fibrin protofibrils. More specifically the cleavage of FPA and FPB exposes the N-terminus in the E region that noncovalently interacts with a complementary C-terminus in the D region of another monomer. The transglutaminase factor XIIIa (FXIIIa) then crosslinks the protofibrils generating stable fibrin polymers. **B)** Fibrin degradation by plasmin generates D-dimer. Figure created with BioRender.com.*

of thrombus formation was unchanged,[105] suggested of a role of GPVI in thrombus growth and stability.

Furthermore, Mammadova-Bach *et al*. demonstrated that recombinant dimeric GPVI binds to fibrin but not to fibrinogen,[104] whereas Alshehri *et al.*

reported that fibrin, but not fibrinogen, binds to monomeric GPVI.[105] Successively, D-dimer, a proteolytic fragment of fibrin, was found to be the region that confers binding to GPVI.[106] This discrepancy led to studies from several groups that questioned whether fibrin binds to monomeric or dimeric GPVI, or not at all[107,108] and the surprising observation that fibrinogen also binds to GPVI.[109] One possible explanation for the conflicting results lies in the GPVI constructs used by these groups, in particular the presence of the stalk region as discussed in Chapter 2. Resolving the crystal structure of GPVI in interaction with a fibrin peptide or fibrinogen may help clarifying current discrepancies. Furthermore, it is of interest to develop ligand specific GPVI antagonists. These aspects are also explored in Chapter 4.

6. GPVI Shedding

Exposure of platelets to GPVI agonists and the activation of intracellular signals is accompanied by the proteolytic cleavage of GPVI and the release of a soluble fragment of 55 kDa while a 10 kDa fragment remains associated to the platelet membrane.[110,111] GPVI shedding may serve to reduce platelet reactivity and thus, to limit thrombosis. In line with this, GPVI shedding in mice has been associated with only a moderate increased bleeding while it conferred a long anti-thrombotic protection in different arterial thrombosis models.[112] Furthermore, pathologic shear rates (3000–10,000 s^{-1}) also trigger GPVI shedding independently of intracellular signal, alongside the active factor X (FXa) which downregulates plasma level of GPVI under procoagulant conditions. GPVI shedding can also be mediated by autoantibodies that either bind GPVI directly or bind other platelet surface antigens and act via FcγRIIa.[113]

While a role of soluble GPVI acting on other cells is unclear, it surely can be used as a quantitative marker for pathological platelet activity in various diseases as elevated plasma level of soluble GPVI have been found in patients

with stroke,[114] disseminated intravascular coagulopathy,[114] microangiopathy[115] and atrial fibrillation;[116] as well as in sepsis, thermal injury[117] and in patients developing deep venous thrombosis.[118]

Platelet receptors shedding is mediated by a class of membrane-associated proteinases that become proteolytically active upon platelet activation. Sheddases of the disintegrin and metalloproteinase (ADAM) family are abundantly expressed on platelets with ADAM10 and ADAM17 being the major protease responsible for GPVI shedding.[119] However, studies in ADAM10- or ADAM17-deficient mice have revealed that GPVI proteolysis is differently regulated by the two sheddases, with ADAM10 responsible for GPVI shedding triggered by the inhibition of calmodulin binding, while ADAM17 acts upon mitochondrial damage.[119] Although GPIbα and GPVI partially share the same sheddases, GPVI shedding does not occur constitutively and interestingly, treating platelets with high doses of aspirin or serotonin has been shown to induce ADAM17-dependent shedding of GPIbα, but not shedding of GPVI.[120,121]

Although the approach of GPVI cleavage might be a promising antithrombotic strategy, it is important to note that this treatment severely compromises normal haemostasis in mice concomitantly treated with aspirin,[122] therefore further studies are needed. However, given the increased levels in multiple human diseases, soluble GPVI could represent an efficient diagnostic and prognostic target.

7. Arterial thrombus build-up and structure

Thrombi formed in the arterial circulation have a dynamic structure. Microscopic observations of mice thrombi revealed a solid base of contracted platelets surrounded by a gradient of less activated platelets extending from the site of injury.[123-126] The currently accepted model of thrombus architecture, based on intravital molecular-based fluorescence imaging in the mice microvasculature, describes it as composed of distinct

core and shell regions, in both the venous and arterial circulation (Figure 5).[127] The core contains strongly activated platelets, it has a restricted permeability for solutes and contains fibrin. By contrast, the shell is an outer region that lies adjacent to the core, which is formed by discoid and minimally activated platelets.[127] Interestingly, a similar organisation was observed in larger mouse arteries.[128] Collagen takes part to the formation of the core region as it mediates adhesion and activation of human platelets via integrin α2β1 and GPVI, respectively. Tissue factor- and GPVI-driven phosphatidylserine exposure, mediates thrombin generation whose signalling is critical for full platelet activation in the core.[26] The diffusion of TxA_2 and ADP from the highly activated platelets in the core support the formation of the shell.[26] However, the absence of fibrin in the shell makes it unclear as how aggregate stability is regulated in this region. A role for the tyrosine kinase Syk has been demonstrated by *in vivo* studies whereby the Syk inhibitor, PRT-060318, induced a rapid thrombus dissolution and platelet disaggregation.[129] On the other side, using anticoagulated human blood, microfluidic experiments have shown that a continuous signalling through ADP-induced PI3K activation, is required to keep the integrin αIIbβ3 in an active state, thereby preventing disaggregation.[130] It is therefore notable that in the same study, disaggregation occurred when platelet aggregates were post-perfused with fibrinogen-depleted plasma,[130] indicating that fibrinogen binding to the integrin contributed to the stability of the aggregates in the absence of fibrin. In addition, a study with the GPVI-blocking Fab, glenzocimab, raised the possibility that aggregate stability in the absence of fibrin is mediated by activation of GPVI by fibrinogen.[131] Together, these *in vitro* and *in vivo* studies, raise the possibility that tyrosine kinase activation by both GPVI and the integrin αIIbβ3, is required for thrombus stability and that blocking signals by both of the receptors may be more effective than inhibiting only one. This is investigated in *Chapter 6*.

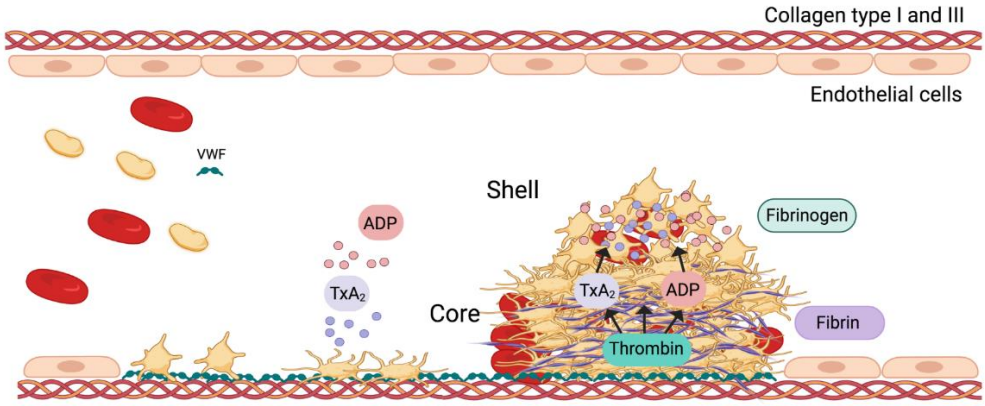

Figure 5. Schematic representation of thrombus core and shell region. *The thrombus core region contains strongly activated platelets, it has a restricted permeability for solutes and contains fibrin. The diffusion of TxA_2 and ADP from the highly activated platelets in the core supports the formation of the shell, which consists of discoid and minimally activated platelets, held together by fibrinogen. Figure created with BioRender.com.*

7. Hypothesis and Aims

In this thesis, human platelet GPVI and its signalling are investigated in the context of thrombus growth and stability. As an overarching hypothesis, I propose that GPVI acts as a signalling receptor for fibrin and fibrinogen in thrombus propagation and thrombus stability. In **Chapter 2**, the controversy on whether GPVI binds to fibrinogen and fibrin, as a monomer or a dimer, is examined. The investigation critically examines the GPVI constructs used by the groups involved in the contradictory results, alongside the binding techniques and key reagents. The analysis furthermore compares the advantages and disadvantages of *in vitro* binding studies and discusses the need for a tool to specifically inhibit the interaction of GPVI with fibrin. In **Chapter 3**, it is investigated whether blood from GPVI-deficient individuals responds differently to platelet activation, aggregation and thrombus formation when flowed over collagen and non-collagen surfaces. From a

clinical perspective, this study contributes to better understand the platelet phenotype of GPVI-deficient individuals. In **Chapter 4**, a range of immobilised surfaces is used to compare shear-dependent platelet adhesion and thrombus build-up via GPVI versus integrin αIIbβ3. These surfaces include fibrinogen, fibrin and collagen. For comparison between the receptors, immunological or pharmacological blockage of GPVI, integrin αIIbβ3, or GPVI-and integrin-dependent tyrosine kinase signalling is used. **Chapter 5** explores the effects of two small molecules, losartan and honokiol, on platelet activation properties, based on earlier reports that these compounds can act as anti-GPVI drugs. **Chapter 6** investigates how signalling downstream GPVI via the tyrosine kinases Syk, Src and Btk contributes to the stability of a thrombus, with particular attention to the thrombus shell. The aim of this study is to explore whether targeting tyrosine kinases downstream GPVI is an effective strategy to induce thrombus instability. The effect of tyrosine kinase inhibitors is compared to inhibition of secondary mediators, ADP and TxA_2. **Chapter 7** provides a first-time review of the possible involvement of GPVI in venous thrombotic complications, including thromboembolism, pulmonary thromboembolism and cancer metastasis. In this chapter, also the putative role of fibrin as a GPVI ligand is discussed. In **Chapter 8** the findings of this thesis are critically discussed.

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Chapter 2

Does fibrin(ogen) bind to monomeric or dimeric GPVI, or not at all?

Based on: A. Slater, G. Perrella, M-B. Onselaer, E.M. Martin, J.S. Gauer, R. Xu, J.W.M. Heemskerk, R.A.S. Ariëns & S.P. Watson

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I co-wrote the article, together with A.S. and S.P.W. M-B.O., E.M.M. and J.W.M.H. edited the manuscript. R.A.S.A., J.S.G. and R.X. supplied the fibrin figure and edited the manuscript.

Abstract

GPVI is the major signaling receptor for collagen on platelets. Dimerization of GPVI is required for collagen binding and initiation of signalling through the associated FcR-γ chain. Recently, fibrin and fibrinogen have been identified as ligands for GPVI and shown to induce signalling in support of thrombus formation and stabilization. Contrasting observations have been reported on whether fibrin binds to monomeric or dimeric GPVI, or to neither form. In this article, we discuss reasons for the contradictory results and how to reconcile these. We conclude that a lack of structural knowledge regarding the GPVI constructs that are being used, along with the use of non-standardized reagents, might be the main cause of the discrepant results. This article aims to highlight some of the key areas that need to be addressed.

Introduction

In 2015, two groups identified fibrin as a ligand for GPVI based on two separate sets of observations, namely (i) platelet procoagulant activity was reduced in patients deficient in GPVI or in the presence of a GPVI monoclonal antibody (mAb) 9012,[1] and (ii) GPVI-deficient mice were unable to form an occlusive thrombus in a $FeCl_3$ injury model whereas the onset of thrombus formation, where platelets are exposed to collagen, was not changed.[2] This suggested the presence of a novel GPVI ligand in platelets and led to the discovery that fibrin binds to GPVI. In 2018, fibrinogen was reported to bind to and activate GPVI based on studies in GPVI-deficient human platelets.[3]

Mammadova-Bach *et al*.[1] demonstrated that recombinant dimeric GPVI, consisting of the D1 and D2 immunoglobulin domains and the stalk region of GPVI linked to the Fc domain of IgG (Figure 1), binds to fibrin, using the mAb 9012 to show that binding was specific. In contrast, there was no specific binding detected to fibrinogen. Separately, Alshehri *et al*.[2] reported that GPVI shed from the surface of platelets (presumed to be monomeric) binds to fibrin but not to fibrinogen. Additionally, the latter authors reported that the binding to immobilized fibrin was not altered in the presence of GPRP (Gly-Pro-Arg-Pro) and was therefore independent of fibrin polymerisation, in contrast to functional results in platelet suspensions. Potential explanation for these contrasting results is that coated fibrin may adopt a different conformation to fibrin in solution or may function as a 'polymerized' reagent in two-dimensions. The explanation for the absence of binding to fibrinogen, as demonstrated in the recent report from Mangin *et al.*,[3] however, is unclear.

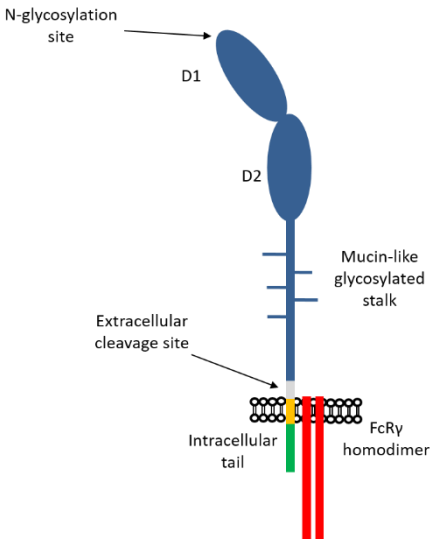

Figure 1.
Structural features of GPVI highlighting the N-terminal D1 and D2 domains, glycosylated stalk, transmembrane and intracellular tail regions. GPVI is associated with the FcR-γ chain through electrostatic interactions within the transmembrane and intracellular tail.

Recently, Onselaer *et al.*[4] reported that recombinant monomeric GPVI, consisting of the D1 and D2 Ig domains but without the stalk region (generated by the group of Andrew Herr) (Figure 2A, 5), binds to fibrin with an affinity constant of 302 nM measured using surface plasmon resonance (SPR). These authors further reported that the binding site lays in the D-region of fibrinogen and D-dimer, but not in the E-region of fibrinogen. Surprisingly, however, no specific fibrin binding was observed to a recombinant dimeric GPVI consisting of the D1 and D2 Ig domains linked to the Fc domain (Figure 2A, 2), or to a dimeric recombinant GPVI that also included the stalk region (again linked by the Fc domain) (Figure 2A, 3). The latter form of recombinant GPVI, named Revacept, is in phase II clinical trials for acute coronary syndromes and transient ischemic attacks.[10]

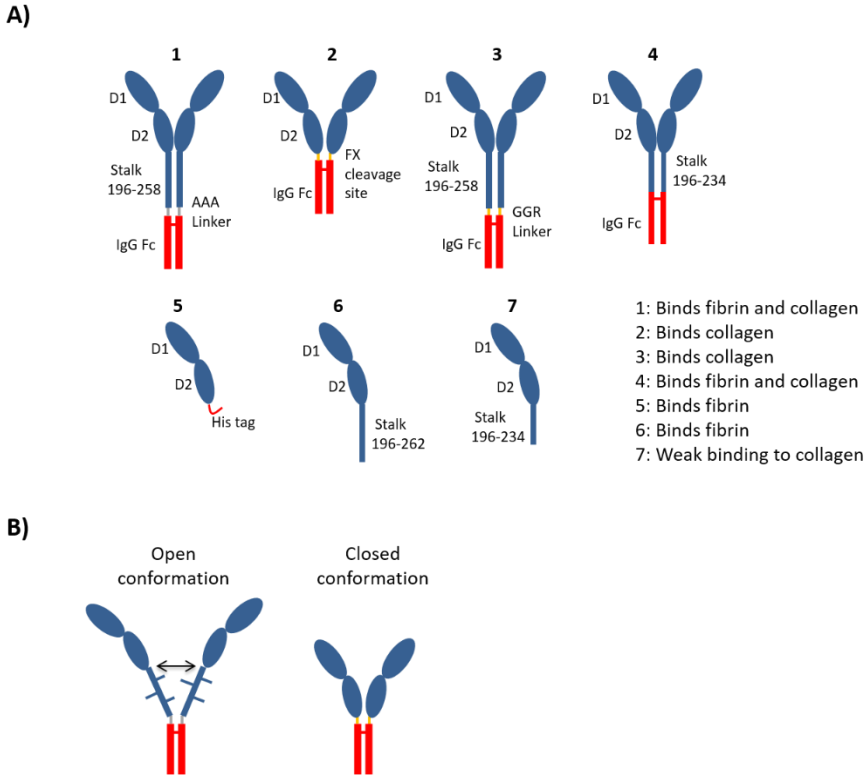

Figure 2. GPVI construct comparison. A, *Cartoon representation of the different GPVI constructs used in GPVI: Collagen/fibrin binding studies. A key of which ligands each construct has been demonstrated to bind is provided on the right. 1) Dimeric GPVI, used by Mammadova-Bach et al.[1] and first reported by Jandrot-Perrus et al.[5] 2) Dimeric GPVI used by Onselaer et al.[4] 3) Dimeric GPVI, known as Revacept, used by Ebrahim et al.[6] and first reported by used by Massberg et al.[7] 4) Dimeric GPVI used by Induruwa et al.[8] and first reported by Miura et al.[5] 5) Monomeric GPVI used by Onselaer et al.[4] 6) Monomeric GPVI used by Alsheri et al[2] and first reported by used by Al-Tamimi et al.[9] 7) Monomeric GPVI used by Induruwa et al. and first reported by Miura et al.[5]* ***B,*** *Hypothetical representation of the repulsion between stalks within dimeric GPVI resulting in a monomeric conformation of GPVI compared to the dimeric form formed in the absence of the stalk.*

Ebrahim *et al*.[6] later confirmed that Revacept (Figure 2A, 3) does not bind to fibrin, in a more extensive and very carefully designed set of studies. Revacept differs from the recombinant dimeric GPVI used by Mammadova *et al*.[1] (Figure 2A, 1) by the short linker sequence connecting the stalk and the Fc domain. In a further twist, Induruwa *et al*.[8] reported that a recombinant dimeric GPVI (Figure 2A, 4), which included the D1 and D2 domains and part of the stalk region, binds weakly to fibrin and fibrinogen, but strongly to the D-region and to D-dimer, calculating the affinity to be similar to that reported by Onselaer *et al.* for binding of fibrin to monomeric GPVI (Figure 2A, 5). Remarkably, however, they did not observe binding of monomeric GPVI to D-dimer. Mangin *et al.* also reported binding of fibrinogen to GPVI, calculating the K_d to be 336 nM, but this was the same monomeric GPVI used by Onselaer *et al*. (2017) based on the D1 and D2 domains, but lacking the stalk region. Moreover, Mangin et al. (2018) reported that, whereas mouse platelets undergo very limited spreading on fibrinogen, transgenic mouse platelets expressing human GPVI form extensive lamellipodia. This led to the conclusion that human but not mouse GPVI is able to bind fibrinogen. On the face of it, there is no easy way to reconcile all these observations. In this opinion article we consider the reasons for the conflicting data and how to develop this emerging field.

GPVI-collagen interaction

Human GPVI has 309 amino acids and consists of a single chain glycoprotein with two extracellular Ig domains (D1 and D2), a mucin rich stalk containing several sites of O-glycosylation, a transmembrane region and cytosolic tail,[12] (Figure 2). GPVI is coupled to the FcR-γ chain homodimer,[13,14] which has disulphide bridges between residues close to the extracellular portion of transmembrane region[14]. Each FcR-γ chain contains an immunoreceptor tyrosine based activation motif (ITAM) defined by the sequence YxxLx6-12YxxL which is phosphorylated on the conserved tyrosines by the Src-family kinases Fyn, Lyn and Src on activation of GPVI.[15,16] Fyn and Lyn but not Src are constitutively associated to GPVI through a proline-rich motif found in

the cytoplasmic tail of GPVI, placing the receptor in a 'ready-to-go' state.[17] Tyrosine phosphorylation of the FcR-γ chain ITAM leads to recruitment of Syk via its tandem SH2 domains and initiation of downstream signalling events that culminate in activation of PLCγ2.[18]

Collagen has long been considered as the major endogenous ligand for GPVI. Collagen is the most abundant protein found in humans and is a major component of the extracellular space. It is made up of three polypeptide chains which form a right-handed triple-helix structure,[19] and multiple collagen triple-helices will self-assemble to form collagen fibers.[20] A distinctive feature of collagen is the presence of glycine-proline-hydroxyproline (GPO) repeats. A synthetic collagen-related peptide (CRP) which consists of multiple GPO repeats was shown to be a powerful platelet agonist when cross-linked,[21-23] either through the formation of disulphide bridges or by glutaraldehyde cross-linking, and has been used extensively to study the role of collagen in platelet activation. This cross-linked peptide activates platelets through GPVI,[12,24] whereas the monomeric non-crosslinked peptide is inactive.[25] These results highlight the importance for the quaternary structure of fibrous collagen and the nearby presence of multiple binding sites for its interaction with GPVI.

Direct binding of collagen to the extracellular region of GPVI fused to the dimeric Fc domain of IgG was first reported by the group of Jung and Moroi.[5] This was the same recombinant form of GPVI later used by Induruwa *et al*. (described above). In contrast, recombinant GPVI without the addition of the Fc domain exhibited no binding. This could be explained either by a loss of avidity or by the adoption of a distinct conformation of dimeric GPVI which promotes binding to collagen. By mutating specific residues within the extracellular D1 and D2 domains of GPVI, D1 was identified as the major collagen binding domain,[26,27] with Val54, Leu56 and Lys79 being identified as key residues for collagen binding to human GPVI. Interestingly, the D1 domain is also the major collagen-binding region of the related Ig receptor LAIR-1 which is homologous to GPVI.[28] The collagen-binding site in LAIR-1

was mapped using NMR and mutagenesis studies, and the conserved D1 residues Arg58, Glu60 and Trp96 were identified as additional binding residues.

GPVI dimerization

Like many traditional tyrosine kinase receptors, there is overwhelming evidence that activation of GPVI requires dimerization in the membrane. Using antibodies specific to the dimeric conformation of GPVI, it was reported that GPVI is expressed predominantly as a monomer in resting platelets with the level of dimeric GPVI increasing upon activation.[29-31] Estimates of the level of dimeric GPVI range from 2% to 29% in resting platelets according to the dimer-specific antibody, suggesting that these antibodies recognize distinct conformations of the receptor. GPVI dimerization has been linked with increased platelet activation, as demonstrated by increased recognition of collagen-activated platelets to dimeric-GPVI antibodies[30,31] and by the powerful response of GPVI to multimeric ligands such as the snake venom toxin convulxin.[32] Collagen have multiple binding sites for GPVI, enabling clustering of GPVI dimers as shown by super-resolution microscopy.[33] Collagen binds to Fc-linked dimeric GPVI with an affinity of 580 nM, with rapid association and dissociation rates.[5] This can explain why GPVI requires the presence of an activated integrin to mediate stable adhesion to collagen (and should therefore not be described as an adhesion receptor). The dimeric GPVI-specific Fabs, m-Fab-F and 204–11, block platelet aggregation on collagen under flow, demonstrating the importance of the dimeric conformation to activation.[30] The increase in GPVI dimerization upon activation by collagen and other receptor ligands[31] is considered to provide a positive feedback pathway for activation by collagen.

The FcR-γ chain is a homodimer and so has the potential to bind to two GPVI receptors[11] and therefore induce GPVI dimerization. Contradicting this, it has been proposed that one FcR-γ homodimer binds to one GPVI, based on the discovery of the formation of a three-helix interface that can only accommodate the binding of one GPVI.[34] The crystal structure of the

extracellular D1 and D2 domains (which lacks the stalk) reveals that two D2 domains form a back-to-back dimer offering an alternative mechanism of dimerization and orientation on the surface of platelets.[35] The orientation of GPVI in the crystal structure generates a groove which could potentially form a binding site for collagen. Elucidating the structure of the collagen-GPVI complex is required to test this possibility. On the other hand, ultra-centrifugation suggested that the D1 and D2 constructs remained monomeric, making it unclear whether the dimeric confirmation is physiologically relevant. The trans-membrane domain likely plays an important role in GPVI dimerization but all recombinant GPVI studies have excluded this region due to solubility issues and difficulties working with membrane proteins. Extracting and purifying full length recombinant GPVI from the cell membrane using styrene-maleic acid co-polymer lipid particles (SMALPs) could provide a method of solving the structure of full length GPVI and determining the role of the trans-membrane domain.[36] The role of GPVI dimerization for ligand binding and signalling remains a very interesting but complex area. Collagen binds specifically to dimeric GPVI and it is likely the specific GPVI conformation adopted in the dimeric structure that is important for ligand binding. It is not known if the stoichiometry of GPVI binding to collagen is 1:1 or 2:1. The role of GPVI dimerization on signaling, however, is still up for discussion as groups have demonstrated that fibrin binds to monomeric GPVI but also induces GPVI signaling.[4] It is unclear how the monomeric GPVI induces cell signaling, and one explanation is that fibrin binding is followed by GPVI dimerization. However, there is currently no data to support this hypothesis.

GPVI-fibrin interaction

Fibrin is a final product of the coagulation cascade and is produced by thrombin-dependent cleavage of fibrinogen[37] to form polymers of insoluble fibrin fibres.[38] Thrombin-activated FXIII cross-links the fibrin polymer to stabilize it mechanically and increase resistance to fibrinolysis by incorporating inhibitors of fibrinolysis.[39] Fibrinogen is a plasma glycoprotein

with a molecular weight of 340 kDa, characterized by two repeats of three polypeptides, Aα, Bβ and the γ chain.[38] The six chains are linked together through the presence of 29 disulphide bonds resulting in a dimeric structure containing a central E-region and two lateral D-regions. Fibrin assembly is initiated when two short N-terminal fibrinopeptide (FPA) are cleaved from the Aα chains by thrombin.[40] Cleavage results in the exposure of two polymerization sites which interact with the two γ-chains from within a neighbouring D region. This results in fibrin protofibrils through a half-staggered arrangement, which branch and link with each other through lateral and bilateral junction.

As discussed above, the evidence that fibrin binds to and activates GPVI derived from functional studies is compelling. The initial experiments demonstrated that binding of fibrin to GPVI was able to support thrombin generation,[1] spreading of platelets[1,2] and tyrosine phosphorylation of Syk and the FcR-γ chain independent of integrin αIIbβ3.[2] These results were later confirmed in patients deficient in GPVI. In addition, Onselaer *et al.* (2017) reported that fibrin-induced platelet aggregation is abrogated in GPVI-deficient platelets[1,4] and that D-dimer inhibits both fibrin and collagen-dependent aggregation.[4] The physiological significance of the fibrin-GPVI interaction is unclear. In the revised model of thrombus growth, proposed by Stalker and Brass, fibrin is found within the core of the thrombus which is composed of fully activated platelets and characterized by poor solute access.[41] However, the degree to which activation of GPVI by fibrin contributes to formation of the core is unclear, and any potential role is likely redundant with that of platelet activation by ADP and TxA_2. The more loosely packed outer shell region of the thrombus consists of partially activated platelets providing good solute access. The shell region is dependent on the $P2Y_{12}$ ADP receptor and the TxA_2 receptor, with diffusion of the two agonists from the core determining the size of the shell.[42,43] Fibrin is believed to be absent from the shell; however, a recent study demonstrated the presence of a fibrin film that covers the entire clot surface and has a distinct structure

compared with fibrin fibres.[44] We question whether this film can also activate platelets in a similar way to fibrin fibers, or if this film plays a different role. In addition, in vivo and in vitro studies of thrombus formation under coagulant conditions showed the presence of growing fibrin network at the thrombus base near the sites of exposed or immobilized tissue factor, respectively.[45,46] Hence, the various experimental models suggest that both vascular and luminal-orientated fibrin can form a substrate for platelet deposition. Alsheri *et al.* reported a failure to form an occlusive thrombus in GPVI-deficient mice, following $FeCl_3$ injury model, even though thrombus onset, whereby collagen is exposed, is not altered. This is consistent with a role for fibrin but not fibrinogen in stimulating thrombus growth/stabilization since mice fibrinogen does not activate GPVI. Furthermore, in a recent model of venous thrombosis, Lehmann *et al*. demonstrated a critical role for GPVI in platelet activation on a fibrin-coated surface in regions of low shear through demonstration of inhibition by D-dimer and the anti-GPVI inhibitory Fab ACT017.[47]

Taken together the functional data is consistent and demonstrates a critical role for GPVI in mediating platelet activation by fibrin in both mice and human. The controversies arise when looking at the structural and mechanistic details regarding the interaction of GPVI and fibrin/fibrinogen. To fully understand this, the structure of the GPVI-fibrin complexes needs to be determined.

Species differences

Although fibrin activates both human and mouse forms of GPVI,[2] Mangin *et al.* have recently reported that fibrinogen only activates human GPVI;[3] thus, explaining the paradoxical observation that human but not mouse platelets generate lamellipodia and stress fibres on fibrinogen, whereas the full spreading observed of platelets from both species on collagen and fibrin. In support of this, it appeared that fibrinogen stimulates phosphorylation of the FcR-γ chain in human but not mouse platelets. In addition, there is no observable defect in adhesion or change in spreading of mouse platelets to

fibrinogen in the absence of GPVI. Fibrinogen binds to monomeric human GPVI with a K_d of 336 nM, which is similar to that of fibrin binding.[3]

Factors that could influence the binding of GPVI and fibrin

1. Fibrin generation

The method of fibrin production is a key variable for binding studies, given that its activity is dependent on the degree of branching and fibre network density (Figure 3). Fibrin is prepared by incubating fibrinogen with thrombin. However, the preparations vary according to the concentration of fibrinogen (1-200 μg/mL), incubation time (15-60 minutes) and the mode of thrombin deactivation (usually hirudin or PPACK), with some groups not reporting the use of an inhibitor. Thrombin concentration is particularly important as this is a major determinant for the number of branch points and fibre network density.[48] Fibrin in suspension is traditionally prepared by the incubation of fibrinogen, thrombin, calcium chloride and FXIIIa. The insoluble fibres can then be solubilized by sonication. For adhesion assays, fibrin is produced on a surface and mimics a polymerized substrate regardless of FXIIIa branching and network formation.

Under these conditions, single platelets have the potential to bind to multiple fibrin subunits. A more controlled method of immobilizing fibrin is to first coat the surface with fibrinogen followed by a wash step to remove unbound fibrinogen. Thrombin is then added to the wells to produce a fibrin monolayer. This produces a more uniform fibrin surface. Even with the inconsistencies of fibrin generation, the discovery that GPVI binds to fibrin through the D-dimer region has been a significant step forward.[4] D-dimer is a 160 kDa degradation product of cross-linked fibrin produced by the action of plasmin.[51,52] D-dimer has an increased solubility and a more uniform structure compared to polymerized fibrin. There are, however, several different forms of D-dimer determined by the enzyme used to digest fibrin (usually plasmin or trypsin) and the conditions of digestion (duration, fibrin

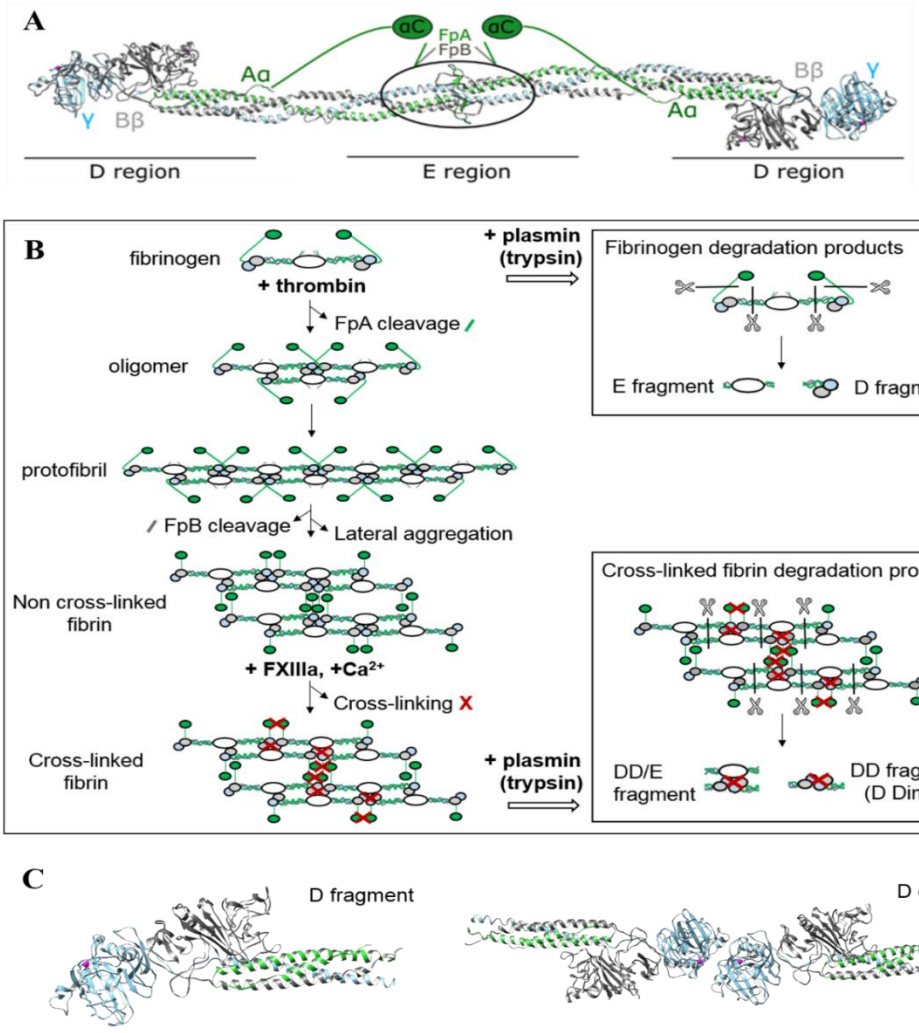

Figure 3. Fibrin formation and fibrin/fibrinogen proteolysis. A*, Crystal structure of fibrinogen molecule[49] (PDB: 3GHG), a plasma glycoprotein consisting of two repeats of three polypeptides, Aα (green), Bβ (grey) and γ chain (blue). The disordered αC regions, FpA and FpB, are modelled.* ***B****, The process of fibrin assembly is initiated by cleavage of fibrinopeptide A (FpA) by thrombin, leading to exposure of a polymerization site which interacts with the D-region of neighbouring fibrinogen molecules. Thrombin activated factor XIII catalyses the cross-linking of fibrin fibrils.*

Proteolysis of fibrinogen molecule by plasmin (or trypsin) yields different protein fragments including the E fragment and D fragment. Similarly, different protein fragments are obtained from cross-linked fibrin proteolysis, such as the DD fragment (D-dimer). ***C****, Crystal structures of D fragment and D dimer*[50] *(PDB: 1FZA/1FZB). Ribbon diagram showing α, β and γ chains in green, grey and blue, respectively. Bound* Ca^{2+} *ions are coloured in magenta.*

and thrombin concentration, temperature). The field requires a standardized preparation of D-dimer to enable comparison of data between groups.

2. Recombinant GPVI

The conflicting data on the binding of monomeric and dimeric forms of GPVI to fibrin emphasise the importance in understanding the relationship between the various recombinant constructs and the conformation of GPVI in the membrane. For example, the GPVI-Fc construct is a dimer due to the Fc domain, and it is unclear as to how this influences the structure of GPVI; for instance, whether the structure consists of a dimeric conformation of GPVI or just two monomeric conformations fused by the Fc domain. Furthermore, the presence of the stalk region could affect the equilibrium between monomeric and dimeric forms, and this this might explain why fibrin has been reported to bind to some but not all forms of Fc-dimeric GPVI. A comparison of the constructs is shown in Figure 2A.

In attempt to explain the differing results, Induruwa et al. proposed that the presence of a short linker region between GPVI and the Fc domain, found in Revacept (Figure 2A, 3) but not within their own construct (Figure 2A, 4), changes the conformation of GPVI and therefore alters the binding to fibrin. This argument is not convincing as the dimeric construct used by Mammadova-Bach *et al.* (Figure 2A, 1) also contained a linker region and was demonstrated to bind fibrin. The absence of the stalk in the construct used by Onselaer *et al.* (Figure 2A, 2) removes a significant anionic interaction. It could be argued that the presence of the negatively charged stalk region

would result in repulsion between both stalks in the Fc-fused constructs. This would push the extracellular regions away from one another resulting in a construct which behaves as a monomer. In contrast, the dimeric GPVI constructs without the stalk regions would not experience this repulsion and therefore the extracellular domains are more likely to form a dimeric interface (Figure 2B). The construct used by Alsheri *et al*. (Figure 2A, 6) represents the most physiologically relevant form of monomeric GPVI as it was purified directly from platelets treated with N-ethylmaleimide.[2] X-ray crystallography and cryo-electron microscopy will be invaluable in gaining critical structural insights which will be required to critically assess the differences in the different constructs. In contrast to the results with fibrin and fibrinogen, all dimeric forms of GPVI bind to collagen while monomeric GPVI shows a much lower level of binding.[4,6]

3. Binding assay conditions

Another potential source of variation are the conditions used for the binding assays. The conditions used in the solid-phase binding assays are given in Table 1. This technique involves the coating of wells with a substrate (collagen or fibrin), and estimation of the binding of a protein of interest to the coated wells. The extent of binding can be quantified using a tagged or labelled antibody specific to the target protein.[53] Each group has used a different method of detection. Mammadova-Bach *et al.* conjugated the GPVI construct with Alexa-488. While this has the advantage of not requiring a secondary antibody Ebrahim *et al.* have suggested that conjugation of GPVI may modify its structure. Alsheri *et al.* and Induruwa *et al.* used the GPVI antibodies 1A12[54] and 1G5,[55] respectively, in combination with an HRP-tagged secondary antibody, to detect binding, but it is also possible that binding of the antibody influences the conformation of GPVI. Onselaer *et al.* and Mangin *et al.* used directly conjugated anti-His (for monomeric His-tagged) GPVI and anti IgG Fc (for dimeric GPVI). This approach has the advantage of detection through a site remote to the D1 and D2 domains, although the use of two different labelling procedures prevents direct

comparison of the results. Ebrahim *et al.* tagged GPVI with biotin and used streptavidin to detect the interaction. Variance in the GPVI concentration, ranging from 100 to 1000 nM, may also influence the ability of GPVI to form dimers or higher order structures.

Additional variables include the incubation time and temperature which influence the time taken to reach equilibrium. Incubation times of GPVI with fibrin(ogen) were comparable between groups and ranged from 60 to 120 minutes at room temperature (Table 1). Although the room temperature should not vary to a significant degree, a controled incubation is strongly recommended.

4. Detection of non-specific binding

A critical issue in all of the mentioned binding studies is the measurement of non-specific binding to the plate or to the blocking agent (most commonly BSA).[56] The gold-standard way to estimate non-specific binding is to use an excess concentration of ligand to block the specific binding site. In the initial studies with fibrin this was not possible, and Mammadova-Bach *et al.* used the 9012 mAb to block the binding site. The observation that the binding region resides in the D-region offers a straight forward way to estimate non-specific binding through the use of D-fragment or D-dimer. D-dimer was used by Onselaer *et al.* and Induruwa *et al.* to demonstrate specific binding of monomeric GPVI to fibrin, with both groups estimating the IC_{50} of the interaction with D-dimer to be submicromolar and therefore similar to the affinities of fibrin and fibrinogen for GPVI.[4,8]

Cell line studies

Another way to demonstrate binding is by detecting adhesion and activation of GPVI-transfected cell lines. Such studies were instrumental in the early years for confirming GPVI as a receptor for collagen.[57] Yet it took several years to develop a robust assay for collagen signaling in transfected cell lines.[58] To-date only Mangin *et al.* have reported increased binding of a GPVI-transfected cell line (mast cell Rbl-2H3) to fibrinogen, and there has been no

report of increased binding to fibrin. The Rbl-2H3 cell line, however, also expresses integrin αIIbβ3 and is adherent. In our hands, GPVI-dependent adhesion to fibrin and fibrinogen has not been observed in several transfected cell lines despite robust adhesion to collagen (unpublished). While the lack of binding of GPVI-transfected cells suggests that the Ig receptor may not bind directly to fibrin and fibrinogen, it may be that the GPVI present in the cell lines is only expressed as a dimer consistent with reports of constitutive signaling by GPVI[58] and firm adhesion to collagen.

Why does fibrinogen not activate platelets in suspension?

A frequently asked question is why platelets are not activated by GPVI in blood, given the submicromolar affinity and high concentration of fibrinogen. The answer almost certainly lies in the fact that binding to monomeric GPVI would not induce GPVI clustering and hence GPVI-signalling. While this could in theory lead to aggregation, the affinity is too low to support aggregation, in the same way that the interaction of collagen with GPVI requires an integrin (αIIbβ3 or α2β1) to mediate stable adhesion under flow.

Dimerization and clustering of GPVI, leading to activation, would occur on a fibrinogen monolayer or in response to fibrin due to the co-location of multiple binding sites. This is illustrated by the results with D-dimer which induces spreading through activation of GPVI when presented as a monolayer, whereas, in suspension it blocks activation of platelets by both collagen and fibrin.[4] When D-dimer is used as a monolayer it essentially mimics a polymerized structure.

These considerations have important implications for the role of fibrin and fibrinogen in thrombus growth. Fibrin could drive thrombus growth through both adhesion and activation of GPVI. Yet it is unclear if fibrinogen could support growth through adhesion and activation, bearing in mind that the thrombus has some resemblance of platelets binding to a protein coated surface

Table 1. *Surface binding assay: all the conditions documented by different groups.*

Reference	Mammadova-Bach *et al.* (2015)[1]	Ebrahim *et al.* (2018)[6]	Induruwa *et al.* (2017)[8]	Onselaer *et al.* (2017)[4]	Alshehri *et al.* (2015)[2]	Mangin *et al.* (2018)[3]
Fibrin formation	Fibrinogen 1 µg/mL with thrombin 1 U/mL	Fibrinogen 100 µg/mL with thrombin 1 U/mL	Fibrinogen 10 µg/mL with thrombin 2 U/mL	Fibrinogen 200 µg/mL with thrombin 1 U/mL	Fibrinogen 200 µg/mL with thrombin 1 U/mL	Fibrinogen (concentration not specified)
Thrombin inhibitors	Not specified	10 U/mL hirudin	Hirudin (concentration not specified))	PPACK (200 µM)	5 U/mL hirudin	n. a.
Incubation time protein on surface	Overnight at 4°C	Overnight at 4°C	Overnight at 4°C	Overnight at 4°C	60 minutes RT	Overnight at 4°C
Blocking buffer	PBS-Tween 20 (0.1%) and 1% Bovine serum albumin (BSA)	PBS 3% milk	PBS 1% BSA	PBS 3% BSA	PBS 1% BSA	PBS 3% BSA
GPVI construct	Dimeric	Dimeric	Dimeric and monomeric	Dimeric and monomeric	Monomeric	Dimeric and monomeric
GPVI concentration	Up to 10 µg/ml	Up to 2000 nM	133 nM	100 nM	Not provided	100 nM
GPVI-detection	Alexa488-conjugated GPVI	Biotin-conjugated GPVI	IG5 mouse anti-pan GPVI	HRP-conjugated goat anti-human IgG-Fc (dimeric) and HRP-conjug-ated anti-His (monomeric)	1A12 mouse anti-GPVI	HRP-conjugated goat anti-human IgG-Fc (dimeric) and HRP-conjugated anti-His (monomeric)
Secondary detection	Secondary not required	HRP-conjugated streptavidin	IRDye 800 CW goat anti-mouse IgG or Alexa 647-conjugated anti-human Fc	Secondary not required	Anti-mouse HRP-conjugated antibody	Secondary not required
Incubation time of GPVI constructs	Not indicated	2 hours	Not indicated	1 hour	1 hour	1 hour

Table 2. *A summary of the observations made and questions that need to be answered regarding GPVI-ligand binding studies.*

Observations	**Open questions and proposals**
The anionic GPVI stalk is absent in some but not all monomeric and dimeric constructs.	• How does this influence the conformation of dimeric GPVI? • How does this influence binding to ligands?
The methods of fibrin generation vary between different the groups.	• How does the ratio of fibrinogen: thrombin effect the structure of fibrin? • D-dimer could be used as a standard reagent.
Monomeric and dimeric GPVI constructs differ between the different groups.	• How does the conformation of monomeric and dimeric GPVI relate to endogenous GPVI in the membrane? • Does GPVI-Fc adopt a different conformation to monomeric GPVI? • Do different GPVI-Fc dimers adopt different conformations?
Fibrin induces GPVI signaling.	• Does fibrin initiate platelet signaling by binding to monomeric GPVI? • How does binding to monomeric GPVI mediate platelet activation?
Antibodies raised against dimeric-GPVI recognize a specific GPVI conformation linked with platelet activation.[30,31]	• Do these antibodies only recognize dimeric GPVI in the membrane? • Can these antibodies also recognize specific conformations of monomeric GPVI?
Soluble D-dimer inhibits collagen/fibrin-induced platelet aggregation but does not inhibit CRP-induced platelets aggregation.[4]	• To what extent does the epitope for D-dimer/fibrin overlap with that for collagen?
The methods for the solid-binding assay differ between the different groups.	• It is necessary to develop a standardized binding assay and determine the effect of variables including time, temperature and buffer for recombinant monomeric and dimer GPVI.

Concluding remarks and where next

A summary of the observations vs current questions within this field are summarised in Table 2. It is evident that greater knowledge is required regarding the structure of GPVI-ligand complexes and the mechanisms of dimerisation and oligomerisation. Without these details, it is difficult to reconcile the conflicting observations in the literature. Solving the structures of GPVI with collagen, fibrin and fibrinogen will map the binding epitopes and provide information on the conformation of GPVI that is adopted to bind collagen, fibrin and fibrinogen, as well as determine the stoichiometry of interaction. It will also provide information relevant to the development of selective inhibitors that can be used to block the interaction with one or several of the above ligands, and in this way determine the physiological role of each interaction. In the meantime, the community needs to unite and share reagents and conditions in order to reconcile the differences described above.

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Declaration of interest

The authors have no conflicts of interest.

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Chapter 3

Flow studies on human GPVI-deficient blood under coagulating and non-coagulating conditions

Based on: M. Nagy*, G. Perrella*, A. Dalby, M.F. Becerra, L.G. Quintanilla, J.A. Pike, N.V. Morgan, E.E. Gardiner, J.W.M. Heemskerk, L. Azócar, J.F. Miquel, D. Mezzano & S.P. Watson
(*equal contribution)

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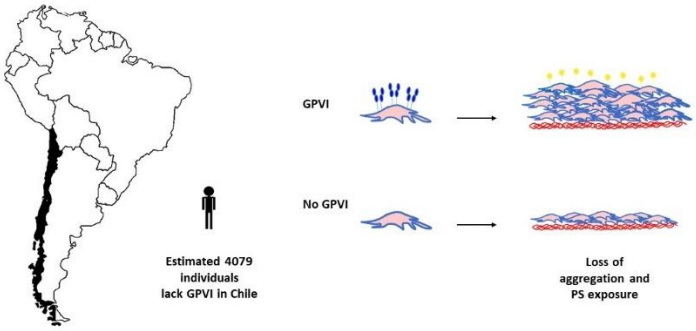
I designed and performed experiments, analyzed and interpreted data, and wrote the manuscript together with M.N.; A.D. performed Exon 6 genotyping; L.G.Q. performed the phosphorylation work; J.A.P. designed spreading analysis; N.V.M. designed the genetic analysis; E.E.G. provided essential reagents; J.W.M.H. edited the manuscript; D.M. diagnosed and recruited patients; S.P.W. supervised research and wrote the manuscript; M.F.B. organized the patient recruitment and performed the thrombin generation assay; and J.F.M. and L.A. selected the subjects representative of the Chilean population.

Visual abstract



Key Points

- GPVI regulates aggregation and PS exposure on collagen and noncollagen surfaces under flow.
- The estimated frequency of the (c.711_712insA) variant in *GP6* in Chile is 2.9%.

Abstract

The role of glycoprotein VI (GPVI) in platelets was investigated in 3 families bearing an insertion within the *GP6* gene that introduces a premature stop codon prior to the transmembrane domain, leading to expression of a truncated protein in the cytoplasm devoid of the transmembrane region. Western blotting and flow cytometry of *GP6*^{hom} (homozygous) platelets confirmed loss of the full protein. The level of the Fc receptor γ-chain, which associates with GPVI in the membrane, was partially reduced, but expression of other receptors and signaling proteins was not altered. Spreading of platelets on collagen and von Willebrand factor (which supports partial spreading) was abolished in *GP6*^{hom} platelets, and spreading on uncoated glass was reduced. Anticoagulated whole blood flowed over immobilized collagen or a mixture of von Willebrand factor, laminin, and rhodocytin (noncollagen surface) generated stable platelet aggregates that express phosphatidylserine (PS). Both responses were blocked on the 2 surfaces in *GP6*^{hom} individuals, but adhesion was not altered. Thrombin generation was partially reduced in *GP6*^{hom} blood. The frequency of the *GP6*^{het} (heterozygous) variant in a representative sample of the Chilean population (1212 donors) is 2.9%, indicating that there are ∼4000 *GP6*^{hom} individuals in Chile. These results demonstrate that GPVI supports aggregation and PS exposure under flow on collagen and noncollagen surfaces, but not adhesion. The retention of adhesion may contribute to the mild bleeding diathesis of *GP6*^{hom} patients and account for why so few of the estimated 4000 *GP6*^{hom} individuals in Chile have been identified.

Introduction

Glycoprotein VI (GPVI) is a member of the immunoglobulin receptor superfamily and a major signaling receptor in platelets for collagen, fibrin, and fibrinogen.[1] GPVI is associated with the Fc receptor (FcR) γ-chain in the membrane. Clustering of GPVI leads to Src family kinase–mediated phosphorylation of a conserved immunoreceptor tyrosine-based activation motif in the FcR γ-chain and binding of the tyrosine kinase Syk through its tandem SH2 domains. This initiates a signaling cascade that culminates in activation of phospholipase Cγ2 (PLCγ2) and Ca^{2+} mobilization, integrin αIIbβ3 activation, granule secretion, and phosphatidylserine (PS) exposure, providing a surface for thrombin generation.[2] Several unrelated patients homozygous for an adenine insertion (c.711_712insA) in exon 6 of the *GP6* gene have been identified in Chile. The insertion generates a premature stop codon prior to the transmembrane region, leading to expression of a truncated protein of ∼50 kDa that is retained in the cytosol. In 2013, Matus *et al.*[3] described the phenotype of 5 *GP6*^{hom} individuals, 3 females (aged 22, 11, and 5 years) and 2 males (aged 23 and 12 years). All of the individuals had a normal platelet count, and 4 had a mild bleeding diathesis, with the fifth, the 5-year-old girl, being asymptomatic. Symptoms started in early childhood and consisted of mild mucosal and skin bleeding. The Ivy bleeding time, recorded for 2 individuals, was 9 and 13 minutes, thus similar or above the normal level (9.5 minutes) and consistent with a mild bleeding diathesis. All hemostatic parameters and plasma von Willebrand factor (VWF) activity were within the normal range.[3] The lack of GPVI surface expression on *GP6*^{hom} platelets was shown by flow cytometry using an antibody against the extracellular region of GPVI.[3] The expression of the truncated protein in the cytosol was shown using immunofluorescence in permeabilized platelets and by western blotting. *GP6*^{hom} platelets failed to aggregate or secrete ^{14}C-serotonin in response to the GPVI agonists collagen, convulxin, and collagen related peptide

(CRP), whereas their response to arachidonic acid and adenosine 5'-diphosphate was unaltered.[3] *GP6*^{het} relatives did not exhibit signs of bleeding[3] and displayed normal aggregation and secretion upon stimulation with collagen and convulxin.[3] In 2 *GP6*^{het} individuals, the response to CRP was reduced, which is in accordance with a previously reported effect in heterozygous mouse platelets.[4] The loss of response to CRP in the *GP6*^{het} individuals is likely due to loss of avidity, whereas for collagen, this is masked by the presence of integrin α2β1. Since 2013, additional *GP6*^{hom} individuals have been identified in Chile, bringing the total to 9 from 8 unrelated families. With no consanguinity and the locations of the families being geographically disperse over several hundred kilometers, the heterozygous (carrier) frequency for c.711_712insA may be relatively high in Chile. These are the only identified individuals worldwide with a homozygous variant that prevents surface expression of GPVI and as such represent a unique resource to study the role of the immunoglobulin receptor in platelet activation.

In this study, we have measured platelet activation on collagen and noncollagen (a mixture of rhodocytin, laminin, and VWF) surfaces under static and flow conditions in 3 *GP6*^{het} and 4 *GP6*^{hom} individuals from 3 unrelated families. The results show that GPVI is critical for spreading under static conditions on a variety of surfaces and for aggregation and PS exposure, but not for adhesion on collagen and non-collagen surfaces under flow. Given the unique appearance of *GP6*^{hom} patients in the Chilean population, we have sequenced exon 6 of 1212 DNA samples representative of the Chilean population[5] and determined a carrier frequency of 2.9% for this variant.

Methods

Ethical statement

Experiments involving blood samples and DNA from Chilean patients were approved by the Ethical Scientific Committee at the Pontificia

Universidad Catolica de Chile and were conducted in accordance with the guidelines of the National Commission on Science and Technology of Chile. Informed consent was obtained according to the guidelines of the local ethics committee and complied with the ethical principles according to the Declaration of Helsinki. The experiments on mice were performed in line with UK Home Office approval (see supplemental Materials and methods).

Patients

The major patient bleeding features, including a Bleeding Assessment Tool,[6] are reported in Suppl. Table 1.

Reagents and samples

The list of reagents including the control and GPVI-null mouse platelets are reported in the supplemental Materials and methods.

Blood withdrawal and platelet preparation

Blood was taken with 3.2% sodium citrate (ratio 1:9) as the anticoagulant. Blood was taken from 3 families with a total of 4 *GP6*^{hom} and 3 *GP6*^{het} individuals. In 2 individuals, blood was drawn on 2 occasions separated by 9 months and considered as an additional n for statistical analysis. All family members had been genotyped for a predicted adenine insertion between positions 711 and 712 of exon 6. Blood was taken from 4 healthy, unrelated individuals on the same experimental days. Washed platelets were separated from plasma by centrifugation, resuspended in a modified Tyrode's buffer, and recentrifuged before final suspension (see the supplemental Materials and methods).

Western blotting

Platelets were mixed with sodium dodecyl sulfate reducing sample buffer and proteins separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes.

The antibodies and concentrations are described in the supplemental Materials and methods.

Platelet spreading

Glass coverslips were coated with collagen (10 μg/mL) and VWF (100 μg/mL) overnight at 4°C and blocked with 1% bovine serum albumin in phosphate-buffered saline (PBS) for 60 minutes at room temperature before washing with PBS. Washed platelets were spread on coated and uncoated coverslips for 45 minutes at 37°C. The coverslips were washed in PBS, and adherent platelets were fixed in 10% formalin, permeabilized with 0.1% Triton X-100 (in PBS), and stained with Alexa Fluor 488-Phalloidin. Platelets were imaged on a Zeiss LSM7 microscope (63x objective). Platelet measurements including area were calculated using a semiautomated machine learning–based workflow[7] and implemented using the open-source software KNIME[8] and ilastik.[9] First, a pixel classifier was trained within ilastik to produce binary segmentations. The classifier was then run on the full data set within KNIME and touching platelets were identified manually by clicking on their center. A watershed transformation was then used to extract cellular boundaries facilitating the calculation of per-platelet measurements including area and circularity. Any objects <1 μm^2 were discarded. The measurements for each platelet were then used to train a random forest classifier to automatically group platelets into predefined subtypes, specifically nonspread, partially spread, and fully spread.

Flow studies

Thrombus formation was assessed in whole blood.[10] Glass coverslips were coated with collagen (50 μg/mL) or a combination of human VWF (50 μg/mL), laminin (100 μg/mL), and rhodocytin (250 μg/mL) before blocking with 1% bovine serum albumin in N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid buffer (pH 7.45). Citrated whole blood, recalcified with $CaCl_2$ (7.5 mM) and $MgCl_2$ (3.75 mM), in the presence of

PPACK was perfused for 4 minutes at 1000 s^{-1} and then labeled by perfusion with AF647-annexin-A5 (1:200) in a modified N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer (pH 7.45) supplemented with $CaCl_2$ (2 mM) and heparin (1 U/mL) for 1.5 minutes. Phase-contrast and fluorescence images were taken for analysis of surface area coverage of adherent platelets and PS exposure. For studies under coagulating conditions,[11] citrated whole blood without PPACK was perfused over slides coated with collagen (50 µg/mL) and recombinant tissue factor (500 pM), and simultaneously, PS exposure was labeled with AF647-annexin-A5. Image analysis was performed by using predefined scripts[12,13] in the open-source software Fiji,[14] and contraction was assessed by visual inspection compared with representative images.[12]

Thrombin generation

Thrombin generation in platelet-rich plasma (PRP) was assessed by continuous assessment of thrombin generation using a calibrated automated thrombogram as described.[15] In brief, the PRP platelet count was adjusted to 150×10^9/L with autologous platelet-poor plasma. PRP (80 µL) was supplemented with tissue factor (20 µL) and fluorogenic substrate plus $CaCl_2$ (20 µL) to trigger thrombin generation. The resultant curves were analyzed, and results are expressed as endogenous thrombin potential (ETP).

Exon 6 genotyping

Genomic DNA was extracted from the whole blood of 1235 individuals, representative of the Chilean population as previously described.[5] PCR amplification was performed across the *GP6* exon 6 variant region using the following primers: 3'-CTCAAAAGGGGAATGGAGATA-5' and 5'-AAG AGAGAGCTCCGTCCTCAC-3' (as used in Matus et al[3]). DNA sequencing was performed using the BigDye Terminator sequencing kit v3.1 and run on the 3730 DNA analyzer. Sequencing results were analyzed using SnapGene Software.

Statistics

Data are represented as means ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism v8 software (San Diego, CA). Significance was determined using unpaired Student *t* test or a 1-way ANOVA with Tukey posttest, as appropriate. Differences with $P < 0.05$ were considered significant.

Results

GP6^{hom} donors have a reduced level of the FcR γ-chain

The presence of a premature stop in *GP6* results in the formation of a truncated protein of ~50 kDa, which lacks the transmembrane and is retained in the cytosol.[3] Consistent with this, the expression of full-length GPVI was reduced and abolished in *GP6*^{het} and *GP6*^{hom} individuals, respectively, as shown by western blotting with an antibody against the cytoplasmic tail of GPVI (Figure 1A). Flow cytometry revealed that surface expression of GPVI was reduced and abolished in *GP6*^{het} and *GP6*^{hom} individuals, respectively (not shown), as previously described.[3] The level of the FcR γ-chain was reduced by 40% to 65% relative to controls in 3 *GP6*^{hom} individuals (Figure 1B). The stimulation of whole tyrosine phosphorylation, including Syk (525/526), LAT (200), and PLCγ2 (1217), by collagen was abolished in *GP6*^{hom} platelets (Figure 1C). The levels of various membrane proteins (GPIbα, GPIIb, and CLEC-2), signaling proteins (Syk, LAT, Btk, RhoA, and PLCγ2), and α-tubulin were similar among control, *GP6*^{het}, and *GP6*^{hom} individuals (Figure 1A-E).

These results confirm the previous reports of reduction and loss of full-length GPVI in *GP6*^{het} and *GP6*^{hom} platelets and demonstrate that the level of the FcR γ-chain is also reduced. This decrease was not anticipated, as the level has been reported to be unchanged in *GP6*^{hom} mouse platelets.[16] However, on repeating these studies, we observed a small reduction (~15%) in *GP6*^{hom} mice platelets, which may be due to increased

degradation (Suppl. Figure 1). It is unclear to what extent the decrease in the FcR γ-chain contributes to the clinical phenotype of the patients.

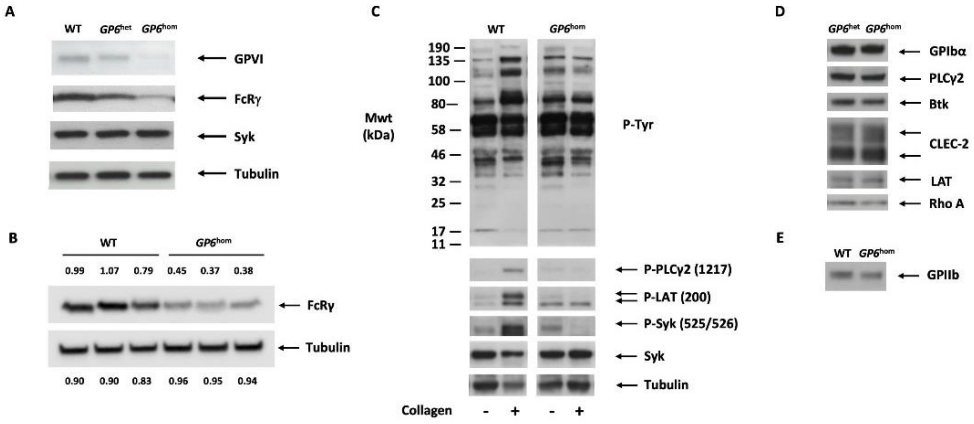


***Figure 1. Protein expression and tyrosine phosphorylation in control and GP6^{hom} platelets. A**, Western blot showing levels of GPVI, FcRγ, Syk, and tubulin (loading control) in control, GP6^{het}, and GP6^{hom} individuals. **B**, Densitometry reading of the level of FcRγ and tubulin (loading control) in a GP6^{hom} individual relative to a control (the GP6^{hom} individual is not related to the donor in panel A). **C**, Collagen (30 µg/mL) stimulation of tyrosine phosphorylation in a control and GP6^{hom} individual (90-second stimulation). **D**, Western blot of representative proteins from GP6^{het} and GP6^{hom} donors. **E**, Western blot of GPIIb from control and GP6^{hom} donors. Results are representative of 3 (**A-C**) or 4 (**D-E**) unrelated GP6^{hom} individuals. Mwt, molecular weight; WT, wild type.*

Spreading on collagen, VWF, and glass

Adhesion and spreading were investigated under static conditions on surfaces coated with collagen, VWF, and glass (uncoated), with the latter serving as a representative charged surface. The majority of platelets from controls generated filopodia and lamellipodia on collagen and on glass (Figure 2A-B). The small number of partially and nonspread platelets on

both surfaces is likely to represent cells that have recently adhered (Figure 2A). In contrast, only one-third of platelets underwent partial spreading on VWF (Figure 2A). Spreading of *GP6*^{hom} platelets on collagen and VWF was abolished and markedly reduced on uncoated glass, whereas adhesion was not altered on all 3 surfaces (Figure 2A-B), indicating involvement of additional receptors, such as integrin α2β1 for collagen and GPIb-IX-V and integrin αIIbβ3 for VWF. Spreading, but not adhesion, was reduced in *GP6*^{het} platelets on all 3 surfaces, although for collagen, this was less marked than for *GP6*^{hom} platelets (Figure 2B). These results demonstrate that GPVI is essential for spreading, but not adhesion, of platelets on collagen, VWF, and a charged surface.

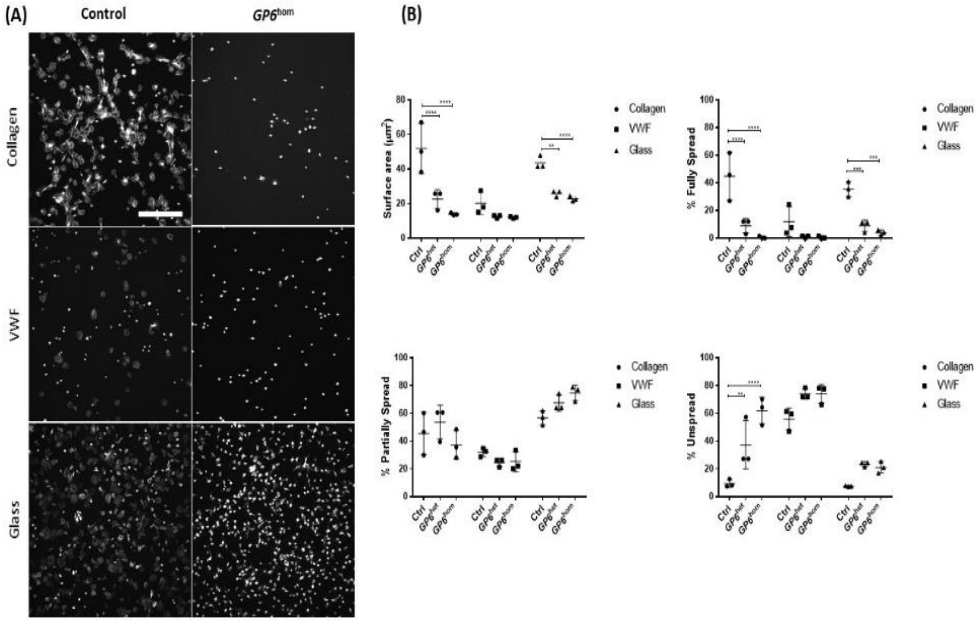


Figure 2. Platelet spreading on collagen, VWF, and glass. A*, Human platelets (2 × 10*^{7}*/mL) were spread on coated and uncoated (not blocked) coverslips as described in "Methods". The images are representative of 3 control and 3 GP6*^{hom} *patients.* ***B****, The graphs illustrate quantification of surface area and the percentage of fully, partially spread, or nonspread platelets (control = 3, GP6*^{het} *=*

*2, GP6^{hom} = 3). Measurements are the mean of 48 882 platelets in a replicate (7 fields of view per replicate), and the figures are representative of part of a field of view. Significance was measured using 2-way ANOVA. **P < 0.01, ***P < 0.001; ****P < 0.0001. The surface area in the field of view is 213 × 213 µm. Scale bar, 50 µm.*

Platelet aggregation under noncoagulating flow conditions

Experiments were undertaken to evaluate the role of GPVI in adhesion and aggregation of noncoagulated blood under arterial shear. Coagulation was inhibited by citrate and PPACK. Whole blood was flowed over collagen and noncollagen (mixture of VWF, laminin, and rhodocytin) surfaces at a shear rate of 1000 s^{-1}, and platelet adhesion and aggregation were analyzed along with contraction score. The contraction score was determined based on a predefined set of example images and depicts the tightness of the aggregate.[10,17] Blood from *GP6*^{het} donors form robust, large aggregates on collagen with a similar surface area coverage and contraction score to that of controls, although PS exposure is reduced by ∼50% (Figure 3A). In contrast, only single platelets adhere and small aggregates form on collagen in *GP6*^{hom} blood (Figure 3A). This is accompanied by a reduction in abrogation of PS exposure, although adhesion is not altered (Figure 3A). This demonstrates a critical role of GPVI in aggregation and PS exposure, but not in adhesion, on a collagen surface. A similar degree of surface coverage to that on collagen was observed for whole blood on a mixture of VWF, laminin, and rhodocytin (Figure 3B). However, in contrast to the collagen surface, coverage consisted of smaller aggregates and single platelets, resulting in a lower contraction score. The proportion of PS-positive platelets was ∼50% of that observed on collagen (Figure 3B). The surface coverage of blood from *GP6*^{het} and *GP6*^{hom} donors was similar to that of controls on the noncollagen surface, although the number of single platelets was increased, notably in *GP6*^{hom} blood (Figure 3B). The exposure of PS was

abrogated on both surfaces (Figure 3B). Thus, GPVI is also important for aggregation and PS exposure, but not adhesion, on a noncollagen surface.

To investigate whether the adhesion of GP6hom platelets on collagen is through the second platelet receptor for collagen, integrin α2β1, experiments were performed using the blocking monoclonal antibody (mAb) 6F1.[18] mAb 6F1 abrogated adhesion of *GP6*^{hom} blood on collagen (Figure 3Ci), but also partially reduced adhesion on the noncollagen surface (Figure 3Cii). Since there are no reports of binding of VWF, laminin, or rhodocytin to integrin α2β1, this may be due to steric hindrance.

These results demonstrate a critical role for GPVI in supporting platelet aggregation and PS exposure in the absence of coagulation on collagen and noncollagen surfaces, with the adhesion of *GP6*^{hom} platelets on collagen being mediated by integrin α2β1.

Platelet aggregation under coagulating flow conditions

Additional flow studies were performed at the same arterial shear rate (1000 s^{-1}) in the presence of recombinant tissue factor and Ca^{2+} (without PPACK) to determine the role of GPVI in the presence of thrombin and fibrin. Under these conditions, fibrin strands were seen to develop in control blood from platelet aggregates on collagen (Figure 4), suggesting that they are catalyzed by platelet-dependent coagulation, as described previously.[11] In contrast, there was no detectable fibrin formation on the noncollagen surface (not shown), and further studies were not performed on this surface. Large platelet aggregates/thrombi are formed when control blood is flowed over collagen under coagulating conditions (Figure PS exposure is also observed and was similar in magnitude to that on collagen in the absence of coagulation (compare Figures 3A and and 4). A similar degree of thrombus formation was observed with *GP6*^{het} and *GP6*^{hom} blood, but fibrin formation was reduced and abolished, respectively (Figure 4).

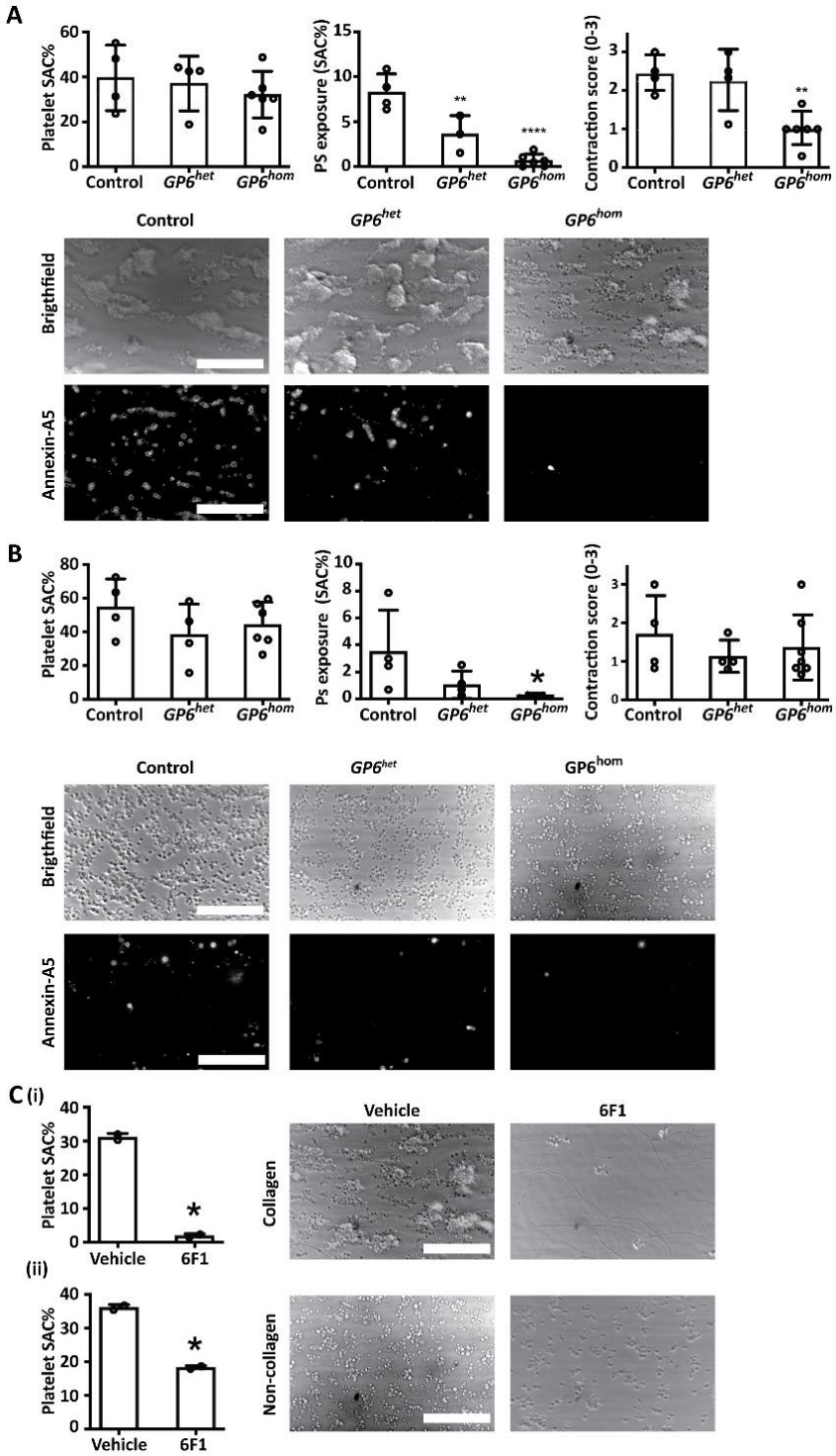

A
Platelet SAC%
PS exposure (SAC%)
Contraction score (0-3)
Control
GP6het
GP6hom
**
****
Brigthfield
Annexin-A5
B
Ps exposure (SAC%)
GP6hom
*
C (i)
Vehicle
6F1
Collagen
(ii)
Non-collagen

Figure 3. GPVI deficiency leads to abolished PS exposure under flow in the absence of coagulation. *Whole blood from control, GP6^{hom}, and GP6^{het} subjects was recalcified in presence of PPACK and perfused over collagen (**A**) and noncollagen (**B**) (laminin, VWF, and rhodocytin) surfaces. Surface area coverage (SAC), PS exposure, and contraction score are presented as mean ± SD; control = 4, GP6^{het} = 4, and GP6^{hom} = 6. ****P <0.0001, **P < 0.01, *P < 0.05. Representative bright-field and Alexa Fluor 647-annexin A5 images are shown. Images were taken at the end point (8 min) after labeling was performed. **C**, Whole blood from GP6^{hom} patients treated with the monoclonal antibody 6F1 (10 µg/mL) was recalcified and perfused over collagen (**i**) and noncollagen (**ii**) surfaces. Quantification of surface area coverage is presented as mean ± SD. GP6^{hom}= 2. Representative bright-field images are shown. Scale bars, 50 µm.*

Exposure of PS was not altered in *GP6*^{het} blood but was abrogated in *GP6*^{hom} blood (Figure 4). These results demonstrate a critical role for GPVI in supporting PS exposure and fibrin formation under coagulating conditions, but not in thrombus formation on collagen at arterial shear.

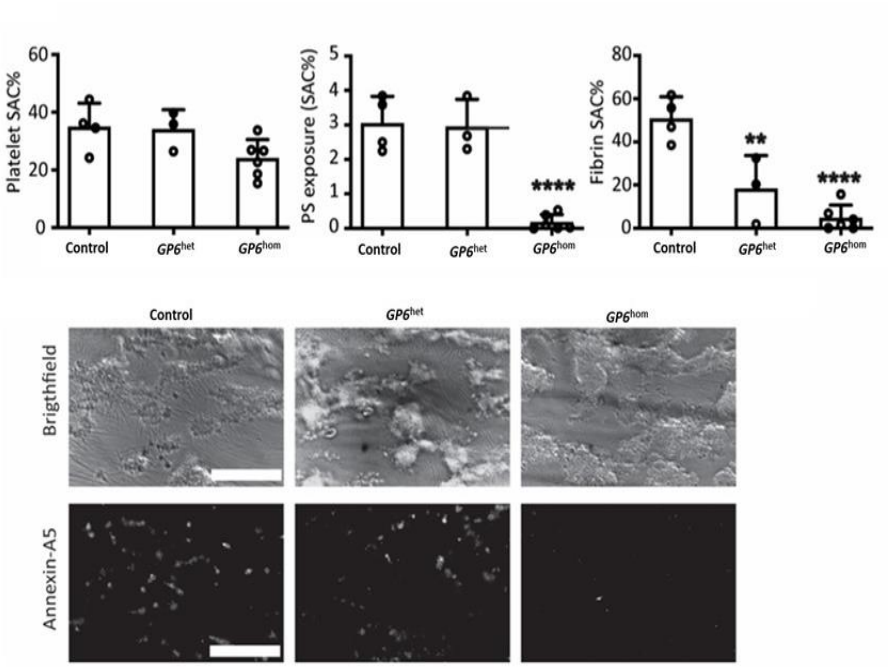


Figure 4. GPVI deficiency leads to abolished PS exposure under flow. *Whole blood from control, GP6^{hom}, and GP6^{het} individuals was recalcified and perfused over a collagen surface cocoated with tissue factor. Quantification of surface area coverage (SAC), PS exposure, and fibrin surface area coverage is shown. Data are shown as mean ± SD; control = 4, GP6^{het} = 3, and GP6^{hom} = 6. ****P < 0.0001, **P < 0.01. Representative bright-field and Alexa Fluor 647-annexin A5 images taken after 6 minutes of blood perfusion are shown from a control and a GP6^{hom} subject on a collagen surface. Scale bars, 50 µm.*

Thrombin generation is reduced in GP6-deficient platelets

Using the calibrated automated thrombogram (also known as the CAT assay), we observed a trend toward a reduction in thrombin generation in PRP isolated from *GP6*^{hom} individuals, depicted as a reduced peak height and ETP (Figure 5A-C). However, this trend was not seen in other parameters (eg, time to peak), and the ETP was not significantly different, suggesting that there is only a slight decrease in the total amount of thrombin formed in *GP6*^{hom} platelets. This partially resembles the results using GPVI-deficient platelets (3 patients with immune thrombocytopenia and 1 compound heterozygote with 2 GPVI variants) and healthy PRP pretreated with GPVI-blocking Fab 9O12. Mammadova-Bach *et al.*[19] reported a significant decrease in peak height in GPVI-deficient patients, while our results only show a trend toward a decrease. This difference is likely to due to the partial nature of the decrease and the low number of patient samples and the variation in response between donors. A similar level of reduction in thrombin generation was seen in PRP from *GP6*^{het} relative to *GP6*^{hom} individuals.

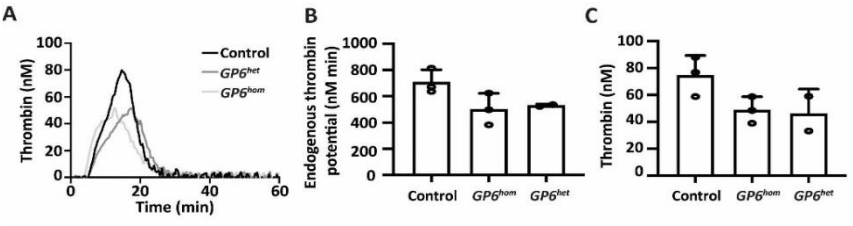


Figure 5. GPVI deficiency leads to a partial reduction in thrombin generation. *Thrombin generation assay was performed in platelets from control, GP6^{het}, and GP6^{hom} patients.* ***A****, Thrombin generation curves from a control, GP6^{het}, and GP6^{hom} individual are shown.* ***B****, ETP is shown.* ***C****, Peak height is shown. Data are resented as mean ± SD; control = 3, GP6^{het} = 2, and GP6^{hom} = 3.*

Frequency of the GP6 c.711_712insA variant in the Chilean population

To investigate the frequency of the c.711_712insA *GP6* variant in the Chilean population, sequencing of *GP6* exon 6 was performed on 1235 DNA samples. Samples represent mixed Chilean Latinos with Mapuche Native American ancestry, as described previously.[5] Of these, 23 samples gave sequencing traces that were of too poor quality to analyze and were excluded. Of the remaining 1212 samples, a total of 36 were found to be heterozygous for the c.711_712insA variant (Suppl. Figure 2), equivalent to 2.9% of the total analyzed. No *GP6*^{hom} samples were identified.

Discussion

This study demonstrates (1) a critical role for GPVI in aggregation and PS exposure, but not adhesion, in human platelets flowed at arteriolar shear over collagen and noncollagen surfaces under noncoagulating conditions; and (2) a critical role for GPVI in PS exposure, but not thrombus formation, on collagen under coagulating conditions. In addition, this study shows that spreading of platelets on collagen, VWF, and uncoated glass, as well as thrombin formation, is reduced or abolished in *GP6*^{hom} platelets. Together, these results show that the role of GPVI in supporting aggregation at arteriolar shear extends to noncollagen surfaces and that GPVI is critical for PS exposure and supporting thrombin formation.

GPVI has long been recognized as a collagen receptor, but more recently, it has been shown to serve as a receptor for further ligands in the vasculature and vessel wall, including fibrinogen, fibrin, fibronectin, vitronectin, and laminin.[1,19-21] In addition, GPVI has been shown to associate with GPIbα in the membrane[22] and support thrombus formation

and platelet adhesion to immobilized VWF at arterial and venous shear.[23] VWF stimulates tyrosine phosphorylation of the FcR γ-chain,[24,25] and treatment with an anti-GPVI antibody reduces FcR γ-chain and Syk phosphorylation upon ristocetin stimulation.[23] One or more of these interactions could explain the reduction in spreading on VWF and platelet aggregation and PS exposure on a mixture of VWF, laminin, and rhodocytin. Alternatively, the reduction in these responses could be due to the ~50% reduction in the FcR γ-chain that is seen in *GP6*^{hom} platelets, and this could be tested in transgenic mice that are heterozygous for the FcR γ-chain. Aggregation to botracetin/VWF is abolished in mouse platelets deficient in FcR γ-chain-deficient but not GPVI-deficient mouse platelets.[26]

The present study also reports that spreading of *GP6*^{hom} platelets is reduced on uncoated glass, suggesting that the GPVI is activated by a charge interaction. Previously, we have shown that GPVI can be activated by a diverse group of charged ligands, including diesel exhaust particles, polysulfated sugars such as fucoidan and dextran sulfate, and histones.[20] These ligands are structurally distinct and have no resemblance to endogenous ligands, consistent with crosslinking of GPVI and platelet activation being mediated by a charge interaction. The role of GPVI in spreading on glass raises the possibility that other endogenous ligands may activate GPVI through a charge interaction, including ligands from the platelet releasate, and this could potentially also contribute to the reduction in aggregation and PS exposure.

The role of GPVI in supporting platelet aggregation on collagen in the absence of coagulation is well documented.[27,28] However, it is only recently that conditions have been developed that permit the study of platelet aggregation on collagen in the absence of thrombin inhibition.[11] The observation therefore that GPVI is critical for platelet aggregation and PS exposure under both coagulating and noncoagulating conditions highlights the critical role of the glycoprotein receptor in thrombus

formation. The retention of adhesion and formation of small aggregates on the 2 surfaces is explained by the presence of a second receptor for collagen, integrin α2β1, and by receptors for rhodocytin (CLEC-2), laminin (α6β1), and VWF (GPIb-IX-V). The observation that adhesion of *GP6*^{hom} platelets is retained on collagen is consistent with previous reports of integrin α2β1 supporting platelet adhesion to collagen in human platelets under flow.[18,29-33] The retention of adhesion to collagen by integrin α2β1, in combination with the vascular wall damage that leads to the exposure of negatively-charged phospholipid on the cell surface thus leading to tissue factor driven thrombin formation, could account for the relatively minor bleeding phenotype of *GP6*^{hom} individuals. The role of integrin α2β1 in supporting adhesion of human platelets in the absence of GPVI is in contrast to mice, in which adhesion to collagen is abolished in the absence of GPVI.[34] This difference could be due to the affinity of nonactivated integrin α2β1 for collagen, the relative density of GPVI and α2β1 on the platelet surface, or the difference in size of mouse and human platelets.

The present results demonstrate that the degree of platelet PS exposure is governed by the level of GPVI expression, as a marked defect was seen in *GP6*^{het} and *GP6*^{hom} individuals under coagulating and noncoagulating conditions. The reduction in platelet procoagulant activity was associated with a slight decrease in thrombin formation. This is in agreement with the results of Mammadova-Bach *et al.* using GPVI-deficient platelets and a GPVI-blocking Fab.[19] GPVI signals synergize with G_q family G protein–coupled receptors to induce PS exposure and a procoagulant surface.[2,35,36]

The potential significance of the present findings should be considered in light of the frequency of the *GP6* c.711_712insA in the Chilean population. The prevalence of the variant in the Chilean cohort was estimated with a carrier status probability of 0.0297 (36/1212). If we consider this observation, and that the allele frequency for the c.711_712insA variant is in Hardy-Weinberg equilibrium, then the theoretical prevalence of *GP6*^{hom} in Chile could be 1/4534 (i.e., the product of 0.0297 × 0.0297 with

division by 4) or greater. This result, combined with population data from the last national census, suggests that 4079 individuals with *GP6*^{hom} could be currently living in Chile, which is more than double the number of registered hemophilia patients. With such a high predicted prevalence, the question remains as to why so few patients have been identified. This may due to the limited genetic testing that has been performed in patients with a bleeding disorder and the relatively mild bleeding diathesis caused by loss of GPVI. A bleeding assessment tool (BAT) in *GP6*^{hom} individuals is shown in supplemental Table 1 and includes an asymptomatic girl (age 12 years) who is the sister of one of the index cases. This suggests that some homozygous carriers of *GP6* c.711_712insA do not present with bleeding issues. The majority of *GP6*^{het} individuals do not have signs of excessive bleeding. This further underscores the interest in targeting GPVI as an antiplatelet therapy.

An important consideration is whether *GP6*^{hom} individuals are protected from thrombosis. As yet, the number and age of the *GP6*^{hom} individuals is too few and too low to ascertain whether this is the case. With the relatively high carrier frequency (2.9%), there is a chance that the heterozygous variant is being selected for in the Chilean population. This is unlikely to have conferred a selection advantage in the context of atherosclerosis, as this is a relatively modern disease and relatively late in onset. Whether this has conferred an advantage in other thrombotic-related or other conditions is not known. Larger, longer-term screening of *GP6*^{het} and *GP6*^{hom} individuals and their cardiovascular clinical manifestations will help to inform their propensity for bleeding and thrombosis.

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Conflict of interest disclosure: J.W.M.H. is a cofounder and shareholder of FlowChamber. The remaining authors declare no competing financial interests.

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Supplementary materials

Reagents

D-Phe-Pro-Arg chloromethyl ketone (PPACK), anti-Syk (sc-1240) monoclonal antibody (mAb), anti-PLCγ2 mAb (sc-5283), anti-GPIbα mAb (sc-59052) and anti-Rho A mAb (sc-418) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Collagen type I was from Nycomed Pharma (Munich, Germany). D-dimer was from HYPHEN BioMed (Neuville-sur-Oise, France). Von Willebrand factor (VWF), anti-P-LAT (Y200) (ab68139) mAb and anti-Btk antibody (ab189434) came from Abcam (Cambridge, UK). Rhodocytin was purified as described.[1] Recombinant tissue factor (rTF; Innovin) was purchased from Siemens (Erlanger, Germany). The antibody to the GPVI tail has been described.[2] Annexin-A5-Alexa Fluor-647 was from Invitrogen (Bleiswijk, The Netherlands). Other reagents including bovine serum albumin (BSA: fatty acid free, ≥96%), formalin, anti-α-tubulin mouse mAb (T6199), anti-FcRγ antibody (06-727), anti-P-Tyrosine mAb (4G10) (05-321) and anti-LAT antibody (06-807) were by Sigma-Aldrich (St. Louis, MO, USA). Anti-P-Syk (Y525/526) mAb (2710), anti-P-PLCγ2 (Y1217) antibody (3871) and GPIIb mAb (13807) were from Cell Signaling (Massachusetts, USA). Anti-CLEC-2 antibody (AF1718) were from R&D Systems (Minneapolis, USA). Z-Gly-Gly-Arg AMC.HCl substrate was from Bachem (Torrance, CA, USA). BigDye Terminator sequencing kit v3.1 and NuPAGETM 4-12% Bis-Tris gel were purchased from Thermo Fisher Scientific (Massachusetts, USA). mAb 6F1 was a kind gift from Barry Coller (Rockefeller, USA).

Mice

Experiments were performed in accordance with UK laws (Animal [Scientific Procedures] Act 1986) with approval of local ethics committee and UK Home Office approval under PPL P0E98D513. The following mice were used: the ITAM-deficient strains. The GPVI-null mice were the kind

gift of Jerry Ware[3] and were bred as homozygous mice with wild type mice purchased. All mice were on a C57Bl/6 background.

Blood and platelet preparation

Washed platelets were obtained by centrifugation using prostacyclin (56 nM) and resuspended in Tyrode (137 mM NaCl, 0.36 mM Na_2HPO_4, 2.68 mM KCl, 11.9 mM $NaHCO_3$, 0.05 g/L glucose, 2 mM $MgCl_2$; pH6.2). They were pelleted by centrifugation at 1500 g for 9 minutes and resuspended at $2x10^7$/mL for static adhesion or $4x10^8$/mL for aggregation and phosphorylation measurements.

Western blotting

Immunoblotting was performed with rabbit anti-human GPVI cytoplasmic tail antibody (1 µg/mL), anti-FcRγ (1:500), anti-Syk (1:200), anti-α-tubulin (1:1000), anti-phosphotyrosine (1:1000), anti-P-PLCγ2 (Y1217) (1:250), anti-P-LAT (Y200) (1:500), anti-P-Syk (Y525/526) (1:500), anti-GPIbα (1:500), anti-PLCγ2 (1:200), anti-Btk (1:500), anti-CLEC-2 (1:500), anti-LAT (1:500), anti-Rho A (1:200) and anti-GPIIb (1:500) antibodies overnight at 4°C.

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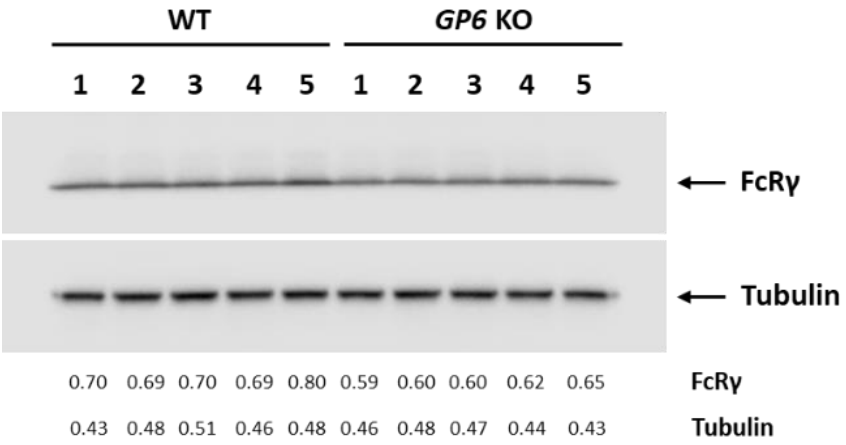
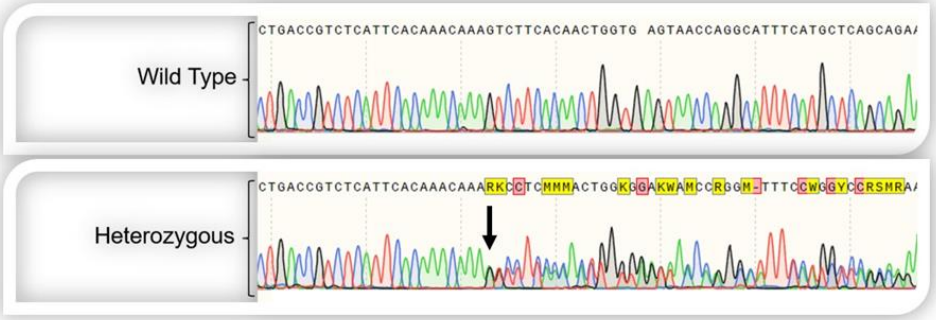
Supplementary figures



Suppl. Figure 1. *Western blot of the FcR γ-chain (FcRγ) in five wild type and five GP6 KO mouse platelets. The densitometry levels are shown. The same amount of platelet protein was loaded in each lane as shown by reprobing for tubulin.*



Suppl. Figure 2. *Representative gene sequence traces of a wild type sample and GP6^{het} sample. Overlapping sequences represent two separate alleles, starting at the insert site of the c.711_712insA mutation (arrowed).*

Suppl. Table 1. Bleeding Assessment Tool (BAT) score of GPVI homozygous and heterozygous individuals. *A bleeding assessment tool (BAT) developed by one of the authors[1] was used to generate a score for GP6^{hom} and GP6^{het} individuals. The asterix illustrates the individuals who donated blood in this study.*

	BAT score	Bleeding symptoms
GP6^{hom}		
Female, age 33*	19	Bleeding after tooth extraction and surgery, heavy menstrual bleeding, abnormal bruising, superficial hematomas, hemoptysis (once).
Female, age 9*	10	Abnormal bruising.
Female, age 22	18	Heavy menstrual bleeding leading to acute anaemia, required multiple blood transfusions, abnormal bruising, epitaxis, excessive bleeding after minor injury.
Male, age 20*	6.5	Ecchymosis in unusual sites, prolonged bleeding after mild injuries, superficial hematomas.
Female, age 12*	1	Easy bruising; no surgery or menarche.
Female, age 22	6	Heavy menstrual bleeding, easy bruising, epistaxis.
Female, age 39	11	Heavy bruising, heavy menstrual bleeding, bleeding after minor injuries; no bleeding during cesarean section.
Male, age 10*	8	Epitaxis (3 cautaries), bruising in unusual sites, prolonged bleeding after mild injuries.
GP6^{het}		
Female, age 66	0	
Female, age 41	0	
Female, age 35*	5.5	Bleeding after totth extraction, ecchymosis, bleeding after delivery.
Male, age 41*	1.5	Bleeding after totth extraction.
Female, age 41*	0	
Male, age 13	0	
Female, age 22	2	Easy brusing.

Reference

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Chapter 4

Non-redundant roles of platelet glycoprotein VI and integrin αIIbβ3 in fibrin-mediated microthrombus formation

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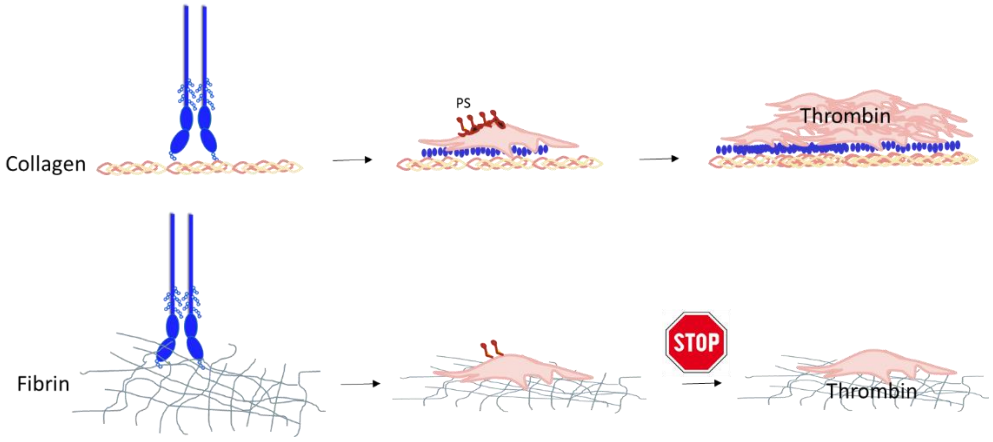
I designed and performed experiments, analyzed and interpreted data, and wrote the article; J. Huang performed experiments and co-wrote the article; I.P. and F.S edited the article; R.A.S.A. and M.J-P. provided reagents and edited the article; S.P.W. edited the article. J.W.M.H. designed experiments, supervised research, interpreted data, and wrote the article.

Visual abstract



Highlights

- Platelet adhesion to a fibrin layer under flow elicits only moderate glycoprotein VI activation.
- The interaction with glycoprotein GPVI (glycoprotein VI) interaction relies on αIIbβ3 and forms small, bilayered thrombi.
- This consolidated thrombus formation is restricted by thrombin binding to fibrin.

Abstract

Objective: Fibrin is considered to strengthen thrombus formation via integrin αIIbβ3, but recent findings indicate that fibrin can also act as ligand for platelet glycoprotein VI.

Approach and Results: To investigate the thrombus-forming potential of fibrin and the roles of platelet receptors herein, we generated a range of immobilized fibrin surfaces, some of which were cross-linked with factor XIIIa and contained VWF-BP (von Willebrand factor-binding peptide). Multicolor microfluidics assays with whole-blood flowed at high shear rate (1000 s^{-1}) indicated that the fibrin surfaces, regardless of the presence of factor XIIIa or VWF-BP, supported platelet adhesion and activation (P-selectin expression), but only microthrombi were formed consisting of bilayers of platelets. Fibrinogen surfaces produced similar microthrombi. Markedly, tiggering of coagulation with tissue factor or blocking of thrombin no more than moderately affected the fibrin-induced microthrombus formation. Absence of αIIbβ3 in Glanzmann thrombasthenia annulled platelet adhesion. Blocking of glycoprotein VI with Fab 9O12 substantially, but incompletely reduced platelet secretion, Ca^{2+} signaling and aggregation, while inhibition of Syk further reduced these responses. In platelet suspension, glycoprotein VI blockage or Syk inhibition prevented fibrin-induced platelet aggregation. Microthrombi on fibrin surfaces triggered only minimal thrombin generation, in spite of thrombin binding to the fibrin fibers.

Conclusions: Together, these results indicate that fibrin fibers, regardless of their way of formation, act as a consolidating surface in microthrombus formation via nonredundant roles of platelet glycoprotein VI and integrin αIIbβ3 through signaling via Syk and low-level cytosolic Ca^{2+} rises.

Introduction

GP (glycoprotein) VI is a platelet immunoglobulin (Ig) receptor, expressed at 3000 to 4000 copies per platelet, and known to be involved in the onset of thrombus formation.[1,2] Common concept is that GPVI mediates the initial activation of platelets in contact with exposed collagen in the vasculature, assisted by platelet integrins and by GPIb-V–IX which interacts with collagen-bound VWF (von Willebrand factor).[3–5] In the platelet membrane, GPVI is constitutively associated with the Fc receptor γ-chain, containing an intracellular immunoreceptor tyrosine-based activation motif. Ligand binding induces clustering of GPVI and ensuing phosphorylation of the immunoreceptor tyrosine-based activation motif via Src-family kinases.[6] This leads to activation of the tyrosine kinase Syk through its tandem SH2 (Src homology2) domain, culminating in activated phospholipase Cγ2 and ensuing Ca^{2+} mobilization.[2,6] Since 2015, it has been recognized that GPVI can also act as a receptor for fibrin and fibrinogen.[7–9] Relevance of this finding comes from the earlier observation that fibrin formation can be both an initial and propagating process in vaso-occlusive thrombus formation upon vascular damage.[10–12] In this setting, the role of GPVI as a functional receptor for fibrin implies a crucial contribution of this receptor interaction in thrombus growth and in the propagation of coagulation. This idea is supported by the observations that (1) GPVI binding to fibrin can trigger platelet procoagulant activity and ensuing thrombin generation[8] and (2) fibrin binds to procoagulant platelets via the cross-linking transglutaminase FXIIIa (factor XIIIa).[13] However, some authors have questioned the role of GPVI as a fibrin receptor in blood.[14]

Integrin αIIbβ3 is known as the conventional platelet receptor for fibrinogen and fibrin, being expressed at 50,000 to 80,000 copies per platelet.[15–17] Similarly to GPVI, integrin αIIbβ3 promotes platelet adhesion and activation via outside-in signaling through Src-family and Syk protein tyrosine kinases.[6,18] Questions then arising are (1) what are the roles of

GPVI and αIIbβ3 in fibrin-dependent platelet activation; (2) how do these receptor interactions contribute to thrombus formation; (3) how can they prevent endless growth of the platelet-fibrin thrombus; and (4) which is the role of blood flow and shear in this process.

In the present article, we aimed to answer these questions and resolving the dispute on the role of GPVI. We studied the relative contribution of GPVI and integrin αIIbβ3 in fibrin-dependent platelet activation and thrombus formation under defined flow conditions. Since fibrin is known to bind VWF,[19] we also explored the contribution herein of the VWF receptor, GPIb-V–IX. Our results show that GPVI provides a weakly activating signal that relies on αIIbβ3-dependent platelet adhesion and Syk activation to form small-sized thrombi. Markedly, our data also indicate that the platelet-activating role of thrombin is dampened on fibrin surfaces.

Materials and methods

The authors declare that materials and data are available upon reasonable request from the authors. An extended version of Materials and Methods is available in the supplement.

Major resources

Please see the Major Resources Table in the supplement.

Blood withdrawal and platelet preparation

Blood was obtained by venepuncture from healthy volunteers (male and female), who had not received antiplatelet or anticoagulant medication for at least 2 weeks. Informed consent was obtained according in compliance with the ethical principles of the Declaration of Helsinki, and studies were approved by the local Medical Ethics Committee (METC 10-30-023, Maastricht University). Blood samples were collected into 3.2% trisodium citrate (Vacuette tubes, Greiner Bio-One, Alphen a/d Rijn, the Netherlands). Blood samples were also obtained from 2 patients with diagnosed Glanzmann thrombasthenia, that is, one homozygous patient

lacking expression of integrin αIIbβ3 on platelets, and one heterozygous patient with 50% of normal αIIbβ3 expression. PRP (platelet-rich plasma) was obtained from the citrated blood by centrifugation at 200g for 15 minutes.[20] After addition of 1:10 vol/vol acid-citrate-dextrose (80 mM trisodium citrate, 183 mM glucose, 52 mM citric acid), the PRP was centrifuged at 2360g for 2 minutes. 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% BSA). After addition of apyrase (1 U/mL) and 1:15 vol/vol acid-citrate-dextrose, another centrifugation step was performed to obtain washed platelets. The platelet pellet was resuspended into Hepes buffer pH 7.45 (10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 5.5 mM glucose, and 0.1% BSA).[20]

Microfluidic flow experiments

Glass coverslips were coated for 1 hour with two 1.5 mm diameter spots, each 3 mm apart, which contained the indicated type of fibrin or fibrinogen (upstream) and, when indicated, collagen type III (50 µg/mL) as a reference spot (downstream), and collagen type I (50 µg/mL). This microspot coating procedure eliminates cross-talk of thrombus formation between the adjacent surfaces.[21] For fibrin spots, fibrinogen (1 mg/mL, 0.5 µL) was applied for 30 minutes, after which α-thrombin (20 nM, 1 µL) was supplemented for additional 30 minutes. Where indicated, mixtures (1 µL) of α-thrombin (2 U/mL), FXIIIa (0.7 µg/mL), and $CaCl_2$ (10 mM) were applied on top of the fibrinogen. Residual FXIIIa activity of the fibrinogen preparation used was determined as 6% in comparison to plasma. Indicated spots were postcoated with the peptide VWF-BP (von Willebrand factor-binding peptide; 100 µg/mL, 1 µL). After the completion of coating, coverslips were blocked with 1% BSA in Hepes buffer pH 7.45 for 30 minutes. As a standard, whole blood was flowed over the coated spots using a microfluidics chamber under conditions allowing coagulation.[12] In brief, 1.0 mL samples of citrated blood were co-perfused with recalcification medium using 2 pulse-free micro-pumps (Model 11

Plus, 70-2212, Harvard Apparatus), and a y-shaped mixing tubing. The recalcification medium (in a second 1 mL syringe) consisted of 32 mM $MgCl_2$ and 63 mM $CaCl_2$ in Hepes buffer pH 7.45. Complete mixing was achieved at a volume ratio of 10 (blood) to 1 (recalcification medium).[22] Flow rates were adjusted to give a total wall-shear rate of 1000 or 100 s^{-1}. Fluorescent labels added per blood sample were $DiOC_6$ (platelet staining), AF568-annexin A5 (phosphatidylserine, exposure), and Alexa Fluor 647-anti-CD62P mAb (P-selectin expression), as described.[23] When appropriate, samples were preincubated for 10 minutes with vehicle, inhibitor PRT-060318 (10 µM, in 0.4 µg/mL pluronic plus 0.5% DMSO) and Fab 9012 (50 µg/mL, in saline). Inhibition of GPVI was achieved with Fab 9O12 (50 µg/mL), which has previously been shown to interfere in the interaction of GPVI and fibrin.[8] Brightfield and multicolor fluorescence images were recorded per spot over time.[24] Per donor, all control and intervention conditions were repeated at least in duplicates. Collected time series of brightfield and fluorescence microscopic images were analyzed by using predefined scripts,[25] formatted in the open-source package Fiji.[26]

Scanning electron microscopy

For electron microscopy, fibrin-coated spots were prepared as for flow studies and coated on a Sefar matrix (sieve mesh, pores: 170 µm; Sefar Pharma) using 96-wells plates.[27] Samples were fixed with 4% paraformaldehyde for 1 hour. After wash with PBS, the samples were dehydrated by a 5-step gradient of ethanol (30–100%), and then dried by 10-minutes treatment with hexamethyl disilizane/ethanol (1:1) and 1 hour exposure to air. Dried samples were mounted onto aluminium pin studs with 12 mm carbon conductive tabs (Ted Pella, Redding, CA, USA), were sputter coated with gold (Quorum Technologies, Ashford, United Kingdom; vacuum pump: Edwards, Crawley, United Kingdom) on carbon tabs, and imaged. Table-top electron microscopy was performed, as before.[27]

Fibrin suspension preparation

Fibrinogen (1 mg/mL) was mixed with $CaCl_2$ (10 mM), FXIIIa (0.7 µg/mL), and thrombin (1 U/mL) and was left to polymerize for 1 hour. Subsequently, D-Phe-Pro-Arg chloromethyl ketone (PPACK; 20 µM) was added to inactivate the thrombin, the clot mixture was agitated until it turned liquid, and it was left for 15 minutes. The gel solution was then ultrasonicated at 20 kHz and amplitude of 80 to 100 µm until clear; this was followed by a centrifugation step at 1000 g for 5 minutes. The obtained pellet was homogeneously resuspended into Hepes buffer pH 7.45.

Platelet aggregometry

Platelet aggregation was monitored by light transmission aggregometry using an automated Chronolog aggregometer (Havertown, PA, USA) at 37°C with stirring at 1200 rpm. Platelet suspensions (2×10^8/mL) were incubated at 37°C for 2 minutes, antagonists were added for 10 minutes, followed by agonists sonicated fibrin, collagen-I (5 µg/mL), or α-thrombin (0.1 U/mL).

Cytosolic Ca^{2+} measurements

Washed human platelets (2×10^8/mL) were loaded with Fluo-4 acetoxy methyl ester (8 µmol/L) and pluronic (0.4 mg/mL) by a 40 minutes incubation in the presence of apyrase (1 U/mL).[28] After centrifugation step in the presence of acid-citrate-dextrose, the Fluo-4-loaded platelets were resuspended into Hepes buffer pH 7.45. Blood samples were supplemented with 10% of autologous Fluo-4-loaded platelets; inhibitors were added after 5 minutes. Changes in cytosolic $[Ca^{2+}]_i$ during flow-dependent adhesion of labeled platelets were recorded for 5 minutes, using a Zeiss LSM 510 confocal microscope, essentially as described before.[29] Time series of fluorescence images were analyzed for changes in fluorescence intensity and for platelet adhesion, using Fiji/image J software.

Data handling and statistics

Data are represented as means ± SD. Statistical analysis was performed using GraphPad Prism v8 software (San Diego, CA, USA). Significance was determined using a 2-way ANOVA (Dunnett and Sidak multiple comparison test) or a 1-way ANOVA (Dunnett multiple comparison test); differences with $P<0.05$ were considered as significant. Heatmaps were generated with the program R. For the heatmap representation, all parameters were univariate scaled to 0 to 10.[21] According to earlier procedures,[30] thrombus values of duplicate or triplicate flow runs from one blood donor were averaged to obtain one parameter set per spot. Mean values of control and inhibitor runs were then compared per blood sample. For subtraction heatmaps, a conventional filter of $P<0.05$ (1-way ANOVA) was applied to determine relevant effects, as described before.[25,30]

Results

Fibrin microstructure of coated spots

To assess the suitability of fibrin-coatings for flow chamber studies, we prepared a series of spotted fibrinogen surfaces which were treated with a thrombin mixture in the presence or absence of the cross-linking transglutaminase, FXIIIa, and a peptide (VWF-BP) capable to capture free VWF from blood.[31] Ultrastructural observation of the different preparations by scanning electron microscopy showed that in all conditions multiple layers of fibrin were formed, which presented as microstructures with both thicker and thinner fibers (Figure 1, arrows). The addition of VWF-BP did not alter the overall fiber structure. However, addition of FXIIIa resulted in fibrin fibers that appeared to be less densely packed with an overall thicker size. The latter observation may be due to the local high transglutaminase concentration upon the fibrin formation.

For comparison, also fibrinogen-only spots were examined with or without VWF-BP. Electron microscopy did not reveal any fibrous structures in this case (Figure 1, Appendix Figure 1).

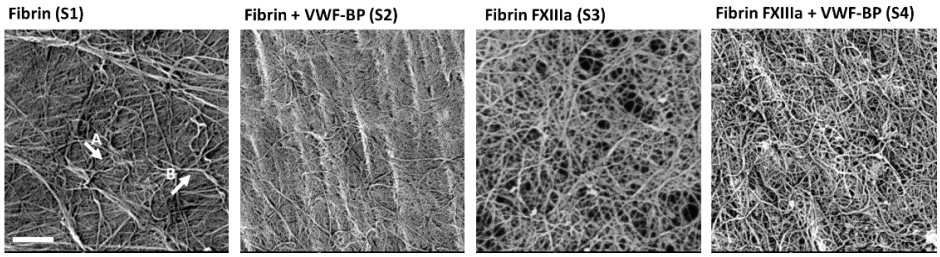


Figure 1. Fibrillar microstructure of immobilized fibrin surfaces. *Representative scanning electron microscopy (SEM) images of immobilized fibrin spots, produced from fibrinogen coatings with or without FXIIIa (factor XIIIa) and VWF-BP (von Willebrand factor-binding peptide). Mixtures were allowed to generate fibrin fibers on a SEFAR (Sefar Pharma) filter for 30 minutes. Representative images are shown; scale bar 2 µm. Arrow A points to a thin fiber, arrow B indicates a thick fiber.*

Formation of only small-sized microthrombi on fibrin surfaces under flow

We examined how the different types of fibrin(ogen) spots formed with or without FXIIIa and VWF-BP (coded as S1-S6, see Table 1) were able to support thrombus formation under flow in microfluidics chambers. Therefore, citrated whole blood (labeled with $DiOC_6$, AF568-annexin A5, and Alexa Fluor 647-anti-CD62P mAb) was flowed over sets of 2 spots at defined conditions, in coagulating condition (absence of PPACK).[22] After 10 minutes of flow at arterial shear rate (1000 s^{-1}), on all fibrin(ogen) surfaces, platelets adhered and formed small aggregates, not extending 2 or 3 cell layers, which we characterized as bilayer aggregates or microthrombi (Figure 2A; representative images of all 6 spots in Suppl. Figure I). In contrast, simultaneous flow experiments using collagen-I

spots produced larger thrombi composed of multi-layered platelet aggregates (see below), such as reported before.[32] No platelets adhered to coverslip areas in between the coated spots.[21]

Capturing of multicolor fluorescence microscopic images at time points of 2, 4, 6, 8, and 10 minutes allowed to assess the kinetics of the process, in terms of 6 parameters. These were $DiOC_6$ platelet adhesion (P1), thrombus morphological score (P2), thrombus contraction score (P3), bi- or multi-layer score (P4), bi- or multilayer size (P5), P-selectin expression (P6), and phosphatidylserine exposure (P7).[25] End stage brightfield and triple-colored microscopic images from fibrin-FXIIIa spots (S3) are shown in Figure 2A. To compare the time-dependent parameter increases per type of fibrin spots (S1–S4) and fibrinogen spots (S5–S6), we scaled all values per parameter across surfaces (scale 0–10) and represented the results in a practical heatmap format (Figure 2B).

Table 1. *Coding of spots (S) and parameters (P) in whole-blood thrombus formation.*

S	Microspot coating	Adhesive receptors	
S1	Fibrin	GPVI, αIIbβ3	
S2	Fibrin + VWF-BP	GPIb, VI, αIIbβ3	
S3	Fibrin FXIIIa	GPVI, αIIbβ3	
S4	Fibrin FXIIIa + VWF	GPIb, VI, αIIbβ3	
S5	Fibrinogen	GPVI, αIIbβ3	
S6	Fibrinogen + VWF-BP	GPIb, VI, αIIbβ3	
S7	Collagen III	GPIb, VI, α2β1	
P	**Image type**	**Description**	**Unit or scaling**
Platelet parameters			
P1	$DiOC_6$	platelet adhesion	%SAC
P6	AF647 α-P-selectin	platelet activation	%SAC
P7	AF568 annexin A5	platelet PS exposure	%SAC
Thrombus parameters			
P2	brightfield	thrombus morphology	score 1-5
P3	brightfield	thrombus aggregation	score 1-3
P4	brightfield	thrombus contraction	score 1-3
P5	brightfield	thrombus multilayer coverage	%SAC

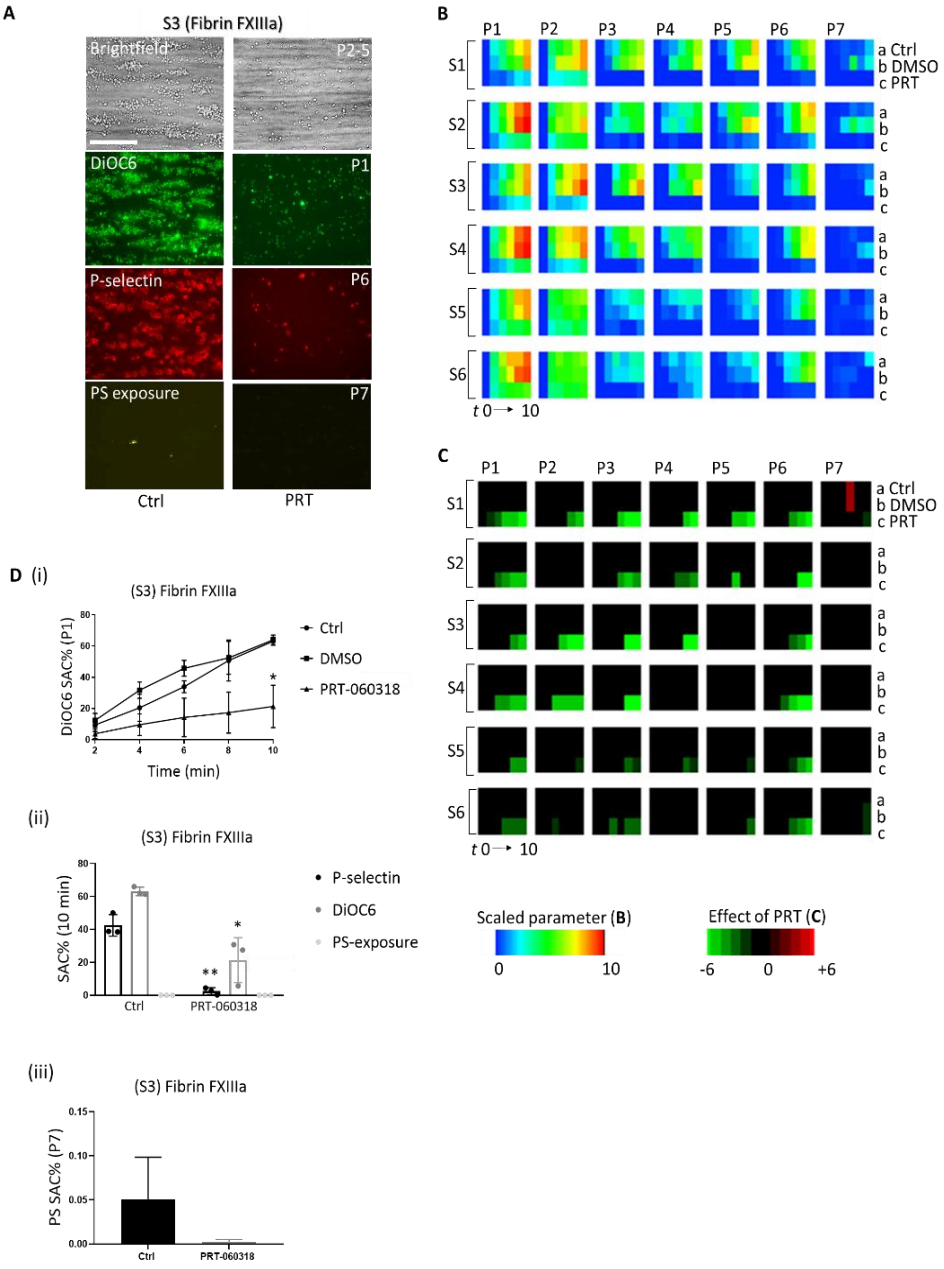


Figure 2. Major role of Syk kinase in microthrombus formation on fibrin or fibrinogen surfaces. *Blood samples preincubated with vehicle (Ctrl) or Syk inhibitor PRT-060318 (PRT, 10 µM), labeled and flowed at 1000 s^{-1} over spots S1–*

S6 (Table). Surfaces were imaged (brightfield and fluorescence) at time points t= 0, 2, 4, 6, 8, 10 minutes to obtain parameters P1 ($DiOC_6$ platelet adhesion), P2 (thrombus morphological score), P3 (contraction score), P4 (bi- or multi-layer score), P5 (bi- or multi-layer size), P6 (P-selectin expression), and P7 (phosphatidylserine [PS] exposure). Effects of PRT were assessed per blood sample, surface, and parameter. ***A****, Representative brightfield and fluorescence images of microthrombi on fibrin-FXIIIa (factor XIIIa) spots (S3) after 10 minutes. Scale bar, 50 µm.* ***B****, Mean parameter values from 3–4 blood samples over time, univariate scaled to 0–10 per parameter. Heatmap of the scaled parameters, demonstrating effects of PRT. Rainbow color code indicates scaled values from 0 (blue) to 10 (red).* ***C****, Subtraction heatmap representing effects of PRT, filtered for relevant differences (n=3–4, P<0.05, 2-way ANOVA). Color code showing relevant decrease (green) or increase (red) in comparison to control runs.* ***D****, Time-dependent increase in platelet adhesion (P1) in the presence or absence of PRT (**D**, **i**). Mean effects of PRT on platelet adhesion (P1) and platelet activation (P6) after 10 minutes (**D**, **ii**); mean effects of PRT on platelet PS exposure (P7) after 10 minutes (**D**, **iii**). SAC indicates surface-area-coverage. Data are means ± SD (n=3–4), *P<0.01, **P<0.001 (2-way ANOVA).*

Overall, the heatmap analysis pointed to similar parameter increases over time for all types of fibrin spots (S1–S4), although rates of platelet adhesion (P1) were slightly higher in spots containing VWF-BP (S2, S4). The latter observation pointed to a moderate enhancement of platelet adhesion but not to bilayer microthrombus formation, by plasma-bound VWF. On all fibrin spots, the adhered platelets gradually increased in P-selectin expression (P6), but remained low in phosphatidylserine exposure (P7), thus indicating an only moderate platelet activation state.[2] Addition of FXIIIa during fibrin formation (S3, S4) slightly increased the formation of platelet aggregates, when compared with no added FXIIIa (S1, S2). Furthermore, the 2 spots with fibrinogen (S5, S6) were even less active in supporting platelet adhesion, bilayer aggregate formation and platelet activation (P-selectin expression) in comparison to the fibrin surfaces (Figure 2A-B).

Given the presence of 6% residual FXIIIa in the fibrinogen preparation used for the coating, we checked if transglutaminase contributed to platelet-activating effects by generating spots of fibrin (S1) and fibrin-FXIIIa (S3) in the presence of the FXIIIa inhibitor T101. However, after 10 minutes of whole-blood flow, both end stage images (Suppl. Figure II A) and scaled time-dependent parameters P1,5-7 did not show significant effects of this inhibitor (Suppl. Figure II B-C). Together, these results pointed to a no more than modest role of FXIIIa-induced cross-linking in fibrin-dependent microthrombus formation.

Major role of Syk kinase in microthrombus formation on fibrin independently of coagulant strength

To assess tyrosine kinase-dependent signaling, blood samples were preincubated with the selective Syk kinase inhibitor PRT-060318, known to completely abolish the thrombus formation on collagen-like surfaces.[30] This inhibitor lowered platelet adhesion and abolished the bilayer aggregate formation and P-selectin expression on all prepared fibrin and fibrinogen spots (Figure 2A-B). Subtraction heatmaps of the scaled treatment effects over time showed for essentially all parameters a relevant reduction with PRT-060318, but not with DMSO vehicle (Figure 2C). For instance, on fibrin-FXIIIa spots, PRT-060318 gradually suppressed platelet adhesion parameters P1 to P2 (Figure 2D i-ii) and even tended to suppress the low phosphatidylserine exposure (Figure 2D iii).

Subsequent flow experiments were performed with fibrin-FXIIIa (S3) spots, which were used as a standard. We first examined how the formation of microthrombi relied on the extent of coagulation, that is, thrombin generation. Therefore, blood samples were incubated either with the thrombin inactivator PPACK, or with 5 or 10 pM tissue factor to trigger the extrinsic coagulation pathway. During 10 minutes of flow at 1000 s^{-1}, microscopic images again were captured and analyzed for $DiOC_6$ platelet adhesion (P1), multilayer score (P5), P-selectin expression (P6), and phosphatidylserine exposure (P7). Markedly, the presence of either

PPACK and tissue factor did not significantly change platelet adhesion, size of microthrombi or the P-selectin expression at fibrin-FXIIIa spots, when compared with control runs (Suppl.Figure III A). In addition, Syk inhibition with PRT-060318 had a similar lowering effect on all parameters, regardless of the presence of PPACK or tissue factor (Suppl.Figure III B).

A marked finding was that, for all variable coagulation conditions, the extent of phosphatidylserine exposure remained low for fibrin-FXIIIa spots (0.1–0.4 SAC%). On the contrary, parallel flow experiments on collagen-I spots caused formation of large-sized platelet thrombi, with high staining for $DiOC_6$ and high phosphatidylserine exposure, with or without tissue factor (Suppl. Figure IV). Hence, the low level of phosphatidylserine exposure observed on fibrin-FXIIIa spots was not due to limitations of the flow set-up, in agreement with earlier findings.[22]

To further assess the apparently limited role of thrombin in the microthrombus formation on fibrin spots, we compared the effects of PPACK with the thrombin receptor antagonist atopaxar and the thrombin inhibitor lepirudin. When added to the blood, neither end stage images nor (subtraction) heatmaps of scaled parameters P1,5-7 indicated any effect of these interventions (Suppl. Figure V A-B). In addition, we measured the ability of thrombi formed on fibrin-FXIIIa and collagen-I spots to support (phosphatidylserine-dependent) thrombin generation, using an earlier described procedure based on the thrombin-induced cleavage of substrate Z-GGR-ACM (Z-Gly-Gly-Arg aminomethyl coumarine).[33] The observed no more than minimal thrombin generation on fibrin-FXIIIa spots supported the conclusion that the procoagulant activity of the fibrin surface is low in comparison to the collagen-I surface (Suppl. Figure V C-D).

Together, these results indicated that the low thrombogenic effect of fibrin surfaces relies on Syk kinase signaling, which is relatively independent of coagulation triggering.

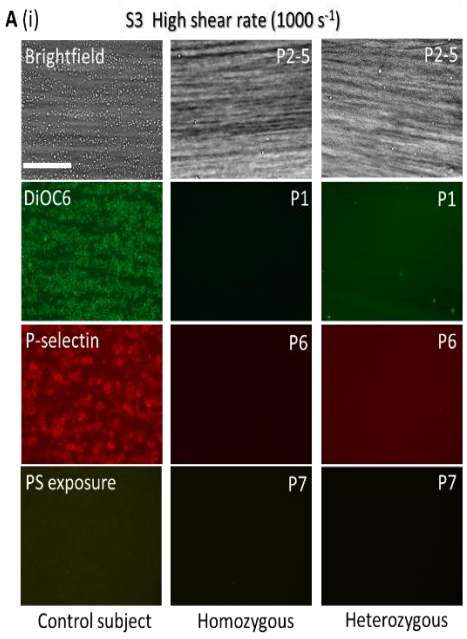
Shear-dependent contribution of integrin αIIbβ3 in microthrombus formation on fibrin

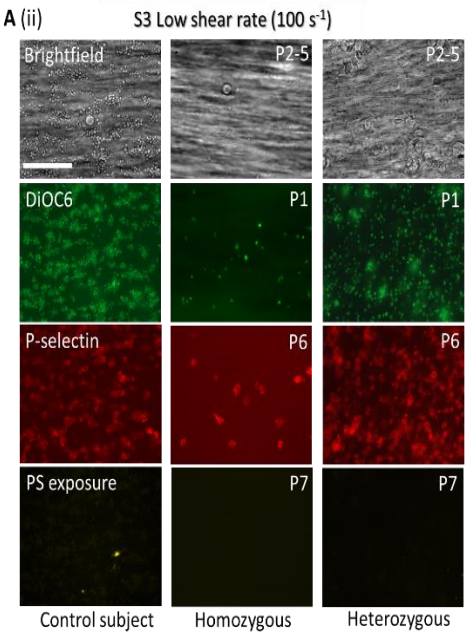
Considering that also integrin αIIbβ3 interaction with fibrin(ogen) can trigger Syk activation,[6,34] we went on to determine the role of this integrin in the microthrombus formation. Therefore, blood samples from 2 patients with Glanzmann thrombasthenia were obtained and flowed over fibrin-FXIIIa spots (S3) at arterial (1000 s^{-1}) or venous (100 s^{-1}) shear rate. Markedly, with blood from the homozygous patient, completely lacking platelet surface expression of αIIbβ3, the platelets failed to adhere to fibrin, regardless of the shear rate (Figure 3A-B). Control experiments indicated that VWF was present on the S3 (fibrin-FXIIIa) surfaces (see below). With blood from the heterozygous patient, presenting with reduced platelet αIIbβ3 expression, the platelets again did not adhere at the high shear rate. However, there was substantial platelet adhesion (P1) at the low shear rate. In the latter case, the normal P-selectin expression (P6) pointed to residual platelet activation. Taken together, these results pointed to a crucial, shear-dependent role of the αIIbβ3 integrin in the flow-dependent platelet interaction with fibrin.

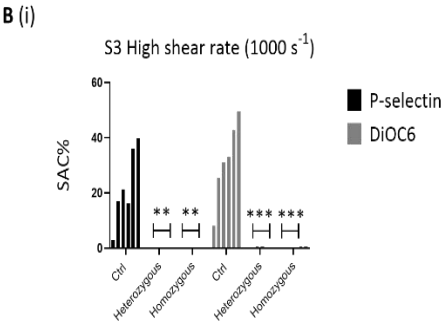
Complementary roles of GPVI, αIIbβ3, and GPIb-V–IX in microthrombus formation on fibrin

Subsequent flow experiments were performed with spots of fibrin-FXIIIa (S3) in combination with downstream collagen-III (S7), which was considered as a reference platelet-activating surface. The comparative analysis of fibrin-FXIII and collagen-III surfaces showed that the microthrombi on fibrin were less contracted and activated than those on the collagen-III (Figure 4A-B). Considering that both GPVI and integrin αIIbβ3 can activate Syk kinase, in this setting we established the role of GPVI in the thrombus-forming process at both surfaces.

Whole-blood samples were treated with anti-GPVI Fab 9012 and flowed over the spots for 10 minutes at high shear rate of 1000 s^{-1}.


A (i)
S3 High shear rate (1000 s-1)
Brightfield
P2-5
P2-5
DiOC6
P1
P1
P-selectin
P6
P6
PS exposure
P7
P7
Control subject
Homozygous
Heterozygous


A (ii)
S3 Low shear rate (100 s-1)
Brightfield
P2-5
P2-5
DiOC6
P1
P1
P-selectin
P6
P6
PS exposure
P7
P7
Control subject
Homozygous
Heterozygous


B (i)
S3 High shear rate (1000 s-1)
SAC%
60
40
20
0
**
**
***
***
Ctrl
Heterozygous
Homozygous
Ctrl
Heterozygous
Homozygous
P-selectin
DiOC6

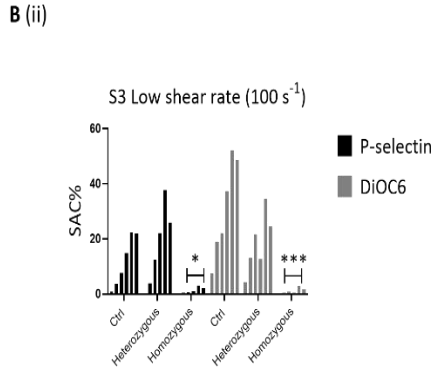

B (ii)
S3 Low shear rate (100 s-1)
SAC%
60
40
20
0
*
***
Ctrl
Heterozygous
Homozygous
Ctrl
Heterozygous
Homozygous
P-selectin
DiOC6

Figure 3. Abolished microthrombus formation on fibrin in Glanzmann thrombasthenia. A*, Representative brightfield and fluorescence images from microthrombi after flow of blood from control subjects or 2 Glanzmann patients over spots of fibrin-FXIIIa (factor XIIIa; 10 minutes). Flow perfusion was at arterial shear rate of 1000 s^{-1} (**A, i**) or at venous shear rate of 100 s^{-1} (**A, ii**). Scale bars 50 µm. **B**, Time-dependent increases in platelet activation (P-selectin expression, P6) and platelet adhesion ($DiOC_6$, P1) for controls (Ctrl, n=4), heterozygous and homozygous patients at time points t=0, 2, 4, 6, 8, and 10 minutes. Graphs at shear rates of 1000 s^{-1} (**B, i**) and 100 s^{-1} (**B, ii**). SAC indicates surface-area-coverage. Mean ± SD (n=4 for controls), **P<0.001, ***P<0.0001 (2-way ANOVA).*

Microscopic images, captured from these surfaces over time, were analyzed for the same parameters as before (P1,5-7). The results showed that, for fibrin-FXIIIa (S3), GPVI inhibition caused significant decreases of platelet adhesion, bilayered aggregation, and P-selectin expression (Figure 4A-B). The effects of Fab 9O12 on platelet adhesion and activation were similar for fibrin-FXIIIa and collagen-III spots. However, these were lower in comparison to Syk inhibition (compare Figures 2C and 4C). This notion agrees with a complementary activation pathway of Syk kinase, involving integrin αIIbβ3. Fibrin has previously been shown to bind to VWF.[19] For the fibrin-FXIIIa spots exposed to flowing blood, we could confirm the binding of plasma-derived VWF by staining with a fluorescein isothiocyanate-labeled anti-VWF antibody (Figure 5A). The presence of VWF was yet lower than on the reference spot, collagen-III. To determine contribution of the VWF-GPIb-V–IX axis to the microthrombus, we used an established blocking anti-GPIbα antibody, RAG35.[35] For both spots S3 (fibrin-FXIIIa) and S7 (collagen-III), the addition of RAG35 antibody resulted in a marked reduction in platelet adhesion, bilayer formation, and P-selectin expression, already observable from the first minutes of flow (Figure 5B-C). Subtraction heatmaps, however, pointed to larger effect for collagen-III than for fibrin-FXIIIa spots (Figure 5D).

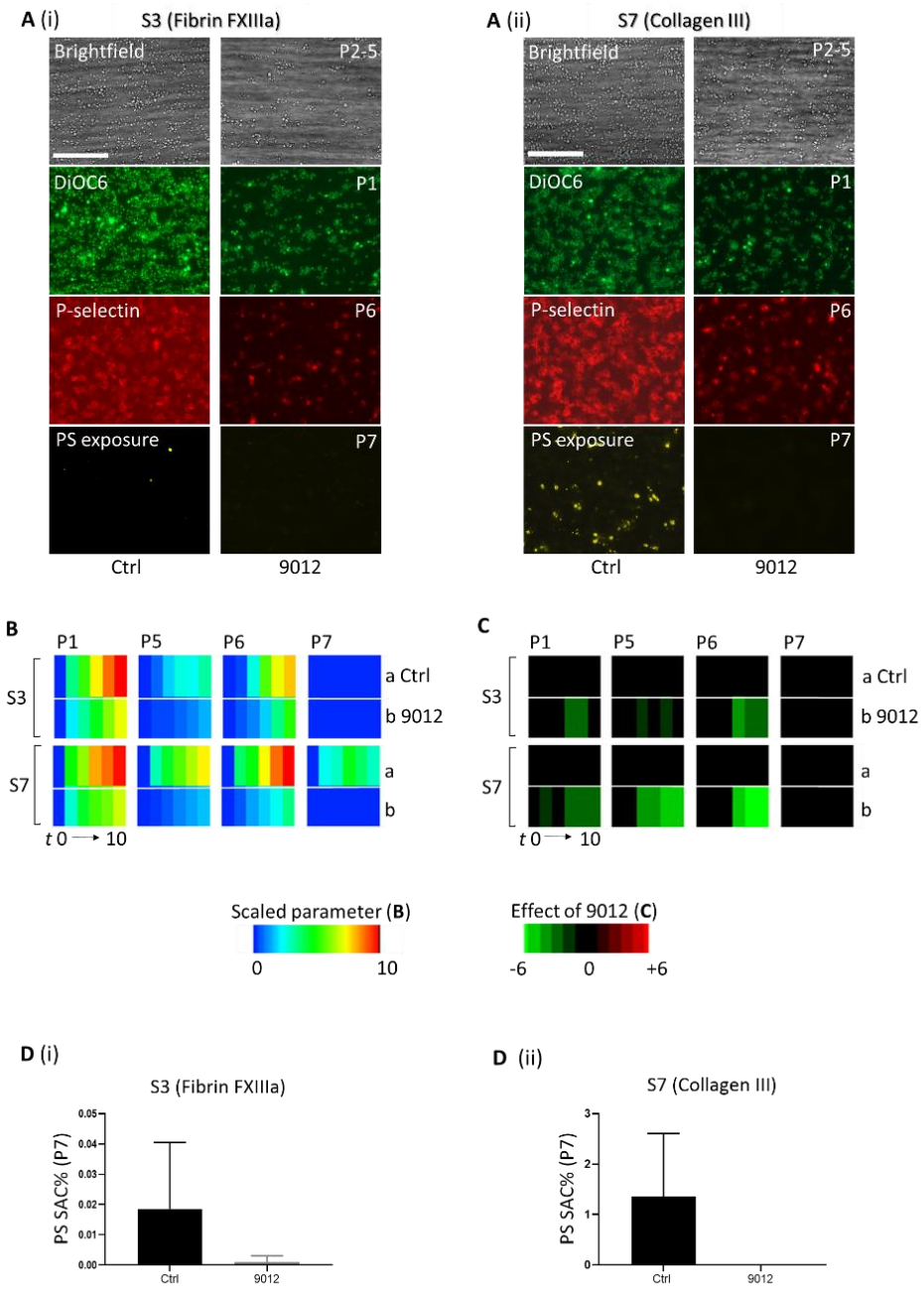


Figure 4. Effect of GPVI (glycoprotein VI) inhibition on parameters of microthrombus formation on immobilized fibrin-FXIIIa (factor XIIIa) or

collagen-III. *Blood samples preincubated with vehicle (Ctrl) or GPVI blocking agent (9O12 Fab, 50 µg/mL) were flowed over spots S3 (fibrin-FXIIIa, upstream) and S7 (collagen-III, downstream) for 10 minutes at 1000 s^{-1}. Microthrombi formed were imaged to obtain parameters P1 ($DiOC_6$ platelet adhesion), P5 (bi- or multilayer size), P6 (P-selectin expression), and P7 (PS exposure).* ***A****, Representative brightfield and fluorescence images from fibrin-FXIIIa (****A****,* ***i****) and collagen-III spots (****A****,* ***ii****) at 10 minutes.* ***B****, Heatmap of scaled parameters, demonstrating mean effects of GPVI inhibition on thrombus formation per spot. Effects of GPVI inhibition were assessed per blood sample, surface, and parameter. Mean values from individual blood samples were univariate scaled to 0–10 per parameter. Rainbow color code indicates scaled values between 0 (blue) and 10 (red).* ***C****, Subtraction heatmaps representing scaled effects of GPVI inhibition, filtered for relevant changes (P<0.05, 2-way ANOVA per surface and parameter). Color code represents decrease (green) or increase (red) in comparison to control runs. Scale bar 50 µm.* ***D****, Graphs representing PS exposure (P7) for fibrin-FXIIIa (****D****,* ***i****) and collagen-III (****D****,* ***ii****) at 10 min. SAC indicates surface-area-coverage. Data are means±SD (n=7), *P<0.05 (2-way ANOVA).*

Role of Syk and GPVI in fibrin-induced platelet aggregation

To further confirm the moderate signaling via Syk kinase and GPVI in fibrin-induced platelet activation, we examined the aggregation response of platelets upon stimulation with a sonicated, homogeneous fibrin suspension. This suspension was treated with PPACK to remove thrombin traces. Similar to the results of microthrombus formation, pretreatment of platelets with Syk inhibitor PRT-060318 abrogated the fibrin-induced aggregation (Figure 6A-B). Similarly, the GPVI blocking Fab 9O12 suppressed fibrin-induced aggregation, but a residual shape change and aggregation remained. In comparison, both inhibitors also antagonized the platelet aggregation induced by collagen-I, but not by thrombin (Figure 6). As expected, treatment with the integrin antagonist tirofiban blocked the fibrin-induced aggregation response by >80% (Figure 2, Appendix Figure 2).

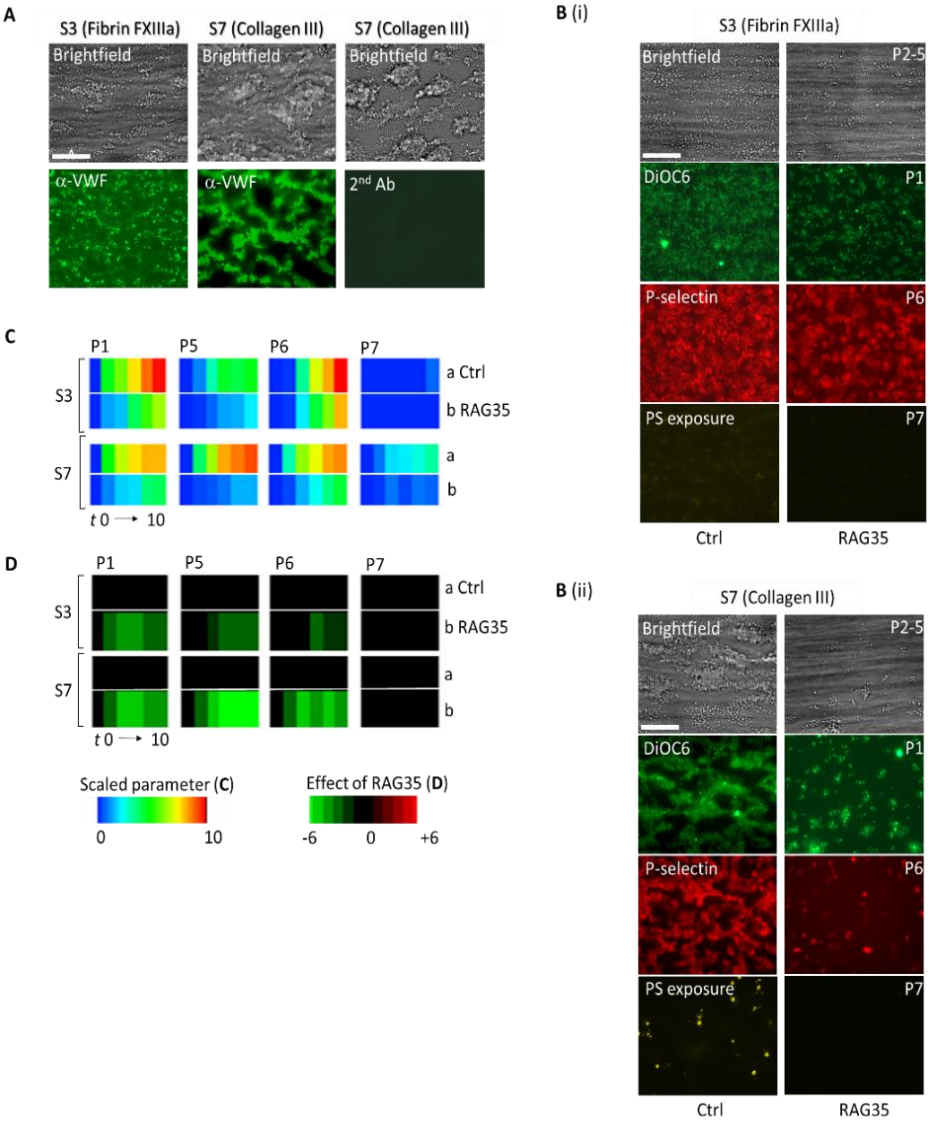


Figure 5. Roles of VWF (von Willebrand factor) and GPIb-V–IX (glycoprotein Ib-V–IX) in microthrombus formation on immobilized fibrin-FXIIIa or on collagen-III. *Blood samples preincubated with vehicle (Ctrl) or GPIb blocking antibody RAG35 (20 µg/mL) were flowed at 1000 s^{-1} over spots S3 (fibrin-FXIIIa, upstream) and S7 (collagen-III, downstream). After 10 minutes, microthrombi formed were imaged to obtain parameters P1 ($DiOC_6$ platelet adhesion), P5 (bi- or multilayer size), P6 (P-selectin expression), P7 (phosphatidylserine [PS] exposure).* ***A****,*

Staining of microthrombi formed on S3 and S7 spots after 10 min for VWF using fluorescein isothiocyanate-labeled anti-VWF antibody (green) or irrelevant control antibody. ***B–E****, Effects of RAG35 antibody on thrombus parameters were assessed over time per blood sample for S3 and S7 surfaces. Scale bar 50 µm. Shown are representative end stage brightfield and fluorescence images for fibrin-FXIIIa (****B, i****) and collagen-III (****B, ii****). Furthermore, heatmap of univariate scaled parameters (0–10), indicating increased build-up of microthrombi over time in the absence or presence of RAG35 antibody (****C****). Rainbow color code shows scaled values between 0 (blue) and 10 (red). In addition, subtraction heatmap representing scaled effects of GPIb blocking (****D****). Filtering was applied for relevant changes (n=6, P<0.05, 2-way ANOVA per surface and parameter). Colour code represents decrease (green) or increase (red) in comparison to control runs. Means ± SD (n=6), P<0.05 (2-way ANOVA).*

Fibrin-induced platelet Ca^{2+} signaling under flow

In collagen-induced platelet activation, GPVI adhesion under flow is known to induce a prolonged and high Ca^{2+} signal, leading to massive P-selectin expression and phosphatidylserine exposure.[21,36] To investigate the Ca^{2+} signal of platelets flowed over fibrin-FXIIIa, blood samples were supplemented with autologous Fluo-4-loaded platelets, and fluorescent $[Ca^{2+}]_i$ rises were measured in real time by confocal microscopy. The results show a consistent, moderate increase in Fluo-4 fluorescence in fibrin-adhered platelets, which was suppressed by Fab 9O12 (Figure 7A). A near complete major reduction in fluorescence increase was observed upon Syk inhibition with PRT-060318. The effects on Fluo-4 fluorescence increases paralleled effects on platelet adhesion (Figure 7B). However, detailed analysis of traces from single adhered platelets confirmed suppression of transient, spiking Ca^{2+} signal generation in the presence of Fab 9O12 or PRT-060318 (Suppl. Figure VI A-C). Of note, no platelet adhesion could be observed in the presence of integrin inhibitor, tirofiban. Taken together, these results confirm that fibrin interaction activates platelets via Syk kinase and GPVI.

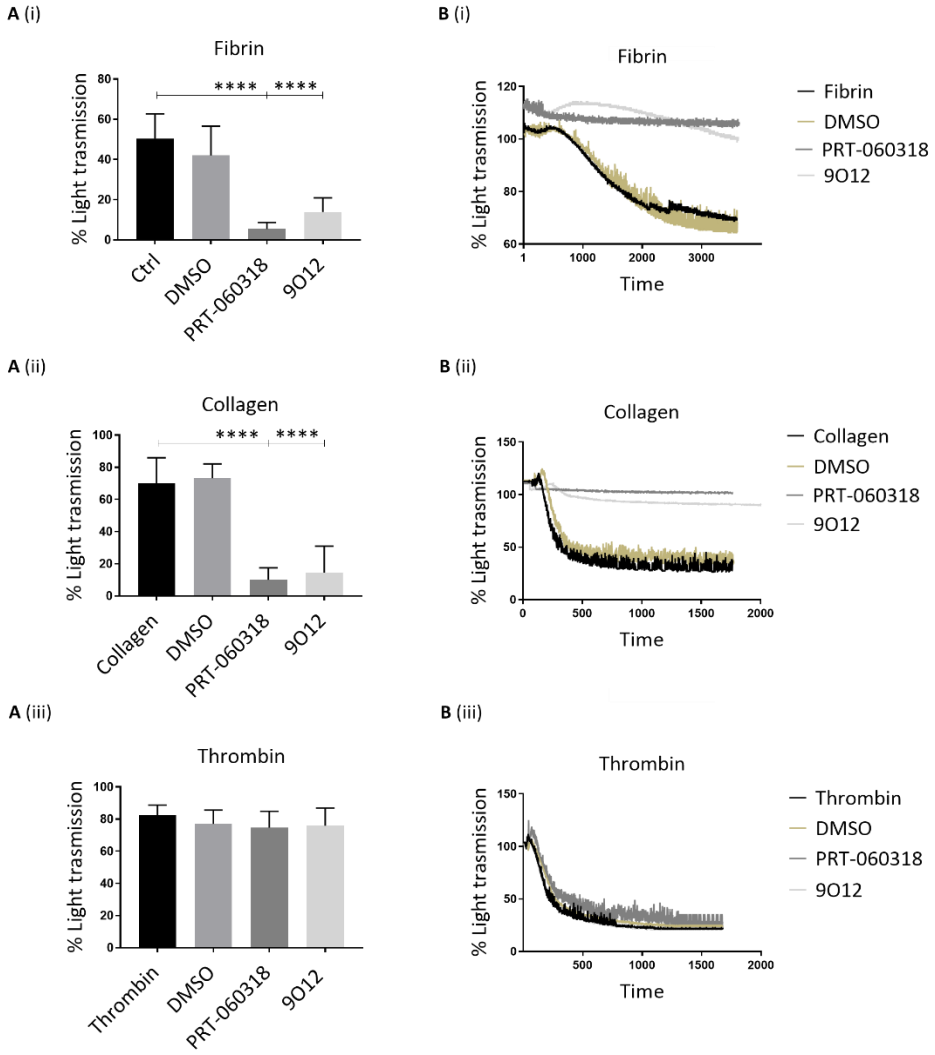


Figure 6. Comparative effects of GPVI (glycoprotein VI) or Syk inhibition on fibrin-mediated platelet aggregation. *Platelets in suspension were treated with Syk inhibitor PRT-060318 (10 µM) or GPVI-inhibitor 9O12 (50 µg/mL) for 10 minutes, before agonist addition. Platelet aggregation was monitored by conventional light transmission aggregometry.* ***A****, Quantitation of maximal aggregation upon stimulation with fibrin, collagen, or thrombin.* ***B****, Representative aggregation traces upon stimulation with indicated agonist. Mean ± SD (n=5), ****P<0.0001 (1-way ANOVA).*

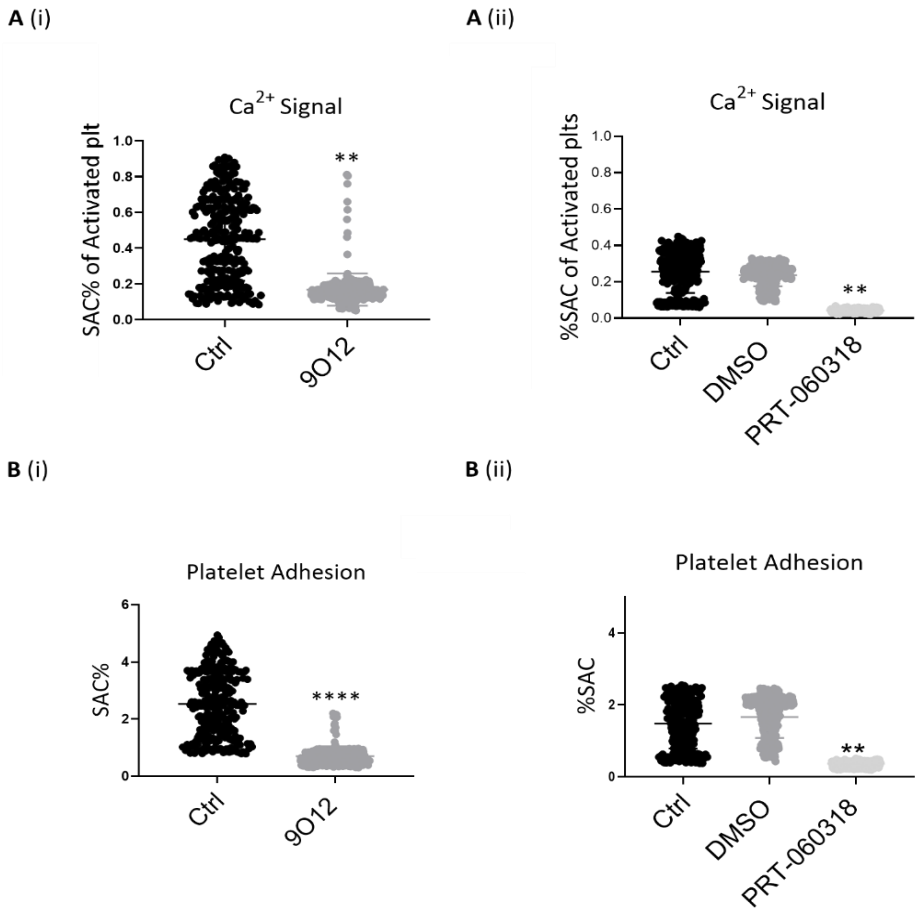


Figure 7. Effects of glycoprotein VI (GPVI) or Syk inhibition on Ca^{2+} signaling in fibrin-adhered platelets under flow. *Blood samples containing autologous Fluo-4-loaded platelets were preincubated with vehicle (Ctrl), GPVI blocking agent 9O12 Fab (50 µg/mL) or Syk inhibitor PRT-060318 (10 µM), and flowed over S3 spots (fibrin-FXIIIa), as for Figure 2. Fluorescence changes in cytosolic $[Ca^{2+}]_i$ of adhered platelets were recorded by confocal microscopy for 5 minutes. Time series of fluorescence images were analyzed for threshold increases in fluorescence intensity representing platelet activation (**A**, **i** and **ii**), and for fluorescence coverage as a measure of platelet adhesion (**B**, **i** and **ii**). Parts present data from parallel flow runs; dots represent values from analyzed images. SAC indicates surface-area-coverage. Bars are means (n=3 experiments), **P<0.001, ****P<0.0001 (1-way ANOVA).*

Fibrin in consolidating microthrombus formation

We explored why the fibrin-adhered platelet were insensitive to thrombin, for instance in flow with tissue factor. As a first approach, we adapted an earlier protocol, where single immobilized platelets in a flow chamber were triggered to generate star-like fibrin fibers.[37]

These fibrin-forming platelets were postperfused for up to 10 minutes with blood samples, again containing labels for platelets ($DiOC_6$), and P-selectin and phosphatidylserine exposure. Recording of brightfield and tri-colour fluorescence images from the same microscopic fields after blood flow showed considerable overlap between the staining (Suppl. Figure VII A). Detailed analysis showed that the stainings concentrated around the immobilized platelets, rather than at the extending fibrin fibers (Suppl. Figure VII B). Thus, the $DiOC_6$-labeled microaggregates showed high overlap with the P-selectin exposing prior immobilized platelets, which was confirmed by measuring the overlap coefficients R (Suppl. Figure VII C). This overlap was further increased by blood flow in the presence of integrin αIIbβ3 antagonist tirofiban. In an earlier study, we observed high binding of Oregon Green 488-labeled thrombin to fibrin-containing thrombi.[11] This could be confirmed using the present coagulant flow conditions for fibrin-FXIIIa and collagen-I spots (Suppl. Figure VIII A-B). As a control, blocking of the coagulation process with PPACK suppressed cleavage of the Oregon Green 488-labeled prothrombin probe, with as a consequence binding to phosphatidyl-serine-exposing platelets only (Suppl. Figure VIII A ii and VIII B ii). Taking together, these data suggest that fibrin fibers provide a relatively poor surface for newly adhering platelets, but can trap locally cleaved prothrombin.

Discussion

While GPVI has been identified as a receptor for fibrin and also fibrinogen,[7–9] the relative strength of the platelet-activating effect of fibrin via GPVI has not been examined in detail. It has been established that blood flow over immobilized collagens or collagen-related peptides

via GPVI causes strong platelet activation responses, that is, a prolonged Ca^{2+} signal, high integrin activation, P-selectin expression, phosphatidyl-serine exposure, and massive thrombus formation.[21,38–40] The present data, using a variety of immobilized fibrin surfaces, point to a weaker GPVI-dependent platelet-activating effect of blood flow over fibrin, in that fewer platelets adhered showing transient Ca^{2+} signals, residual P-selectin expression and limited phosphatidylserine exposure, altogether resulting in the formation of only small-sized microthrombi.

The microthrombus formation under flow appeared to be hardly influenced by producing the fibrin with or without added VWF-BP or FXIIIa. However, this does not rule out a role of VWF or FXIIIa in the thrombus-forming process, because fibrin can capture VWF from the blood plasma,[19] and FXIIIa can also be produced in coagulating plasma and released from activated platelets.[13] Evidence for a relative weakness of fibrin (in comparison to collagen) as platelet-activating surfaces was further corroborated by experiments showing that fibrin fibers extending from immobilized platelets are relatively ineffective in trapping newly perfused platelets, when compared with the immobilized platelets themselves. Comparative analysis of the microthrombi on fibrin-FXIIIa and collagen-III spots indicated that the platelets on fibrin had a lower activation state than those on collagen. Nevertheless, on both surfaces, inhibition of Syk (PRT-060318) or blockade of GPVI (Fab 9O12) suppressed the flow-dependent platelet adhesion, aggregate formation, and activation (P-selectin expression). In agreement with these findings, also light transmission aggregation studies using stirred platelet suspensions showed that the fibrin-induced aggregation process is abolished by both PRT-060318 and Fab 9O12.

Complete or partial defects in expression of integrin αIIbβ3 (Glanzmann patients) resulted in an annulled platelet adhesion to fibrin under flow, which in case of partial deficiency was limited to the high shear rate condition. In addition, we could establish a role of GPIb-V-IX by using the

blocking anti-GPIbα antibody RAG35.[35] This antibody substantially but not completely decreased platelet adhesion to both fibrin-FXIIIa and collagen-III spots, while the remaining adhered platelets still displayed P-selectin expression. Together, these findings point to complementary and nonredundant roles of GPVI, αIIbβ3, and GPIb-V-IX complex in the microthrombus formation on fibrin surfaces. Since the tyrosine kinase Syk is known to be phosphorylated and activated downstream of both GPVI and αIIbβ3,[6,30,41] our results suggest that concomitant activation via both receptors is required for formation of the microthrombi. This idea is supported by a previous study showing that Syk phosphorylation is a continuous process in murine thrombus growth, and that secondary Syk inhibition can annul platelet adhesion even on preformed thrombi under flow.[42] A nonredundant contribution of GPVI and αIIbβ3 can also be derived from the observation that perfusion of blood from patients with GPVI deficiency over fibrin spots resulted in an abolished aggregate formation, although individual platelets still adhered (unpublished data, but see Ref.[43]).

Novel related observations were (1) the low phosphatidylserine exposure of platelets on fibrin (although still dependent on low-level GPVI), (2) the relative inability of thrombin to alter fibrin-dependent microthrombus formation, and (3) a low-level thrombin generation of platelets on fibrin in comparison to collagen. An explanation for these observations is the finding, supported by earlier studies,[11] that fibrin captures (fluorescent-labeled) thrombin, apparently without ability to cleave its substrate Z-GGR-ACM. This agrees with the earlier notion of irreversible thrombin binding to a fibrin network.[44] A suggestion then is that under the present microfluidic conditions fibrin-bound thrombin is unable to activate platelets. Clearly, more research needs to be done to better understand this phenomenon. Hence, our present findings lead to the concept that on fibrin a low platelet GPVI activation and an inactivation of thrombin induces only weak support of thrombus formation; or in other words, that

platelet interaction with fibrin in particular consolidates the process of thrombus formation. However, we cannot rule out that under certain (patho)physiological static or flow conditions the role of fibrin is enlarged.[45] The overall observation of fibrin-induced microthrombus formation suggests that fibrin fibers act as consolidating elements of the thrombus shield, such in contrast to vascular collagens which trigger the formation of larger size thrombi. Given that thrombus growth is regulated by secondary mediators, such as ADP and thromboxane A_2, which activate platelets in the thrombus core, it is not evident that the fibrin-GPVI interaction substitutes the high GPVI activation induced by collagens. A local inactivation of thrombin by fibrin may further contribute to this dampening process.

Nonstandard abbreviations and acronyms

FXIIIa	factor XIIIa
GPVI	glycoprotein VI
PPACK	D-Phe-Pro-Arg chloromethyl ketone
PRP	platelet-rich plasma
VWF	von Willebrand factor
VWF-BP	von Willebrand factor-binding peptide

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Disclosures J.W.M. Heemskerk is founder and co-owner at FlowChamber b.v. The other authors report no conflicts.

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Supplemental materials

Flow studies with FXIIIa or thrombin (receptor) inhibitors

Coverslips were coated for 1 hour with two spots, each 3-mm apart, that consisted of fibrin (upstream) and fibrin-FXIIIa (downstream), as described in the Methods. In short, fibrinogen (1 mg/mL, 0.5 µL) was applied for 30 minutes, after which α-thrombin (20 nM, 1 µL) was added for an additional 30 minutes. Where indicated, the thrombin was mixed with $CaCl_2$ (10 mM), FXIIIa (0.7 µg/mL) and/or inhibitor T101 (20 µM); and the mixture was applied on the fibrinogen spot. Whole blood flow was performed under conditions allowing coagulation at a total wall-shear rate of 1000 s^{-1}. To compare the effects of thrombin inhibitors, blood samples were preincubated for 10 minutes with vehicle or atopaxar (5 µg/mL), recombinant hirudin (lepirudin 10 5 µg/mL) and/or PPACK (40 µM), then recalcified with 3.2 mM $MgCl_2$ (f.c.) and 6.3 mM $CaCl_2$ (f.c.) in Hepes buffer pH 7.45, and perfused through the flow-chamber at arterial shear rate (1000 s^{-1}). Fluorescent labels added per blood sample were $DiOC_6$ (platelet staining), AF568-annexin A5 (PS exposure), and AF647-anti-CD62P mAb (P-selectin expression). Brightfield and multicolor fluorescence images were taken every 2 minutes. Per donor, control and intervention conditions were repeated at least in duplicates. Collected time series of brightfield and fluorescence microscopic images were analysed by using pre-defined scripts.[1] The scripts were formatted in the open source package Fiji.[2]

Thrombin generation measurement and prothrombin staining

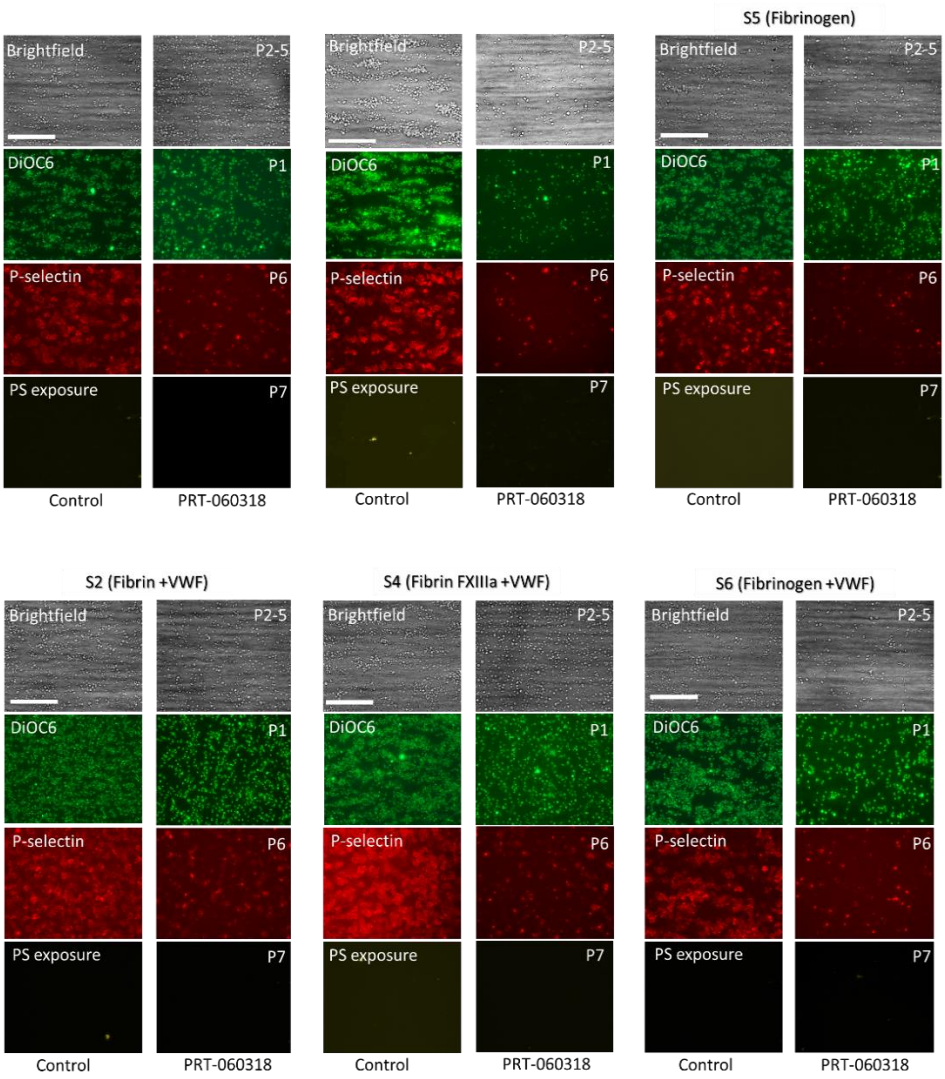
Coverslips were coated with spots of fibrin-FXIIIa (upstream) and collagen-I (downstream). Whole blood flow was perfused for 10 minutes under conditions allowing coagulation a total wall-shear rate of 1000 s^{-1}. Recalcified blood was perfused for 10 minutes, after which the thrombin-specific fluorogenic substrate Z-GGR-AMC (Z-Gly-Gly-Arg-AMC, 0.5 mM)

was added flow was continued for another 2 minutes. Fluorescence images were then taken under stasis every 2 minutes using a DAPI cube. To stain for labeled (pro)thrombin, thrombi were formed on similar spots under condition allowing coagulation, as described above. After 10 minutes of flow, the thrombi were post-perfused with Hepes buffer pH 7.45 containing $CaCl_2$, 0.3 µM active-site labeled OG488-prothrombin (cleavable by factor Xa into OG488-thrombin)[3] and TF (30 pM). Brightfield and fluorescence images were taken after rinse with Hepes buffer. Fluorescence microscopic images were analysed using pre-defined scripts.

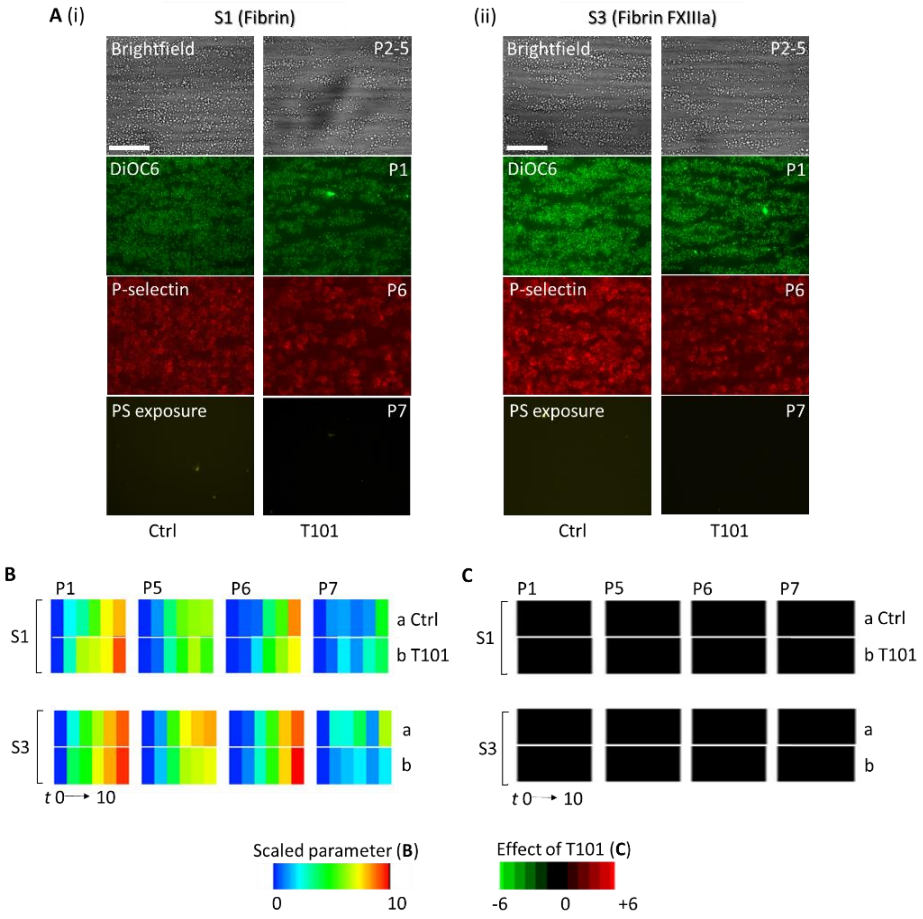
Platelet adhesion to fibrin-forming spread platelets

Washed platelets (100 x 10^9/L) were allowed to spread on glass coverslips for 10 minutes, before blocking with 1% BSA in Hepes buffer pH 7.45. Plasma (citrate-anticoagulated) and $CaCl_2$-containing medium (63 mM $CaCl_2$ and 32 mM $MgCl_2$ in Hepes buffer pH 7.45) were then co-perfused at low shear rate (250 s^{-1}) for 4 minutes, until fibrin fibres were appearing from the spread platelets. After stopping fibrin formation with heparin (1 U/mL), PPACK-anticoagulated whole blood was flowed over at 1000 s^{-1}, labeled with $DiOC_6$ (platelet staining), AF568-annexin A5 (PS exposure) and AF647-anti-CD62P mAb (P-selectin expression). During flow for 10 minutes, representative brightfield and tricolor fluorescence images, taken as overlays, were analyzed for fluorescence overlays after thresholding using the Colocalization Finder plugin of Fiji.

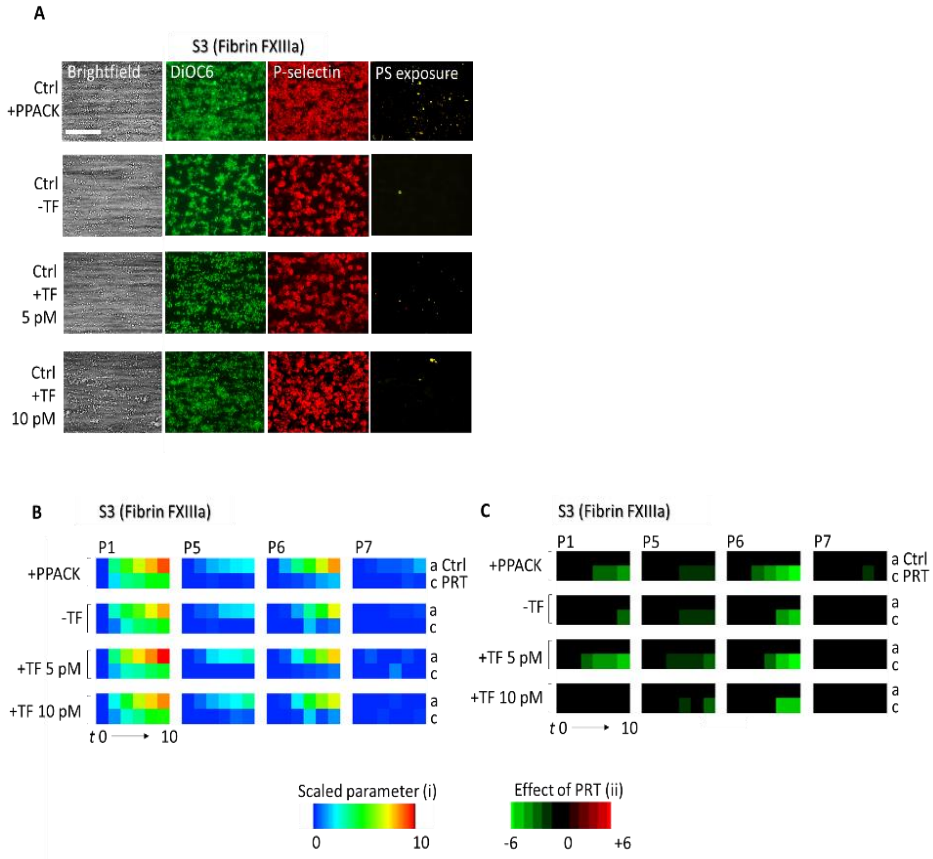
Supplemental figures



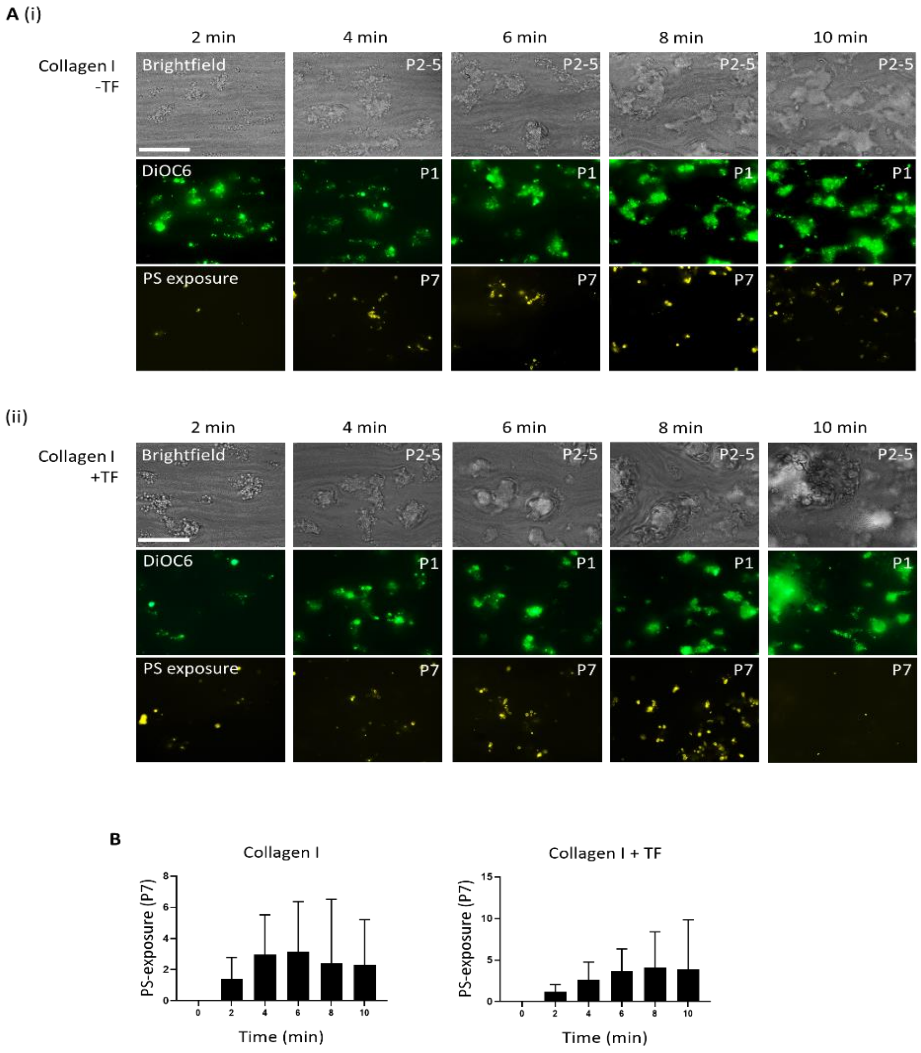
Suppl. Figure I. Representative images of bilayered thrombi formed on various fibrin and fibrinogen surfaces with/without FXIIIa or VWF. *Representative brightfield and fluorescence images (n = 3-4 donors) of fibrin (S1), fibrin + VWF-BP (S2), fibrin FXIIIa (S3), fibrin FXIIIa+ VWF-BP (S4), fibrinogen (S5) and fibrinogen + VWF-BP (S6) with or without Syk inhibitor (PRT-060318, 10 µM) after 10 minutes of flow at arterial shear rate (1000 s^{-1}). Scale bar 50 µm.*



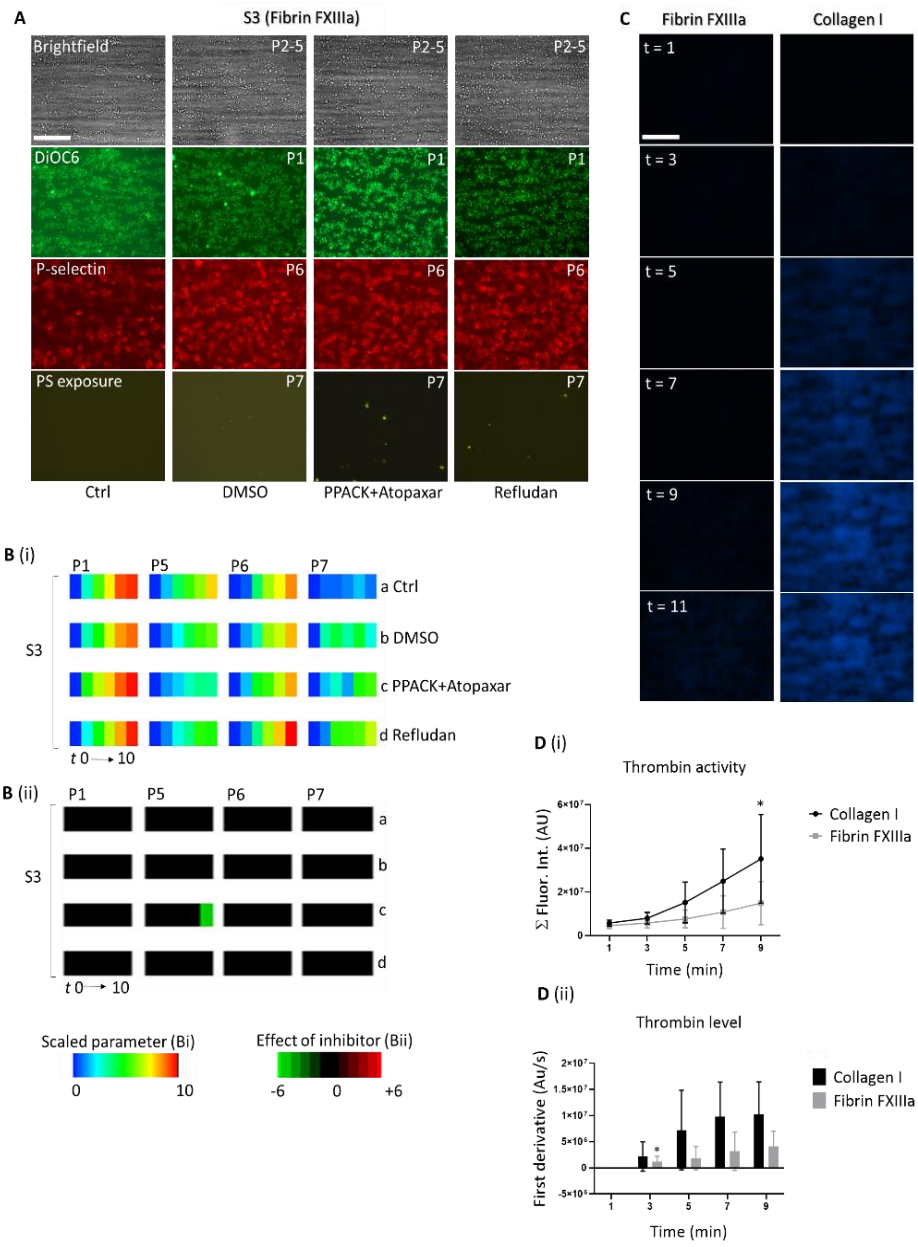
Suppl. Figure II. Effect of factor XIIIa inhibition during fibrin formation on microthrombus formation. *Spots of fibrin without (S1) or with co-coated FXIIIa (S3) were prepared, as in Methods. Inhibitor T101 (20 µM) was added during fibrin formation, where indicated. Recalcified whole blood containing labels was flowed and parameters of thrombus formation were measured.* ***A****, Representative brightfield and fluorescence images after 10 minutes of flow over S1 (fibrin) (****A, i****) or S3 (fibrin-FXIIIa) (****A, ii****).* ***B****, Heatmaps of scaled image parameters (SAC%): P1 (DiOC$_6$), P5 (bi- or multilayer coverage), P6 (P-selectin) and P7 (PS exposure), demonstrating average effect of T101 per spot. Rainbow color code of scaled values from 0 (blue) to 10 (red).* ***C****, Subtraction heatmap showing effect of T101, for spots S1 and S3, filtered for significant changes (n=3; P<0.05, two-way ANOVA); black color indicates no significance.*



Suppl. Figure III. Role of Syk in regulation of microthrombus formation on fibrin spots at (non)coagulant conditions. *Blood samples pre-incubated with vehicle (Ctrl) or Syk inhibitor (PRT, 10 µM) were flowed over spots S3 (fibrin-FXIIIa) at 1000 s^{-1}. Microthrombi formed were imaged to obtain parameters P1 (adhesion via $DiOC_6$), P5 (bi- or multilayer size), P6 (P-selectin expression), and P7 (PS exposure). Effects of Syk inhibition were assessed per blood sample and parameter.* ***A****, Representative brightfield and fluorescence images per surface in the presence of PPACK or TF with/without PRT after 10 minutes of flow.* ***B****, Mean values from n = 6 blood samples were univariate scaled to 0-10. Heatmap of scaled parameters, demonstrating mean effects of Syk inhibition. Rainbow colour code indicates scaled values between 0 (blue) and 10 (red).* ***C****, Subtraction heatmap, representing effects of Syk inhibition, filtered for relevant changes (n = 6, P<0.05, two-way ANOVA per surface and parameter). Color code represents decrease (green) or increase (red) in comparison to control. Scale bar 50 µm.*

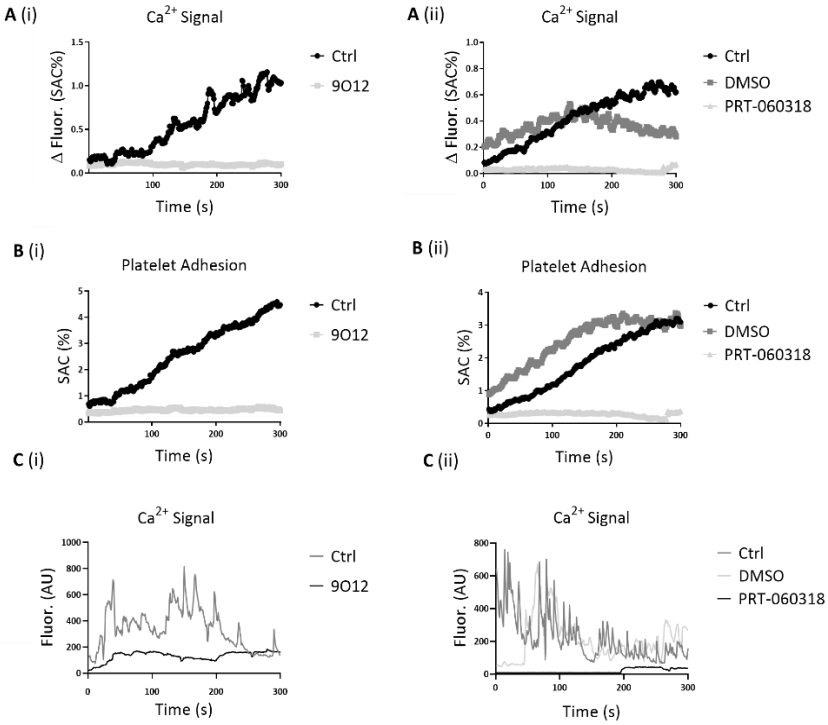


Suppl. Figure IV. Formation of multi-layered thrombi with phosphatidylserine (PS) exposure on collagen-I. *Blood was flowed over collagen-I spots at shear rate of 1000 s^{-1} under coagulant conditions; tissue factor (TF) (10 pM, f.c.) was added to the recalcification buffer, where stated. Spots were imaged to obtain parameters P1 ($DiOC_6$) and P7 (PS exposure) over a time lapse of 10 minutes.* ***A****, Representative brightfield and fluorescence images of multilayered platelet thrombi formed on collagen-I (**A, i**), and collagen-I + TF (**A, ii**). Scale bar 50 µm.* ***B****, Graphs of PS exposure (P7) on collagen-I and on collagen-I + TF. Data are means ± SD (n = 10).*

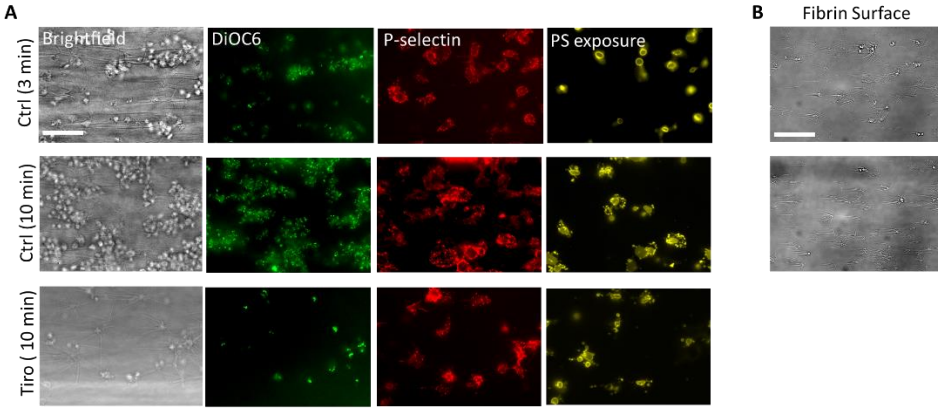


Suppl. Figure V. Low contribution of thrombin to thrombus formation on fibrin surfaces. A, B*, Recalcified, labeled whole blood was perfused over fibrin-FXIIIa spots at 1000 s^{-1} for 10 minutes, as for Figure 2. Blood samples were pre-treated with DMSO vehicle, lepirudin (10 µg/mL) or atopaxar (5 µg/mL) + PPACK (40 µM),*

as indicated. ***A****, Representative images after 10 minutes of flow. Scale bar 50 µm.* ***B****, Microthrombi were analysed for parameters P1 ($DiOC_6$), P2 (bi- and multilayer score), P3 (P-selectin expression) and P4 (PS exposure) as a function of time. Scaled heatmaps of time-dependent changes, presented per parameter (**B**, **i**); and presented as subtraction heatmaps showing intervention effects after filtering for significance (green, P<0.05 two-way ANOVA; black, not significant) (**B**, **ii**).* ***C-D****, Recalcified whole blood containing fluorogenic thrombin substrate Z-GGR-AMC (0.5 mM) was perfused over fibrin-FXIIIa and collagen-I spots at 1500 s^{-1} for 10 minutes. Subsequently, thrombin generation was measured under stasis from fluorescence accumulation per spot.* ***C****, Representative images taken every 2 minutes starting from 1 minute.* ***D****, Graph of integrated fluorescence intensity (**D**, **i**) and of first derivative indicating thrombin activity (**D**, **ii**). Thrombin generation comparing collagen and fibrin was measured for each time point. Means ± SD (n = 4, *P<0.1, Two-way ANOVA).*



Suppl. Figure VI. Inhibition of GPVI or Syk affecting fibrin-induced Ca^{2+} fluxes in platelets under flow. *Blood samples supplemented with autologous Fluo-4-loaded platelets were pre-incubated with vehicle (Ctrl), GPVI blocking antibody 9O12 or Syk inhibitor PRT-060318, and flowed over S3 spots (fibrin-FXIIIa). Fluorescence from adhered platelets was continuously recorded by confocal microscopy for 5 minutes.* ***A-B****, Movies analysed for fluorescence intensity above threshold fluorescence (Fluor.), i.e. representing elevated $[Ca^{2+}]_i$ above resting level (****A i*** *and* ***ii****), and for platelet adhesion as SAC% (****B i*** *and* ***ii****). Also shown are time traces of fluorescence changes in representative single platelets (****C i*** *and* ***ii****).*



C

Control (overlap R)

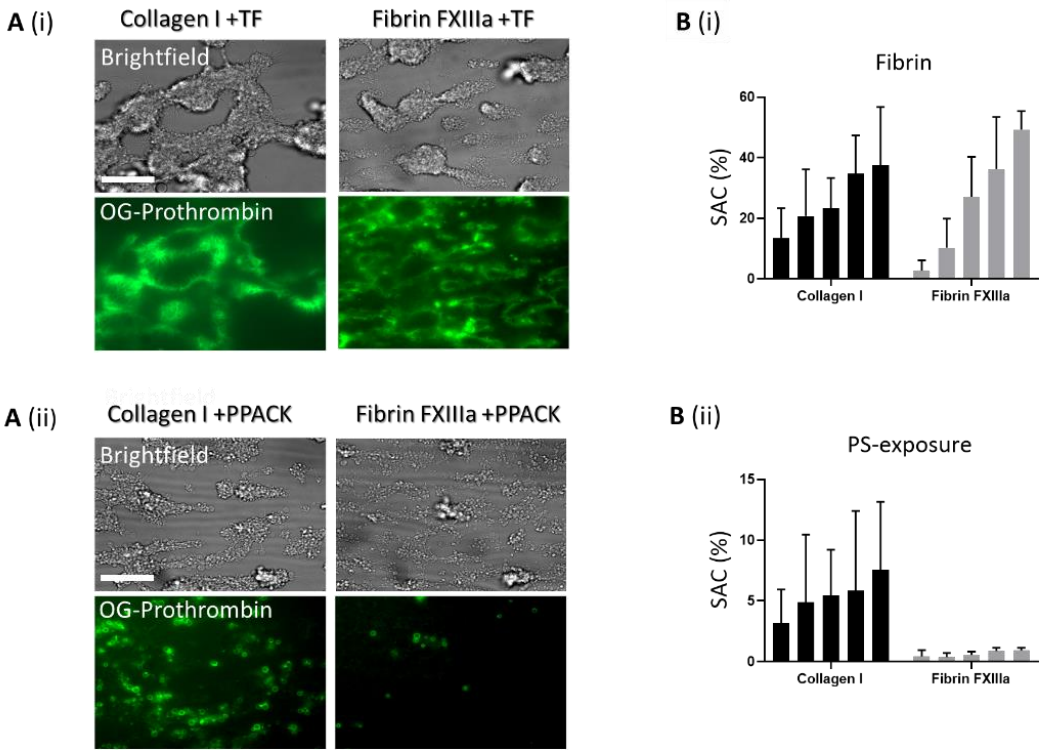
	Brightfield	PS expos.	P-selectin	DiOC6	
Brightfield	1.00	0.97	0.94	0.97	3 min
PS exposure	0.95	1.00	0.94	0.96	
P-selectin	0.87	0.89	1.00	0.97	
DiOC6	0.91	0.90	0.87	1.00	
	10 min				

Tirofiban (overlap R)

	Brightfield	PS expos.	P-selectin	DiOC6	
Brightfield	1.00	0.99	0.97	0.99	3 min
PS exposure	0.98	1.00	0.97	0.99	
P-selectin	0.96	0.97	1.00	0.97	
DiOC6	0.99	0.99	0.97	1.00	
	10 min				

Suppl. Figure VII. Platelet adhesion and aggregation preferentially to immobilised platelets rather than to fibrin. *Washed platelets were allowed to spread to glass coverslips, which were blocked with BSA-containing Hepes buffer pH 7.45, and then perfused with recalcified plasma to stimulate radial fibrin formation from the immobilized platelets. Subsequently, recalcified labeled ($DiOC_6$, anti-P-selectin and annexin A5) blood was flowed at shear rate 1000 s^{-1}, as for Figure 2. Integrin antagonist tirofiban (1 µg/mL) was present, where indicated. After 3 or 10 minutes, microscopic overlay images were taken from the*

*same field. **A**, Representative brightfield and fluorescence images. **B**, Brightfield images of fibrin formed by the immobilized platelets. **C**, Matrix of overlap coefficients R of thresholded images, indicating similarity of positive pixels, for control and tirofiban conditions (n = 3). Note high pixel overlap of fluorescence from flowed $DiOC_6$ platelets with the P-selectin staining from the immobilized platelets; overlap further increased in the presence of tirofiban.*



Suppl. Figure VIII. Binding of cleaved OG488-prothrombin to fibrin in thrombi. *Recalcified blood was perfused over fibrin-factor XIIA (FXIIIa) and collagen-I spots under coagulant (30 pM tissue factor, TF) or non-coagulant (20 µM PPACK) conditions for 6 minutes at 1000 s^{-1}. The formed platelet thrombi were stained with OG488-prothrombin (green) in the presence of $CaCl_2$ and TF or PPACK, respectively. **A**, Representative brightfield and OG488-(pro)thrombin fluorescence images from fibrin FXIIIa (**A**, **i**) and collagen-I (**A**, **ii**) spots. Scale bars 50 µm. Note that with TF present the cleaved OG488-thrombin binds to fibrin-*

containing thrombi, whereas in the absence of coagulation (PPACK) the non-cleaved OG488-prothrombin only binds to balloon-shaped, PS-exposing platelets. ***Bi-ii****, Graphs showing time-dependent increases in coverage of label in the presence of TF (OG488-thrombin) or PPACK (OG488-prothrombin). Mean + SD (n=3, P<0.05, two-way ANOVA).*

Supplemental tables

Major resource table

Antibodies

Target antigen	Vendor or Source	Catalog #	Working concentration	Lot #	Persistent ID / URL
Polyclonal anti-VWF-FITC Ab rabbit	DAKO (Michigan, MI, USA)	N.a.	20 μg/mL	N.a.	N.a.
AF488 donkey anti-rabbit IgG	Invitrogen ThermoFisher (Eindhoven, NL)	R37118	20 μg/mL	N.a.	https://www.thermofisher.com
mAb RAG35	Dr. J. van Mourik (CLB, Transfusion Service, Amsterdam, NL)	N.a.	20 μg/mL	N.a.	N.a.
Anti-GPVI Fab 9O12	Dr. M. Jandrot-Perrus (INSERM, University Paris Diderot, Paris, F)	N.a.	50 μg/mL	N.a.	N.a.
AF647-anti-human CD62P mAb	Biolegend (London, UK)	304916	0.5 μg/mL	N.a.	https://www.biolegend.com/en-us
AF568-annexin A5	ThermoFisher (Eindhoven, NL)	A13201	0.5 μg/mL	N.a.	https://www.thermofisher.com

Other

Description	Source / Repository	Persistent ID / URL
Membrane probe $DiOC_6$	ThermoFisher (Eindhoven, NL)	https://www.thermofisher.com/order/catalog/product
Fluo-4 acetoxy methyl ester	ThermoFisher (Eindhoven, NL)	https://www.thermofisher.com/order/catalog/product/F14201
Pluronic	Invitrogen (Carlsbad CA, USA)	https://www.thermofisher.com/order/catalog/product/P3000MP
D-Phe-Pro-Arg chloromethyl ketone (PPACK)	Santa Cruz Biotechnology (Santa Cruz, CA, USA)	https://www.scbt.com/p/ppack-dihydrochloride
Human fibrinogen	Enzyme Research Laboratories (Swansea, UK)	https://www.enzymeresearch.co.uk/product/human-fibrinogen--von-willebrand-factor-and-plasminogen-depleted
Human α-thrombin	Stago (Saint-Ouen-l'Aumone, F)	N.a.
Collagen-I Horm	Nycomed (Hoofddorp, NL	https://www.takeda.com/de-at/hcps/diagnostika
Human collagen-III	Southern Biotechnology (Birmingham, AL, USA)	https://www.southernbiotech.com
VWF-BP	CambCol (Cambridge, UK)	http://www.cambcollabs.com
Tissue factor (rTF; Innovin)	Siemens (Erlangen, D)	https://www.yumpu.com/es

Table ccontinuing on next page

Description	Source / Repository	Persistent ID / URL
Syk inhibitor PRT-060318	Bio-Connect, (Huissen, NL)	https://www.bio-connect.nl/chemicals/prt-060318
Z-Gly-Gly-Arg-AMC	Bio-Connect (Huissen, NL)	https://www.bio-connect.nl/chemicals/z-gly-gly-arg-amc-acetate
Atopaxar	Axon Medchem (Groningen, NL)	https://www.axonmedchem.com/product/2030
Lepirudin	Abcam (Cambridge, UK)	https://www.abcam.com/recombinant-hirudin-protein-ab73660
FXIIIa inhibitor T101	Zedira (Darmstadt, D)	https://zedira.com/Blood-coagulation/FXIII-Inhibitors
OG488-prothrombin	Gift from Dr. P. Bock (Vanderbilt USM, Nashville, TE, USA	N.a. Ref. [3]

Supplemental references

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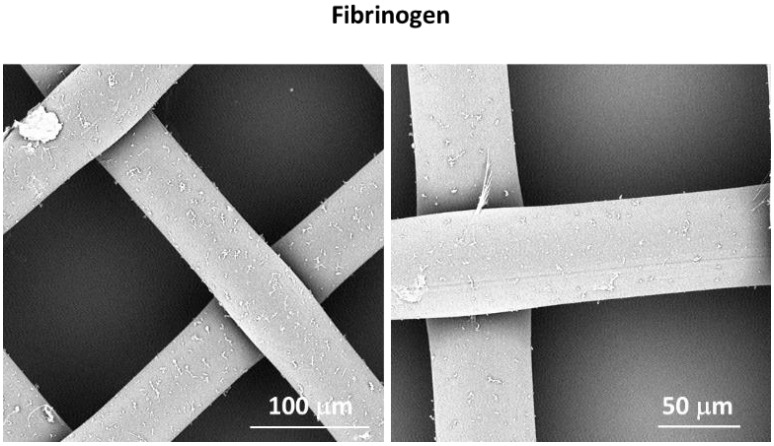
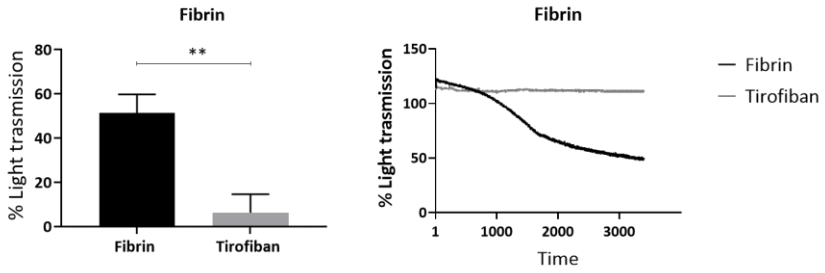
3. Berny MA, Munnix IC, Auger JM, Schols SE, Cosemans JM, Panizzi P, Bock PE, Watson SP, McCarty OJ and Heemskerk JW. Spatial distribution of factor Xa, thrombin, and fibrin(ogen) on thrombi at venous shear. *Plos One*. 2010;5:e10415.

Appendix



Appendix Figure 1. Representative images of fibrinogen. *Scanning electron microscopy (SEM) images of immobilised fibrinogen spot coated on a SEFAR filter.*



Appendix Figure 2. Inhition of intregrin αIIbβ3 in fibrin-activated platelets. *Platelet in suspension were treated with intregrin αIIbβ3 inhibitor, tirofiban (5 µg/mL) for 10 minutes, before stimulation with fibrin. Platelet aggregation was monitored with conventional light transmission aggregometry.* ***A****, Quatitation of maximal aggregation upon stimulation with fibrin.* ***B****, Representative aggregation traces. Mean + SD (n = 3), **P<0.005 (two-tailed Student's unpaired t-test).*

Chapter 5

Comparison of the GPVI inhibitors losartan and honokiol

M-B. Onselaer, M. Nagy, C. Pallini, J.A. Pike, G. Perrella, L.G. Quintanilla, J.A. Eble, N.S. Poulter, J.W.M. Heemskerk & S.P. Watson

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I contributed to the phosphorylation work, M-B.O. designed and performed research, analysed and interpreted data and wrote the manuscript; M.N., C.P., and J.A.P. performed experiments, and interpreted data; L.G.C. and N.S.P. performed experiments; J.A.E. purified rhodocytin and edited the manuscript; J.W.M.H. interpreted data and edited the manuscript; S.P.W. supervised research, analysed and interpreted data, wrote the manuscript.

Abstract

Losartan and honokiol are small molecules which have been described to inhibit aggregation of platelets by collagen. Losartan has been proposed to block clustering of GPVI but not to affect binding of collagen. Honokiol has been reported to bind directly to GPVI but only at a concentration that is three orders of magnitude higher than that needed for inhibition of aggregation. The mechanism of action of both inhibitors is so far unclear. In the present study, we confirm the inhibitory effects of both agents on platelet aggregation by collagen and show that both also block the aggregation induced by the activation of CLEC-2 or the low affinity immune receptor FcγRIIa at similar concentrations. For GPVI and CLEC-2, this inhibition is associated with a reduction in protein tyrosine phosphorylation of multiple proteins including Syk. In contrast, on a collagen surface, spreading of platelets and clustering of GPVI (measured by single molecule localisation microscopy) was not altered by losartan or honokiol. Furthermore, in flow whole-blood, both inhibitors suppressed the formation of multi-layered platelet thrombi at arteriolar shear rate at concentrations that hardly affect collagen-induced platelet aggregation in platelet-rich plasma. Together, these results demonstrate that losartan and honokiol have multiple effects on platelets which should be considered in the use of these compounds as anti-platelet agents.

Introduction

Haemostasis is a finely regulated process triggered after vessel wall injury serving to curtail blood loss and to restore vascular integrity. One of the key components of haemostasis are platelets, which rapidly adhere and aggregate at sites of a lesion.[1] Glycoprotein VI (GPVI), a member of the immunoglobulin superfamily, is the major signalling receptor for collagen and fibrin.[2-5] GPVI is expressed on the surface membrane in complex with the FcRγ chain which contains an immunoreceptor tyrosine activation motif (ITAM) characterized by two YxxL/I sequences (where x is any amino acid) separated by 12 amino acids. Src kinases associated with the cytoplasmic tail of GPVI initiate phosphorylation of two conserved tyrosines in the ITAM of the FcRγ chain.[6,7] This leads to the recruitment and activation of Syk via its SH2 domains, and triggering of a phosphorylation complex consisting of adapter and signalling proteins that culminates in Ca^{2+} mobilisation and platelet activation.[8,9]

The C-type lectin-like receptor, CLEC-2, has a single copy of a YxxL sequence known as a hemITAM. Phosphorylation of two CLEC-2 receptors leads to binding of Syk and initiation of a signalling cascade that is similar to that driven by the GPVI-FcRγ–chain.[10-12] To date, podoplanin is the only established endogenous ligand for CLEC-2. Podoplanin is expressed on the surface of a variety of cells including epithelial and stromal cells but is absent from vascular endothelial cells. Although its function in haemostasis is not clear, there is evidence that CLEC-2 play a pivotal role in arterial and venous thrombosis.[13,14]

Losartan, an angiotensin II receptor antagonist, has been proposed to inhibit clustering but not binding of GPVI to collagen,[15,16] leading to inhibition of platelet activation in vitro and reduced platelet accumulation after carotid injury in mice.[17–20] Honokiol is a natural bioactive molecule isolated from *Magnolia* species, which is used in traditional Chinese medicine. Honokiol is a multifunctional compound with many potential therapeutic properties, including antioxidant, anti-inflammatory, anti-cancer, anti-depressant and anti-neurodegeneration activities.[21–23]

Honokiol also has anti-thrombotic effect, and has been shown to bind to GPVI at concentrations that are three orders of magnitude higher than those required for inhibition of platelet aggregation, suggesting an alternative mechanism of inhibition.[24,25] In the present study, we have further interrogated the mechanism of action for both inhibitors.

Material and methods

Reagents

Horm collagen and collagen diluent were purchased from Nycomed (Munich, Germany). CRP (ten glycine-proline-hydroxproline [GPO] repeats) was crosslinked as described.[26] Rhodocytin was purified in the Eble lab (University of Münster, Germany) from the crude venom of Calloselasma rhodostoma. The mouse monoclonal antibodies (mAbs) anti-phosphotyrosine clone 4G10 (05–321) and rabbit polyclonal anti-FcR γ-chain (06–727) were purchased from Merck Millipore (Watford, UK). The rabbit polyclonal antibody anti-Syk (sc-1077), the mouse mAbs anti-Syk 4D10 (sc-1240) and anti-FcR γ-chain (sc-390222) were purchased from Santa Cruz (Wembley, UK). All other reagents including losartan, honokiol and the anti-mouse IgG (Fc specific) $F(ab')_2$ fragment antibody were purchased from Sigma-Aldrich (Poole, UK), or came from described sources.[3] Losartan was dissolved in water and honokiol in DMSO. The mouse monoclonal mAb IV.3 against the low affinity immune receptor FcγRIIA was purified from the hybridoma obtained from the American Type Culture Collection. 1G5-Fab against Pan-GPVI was gift from Elizabeth Gardiner (Australian National University, Canberra, Australia).

Platelet isolation

Venous blood was taken from healthy volunteer using 3.8% (v/v) sodium citrate (1:9) as the anti-coagulant with informed consent according to the guidelines of the local ethics committee (ERN_11-0175). All steps of this study complied with the ethical principles according to the Declaration of Helsinki. Acid citrate dextrose (ACD, 1:10) was added to the blood. Platelet-rich plasma (PRP) was obtained by centrifugation at 200 g for 20 minutes at room

temperature. Washed platelets were obtained by centrifugation at 1000 g for 10 minutes at room temperature using prostacyclin (2.8 μM) and resuspended in modified Tyrode's-HEPES buffer (134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES, 5 mM glucose, 1 mM $MgCl_2$; pH7.3) Washed platelets were used at 2×10^7/mL for static adhesion or 5×10^8/mL for other studies.

Platelet aggregation

Washed platelets at 5×10^8/ml were pre-treated for 5 minutes with different concentrations of losartan, honokiol or solvent controls prior to stimulation by collagen, rhodocytin, thrombin or mAb IV.3 crosslinked with $F(ab')_2$. Light transmission was recorded at 37°C with stirring (1200 rpm) in an aggregometer (Chrono-Log Stago, Havertown, PA, USA). ATP secretion was monitored in washed platelets in parallel with platelet aggregation by adding firefly luciferase and luciferin (2 μM) and comparing the luminescence generated by platelet ATP release with an ATP standard.

Platelet spreading

Glass coverslips were coated in the presence of 10 μg/mL of collagen or fibrin generated as described previously.[5] Following washing with PBS, the coverslips were blocked with 5 mg/mL heat-inactivated bovine serum albumin (BSA) in PBS for 60 minutes. Washed platelets 2×10^7/mL were incubated with honokiol (25 μM), losartan (25 μM) or solvent controls prior to be allowed to spread for 30 or 45 minutes, for human or mouse platelets respectively, at 37°C. The cells were then washed with PBS followed by fixation with paraformaldehyde (3.7%) for 10 minutes. For actin staining, the platelets were permeabilised with 0.1% Triton X-100 for 5 minutes and stained with Alexa-488-labelled phalloidin for 45 minutes in the dark. Platelets were imaged on a Zeiss Axiovert 200 M microscope. Fluorescence from platelets was analysed using ImageJ (NIH, Bethesda, USA). In each independent experiment, 5 random fields of view per condition were analysed, with at least 100 platelets in total per condition.

Protein phosphorylation study

In presence of eptifibatide (9 µM), washed platelets 5×10^8/mL were pre-treated for 5 min with honokiol (25 µM), losartan (25 µM) or solvent controls prior to activation by collagen (3 µg/mL), rhodocytin (150 µM) or thrombin (1 U/mL) with or without fibrinogen (200 µg/mL) at 37°C with stirring (1200 rpm) in an aggregometer for 60 seconds. Activation was terminated with 2x ice-cold lysis buffer (300 mM NaCl, 20 mM Tris, 2 mM EGTA, 2 mM EDTA, and 2% IGEPAL CA630 [NP-40 equivalent], pH 7.4, plus 2.0 mM Na_3VO_4, 100 mg/mL AEBSF [4-{2-aminoethyl} benzenesulfonyl fluoride hydrochloride], 5 mg/mL leupeptin, 5 mg/mL aprotinin, and 0.5 mg/mL pepstatin). Whole cell lysates (WCLs) were prepared by boiling a sample of lysate with sodium dodecyl sulfate (SDS) sample buffer. Syk was immunoprecipitated with 2 µg of Syk antibody (4D10) and incubated protein A-Sepharose beads overnight at 4°C. The beads were then washed, and proteins were eluted by boiling in SDS sample buffer. Immunoprecipitates (IPs) and WCLs were separated by SDS-polyacrylamide gel electrophoresis, electrotransferred, and western blotted. All primary antibodies were used diluted at 1/1000 in TBS-Tween-BSA 5% for overnight at 4°C. The secondary antibodies were used diluted at 1/10000 in TBS-Tween for 1 hour at room temperature. Western blots were imaged with autoradiographic film.

Cell/NFAT assay

The following constructs and plasmids were used for GPVI transfections into cells for NFAT-luciferase assays as previously described.[27] Human GPVI sub-cloned into pcDNA3 with a C-terminal Myc tag and human FcRγ (untagged) in pEF6 were generated. The nuclear factor of activated T cells (NFAT)-luciferase reporter containing three copies of the distal NFAT site from the IL-2 promoter as described previously.[28] In brief, indicated amounts of DNA of each construct and 15 µg of NFAT-luciferase reporter construct were transfected by electroporation at 350 V and 500 µF microfarads into 2×10^7 DT40 cells. The transfected cells were incubated with losartan at 25 µM or 250 µM and stimulated by collagen at 10 µg/mL for 6 hours at 37°C. Luciferase activity was

measured with a Centro LB960 microplate luminometer (Berthold Technologies, Germany).

Platelet spreading and staining for STORM imaging

For STORM imaging, 35 mm #1.5 (0.17 mm) glass bottomed MatTek dishes (MatTek Corporation, USA) were coated with 10 µg/mL Horm collagen diluted in manufacturer-supplied diluent or in 10 µg/mL cross-linked collagen related peptide (CRP-XL) using phosphate-buffered saline (PBS) and stored overnight at 4°C. Dishes were blocked with 5 mg/mL BSA for 1 hour at room temperature then washed with PBS. Washed and rested human platelets, diluted to 2×10^7/mL in modified Tyrode's buffer were incubated with 2 µg/mL 1G5-Fab against Pan-GPVI for 5 minutes at 37°C. The labelled platelets were then incubated with 25 µM losartan or 25 µM honokiol or vehicle (PBS for losartan or DMSO for honokiol) for another 5 minutes at 37°C before being transfered onto the coated MatTek dishes. Platelets were allowed to spread for 45 minutes at 37°C and rinsed once with PBS to remove unbound platelets. Adhered platelets were fixed for 10 minutes with 10% neutral buffered formalin solution (Sigma, Poole, UK), followed by 5 minutes permeabilisation with 0.1% Triton X-100 in PBS. After PBS washes, the platelets were blocked for 1 hour at room temperature with 1% BSA + 2% goat serum (in PBS). 1G5-Fab-labelled platelets were secondary labelled with anti-mouse-Alexa647 antibody and stained for actin with Alexa488-phalloidin (both from ThermoFisherScientific; 1:300 dilution in block buffer) for 1 hour at room temperature. Platelet samples were washed at least three times with PBS prior to imaging.

STORM Imaging

Single-molecule images of platelet GPVI were acquired using a 100 × 1.49 N.A. TIRF objective lens on a Nikon N-STORM system in TIRF and dSTORM mode. The system was equipped with a Ti-E stand with Perfect Focus, Agilent Ultra High Power Dual Output Laser bed (containing a 170-mW 647-nm laser and a 20-mW 405 laser) and an Andor IXON Ultra 897 EMCCD camera. Fluorophore

blinking was achieved by imaging the samples in a PBS based buffer containing 100 mM MEA-HCl, 50 µg/mL glucose oxidase and 1 µg/mL catalase as detailed in Metcalf et al.[29] Single colour (Alexa647) imaging was performed using the N-STORM emission cube with reactivation of fluorophore blinking achieved by increasing the 405 nm laser power by 5% every 30 seconds. For each image, 20,000 frames were acquired using the Nikon NIS Elements v4.5 software with an exposure time of 9.2 ms, gain 300 and conversion gain 3. Image reconstruction to obtain localisation co-ordinates for each identified fluorescence blink was undertaken with the Nikon STORM analysis module 3.2 using drift correction and Gaussian rendering. For each condition, 5 different fields of view (FOV) from 3 independent experiments were captured. Only data points with a photon count>500 were included in the cluster analysis. For the analysis of dSTORM data, localised data points within each FOV were grouped into clusters using density-based clustering of applications with noise (DBSCAN). The clustering was executed using the open-source software KNIME and the R package 'dbscan' (workflow available on request).[30] The radius of local neighbourhood was set to 40 nm and 50 nm, and the minimum number of directly reachable points was set to 15 and 10, for CRP and collagen respectively. Cluster area was calculated by placing a circle of radius 30 nm over every detection in a cluster and calculating the union of these circles. This was estimated using a grid with pixel size 5 nm and image based dilation. The analysis was performed on whole FOVs and the quantitative cluster data was outputted as a spreadsheet. Graphs and statistics of the clustering data were calculated in Graphpad Prism 7.

Whole blood thrombus formation

Whole blood thrombus formation was assessed under flow conditions as described elsewhere.[31] In brief, glass coverslips were coated with 2 µL of 50 µg/mL collagen type I and mounted onto a transparent parallel-plate flow chamber. Blood samples treated with honokiol or losartan or vehicle were re-calcified with 3.75 mM $MgCl_2$ and 7.5 mM $CaCl_2$ in the presence of 40 µM active-site thrombin inhibitor PPACK, prior to experimentation. Recalcified

blood samples were perfused for 6 minutes at wall shear rate of 1000 s^{-1} over the microspot surface. Representative phase-contrast microscopic images were captured using a fast line-scanning Zeiss LSM7 microscope equipped with 63x oil-immersion objective. Images were analysed using Fiji software.[32] Four outcome parameters were assessed: platelet surface area coverage, thrombus morphological score (0–5), thrombus contraction score (0–3) and platelet multilayer score (0–3) as previously explained.[31,33] Scoring was done by visual inspection of brightfield images based on a pre-defined score system. Morphological score indicates the aggregate formation by using a 5-point scale: 0: no platelet adhesion; 1: single platelet (>15) adhesion, 2: platelet monolayer; 3: small aggregates; 4: medium size aggregates; 5: large aggregates respectively. Thrombus contractility is appreciated based on a 3-point scale, where 0: no aggregate formation, 1: loose aggregates; 2: aggregates started to contract; 3: tightly packed, dense aggregates respectively. Multilayer score refers to thrombus volume on a 3-point scale, where 0: monolayer of platelets; 1: 2 layers of platelets; 2: multiple layers of platelets; 3: large, really high thrombi respectively.

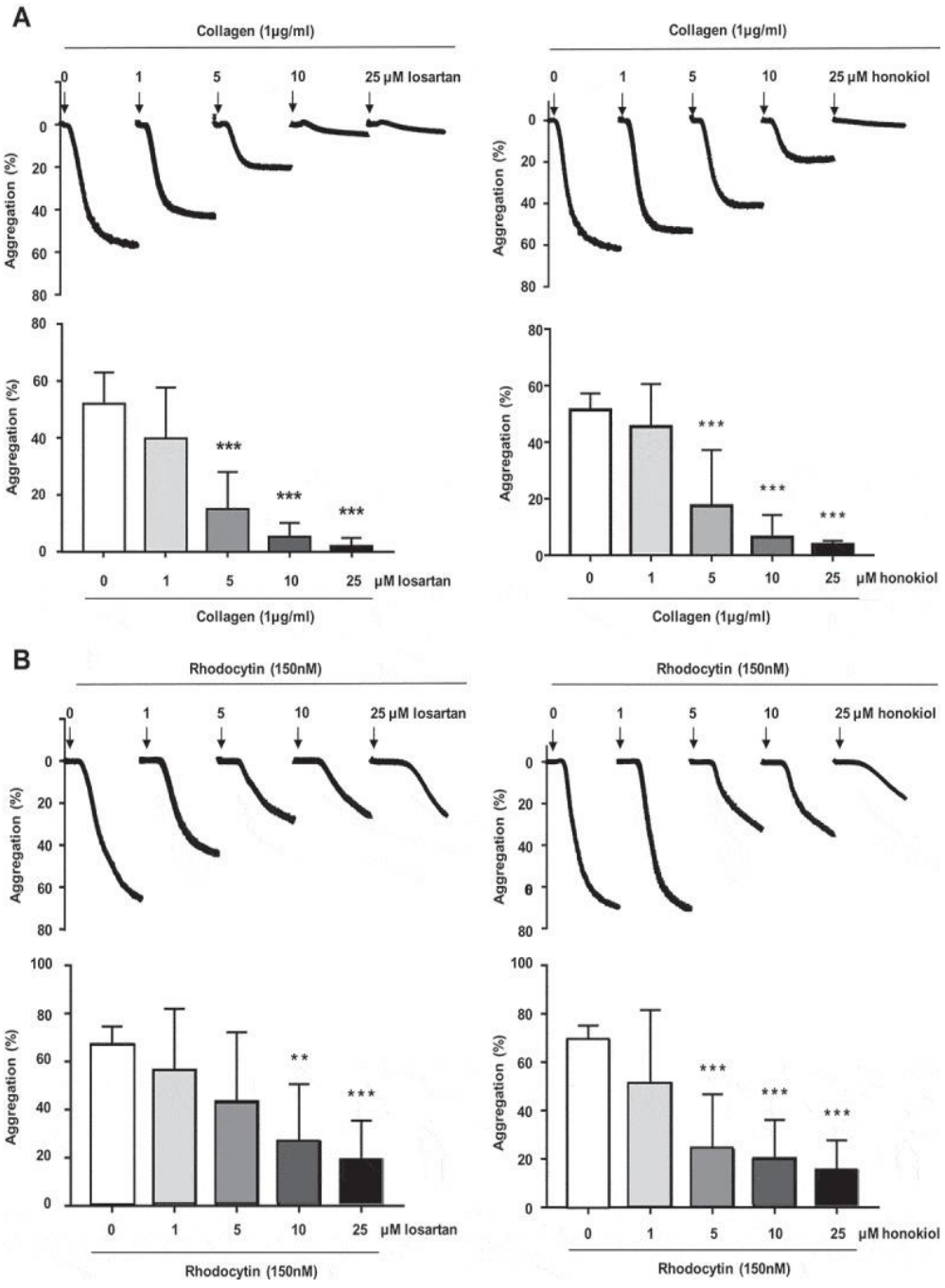
Data analysis

Statistical analysis was realised by ANOVA with Tukey post-test. A *P* value of <0.05 was considered to be significant.

Results

Comparison of losartan and honokiol in platelet aggregation

In a previous study, platelet aggregation induced by 10 µg/mL of collagen was found to be inhibited by losartan with an IC_{50} of 6.3 µM[21] and by honokiol with an IC_{50} of 0.6 µM.[26] In Figure 1, we demonstrate, by comparing the two compounds, that losartan and honokiol dose-dependently inhibited aggregation of washed platelets induced by 1 µg/mL collagen with an IC_{50} of 3.7 µM for losartan and of 4.6 µM for honokiol. At these concentrations, neither inhibitor had an effect on platelet activation by thrombin or the thromboxane mimetic U46619 (data not shown).


A
Collagen (1μg/ml)
0
1
5
10
25 μM losartan
Aggregation (%)
0
20
40
60
80
***
***
***
0
1
5
10
25 μM losartan
Collagen (1μg/ml)
Collagen (1μg/ml)
0
1
5
10
25 μM honokiol
Aggregation (%)
***
***
***
0
1
5
10
25 μM honokiol
Collagen (1μg/ml)
B
Rhodocytin (150nM)
0
1
5
10
25 μM losartan
Aggregation (%)
100
**
***
0
1
5
10
25 μM losartan
Rhodocytin (150nM)
Rhodocytin (150nM)
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1
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25 μM honokiol
Aggregation (%)
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1
5
10
25 μM honokiol
Rhodocytin (150nM)

Figure 1. Losartan and honokiol dose-dependently reduce aggregation of washed platelets induced by collagen or rhodocytin. *Washed platelets were pretreated with different concentrations of losartan or honokiol for 3 minutes before stimulation with collagen (**A**) 1 µg/mL or rhodocytin (**B**) 150 nM. Bar graphs represent results from a total of 5 independent experiments and results are shown as means ± SD **P <0.01, ***P <0.001.*

On the other hand, both losartan and honokiol delayed and reduced aggregation to the CLEC-2 agonist rhodocytin with IC_{50} values of 3.5 µM and 2.1 µM, respectively (Figure 1) and furthermore inhibited the platelet response to the low affinity immune receptor FcγRIIA over the same concentration range (Suppl. Figure 1).

Collagen and rhodocytin induce a similar pattern of increase in tyrosine phosphorylation, which was abrogated by losartan or honokiol at approximately 10x their IC50 values (Figure 2). In addition, both inhibitors blocked tyrosine phosphorylation of the tyrosine kinase Syk (Figure 2). Losartan and Honokiol were less potent in the presence of plasma, due to protein binding, requiring a concentration of 250 µM to achieve inhibition of aggregation (Suppl. Figure 2).[32] At this concentration, losartan inhibited NFAT activation by GPVI and CLEC-2 in a transfected cell line model in the presence of serum (Figure 3A), but did not have an effect on constitutive signalling (i.e. ligand-independent signalling) of either receptor (Figure 3B). Honokiol could not be tested in the cell line model due to the effects of vehicle DMSO on NFAT activity.

Losartan and honokiol do not block platelet spreading or ligand binding to GPVI

Losartan and honokiol (both used at 25 µM in Tyrode's buffer) did not have significant effects on the adhesion or spreading of platelets on collagen (Figure 4). Similar results were observed at 100 µM (data not shown). Further, neither compound altered clustering of GPVI by CRP (Figure 5) or by collagen (not shown) as measured by single molecule localisation microscopy (dSTORM).

This result is consistent with the lack of interference of either compound with GPVI-collagen binding, in an ELISA competitive binding assay using recombinant GPVI (Suppl. Figure 3).[21]

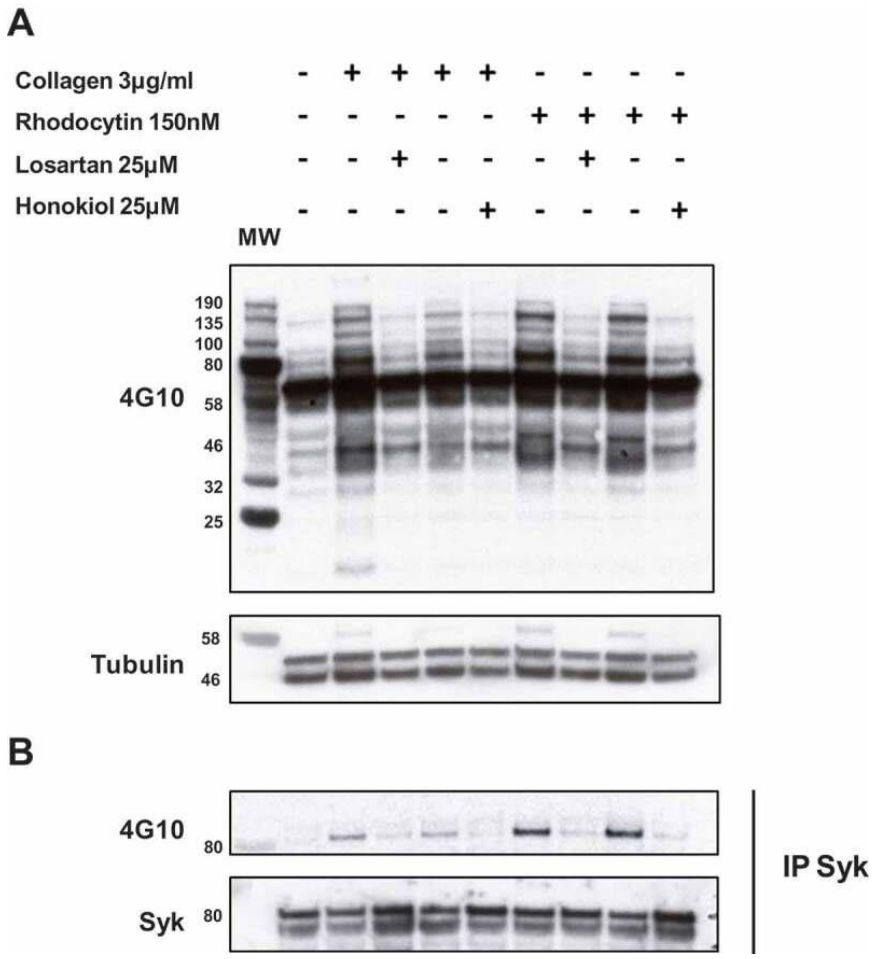


Figure 2. Losartan and honokiol inhibit platelet protein tyrosine phosphorylation, including Syk, induced by collagen or rhodocytin. *Washed platelets were pre-treated with 25 µM of losartan or honokiol for 3 minutes before stimulation with collagen 3 µg/mL or rhodocytin 150 nM. Stimulations were stopped with addition of lysis buffer.* ***A,*** *whole-cell lysate (WCL) and* ***B,*** *immunoprecipitate (IP) of Syk. WCL and IP were*

separated by SDS-polyacrylamide gel electrophoresis and Western blotted for pTyr and Syk, respectively. The first lane shows the molecular weight markers (MW). The results are representative of 3 independent experiments.

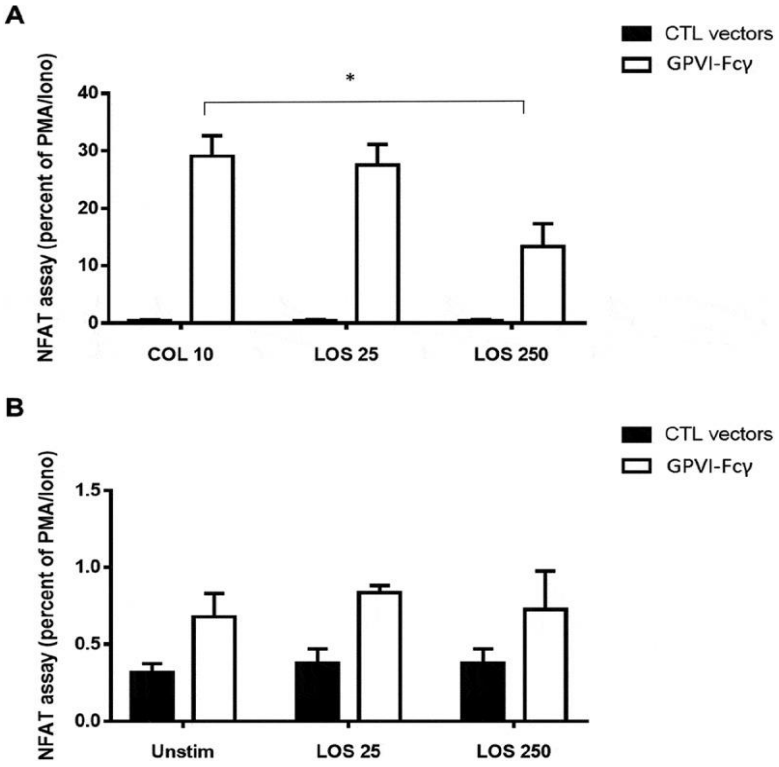


Figure 3. Losartan reduces signalling induced by collagen in transfected DT40 cells. *DT40 B-cells were transfected with a NFAT-luciferase reporter construct, a β-galactosidase construct to control for transfection efficiency, and two GPVI and FcRγ-chain(Fcγ) expression constructs or empty control (CTL) vectors. Sixteen hours post-transfection, expression of GPVI was confirmed by flow cytometry (data not shown). The transfected cells were pre-treated with losartan (LOS) at 25 µM or 250 µM. They were either (**A**) stimulated with collagen (10 µg/mL) or PMA (50 ng/mL) plus ionomycin (1 µM) or (**B**) unstimulated. Six hours later, cells were lysed and assayed for luciferase and β-galactosidase. Luciferase data were normalized for β-galactosidase expression. Error bars represent the SEM from three independent experiments. * P <0.001.*

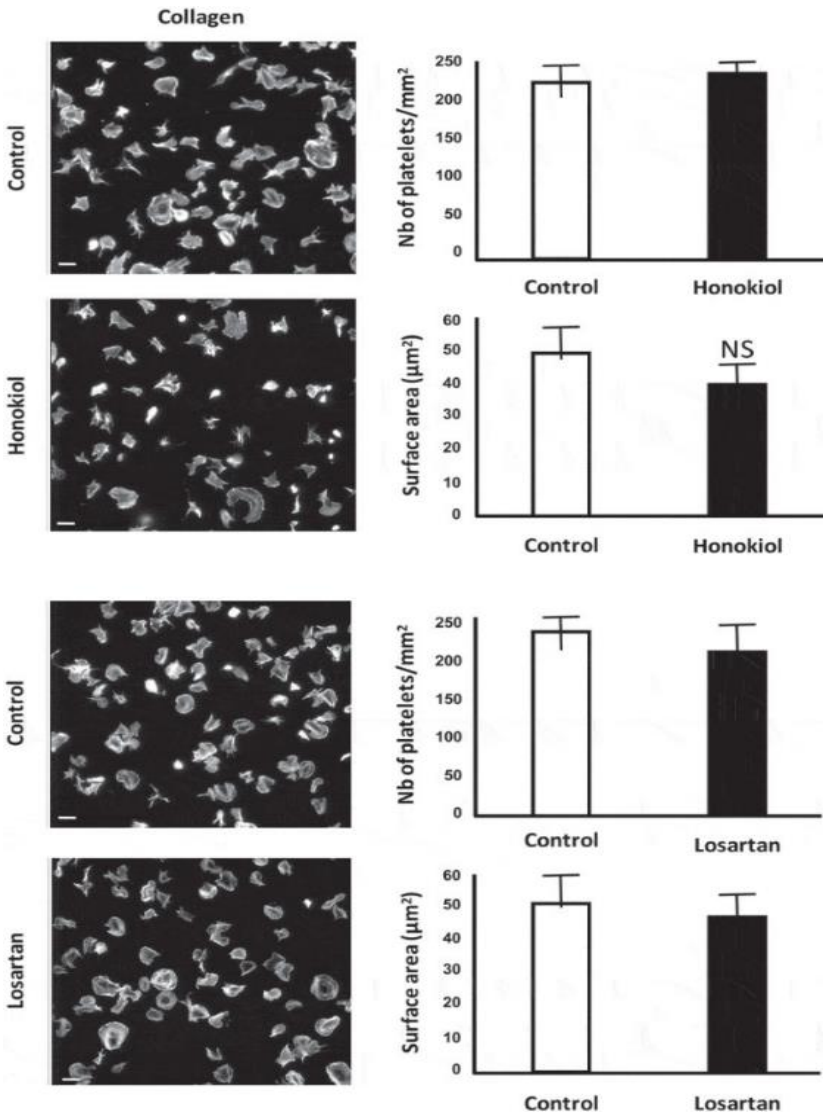
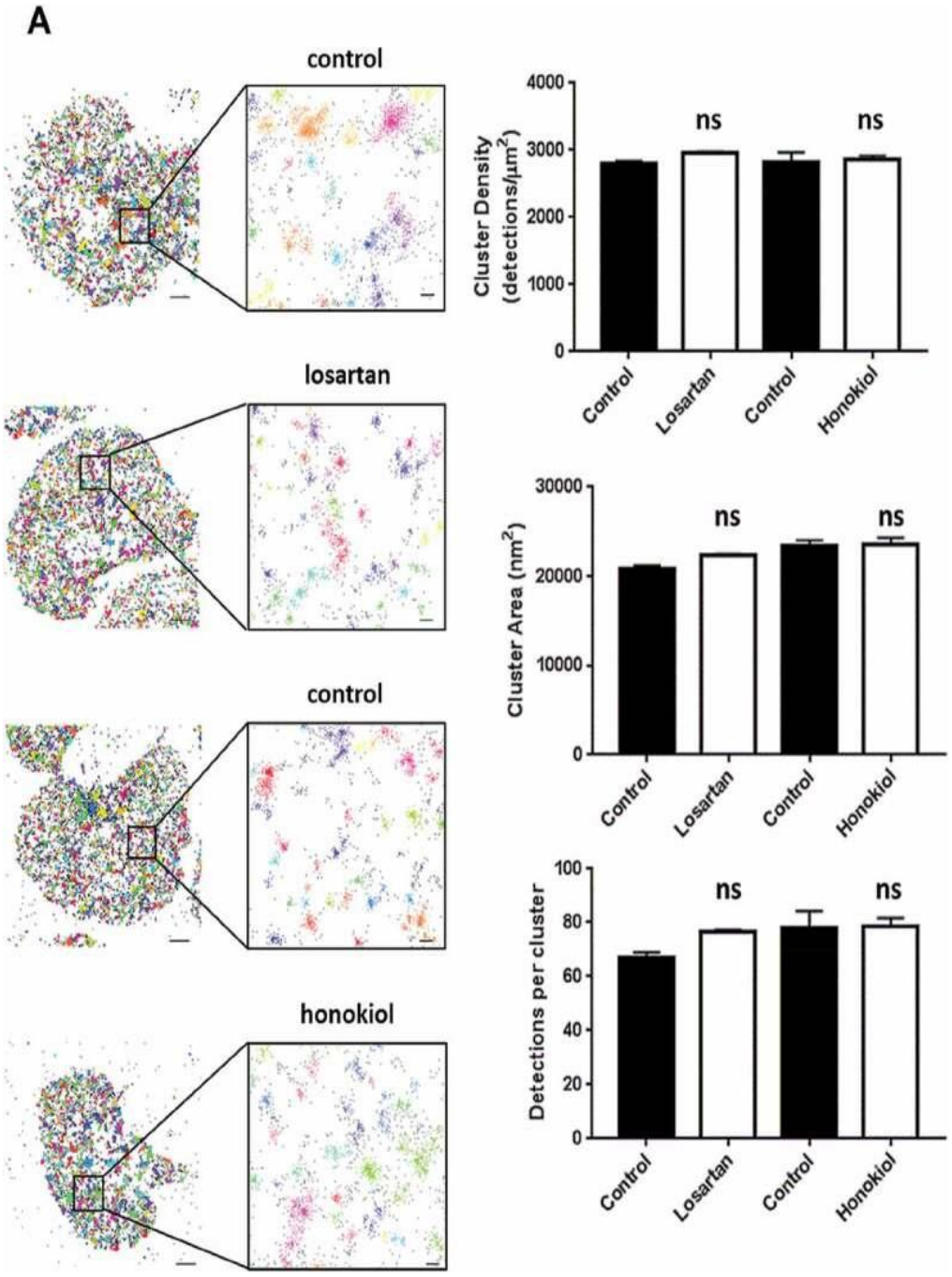


Figure 4. Losartan and honokiol do not affect platelet spreading on collagen. *Glass coverslips were coated with collagen. Washed platelets were pre-treated with 25 µM of (**A**) losartan or (**B**) honokiol before spreading on collagen, followed by fixation and staining for actin with Alexa-488 phalloidin. Scale bar, 5 µm. Bar graphs illustrate quantification of the surface area covered with platelets per field, and the numbers of platelets counted per field. Data are shown as means ± SD and are representative of three experiments.*


A
control
losartan
control
honokiol
Cluster Density (detections/μm²)
4000
3000
2000
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Losartan
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Honokiol
Cluster Area (nm²)
30000
20000
10000
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ns
ns
Control
Losartan
Control
Honokiol
Detections per cluster
100
80
60
40
20
0
ns
ns
Control
Losartan
Control
Honokiol

Figure 5. Losartan and honokiol do not affect clustering of GPVI. *Glass coverslips were coated with CRP. Washed platelets were pre-incubated with 1G5 Fab (pan-GPVI), then pre-treated with 25 µM losartan, 25 µM honokiol or vehicle before spreading on CRP. Following fixation, GPVI was secondary labelled with mouse-Alexa647 and actin was labelled with Alexa488-phalloidin. The single molecule localisation data acquired by STORM was analysed for clustering using DBSCAN. Different colours represent different clusters (with black dots representing noise). For each condition, 5 different fields of view (FOV) from 3 independent experiments were captured. Representative cropped scatter plots for each condition show the output of the DBSCAN clustering algorithm (left panel). Scale bars: 1 µm and 0.1 µm. Bar graphs show quantification of cluster density, cluster area and number of detections per cluster. Mean ± SEM (n = 3) (right panel).*

Losartan and honokiol inhibit thrombus formation

Whole blood perfusion experiments were performed on collagen-coated microspots to assess the effects of platelet pre-treatment with losartan or honokiol (25–100 µM). Thrombi were analysed for platelet deposition (% surface area coverage) and by visual inspection using a pre-defined score system (morphological, contraction and multilayer score).[33] Losartan treatment did not affect platelet deposition (Figure 6A-B) but decreased thrombus contraction and multilayer scores (Figure 6D-E) as an indication of lower thrombus integrity and the presence of loose platelet aggregates. In contrast, honokiol pre-treatment resulted in a dose-dependent decrease in platelet adhesion (lower surface area coverage of platelets) to collagen and significantly decreases the contraction and multilayer scores (Figure 7), pointing to a suppressed formation of tightly packed thrombi. These results show that both losartan and honokiol affect formation of thrombi in whole blood but in different ways. These concentrations of the two inhibitors have little effect however on the response to GPVI activation of platelets in platelet rich plasma due to protein binding (see above).

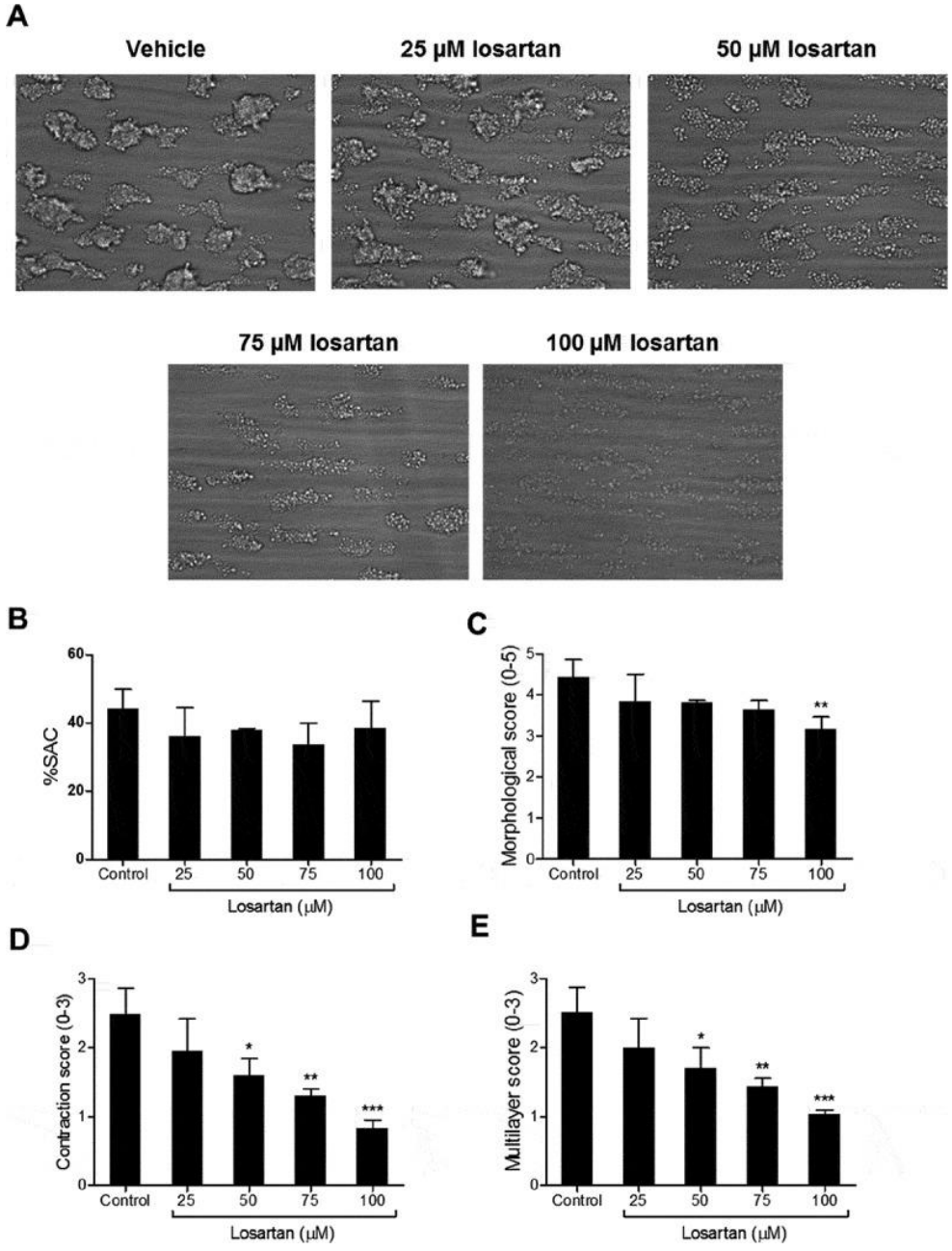

Figure 6. Losartan inhibits the formation of contracted, multilayered platelet thrombi under flow. *Whole blood treated with vehicle solution or losartan was perfused over collagen surface for 6 minutes at wall shear rate of 1000 s^{-1}.* ***A,*** *Phase*

contrast images were captured and shown as representative images. ***B-E,*** *Platelet surface area coverage (% SAC), thrombus morphological score (range 0–5), thrombus contraction score (0–3) and platelet multilayer score (0–3) were determined. Data are presented as mean ± SEM (n = 4). *P <0.05, **P <0.01, ***P <0.001.*

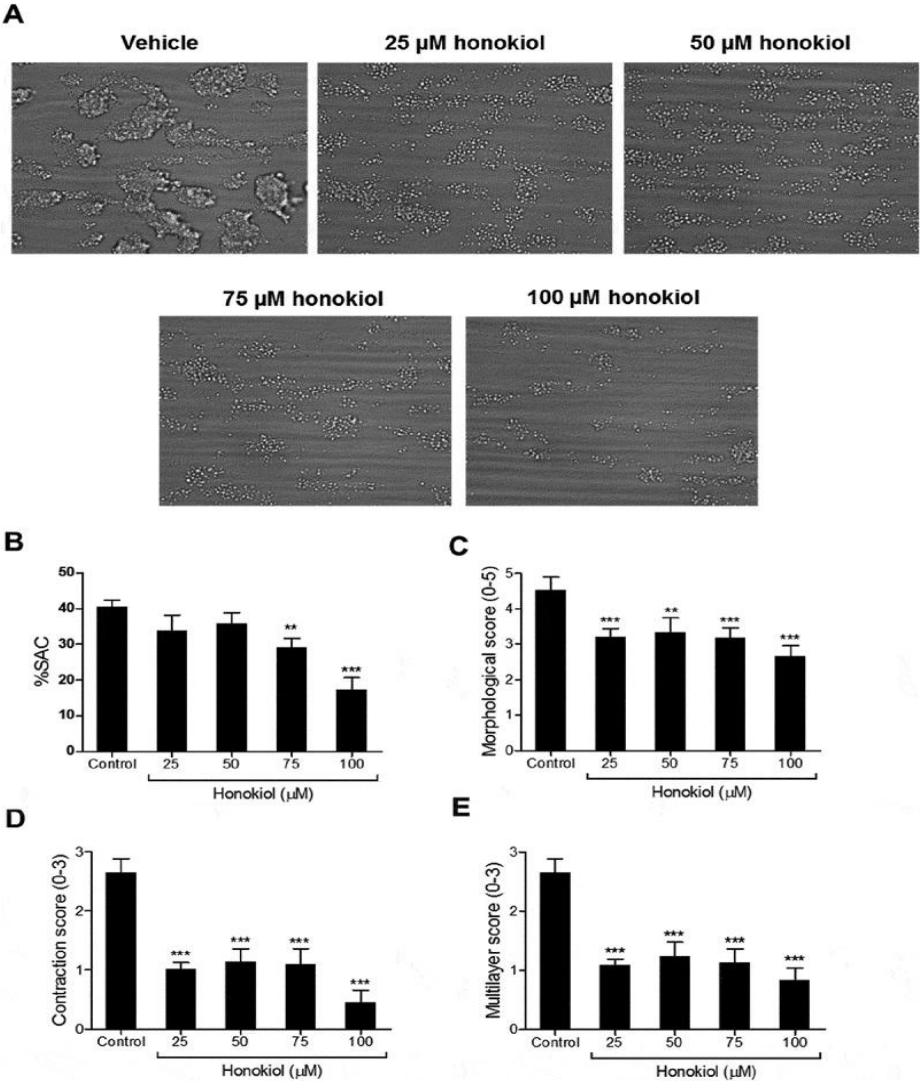

Figure 7. Honokiol inhibits both platelet adhesion and formation of multilayered platelet thrombi under flow. *Whole blood treated with vehicle solution or honokiol*

*was perfused over collagen type I for 6 minutes at wall shear rate of 1000 s^{-1}. **A**, Phase contrast images were captured and shown as representative images. **B-E**, Platelet surface area coverage (% SAC), thrombus morphological score (range 0–5), thrombus contraction score (0–3) and platelet multilayer score (0–3) were determined. Data are presented as mean ± SEM (n = 4). *P <0.05, **P <0.01, ***P <0.001.*

Discussion

This work provides new information on the platelet inhibition mechanisms of losartan and honokiol. We confirmed that losartan and honokiol inhibit platelet aggregation by collagen. However, while the IC50 for losartan was similar to that previously reported,[21] the IC50 for honokiol is almost one order of magnitude higher than in the original report.[26] The explanation for this difference is unclear. Interestingly, both losartan and honokiol inhibited platelet activation by the (hem)ITAM receptors rhodocytin and FcγRIIA at similar concentrations to those for inhibition by collagen. This effect was mediated by inhibition of tyrosine phosphorylation, including Syk which plays a proximal role in signalling by all three receptors. Inhibition of the response to GPVI was not due to a direct competition with collagen since losartan did not block binding of recombinant GPVI to collagen[15] and honokiol inhibited this binding at a concentration that is three orders of magnitude greater than required for inhibition of aggregation.[25] Although both losartan and honokiol inhibited platelet aggregation and Syk phosphorylation, they were unable to prevent clustering of GPVI or spreading on collagen at concentrations up to 100 µM. This may be related to the relatively high concentration of collagen on the surface thus overcoming the inhibitory effect of the two reagents. Alternatively, neither agent may be able to inhibit clustering of GPVI on a surface and therefore inhibit activation, in contrast to previous results in suspension using an antibody that recognises the dimeric form of GPVI (9E18) conjugated with Duolink PLA probes.[15] Losartan had no effect on constitutive signalling by GPVI in transfected DT40 cells, which may be a functional readout for clustering of the immunoglobulin receptor. Losartan and honokiol are highly protein bound in plasma[18,32,34] necessitating use of higher

concentrations in the presence of plasma to achieve the same level of inhibition as seen in washed platelets. Unexpectedly however, both inhibitors suppressed collagen-dependent platelet aggregation in whole blood under flow conditions at concentrations that hardly affect platelet aggregation in plasma as measured by light transmission aggregometry. Furthermore, the mode of inhibition under flow was distinct for the two inhibitors: while both suppressed the formation of tight platelet thrombi, honokiol also interfered with platelet adhesion. One explanation for the inhibition observed in whole blood could be based on their lipophilic characteristic causing disruption of lipid-to-lipid or lipid-protein interactions[35,36] or blocking protein-protein interactions. In addition, honokiol has been reported to disrupt the integrity of the inner membrane of mitochondria.[37] These data could explain the pleiotropic impacts on various cells in different pathologies.[38]

In this study, we compared two small molecules described as specific inhibitors for GPVI on different aspects of platelets activation and function. Although both compounds were able to inhibit platelet aggregation induced by collagen, we could not confirm the original potency of honokiol on this response and we found that neither agent inhibited spreading of platelets on collagen. The likely explanation is that both compounds are unable to inhibit clustering of GPVI of platelets on a surface. Both inhibitors also blocked rhodocytin and FcγRIIA-induced CLEC-2 activation demonstrating that they are not specific to GPVI at the concentrations used. This is illustrated by the inhibition of platelet aggregation on whole blood under flow conditions at concentrations that have a minimal effect on platelet activation by collagen in plasma. The multiplicity of effects of the two inhibitors should be considered in the context of their use as anti-platelet agents.

Funding statement

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Disclosure statement

The authors declare no competing financial interests.

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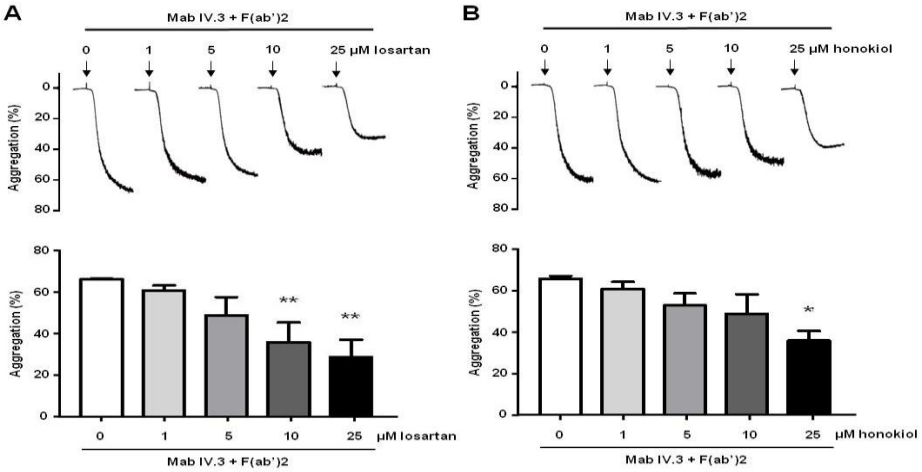
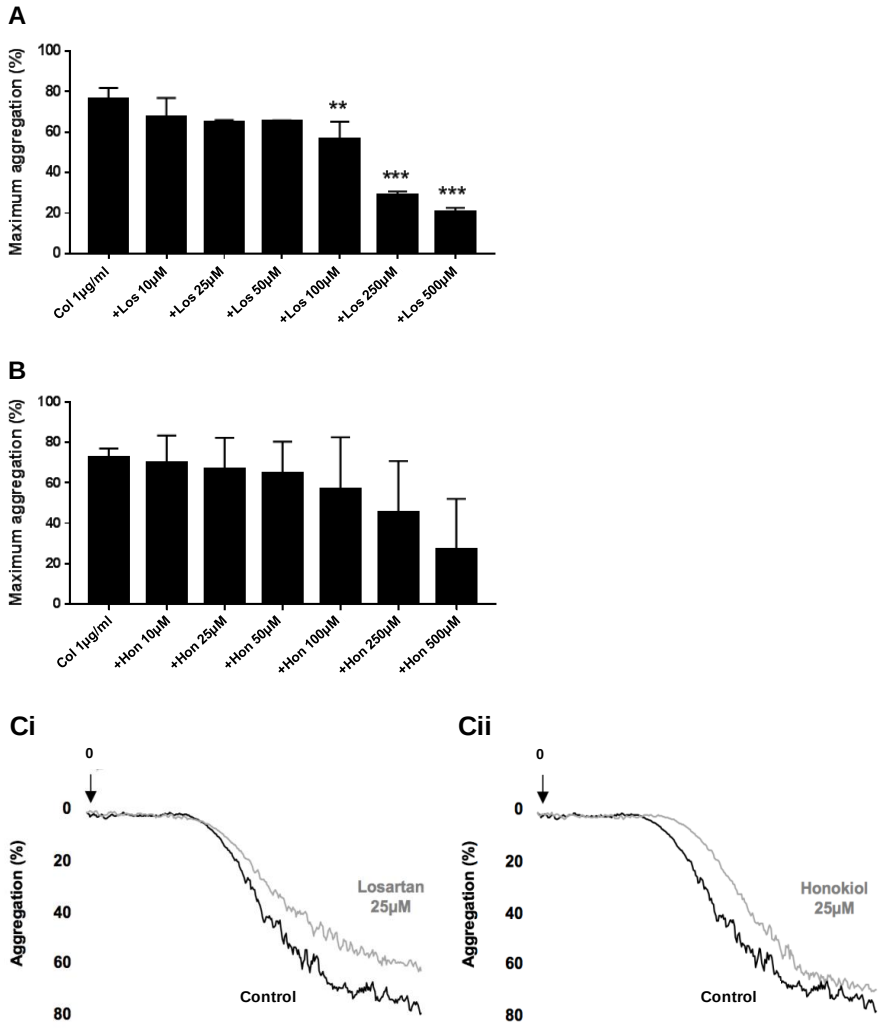
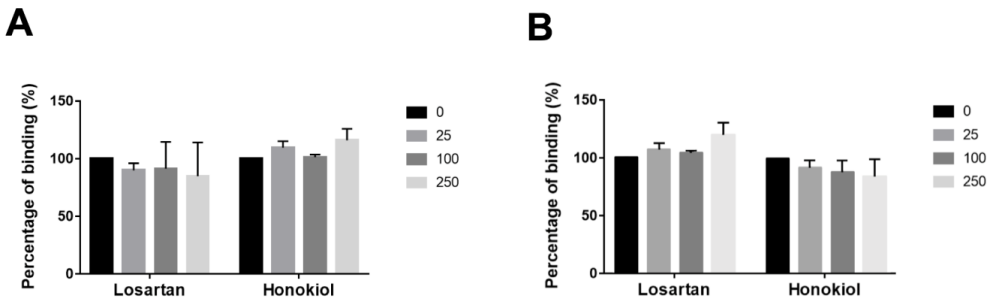
Supplemental figures



Suppl. Figure 1. Losartan and honokiol dose-dependently reduce aggregation of washed platelets induced by FcγRIIA activation. *Washed platelets were pretreated with different concentrations of losartan (**A**) or honokiol (**B**) for 3 minutes before addition of mab IV.3 (1 µg/mL) for 60 seconds followed by stimulation with F(ab')$_2$ (30 µg/mL). Bar graphs represent a total of 3 independent experiments and results are shown as mean ± SD. *P <0.05, **P <0.01.*



Suppl. Figure 2. Losartan and honokiol dose-dependently reduce platelet aggregation in plasma, induced by collagen. *Platelet-rich plasma was pretreated with different concentrations of losartan (**A**) or honokiol (**B**) for 3 minutes before stimulation with collagen 1 µg/mL. Total of 5 experiments, means ± SD. **P <0.01, ***P 0.001. Aggregation traces are representative for the effect of 25µM of losartan (**C, i**) or honokiol (**C, ii**) on collagen-induced platelet stimulation. The slight delay in aggregation onset observed with honokiol was not seen in all experiments.*



Suppl. Figure 3. Losartan and honokiol do not inhibit binding of monomeric GPVI to collagen. *Recombinant GPVI monomeric protein (**A**) or GPVI dimeric protein (**B**) was incubated with different concentrations of losartan or honokiol (in µM) before addition onto a collagen coated-surface (10 µM). The bar graph represents the binding of the recombinant GPVI to collagen from an ELISA inhibition assay (n = 4). Data are shown as means ± SD.*

Chapter 6

Role of tyrosine kinase Syk in thrombus stabilisation at high shear

G. Perrella, S. Montague, H.C. Brown, L.G. Quintanilla, A. Slater, D. Stegner, M. Thomas, J.W.M. Heemskerk & S.P. Watson

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I designed and performed experiments, analysed and interpreted data, and wrote the manuscript; S.J.M. performed experiments and edited the manuscript; H.C.B., D.S., M.T., J.W.M.H. edited the manuscript; L.G.Q. performed experiments; A.S. produced the nb21 and edited the manuscript; S.P.W. designed experiments, supervised research, interpreted data, and wrote the manuscript.

Graphic Abstract

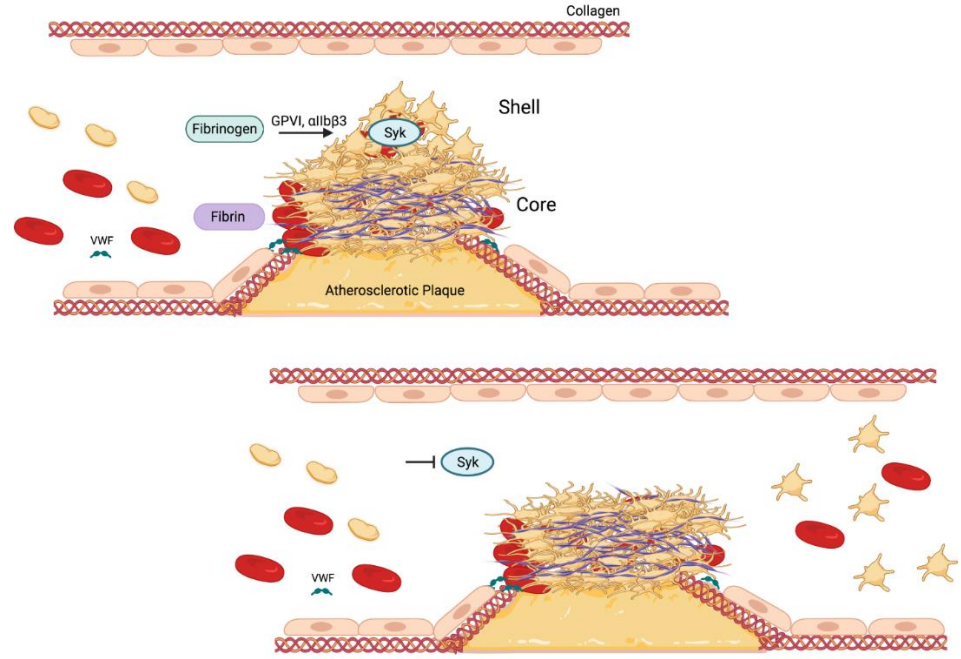


Figure created with BioRender.com.

Abstract

Understanding the pathways involved in the formation and stability of the core and shell regions of a platelet-rich arterial thrombus may result in new ways to treat arterial thrombosis. The distinguishing feature between these two regions is the absence of fibrin in the shell which indicates that in vitro flow-based assays over thrombogenic surfaces, in the absence of coagulation, can be used to resemble this region. In this study, we have investigated the contribution of Syk tyrosine kinase in the stability of platelet aggregates (or thrombi) formed on collagen or atherosclerotic plaque homogenate at arterial shear (1000 s^{-1}). We show that post-perfusion of the Syk inhibitor PRT-060318 over preformed thrombi on both surfaces enhances thrombus breakdown and platelet detachment. The resulting loss of thrombus stability led to a reduction in thrombus contractile score which could be detected as early as 3 min after perfusion of the Syk inhibitor. A similar loss of thrombus stability was observed with ticagrelor and indomethacin, inhibitors of platelet adenosine diphosphate (ADP) receptor and thromboxane A_2 (TxA_2), respectively, and in the presence of the Src inhibitor, dasatinib. In contrast, the Btk inhibitor, ibrutinib, causes only a minor decrease in thrombus contractile score. Weak thrombus breakdown is also seen with the blocking GPVI nanobody, Nb21, which indicates, at best, a minor contribution of collagen to the stability of the platelet aggregate. These results show that Syk regulates thrombus stability in the absence of fibrin in human platelets under flow and provide evidence that this involves pathways additional to activation of GPVI by collagen.

Introduction

Platelet-rich thrombi formed in vivo in mice have been shown to be composed of a core and shell region in both the arterial and venous microcirculation.[1] In the core region, platelets and fibrin are densely packed, and permeability is heavily restricted,[2] necessitating the use of a fibrinolytic to dissolve the platelet aggregate.[3,4] The outer, more permeable, shell region however, consists of weakly activated and loosely packed platelets and does not contain fibrin,[2] suggesting that a different strategy is needed to promote disruption of this region and thus help to prevent the build-up of an occlusive thrombus.

Glycoprotein VI (GPVI) is a platelet immunoglobulin receptor which is known as a receptor for collagen,[5] but in recent years has also been shown to be a receptor for fibrin and fibrinogen, among other predominantly charged ligands.[6] In vitro flow studies have shown that GPVI contributes to the stability of newly formed platelet aggregates on collagen at high shear through use of the blocking anti-GPVI Fab ACT017, which has more recently been named glenzocimab.[4] Since the activation of GPVI by fibrinogen is also dependent on integrin αIIbβ3, with the interplay of the two receptors driving platelet adhesion and activation;[7] this suggests that blocking signalling pathways common to both receptors may have a greater antithrombotic effect than blocking GPVI alone.

The tyrosine kinase Syk plays a critical role in signalling by integrin αIIbβ3 and GPVI[8,9] and an inhibitor of Syk, fostamatinib, is clinically used for the treatment of patients with refractory immune thrombocytopenia (ITP) without increasing the risk of bleeding despite the marked reduction in platelet count in this patient group.[10] Moreover, a retrospective analysis provides evidence of a notably low incidence of thrombotic events in patients treated with the Syk inhibitor.[10] Thus, Syk inhibitors represent a new class of antiplatelet agent with reduced bleeding risk compared to current drugs. The involvement of Syk in thrombus formation and

thrombus growth and stabilisation suggests that inhibitors will be effective against multiple stages in thrombus formation.

In the present study, we have investigated the role of Syk on the stability of preformed thrombi formed on collagen or human atherosclerotic plaque homogenate at arterial shear. Furthermore, we have compared the effect of Syk inhibition to that of the feedback agonists, adenosine diphosphate (ADP) and thromboxane A_2 (TxA_2), and of the tyrosine kinases Src and Btk. We have also investigated the effect of a recently described nanobody, Nb21, to GPVI that blocks platelet activation by collagen.[11] The results show that inhibition of Syk promotes thrombus breakdown on collagen and plaque homogenate thereby demonstrating a critical role for the kinase in the stability of this region.

Materials and methods

Materials

The complete list of reagents is described in supplementary materials. The anti-GPVI nanobody 21 (Nb21) was generated as described.[11] The human plaque homogenate was obtained from 10 patients with symptomatic carotid artery stenosis undergoing carotid endarterectomy, then homogenised and pooled as described.[12] All experiments involving human subjects were performed in accordance with the declaration of Helsinki and Good Clinical Practice and approved by NHS research and ethics committees (North West – Haydock Research Ethics Committee 20/NW/0001 and West Midlands – South Birmingham Research Ethics Committee 18/WM/0386). Use of blood from healthy volunteers was approved by the University of Birmingham Ethics Review (ERN_11-0175AP10). An informed consent was obtained from the healthy volunteers and patients to participate in the study.

Blood withdrawal and platelet preparation

Blood was taken into 4% sodium citrate from consenting healthy volunteers, who had not taken anti-platelet agents in the previous ten

days. Washed human platelets were obtained by centrifugation of platelet-rich plasma (PRP) in the presence of prostacyclin (0.56 μM) and resuspended in modified Tyrode's buffer (134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM Hepes, 5 mM glucose, 1 mM $MgCl_2$, pH 7.3) as described.[13] The isolated platelets were used at a cell density of $5x10^8$/mL for protein phosphorylation studies and of $2x10^8$/mL for aggregometry.

Platelet aggregation

Changes in light transmission were recorded at 37°C with stirring (1200 rpm) in an optical aggregometer (PAP-8E, Milan, Italy). Washed platelets, in presence or absence of eptifibatide (9 μM), were warmed for 2 minutes prior to activation by Horm collagen (30 μg/mL), thrombin (1 U/mL), fibrinogen (200 μg/mL) or TRAP (10 μM).

Western blotting

Platelet activation was terminated with a sodium dodecyl sulphate (SDS) reducing sample buffer. Whole cell lysates (WCL) were prepared and immunoblotted as previously described.[14] Syk and PLCγ2 were immunoprecipitated as previously described.[14]

Flow adhesion

Glass coverslips were coated overnight at 4°C with Horm collagen (50 μg/ml) or plaque homogenate (0.5 mg/mL) before blocking with 10% BSA for 30 min in Hepes buffer (10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 5.5 mM glucose, and 0.1% BSA), pH 7.45.[7] Citrated whole blood, recalcified with $CaCl_2$ (7.5 mM) and $MgCl_2$ (3.75 mM), in the presence of PPACK (40 μM), was perfused for 7 minutes at 1000 s^{-1}.[15] Aggregates were successively post-perfused with Hepes buffer with vehicle (DMSO, 0.01%) or an inhibitor of Syk (PRT-060318, 10 μM), Src (dasatinib, 10 μM), Btk (ibrutinib, 7 μM), GPVI (Nb21, 500 nM), $P2Y_{12}$ (ticagrelor, 10 μM), or cyclooxygenase (indomethacin, 10 μM), for 10 minutes at 1000 s^{-1}. Inhibitors were added to the Hepes buffer pH 7.45,

supplemented with 0.1% BSA, 0.1% glucose, 2 mM $CaCl_2$ and 1 U/mL heparin. Brightfield images of a same field of view, which included multiple aggregates, were taken every 60 seconds for 10 minutes. Aggregate morphology, contraction and multilayer scores were assessed blind by visual inspection compared to a standard set of representative images.[16] This scoring system distinguishes single adhesive platelets from those organized in aggregates (morphological score) and monitors the tightness (contraction score) and size of the aggregates (multilayer score). Where stated experiments were performed at 37°C.

Epifluorescence microscopy

Recalcified blood, treated with PPACK and labelled with PE anti-GPVI mAb, APC anti-CD41a mAb and FITC anti-fibrinogen mAb was flowed for 7 minutes at arterial shear (1000 s^{-1}) over coverslips coated by Horm collagen (50 μg/mL) or plaque homogenate (0.5 mg/mL). The formed aggregates were post-perfused with vehicle (DMSO, 0.01%) or PRT-060318 (10 μM) for 3 minutes at arterial shear (1000 s^{-1}), and then fixed in formalin for 15 minutes, before washing in PBS and mounting in between glass slides. Seven images per fields of view were taken using an epifluorescence microscope. Image analysis was performed by measuring fluorescence intensity in the open source software Fiji.[17]

Statistical analysis

Data are presented as means ± SEM unless stated otherwise, with statistical significance taken at $P<0.05$ (one-tailed Student's paired t-test). Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software Inc. La Jolla, CA, USA).

Results

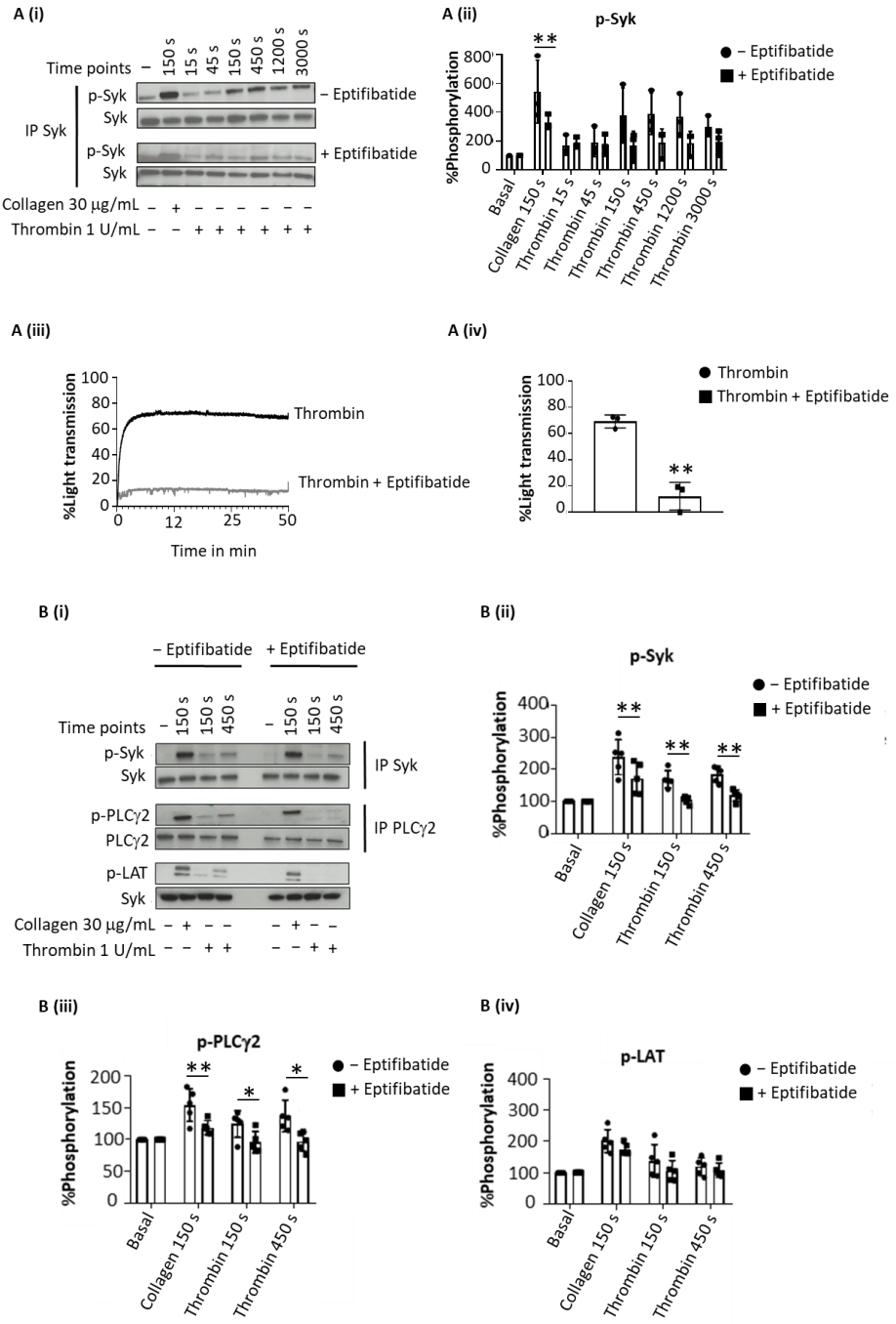
Thrombin stimulates sustained phosphorylation of Syk via integrin αIIbβ3 during platelet aggregation

The outer shell of an arterial thrombus is composed of aggregated

platelets held together by the interaction of fibrinogen with integrin αIIbβ3, with activation of the integrin mediated by the autocrine feedback mediators, ADP and TxA_2.[18, 19] It has been proposed that the two mediators diffuse from the highly activated platelets in the thrombus core region to support platelet aggregation in the shell.[20, 21] In this model, it is unclear how the activation of the integrin is maintained over time as the levels of the two mediators decline due to exhaustion of intracellular stores and reduced activation of phospholipase A_2. One explanation is that integrin activation is maintained by a positive feedback pathway mediated through the activation of αIIbβ3 and GPVI by fibrinogen.[7] In this case, activation of Syk during platelet aggregation should be sustained.

To investigate this, we activated platelets in suspension by thrombin which signals through the G_q protein-coupled receptors, PAR-1 and PAR-4.[22] Thrombin was selected for the experiments because it is the most powerful G_q protein-coupled receptor ligand in platelets. We performed the experiments in the absence or presence of the αIIbβ3 antagonist, eptifibatide, to establish the role of the integrin in mediating Syk activation. Thrombin evoked sustained tyrosine phosphorylation of Syk over 150-3,000 seconds (as concluded from 4G10 staining after western blotting), which was inhibited by eptifibatide at all time points (Figure 1A i-iv). In contrast, collagen-induced tyrosine phosphorylation of Syk, was reduced but not blocked by eptifibatide (Figure 1A i-ii). Eptifibatide also blocked the increase in tyrosine phosphorylation of LAT and PLCγ2 by thrombin but not by collagen (Figure 1B i-iv). To rule out a possible role of fibrin generation by thrombin in these experiments, the studies were repeated with the PAR-1 peptide agonist TRAP (Figure 1C i). TRAP also induced a sustained phosphorylation of Syk, LAT and PLCγ2 (Figure 1C ii-iv), whereas fibrinogen had no effect, as expected.

These data show a sustained activation of Syk downstream of integrin αIIbβ3 raising the possibility that this contributes to the maintenance of integrin activation during platelet aggregation.


A (i)
Time points – 150 s 15 s 45 s 150 s 450 s 1200 s 3000 s
IP Syk
p-Syk
Syk
– Eptifibatide
p-Syk
Syk
+ Eptifibatide
Collagen 30 µg/mL – + – – – – – –
Thrombin 1 U/mL – – + + + + + +
A (ii)
p-Syk
%Phosphorylation
– Eptifibatide
+ Eptifibatide
Basal
Collagen 150 s
Thrombin 15 s
Thrombin 45 s
Thrombin 150 s
Thrombin 450 s
Thrombin 1200 s
Thrombin 3000 s
A (iii)
%Light transmission
Thrombin
Thrombin + Eptifibatide
Time in min
A (iv)
%Light transmission
Thrombin
Thrombin + Eptifibatide
B (i)
– Eptifibatide
+ Eptifibatide
Time points – 150 s 150 s 450 s – 150 s 150 s 450 s
p-Syk
Syk
IP Syk
p-PLCγ2
PLCγ2
IP PLCγ2
p-LAT
Syk
Collagen 30 µg/mL – + – – – + – –
Thrombin 1 U/mL – – + + – – + +
B (ii)
p-Syk
%Phosphorylation
– Eptifibatide
+ Eptifibatide
Basal
Collagen 150 s
Thrombin 150 s
Thrombin 450 s
B (iii)
p-PLCγ2
%Phosphorylation
– Eptifibatide
+ Eptifibatide
Basal
Collagen 150 s
Thrombin 150 s
Thrombin 450 s
B (iv)
p-LAT
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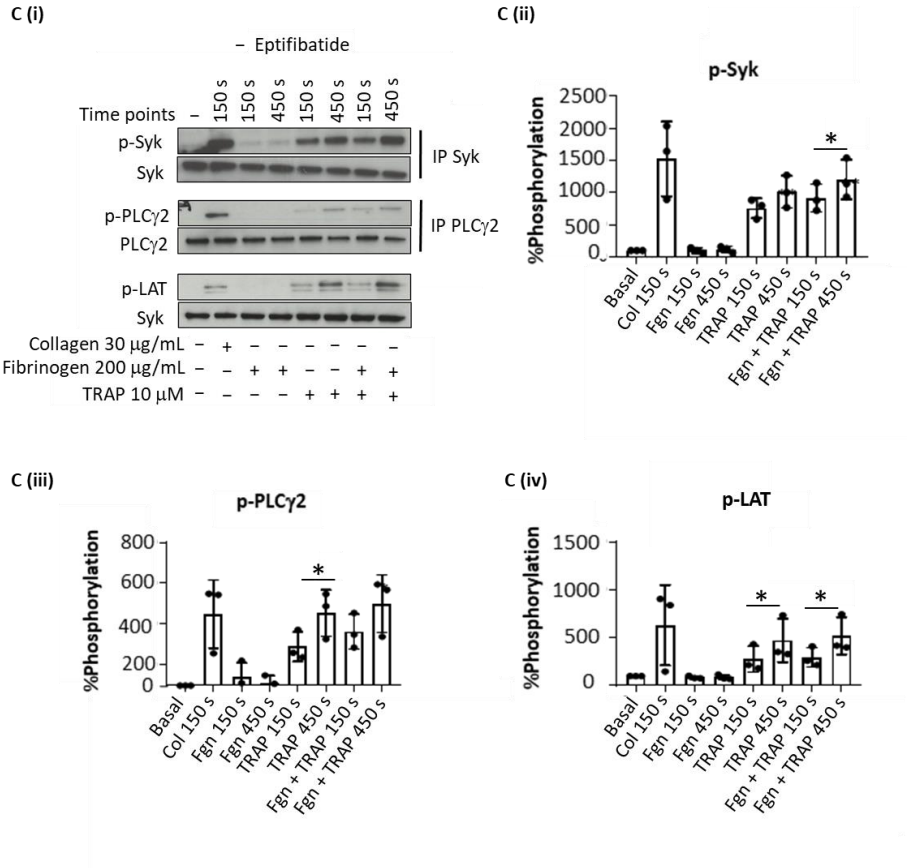


Figure 1. Sustained Syk phosphorylation in thrombin-stimulated platelet is integrin-dependent. *Washed platelets at 5×10^8/mL were stimulated with Horm collagen (30 µg/mL), thrombin (1 U/mL), TRAP (10 µM) or fibrinogen (200 µg/mL) in the presence or absence of eptifibatide (9 µM). Activation was stopped at stated time after addition of the agonist.* ***A****, Representative blot of Syk tyrosine phosphorylation from 6 donors. (****A****,* ***i****) Whole cell lysates were immuno-precipitated with anti-Syk mAb and probed with anti-phospho-tyrosine mAb 4G10; (****A****,* ***ii****) Percentage of Syk tyrosine phosphorylation, based on Western blots, from 6 donors (mean ± SD); (****A****,* ***iii****) Aggregation trace of platelets stimulated with thrombin (1 U/mL), representative of 3 donors, and (****A****,* ***iv****) graph showing aggregation as % light transmission (mean ± SD) after 50 minutes of agonist stimulation.* ***B****, Representative Western blot (****B****,* ***i****), and calculated percentage of Syk (****B****,* ***ii****), PLCγ2 (****B****,* ***iii****) and LAT (****B****,* ***iv****) phosphorylation in response to thrombin (Thromb) from 5 donors (mean ± SD). Horm collagen (Col) was used as positive*

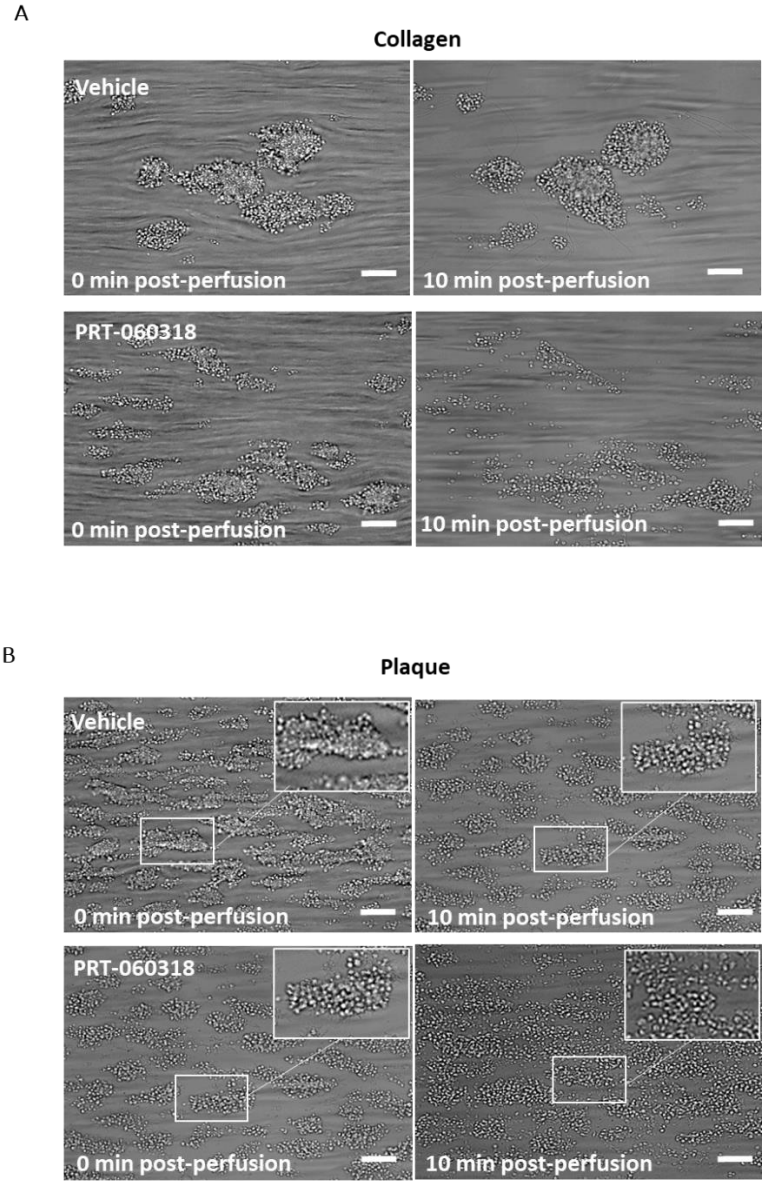
*control. **C**, Representative blot (**C, i**); mean ± SD of Syk (**C, ii**), PLCγ2 (**C, iii**) and LAT (**C, iv**) phosphorylation in response to collagen (Col), TRAP or fibrinogen (Fgn) from 3 donors. * P <0.05, ** P <0.005, one-tailed paired Student's t-test. Where stated, proteins were immunoprecipitated (IP).*

Inhibition of Syk on collagen and plaque homogenate causes thrombus shell instability

Experiments were designed to investigate the contribution of Syk to GPVI-dependent thrombus stability. In these experiments, recalcified blood was perfused for 7 min at arterial shear (1000 s^{-1}) over Horm collagen or human plaque material. Both ligands stimulate platelet activation through GPVI.[5, 12] The thrombin inhibitor, PPACK, was used to prevent fibrin formation. Aggregates were post-perfused for 10 min with vehicle (DMSO) or the Syk inhibitor, PRT-060318, at a concentration (10 μM) that has been shown to be effective in whole blood.[23, 24]

Heparin was added to the post-perfusion buffer to prevent residual formation of fibrin. Thrombus disaggregation was monitored by taking brightfield images at 60 sec intervals. Aggregates formed after 7 minutes of blood perfusion on both collagen and plaque material were tightly contracted and consisted of multiple platelet layers as illustrated in Figure 2A-B. Post-perfusion with vehicle for 10 minutes had a minor effect on the architecture, as reflected by the small reduction in morphological and contraction scores (Figure 2C i,ii and D i,ii). The aggregates were still composed of multiple layers of platelets as shown by the multilayer score (Figure 2C iii and D iii). In contrast, perfusion with PRT-060318 on both surfaces promoted aggregate breakdown which could be clearly seen by eye (Figure 2A-B) and was confirmed by measurement of morphology (Figure 2C i and D i). The increased breakdown could be detected within 3 min as shown by the reduction in the contraction score at this time (Figure 2C ii and D ii). The contraction score had return to baseline by 6-

10 minutes. The loss of stability led to a slow detachment of platelets eventually forming a monolayer on the surface (Figure 2C iii and D iii).



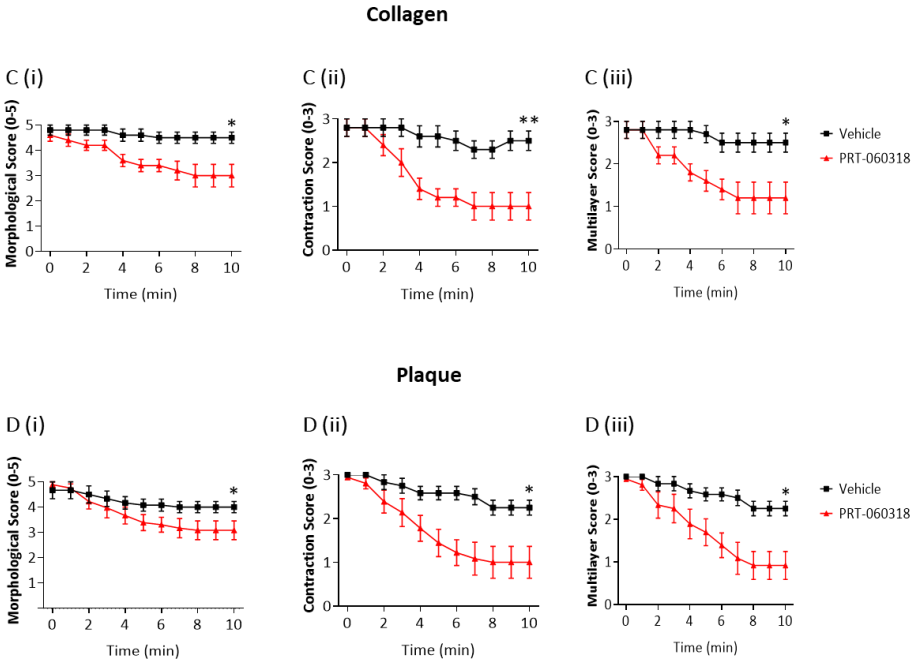


Figure 2. Inhibition of Syk causes loss of contraction and platelet adhesion on collagen and plaque material. *Recalcified blood from 6 donors was perfused for 7 minutes on Horm collagen and plaque at room temperature at shear rate of 1000 s^{-1}.* ***A****, Representative images of aggregates formed on collagen and plaque (****B****), taken during post-perfusion at times 0 and 10 minutes with vehicle or with PRT-060318 (n = 6). Scale bar = 100 µm.* ***C****, Disaggregation on collagen or on plaque (****D****) was monitored by taking brightfield images every 60 seconds and measured using morphological (****C, i*** *and* ***D, i****), contraction (****C, ii*** *and* ***D, ii****) and multilayer scores (****C, iii*** *and* ***D, iii****).*[16] *Aggregates were post-perfused with rinse buffer and vehicle (DMSO) or the Syk inhibitor PRT-060318 (10 µM). Data are shown as means ± SEM.*

To further explore the effect of Syk inhibition on thrombus characteristics, recalcified blood was triple labelled with PE-anti-GPVI mAb, APC-anti-CD41a mAb and FITC-anti-fibrinogen prior to perfusion over collagen and plaque, and then post-perfused for 3 minutes in the presence of buffer or PRT-060318 (Figure 3A i-ii). PPACK and heparin

were included as above to prevent thrombin formation. At this early time, perfusion with the Syk inhibitor caused a significant impairment in morphological, contraction and multilayer scores (Figure 3B). Fluorescence images of the aggregates indicated a reduced labelling of fibrinogen and GPVI staining in the presence of PRT-060318 although this did not reach significance (Figure 3C). Imaging was not performed at later timepoints due to the reduction in intensity through loss of platelets.

Taken together, the above results show that post-inhibition of Syk promotes disaggregation of the preformed thrombi on surfaces of collagen or plaque which eventually leads to the formation of a platelet monolayer.

Inhibition of Syk promotes thrombus breakdown at 37°C

We next asked if the contribution of Syk to thrombus shell architecture is also seen at the body temperature of 37°C. The thrombi formed at 37°C were larger and more tightly packed than those formed at room temperature (Figure 4A). In the presence of PRT-060318, the aggregates could be seen to loosen and to spread out from approximately 2 minutes of perfusion with the Syk inhibitor relative to vehicle (Figure 4B i-ii). However, the majority of platelets was retained in the aggregate for up to 5 minutes and only at later times could they be seen to detach resulting in a significant decrease in the multilayer score (Figure 4B i-ii).

This shows that Syk contributes to thrombus stability at both room temperature and at 37°C temperature despite the increased stability of the aggregates at the higher temperature.

Inhibition of Src and secondary agonists but not Btk promotes thrombus breakdown

We then extended the studies at 37°C to investigate the role of Src and Btk tyrosine kinases in aggregate stability, alongside the secondary mediators ADP and TxA_2.

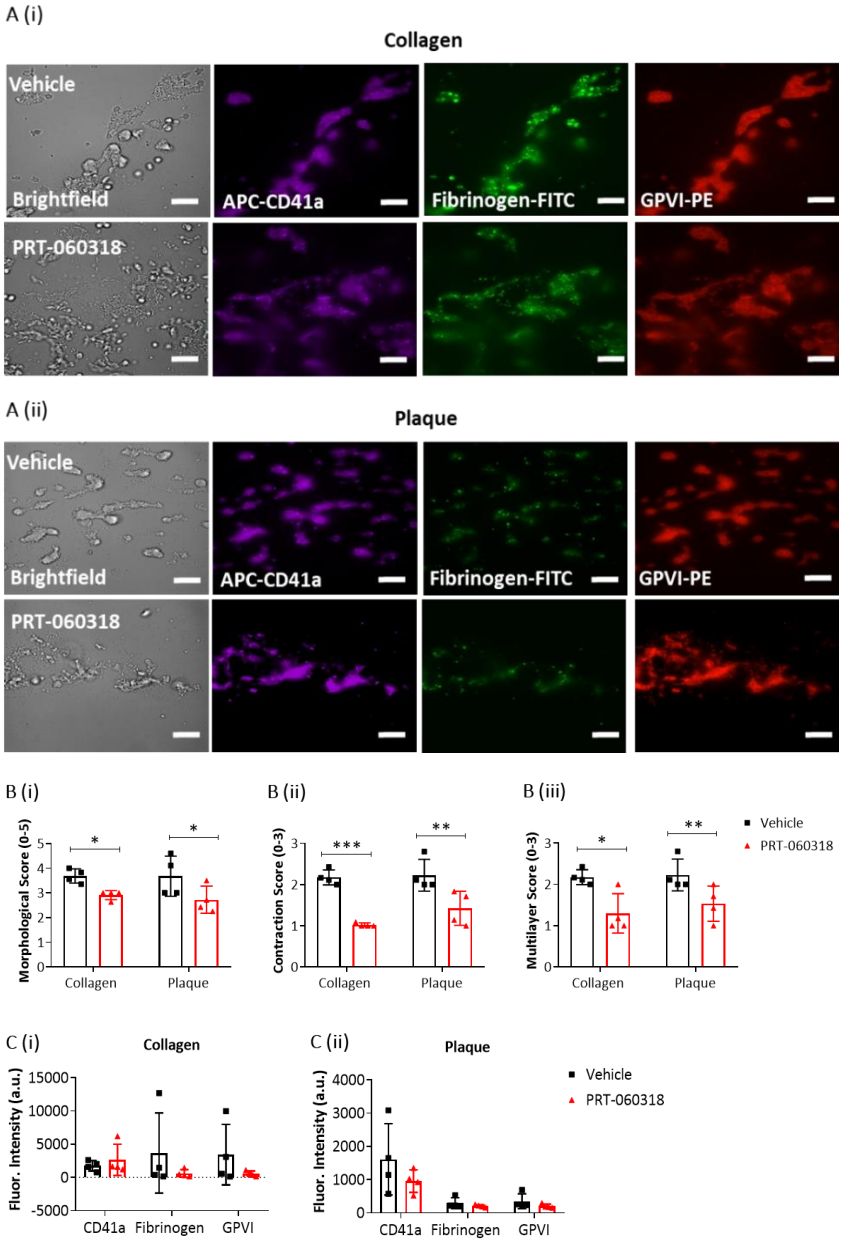


Figure 3. Platelet activation imaging of thrombi postperfused under condition of Syk inhibition. *Recalcified blood samples labelled with APC-αCD41a mAb (purple), FITC-fibrinogen (green) and PE-αGPVI mAb (red) was*

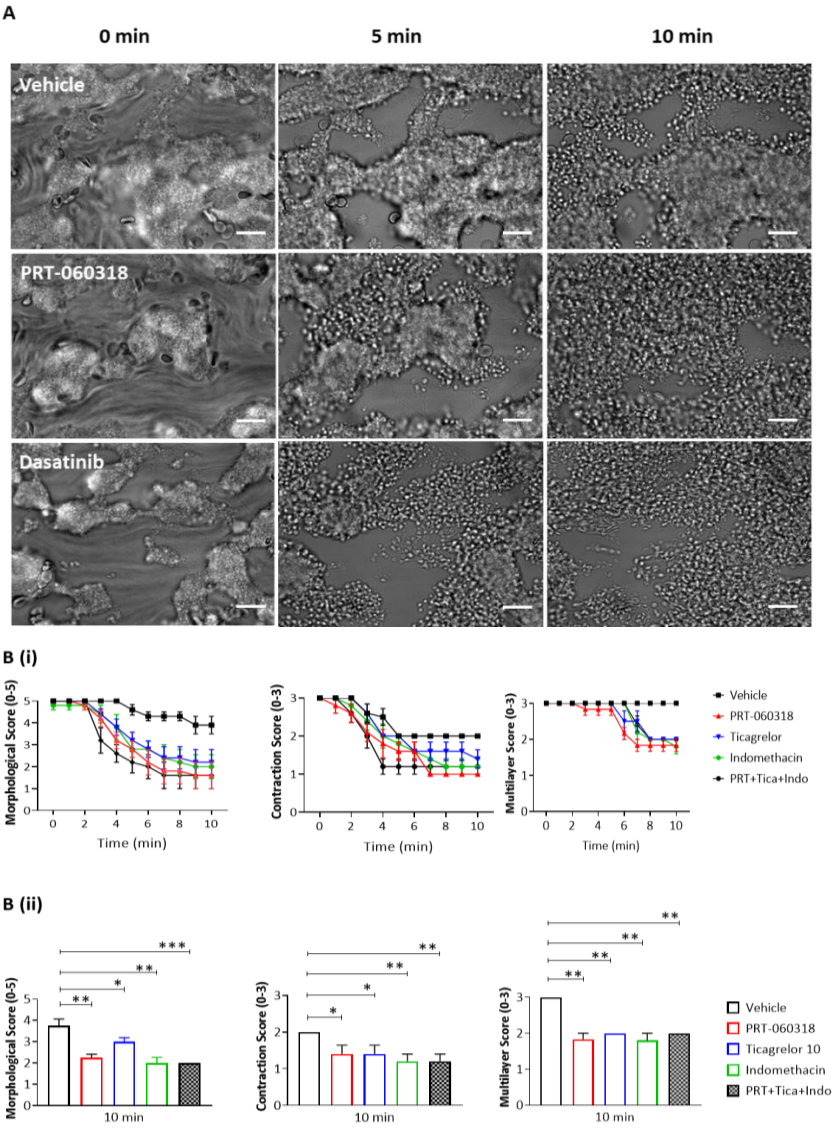
perfused for 7 min over immobilised collagen or plaque material at room temperature and arterial shear rate (1000 s^{-1}). Post-perfusion for 3 minutes was with rinse buffer containing vehicle (DMSO) or Syk inhibitor PRT-060318 (10 µM). ***A****, Representative microscopic images, showing remaining aggregates on collagen (****A****,* ***i****) or plaque (****A****,* ***ii****) after 3 minutes of post-perfusion with vehicle or PRT-060318 (n=4 donors).* ***B****, Graphs showing scores of thrombus morphology (****B****,* ***i****), thrombus contraction (****B****,* ***ii****) and thrombus multilayer (****B****,* ***iii****).*[16] ***C****, Graphs of fluorescence intensity (arbitrary units, a.u.) of platelet aggregates on collagen (****C****,* ***i****) or plaque material (****C****,* ***ii****) fixed after 3 minutes of post-perfusion. Scale bar= 50 µm. Data are shown as mean ± SD, *p < 0.05, **P < 0.005, ***P < 0.0005, one-tailed Student's paired t-test.*

Src kinases lie both upstream and downstream of Syk in the GPVI signalling cascade, and Btk lies downstream of both kinases.[25] As observed with the Syk inhibitor PRT-060318, the Src inhibitor dasatinib (10 µM) induced an increase in aggregate breakdown at 2 minutes of post-perfusion (Figure 4C i-ii), with a marked loss of aggregate architecture after 6 min of post-perfusion similar to that observed in the presence of PRT-060318 (Figure 4B-C). In contrast, the Btk inhibitor ibrutinib (7 µM), at a concentration that is effective in plasma,[26] caused only a mild decrease in the contractile score and no apparent change in morphology (Figure 4D). The much weaker effect may be due to redundancy with the Tec family kinase, Tec, or the later role of Btk in the GPVI signalling cascade.

The dependency of thrombus stability on the secondary mediators, ADP and TxA_2, was investigated using the the $P2Y_{12}$ receptor antagonist ticagrelor (10 µM) and cyclooxygenase inhibitor indomethacin (10 µM), respectively. The two inhibitors had a similar effect on thrombus stability to that of PRT-060318 and dasatinib (Figure 4B ii-Cii). Moreover, the combination of indomethacin, ticagrelor and PRT-060318 caused only a slight increase in disaggregation suggesting that the two mediators work in concert with Syk to promote aggregation (Figure 4B ii and 4C ii). This

could reflect either synergy of intracellular signals or a role of Syk in stimulating release of ADP and TxA_2.

Together, the results show a critical role for Syk and Src kinases alongside the two secondary mediators ADP and TxA_2 in supporting aggregate stability on collagen.



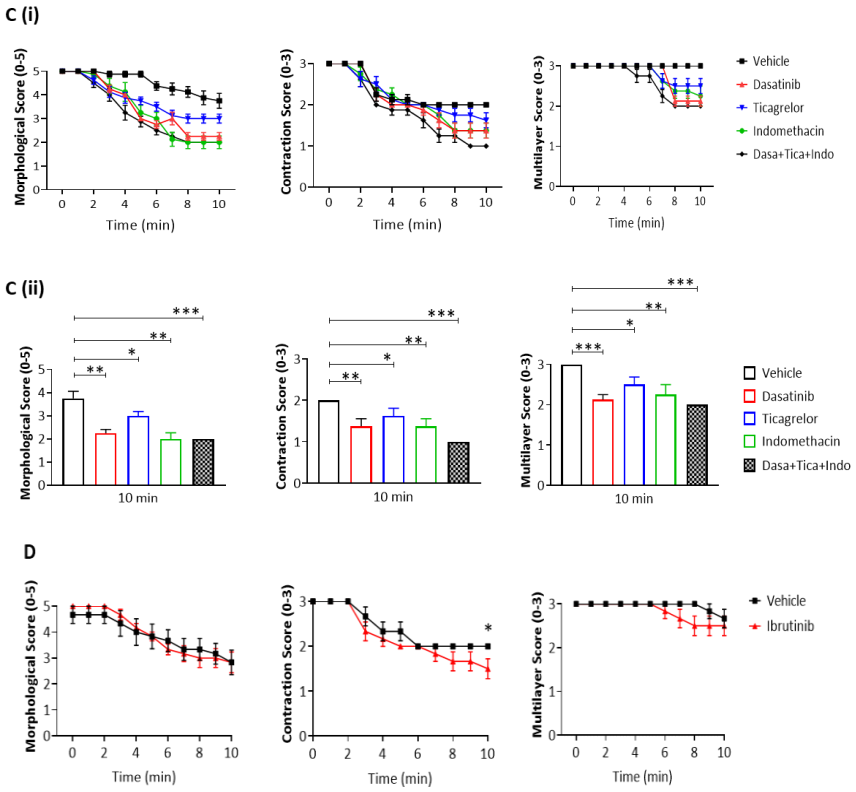


Figure 4. Inhibition of Syk causes the same extent of disaggregation observed with antagonists of adenosine diphosphate (ADP) and thromboxane A_2 (TxA_2). *Recalcified blood was perfused for 7 min on Horm collagen at 37 °C at arterial shear (1000 s^{-1}), and post-perfused for 10 minutes with rinse buffer with vehicle (DMSO) or inhibitor of Syk, Src or Btk (PRT-060318 10 µM, dasatinib 10 µM or ibrutinib 7 µM, respectively).* ***A****, Representative images of aggregates at 0, 5, and 10 minutes of post-perfusion with vehicle, PRT-060318 (n = 5) or dasatinib (n = 6).* ***B-D****, Graphs showing extent of disaggregation, as measured from scores of thrombus morphology, contraction and multilayer,[16] at every 60 seconds (**B**, **i** and **C**, **i** and **D**) or at 10 minutes (**B**, **ii** and **C**, **ii**) of post-perfusion with either vehicle, PRT-060318 (PRT) (**B**, **i** and **ii**), dasatinib (**C**, **i** and **ii**) or ibrutinib (**D**), alone or combined with indomethacin (Indo, 10 µM) and ticagrelor (Tica, 10 µM). Scale bar = 50 µm. Data are shown as mean ± SEM. *P <0.05, ** P <0.005, *** P <0.0005, one-tailed paired Student's t-test.*

Blocking of GPVI with Nanobody 21 causes only a minor thrombus breakdown on collagen

The nanobody Nb21 was raised against human GPVI and shown to block platelet activation by collagen as measured by light transmission aggregometry and flow adhesion over collagen at arterial shear.[11] Post-perfusion with Nb21 however caused only a small impairment in thrombus stability over collagen at longer times of flow (8 minutes; Figure 5B), suggesting that loss of stability in the presence of Syk and Src inhibitors is largely independent of blockade of GPVI activation by collagen.

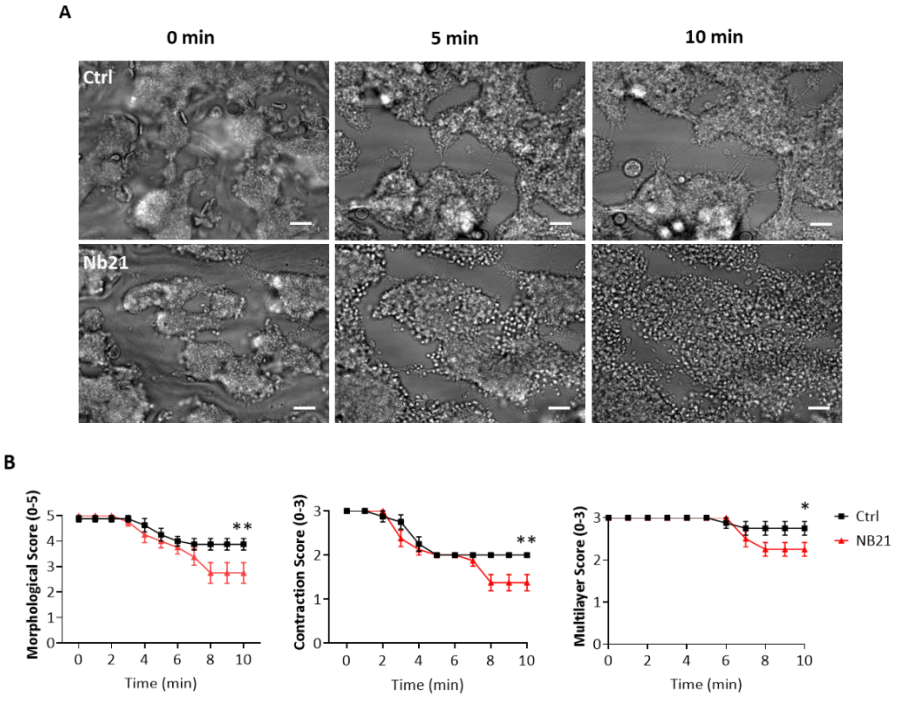


Figure 5. Blockage of GPVI with the anti-GPVI nanobody 21 (Nb21) causes minor thrombus instability. *Recalcified blood was perfused for 7 minutes on Horm collagen at 37°C at arterial shear (1000 s^{-1}), and successively post-perfused for 10 minutes with rinse buffer and blocking anti-GPVI nanobody Nb21 (500 nM).* ***A****, Representative images after perfusion of blood from 6*

donors, taken during post-perfusion at time 0, 5 and 10 minutes. ***B****, Graphs showing extent of disaggregation, monitored from recorded brightfield images every minute, and measured as morphological, contraction and multilayer scores.*[16] *Scale bar= 50 µm. Data are shown as means ± SEM. *P <0.05, **P <0.005 one-tailed Student's paired t-test.*

Discussion

Platelets in the shell region of aggregates formed in vivo are held together by the binding of fibrinogen to the major platelet integrin αIIbβ3, with the diffusion of secondary mediators from the thrombus core region mediating inside-out activation of the integrin and binding to fibrinogen. The secretion of the two mediators diminishes over time leading to the question as to whether alternative pathways exist to support integrin activation in the shell.

In the present study, we demonstrate a critical role for Src and Syk tyrosine kinases in supporting the stability of preformed shell-type thrombi on collagenous surfaces in the absence of fibrin formation, and show that this is mediated in concert with the signals from ADP and TxA_2. We propose that activation of Src and Syk kinases is mediated through outside-in signalling by αIIbβ3 and GPVI through their interaction with fibrinogen leading to a feedback pathway that reinforces integrin activation in combination with ADP and TxA_2. This is supported by the observation in the present study that activation by the G protein-coupled receptor ligands thrombin or TRAP induces weak but sustained activation of Syk through an αIIbβ3-dependent pathway. Moreover, the initial activation of GPVI by collagen appears to play little role in thrombus stability as shown using a nanobody, Nb21, that blocks activation of GPVI by matrix proteins. The effect on thrombus stability is also independent of adhesion as a single layer of platelets is retained in the presence of the inhibitors. The results emphasise the critical interplay of tyrosine kinase-based and G protein-coupled receptors in supporting the stability of

preformed thrombi under containing a shell-type of aggregated platelets under higher shear.

An important consideration is the extent to which results from the in vitro flow model can be extrapolated to formation of the shell region in vivo. The shell region is distinguished from the core by the absence of fibrin, and is composed of loosely packed, aggregated platelets which, at the extremes of the shell, retain their discoid shape suggesting that they are less active. It is this region that has been modelled in the flow experiments in this study due to the presence of PPACK which prevents thrombin and fibrin formation. However, in the in vitro flow model, the aggregate is formed on a layer of collagen or plaque material and not on a fibrin-rich aggregate.

A critical role for TxA_2 and the ADP $P2Y_{12}$ receptor in the formation of the shell in vivo has been reported[18] and has been replicated here in the flow model. Under arterial flow in vitro and in the absence of fibrin, inhibition of Syk has been shown to impair thrombus formation on collagen[23] and on fibrinogen,[7] suggesting a critical inter-play of integrin αIIbβ3 and GPVI in platelet activation. Further, continuous signalling through ADP has been shown to prevent platelet disaggregation in vitro by supporting binding of integrin αIIbβ3 to fibrinogen.[27] There exists a wealth of reports of synergy between G protein-coupled and tyrosine kinase-linked receptors in mediating platelet activation suggest that this is also likely to be replicated in vivo. However, fibrinogen does not activate GPVI in mouse platelets,[28] and the important role of Syk in thrombus formation[29,30] makes it difficult to investigate the role of Syk in thrombus stability *in vivo* in mice.

The present results delve deeper into the recent observations of Ahmed et al.[4] that the GPVI antagonist, ACT017, destabilises preformed thrombi through a pathway that is dependent on downstream signalling. The inability of Nb21 to mimic the effect of Syk inhibition is in contrast to the result with ACT017 and can be explained by the lack of effect of Nb21 on

fibrinogen-dependent activation of GPVI (GP, unpublished observation), in contrast to that of ACT017.[4] This provides indirect evidence that the two inhibitors bind to distinct sites on GPVI.

GPVI has long been recognised to be a promising anti-thrombotic target for many years,[31] but as of today, ACT017 (glenzocimab) is the only anti-GPVI blocker currently in clinical trial, where it is being used in combination with best treatment for acute stroke.[32] Inhibitors of αIIbβ3 are widely used against acute coronary syndrome but are not suitable for long-term therapy due to the risk of excessive bleeding.[33] Src, Syk and Btk inhibitors are currently available in clinic for the treatment of patients with solid tumour, ITP and haematological malignancies, and have been shown to be well tolerated.[34] Both GPVI and αIIbβ3 activate Src and Syk kinase suggesting that blocking either kinase may significantly impair thrombus formation while overcoming limitations concerning inhibitors of both receptors. Moreover, neither the Src or Syk inhibitors alter the response to G protein-coupled receptors signals which are critical for haemostasis.

The present results show similar effects with inhibitors of Syk and Src kinases but not with ibrutinib which blocks Btk. The latter may be due to redundancy with Tec or its position further down the GPVI signalling cascade. Src kinases are ubiquitous and as yet selective inhibitors have not been identified, and current inhibitors have a wide spectrum of off-target effects. On the other hand, Syk is restricted to haematopoietic cells. Further, the only inhibitor in the clinic, fostamatinib, has been shown to be well tolerated even in ITP patients with low platelet counts,[35] and there is evidence of a reduction in thrombus formation in patients on prolonged treatment with fostamatinib.[10]

In conclusion, the present results show a critical role for Syk in supporting thrombus stability under arterial shear and provide evidence that this is independent of activation of GPVI by collagen. The findings further emphasise Syk as a target for development of a new class of antiplatelet

agent that may prevent formation of occlusive thrombosis by both inhibiting thrombus formation and by promoting thrombus breakdown in areas of weak platelet activation.

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Supplementary materials and methods

Antibodies

Target antigen	Vendor or Source	Working concentrations
HRP-conjugated sheep α-mouse and donkey α-rabbit IgG	GE Healthcare (Little Chalfont, UK)	1:10000
phospho-specific pAb against LAT (pY200)	Abcam (Cambridge, UK)	1:500
anti-Syk mAb (4D10)	Cambridge Bioscience (Cambridge, UK)	1:200
phospholipase C (PLC)γ2 mAb (Q-20)	Cambridge Bioscience (Cambridge, UK)	1:200
anti-P-tyrosine mAb (4G10)	Cambridge Bioscience (Cambridge, UK)	1:1000
phycoerythrin (PE)-labelled mouse anti-human platelet GPVI mAb (clone HY101)	BD Pharmingen (San Diego, CA, USA)	1:200
APC-labelled mouse anti-human CD41a mAb (clone HIP8)	BD Pharmingen (San Diego, CA, USA)	1:100
fluorescein isothiocyanate (FITC)-labelled polyclonal rabbit anti-human fibrinogen	Dako (Santa Clara, CA, USA)	1:100

Other reagents

Target antigen	Vendor or Source
D-Phe-Pro-Arg chloromethyl ketone (PPACK)	Cambridge Bioscience (Cambridge, UK)
protein Sepharose A, bovine serum albumin (BSA, fatty acid free, ≥96%)	Thermo Fisher Scientific (Waltham, MA, USA)
NuPAGETM 4-12% Bis-Tris gel from	Thermo Fisher Scientific (Waltham, MA, USA)
PRT-060318	Caltag Medsystems (Buckingham, UK)
Ibrutinib	Toronto Research Chemicals (North York, ON, Canada)
Dasatinib	LC Laboratories (Woburn, MA, USA)
Ticagrelor	Sigma-Aldrich (Zwijndrecht, NL)
Thrombin	Sigma-Aldrich (Zwijndrecht, NL)
Indomethacin	AstraZeneca (The Hague, NL)
Eptifibatide	GSK (Brentford, UK)
Horm collagen	Nicomed (Munich, Germany)
Collagen diluent	Nicomed (Munich, Germany)
Thrombin-activating peptide (TRAP, SFLLNR)	Alta Bioscience (Redditch, UK)
Fibrinogen	Enzyme Research (Swansea, UK)
Heparin	Wockhardt (Wrexham, UK)

Chapter 7

Review: Platelet GPVI (glycoprotein VI) and thrombotic complications in the venous system

G. Perrella, M. Nagy, S.P. Watson & J.W.M. Heemskerk

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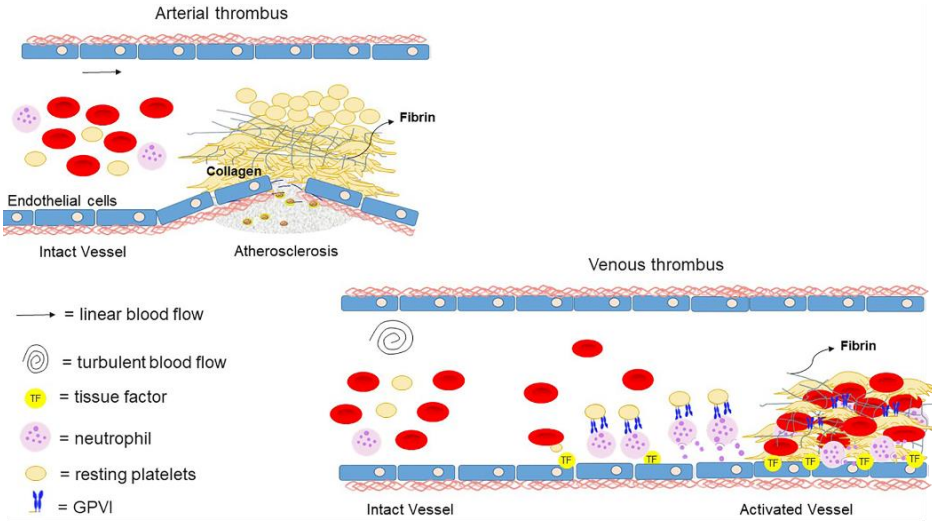
I co-wrote, revised and reviewed the manuscript together with M.N., S.P.W. and J.W.M.H.

Graphic Abstract



Highlights

- Platelets actively contribute to venous thrombosis with a suggested role of GPVI (glycoprotein VI).
- Platelet GPVI contributes to thrombosis in inflammation and cancers.
- Genetic variation in GP6 is a modest risk factor for venous thrombosis.

Abstract

The immunoglobulin receptor GPVI (glycoprotein VI) is selectively expressed on megakaryocytes and platelets and is currently recognized as a receptor for not only collagen but also a variety of plasma and vascular proteins, including fibrin, fibrinogen, laminin, fibronectin, and galectin-3. Deficiency of GPVI is protective in mouse models of experimental thrombosis, pulmonary thromboembolism as well as in thromboinflammation, suggesting a role of GPVI in arterial and venous thrombus formation. In humans, platelet GPVI deficiency is associated with a mild bleeding phenotype, whereas a common variant rs1613662 in the GP6 gene is considered a risk factor for venous thromboembolism. However, preclinical studies on the inhibition of GPVI-ligand interactions are focused on arterial thrombotic complications. In this review we discuss the emerging evidence for GPVI in venous thrombus formation and leukocyte-dependent thromboinflammation, extending to venous thromboembolism, pulmonary thromboembolism, and cancer metastasis. We also recapitulate indications for circulating soluble GPVI as a biomarker of thrombosis-related complications. Collectively, we conclude that the current evidence suggests that platelet GPVI is also a suitable co-target in the prevention of venous thrombosis due to its role in thrombus consolidation and platelet-leukocyte complex formation.

Interfaces of platelet and coagulation activation

It is established that the processes of platelet activation and coagulation are highly interconnected in hemostasis and (arterial) thrombosis.[1,2] The reported connection mechanisms are multiple, and involve in particular: (i) collagen- and thrombin-induced exposure on platelets of the procoagulant phospholipid phosphatidylserine, (ii) subsequent enhanced generation of factor Xa and thrombin on these procoagulant platelets, (iii) enforcement of platelet activation by the coagulation products thrombin and fibrin, and (iv) thrombin-mediated fibrinogen proteolysis to form fibrin and stabilize the platelet thrombus.[1,3,4] This concept of interactive thrombus-and-clot-formation is supported by static and flow studies, and it depicts platelets as thrombin- and fibrin-responsive cells, capable to aggregate and to generate massive amounts of thrombin and fibrin. Both the extrinsic (triggered by tissue factor) and intrinsic (activated by factor XIIa) coagulation pathways are considered to contribute to the thrombin generation process.[5] Modeling studies furthermore support the concept of tissue factor and factor XII playing critical roles in the thrombus formation at both arterial and venous flow conditions.[6,7] In the human body, the process is considered to start with the GPVI-dependent platelet adhesion to collagen present in the subendothelial matrix or ruptured atherosclerotic plaque.[8] In the context of thromboinflammation, that is when inflammation leads to thrombosis, also other cells come into the play. On the inflamed endothelium, circulating platelets interact with leukocytes (neutrophils, monocytes) again resulting in thrombin and fibrin generation.[9] In this article, we critically review the evidence for a role of GPVI in venous thrombosis alongside its contribution to arterial thrombosis.

GPVI and thrombus formation

GPVI is expressed exclusively on platelets and megakaryocytes. In the platelet membrane, GPVI is associated with the FcR γ (Fc receptor γ)-chain, which is responsible for the signaling via its immunoreceptor-

tyrosine-based-activation-motif.[10] Upon GPVI-ligand interaction and dimerization, the 2 tyrosine residues in the immunoreceptor-tyrosine-based-activation-motif become phosphorylated by Src-family kinases, which results in the binding and phosphorylation of the tyrosine kinase Syk (spleen tyrosine kinase) through its tandem SH2 (Src homology 2) domains. The ensuing phosphorylation cascade in the LAT signalosome leads to activation of phospholipase Cγ2 and phosphoinositide 3-kinases, culminating in a prolonged intracellular Ca^{2+} increase and other platelet responses.[11] As a main signaling receptor for collagen, GPVI has widely been studied in the context of arterial thrombosis, whereas patient examinations suggest a limited but non-negligible role in hemostasis.[12-14] An explanation is that in the absence of GPVI, hemostasis is preserved by parallel platelet pathways, for example, by platelet adhesion to collagen by the integrin α2β1 receptors and indirectly by GPIb-V-IX (glycoprotein Ib-V-IX) interacting with collagen-bound VWF (von Willebrand factor). Both GPVI and the FcR γ-chain have been found to be essential in murine arterial thrombosis models triggered by vascular injury and collagen exposure, regardless of the vascular bed, with a limited contribution to tail bleeding.[15]

The human *GP6* gene contains 8 exons, of which the last encodes for a short intracellular and transmembrane domain (Figure 1A-B). Nine *GP6* variants have been identified so far, the majority of which associates with a loss of receptor expression or function (Figure 1B-D). Individuals with platelet GPVI deficiency, carrying a common homozygous insertion in *GP6* that prevents protein expression, were identified in 11 unrelated families in Chile.[22] They all have normal platelet counts and no more than mild bleeding diathesis.[12] The platelets were shown to be dysfunctional in flow-dependent thrombus formation, but not in adhesion to collagen surfaces.[22] Gene mapping of a cohort of 1212 blood donors set the mutation incidence at 2.9% in the Chilean population, suggesting the existence of a large number of mutation carriers without clear

symptoms.[22] Whether these individuals are protected from thrombosis is unknown but of great interest. Furthermore, few patients have been identified with an acquired immune deficiency in platelet GPVI and associated thrombocytopenia.[13] In recent years, it has become clear that additional adhesive proteins in vessel wall or blood can function as ligands and agonists for GPVI, usually in conjunction with an integrin. These include vascular laminins (with integrin α6β1 as coreceptor),[23,24] fibrillar fibronectin,[25] and the basement membrane protein nidogen-1,[26] leading to the suggestion that it functions as a pattern recognition receptor.[27] In addition, it appeared that fibrinogen and fibrillar fibrin can bind and activate platelet GPVI.[28-30] However, in comparison to collagens or synthetic collagen-like peptides, the fibrin- or fibrinogen-mediated GPVI activation was found to have limited Syk-mediated signaling capacity and to rely on integrin αIIbβ3.[31]

Additive roles of GPVI and thrombin

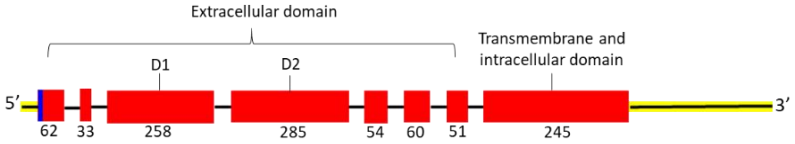
As a general scheme, in vitro flow perfusion studies point to a synergy of GPVI- and collagen-dependent platelet activation, aggregation, and procoagulant activity, together with a tissue factor-mediated generation of thrombin and fibrin.[2,5-6] Similarly, mouse models of in vivo thrombus and fibrin formation have elucidated an additive contribution of GPVI-induced platelet activation and tissue factor–induced coagulation, in the mesenteric arterioles and venules.[32,33] Complementarity of GPVI and thrombin activities also appeared from the structure of in vivo thrombi raised by collagen exposure, with an inner core of highly activated platelets, a transition zone, and an outer shell of loosely packed platelets, with fibrin fibers stabilizing the inner core.[34] Similarly, GPVI was found to regulate the stability and hence thrombus structure.[35] Mouse studies furthermore revealed a role of GPVI in the thrombin-sensitive ischemic stroke models, for example, via transient middle cerebral artery occlusion, in which GPVI depletion suppressed arterial platelet adhesion and drastically reduced infarct size in the brain, independently of platelet

aggregation.[36-38] Together, these findings draw attention to roles of platelet GPVI beyond the classical collagen-induced aggregation.

Platelets and venous thrombosis

The effective treatment of venous thrombotic complications by a wide spectrum of anticoagulants implicates that especially thrombin (fibrin clotting) has an overall controlling role in venous thrombus formation, thus leaving a subordinate role for platelets. The spectrum of VTE includes deep venous thrombosis (DVT) and pulmonary thromboembolism. Worldwide VTE is the third common cause of cardiovascular mortality after coronary artery disease and stroke. Importantly, VTE is considered a long-term and phased disease since an initial (unprovoked) thromboembolism in the venous system can be followed by recurrent VTE, life-threatening emboli in the lungs, and a post-thrombotic syndrome.[39] Phenotypically, the accepted model is that impaired blood flow together with hypercoagulability and endothelial dysfunction (Virchow's triad) gradually lead to the formation of large-size venous red thrombi, composed of fibrin, platelets, and red cells; a process that is driven by thrombin and can start in the valve pockets of large veins.[39,40] Activated platelets contribute to the venous thromboembolic events. Although less predominant than in arterial thrombi, platelet aggregates were found to comprise a relevant part of analyzed venous clots.[41,42] This agrees with the outcome of in vitro microfluidic studies, which assign a role of GPVI and other platelet receptors in thrombus formation at both venous and arterial flow conditions.[43] In adapted microfluidic chambers simulating venous flow disturbances around valves, it was found that platelets contribute here to the thrombus and clot growth by interacting with fibrin.[7] Evidence for a consistent role of platelets in venous thrombosis furthermore comes from in vivo mouse studies.

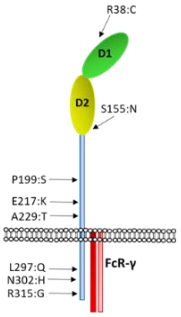
A)



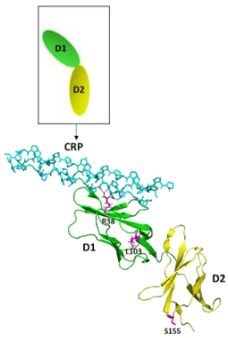
= 5' upstream and 3' downstream sequence
= Promoter
= Exon
= Intron

Reported are number of nucleotides

B)



C)



D)

GPVI Natural variants	Position in the translated protein	Description
R58:C	R38:C	Abnormal protein migration and a loss of collagen binding.[117]
L123:V	L103:V	Reduced collagen binding.[118]
S175:N	S155:N	Reduced membrane expression and reduced snake toxin convulxin binding.[119]
P219:S	P199:S	Reduced expression on platelet, impaired collagen binding, reduced VTE risk in healthy subjects.[52]
E237:K	E217:K	Fetal loss in patients with sticky platelet syndrome.[120]
A249:T	A229:T	Fetal loss in patients with sticky platelet syndrome. [120]
L317:Q	L297:Q	Results in a modified protein.[118]
N302:H	N322:H	Positive association between the GG genotype and type 2 diabetes.[121]
R335:G	R315:G	Results in a modified protein.[118]

Figure 1. GPVI (glycoprotein VI) structure and natural variants. A*, Schematic presentation of the human GP6 gene structure. The first seven exons encode for the protein extracellular domain, with exons 3-4 encoding for domains D1 and D2, respectively. Exon 8 encodes for the transmembrane and short intracellular region.* ***B****, Cartoon showing the human GPVI protein domains with amino acid positions of the nine GPVI natural variants and mutations indicated.* ***C****, Representation of crystal structure of the GPVI binding site interacting with CRP (collagen-related peptide).* ***D****, Table of reported effects of GPVI variants on protein expression and platelet functions.*[16–21] *FcR γ indicates Fc receptor γ; and VTE, venous thromboembolism.*

We note here that the present mouse venous thrombosis models have limitations in the way of triggering thrombus formation (usually flow restriction) and a short time of thrombus development (days to weeks). Nevertheless, it was shown that following veins flow restriction in such DVT models, platelets promote the recruitment of leukocytes (monocytes and neutrophils) to the activated endothelium. This resulted in a leukocyte-dependent coagulation process and the buildup of a venous thrombus or clot.[44] It was proposed that leukocyte-expressed tissue factor was instrumental in the induction of thrombin generation. In a similar mouse model of venous flow restriction, the amyloid precursor protein was identified as negative regulator of platelet-neutrophil interactions, fibrin-thrombus formation, and embolization; this led to the suggestion that the platelet-derived amyloid precursor protein may limit VTE.[45] However, other mouse studies indicated that also platelet-expressed P-selectin and chemokines regulate leukocyte interactions.[46] Furthermore, mouse platelet deficiency in the secretion-controlling protein SNAP23 (a condition leading to thrombocytopenia) resulted in an impaired thrombosis tendency in both arteries and veins.[47] Collectively, these rodent studies suggest a partly leukocyte-dependent and partly fibrin-dependent role of platelets in the development of experimental VTE.

In humans, recent clinical studies to compare treatments of coronary artery disease advocate the combined use of antiplatelet and anticoagulant medication (dual pathway therapy). In the COMPASS trial (Cardiovascular Outcomes for People Using Anticoagulation Strategies), it was concluded that the combination of aspirin (weak platelet inhibitor) and rivaroxaban (factor Xa antagonist) resulted in a better cardiovascular outcome, when compared with single aspirin or rivaroxaban alone.[48] Although the rivaroxaban treatment led to more bleeding events than aspirin intake, the dual pathway therapy resulted in less VTE events when compared with the monotherapies. This suggested a favorable effect of aspirin also for patients with only venous thrombosis. The INSPIRE initiative with 1200 patients concluded that aspirin plus anticoagulant treatment reduced the overall risk of recurrent VTE by more than one-third.[49] However, later meta-analyses and post hoc studies did not confirm risk reduction of VTE by aspirin on top of anticoagulant.[50-52] It is remarked here that aspirin in vitro is a weak platelet inhibitor, suppressing collagen-induced, but not thrombin-induced platelet responses. Altogether, this led us to speculate that stronger antiplatelet drugs are more effective in preventing venous thrombotic events, for instance in VTE or in atrial fibrillation.

Genetic variation in GP6 and venous thrombosis

In 2007, the incidence of DVT was estimated at 1 per 1000 individuals per year, with a 10-year recurrent risk of about 30%.[53] Several risk factors for DVT have been recognized, including age, hospitalization, cancer, pregnancy, anticonception, and surgery.[53] Family studies have estimated that approximately half of the DVT cases are heritable.[54] Yet, the identified genetic factors, although of large effect sizes, account for only a minority of all DVTs. Genetic factors link to deficits in the anticoagulant proteins, antithrombin protein C and protein S, as well as to a gain-of-function in the procoagulant proteins factor V (factor V Leiden or Padua) and prothrombin (G20210A mutation).[54,55] Next to the genes of these

(anti)coagulant factors, efforts have been made to search for additional (common) variants that associate with DVT, for a better risk prediction and understanding of the disease.

In a first population-based case-control study, the Leiden group examined 20 000 single nucleotide variants, which after a 4-stage refinement protocol resulted in 3 single nucleotide variants that significantly associated with DVT.[53] A minor allele of the antithrombin gene *SERPINC1* (T, rs2227589) had a modest prothrombotic tendency (Table 1). Interestingly, the same held for a major allele of *GP6* (G, rs1613662), which is linked to a higher GPVI expression on platelets. A meta-analysis of 5 studies encompassing 4000 VTE cases and 6100 controls indicated that the 2 single nucleotide variants of *SERPINC1* and *GP6* counted as 15% higher risk factors for venous thrombosis in White patients (Table 1). Although was not seen for Black patients, the sample size was underpowered here for a definitive conclusion.[64] In a prospective Danish case-cohort study, it was later confirmed that the heterozygous presence of this *GP6* allele (G, rs1613662) moderately, but significantly, increased the hazard ratio for VTE.[65] Since 2015, the research has identified 17 VTE-associated genes, predominantly of (anti)coagulation factors, including *GP6*, *ABO*, and secretion-regulating genes appearing as network disconnected entities.[54] In a recent study of the association between this *GP6* SNP and VTE risk in cancer, it was found that cancer-free AA-allele carriers had a 12-29% higher risk of VTE or DVT (nonsignificant) and a 53% to 61% higher risk of pulmonary thromboembolism.[16] The different risk between genotypes, however, disappeared for the (lower numbers of) patients with active cancer, which suggested that the cancer state overruled mild effects of a variable GPVI expression.[16]

The risk *GP6* variant rs1613662 consists of an A/G conversion, which introduces a serine to proline substitution in amino acid 219 and is considered to affect the expression of GPVI on platelets (Figure [B] and [D]).[16]

Table 1. *Epidemiological support for a role of variation in genes of GPVI, FcR γ-chain and antithrombin in the venous thrombosis risk.*

Study	Disease (end-term)	Cases	Population	Gene SNV (odds* or hazard†) GP6‡ rs16113662	FCER1G§ rs3557	SERPINC1‖ rs2227589	Reference
MEGA 2	DVT	1314	2877	1.14*	-	1.29*	Bezemeret et al[53]
GWAS	VTE	419	1228	1.18*	-	1.23*	Austin et al,[64] Trégouët et al[70]
MARTHA	VTE	1150	1150	1.08*	-	1.00*	Austin et al,[64] Trégouët et al[70]
FARIVE	VTE	607	607	1.27*	-	1.00*	Austin et al,[64] Trégouët et al[70]
GATE	VTE	544	661	1.04*	-	1.07*	Austin et al[64]
Meta-analysis 5 studies VTE		4021	6147	1.17* (A)	-	1.15* (T)	Austin et al[64]
DCCS	VTE	600	1742	1.18†	-	1.17†	El-Galaly et al[65]
TS	DVT	1071	12 446	1.29†	-	-	Skille et al[16]
TS	VTE	144	12 446	1.14†	-	-	Skille et al[16]
TS	PE	89	12 446	1.61†	-	-	Skille et al[16]
GWAS	in vitro thrombus	94	(173 480)	1.10†	1.07†	-	Petersen et al,[68] van Geffen et al[69]

Abbreviations: DCCS indicates Danish case-cohort study; DVT, deep venous thrombosis; FcR γ, Fc receptor γ; GATE, Genetic Attributes and Thrombosis Epidemiology; GPVI, glycoprotein VI; GWAS, Genome Wide Association Study; MARTHA, Marseille Thrombosis Association study; MEGA, Multiple Environmental and Genetic Assessment of Risk Factors for Venous Thrombosis study; PE, pulmonary thromboembolism; SNV, single nucleotide variant; TS, Thrombosis study; and VTE, venous thromboembolism.

* Hazard ratio with variable adjustments (none, age, sex, body mass, cancer site, cancer stage).

† Odds ratio with variable adjustments (none, age, sex, body mass, cancer site, cancer stage).

‡ GP6 rs16113662 (Ser219Pro, A/G). GP6 risk allele frequency A 0.73-0.84, GG lower GPVI expression.

§ FCER1G rs3557 (promoter SNV, T/G). FCER1G risk allele frequency T 0.85, G lower GPVI expression.

‖ SERPINC1 rs2227589 (intronic SNV, C/T); Risk allele frequency T 0.10–0.17, lower antithrombin activity); unclear relation with rs2227624 (Val30Glu, A/T) with conflicting link to thrombotic diseases (GeneCards).

Platelets carrying the minor G-allele thus express less GPVI receptors and can be impaired in collagen-dependent adhesion and activation properties.[66,67] Shear-dependent thrombus formation was found to be reduced in these individuals to a similar extent as in subjects carrying a variant (rs3557) of *FCER1G* encoding for the FcRγ-chain, which also associates with lower GPVI expression (Table 1).[68,69] In agreement with the linkage between GPVI and FcRγ-chain, it appears that human platelet deficiency in GPVI is accompanied by a lower expression of the FcRγ-chain.[22]

In addition to the common variant P219S of GPVI, several other rare mutations in the *GP6* gene are described that associate with an altered collagen-receptor binding or a reduced GPVI expression level on platelets (Figure 1D). Markedly, heterozygous deficiency in human *GP6* seems to be accompanied by a no more than subtle effect on platelet function.[12,22] Similarly, in heterozygous *GP6*$^{+/-}$ mice, no obvious platelet phenotype was found.[71]

Mouse GPVI and venous thrombosis

In mice, it is well established that antibody-mediated or genetic deficiency of either GPVI or FcRγ-chain results in abrogation of thrombus formation in the arterial circulation, with limited effects on tail bleeding.[15,72] A few available reports also point to a role of mouse GPVI in venous thrombosis, although knowledge is still scarce here. In the murine microcirculation, it was observed that injury-induced thrombus formation in the venules required collagen-dependent platelet activation and tissue factor–induced thrombin generation, with a procoagulant role herein of phosphatidylserine-exposing platelets.[32] Upon injury of the mouse mesenteric tissue, thrombus formation was reduced to a similar extent in the venules and arterioles, after the depletion of platelet GPVI with JAQ1 monoclonal antibody against GPVI or after genetic deletion of the FcRγ-chain.[73]

Acknowledging the limitations of current large-vein thrombosis models in mouse (eg, artificial flow restriction), these do point to a key role of platelets in general and of GPVI in particular. In transgenic mice with low levels of anticoagulant factors (antithrombin or protein C), it was established that a (fatal) thrombotic occlusion of large vessels was dependent on platelet activity next to tissue factor.[74] In this context, considering that galectin-3–binding proteins can also act as GPVI ligands,[75] an interesting finding is that the injection of galectin-3 enhanced the venous thrombosis induced by stasis.[76] It should be stated, however, that next to GPVI, also platelet GPIb-V-IX and its ligand VWF can act as functional players in mouse venous thrombosis models.[77,78] Moreover, in mouse embolization models, it was reported that GPVI depletion causes a transient protection against tissue factor–induced pulmonary thromboembolism.[79]

Collectively, these mouse studies lead to the attractive, but still unproven, supposition that GPVI acts as a central platelet receptor in the context of venous thrombus formation and embolization and by implication that anti-GPVI cotreatment might provide antithrombotic protection by affecting thrombus formation in a both collagen- and thrombin-dependent way.

Soluble GPVI as a biomarker in thrombotic diseases

Both in humans and mice, GPVI is stably expressed on resting platelets, but it can be extracellularly cleaved in the presence of antibodies or agonists, resulting in shedding of the GPVI extracellular ectodomain. The proteolysis results in a platelet population that is essentially devoid of functionally active GPVI.[80,81] Shedding of GPVI is primarily mediated by ADAM proteases (a disintegrin and metalloprotease), which become enzymatically active upon platelet activation. From experiments with mice deficient in ADAM10 or ADAM17, it was postulated that yet another enzyme can contribute to antibody-induced GPVI shedding.[82] In humans, ADAM10 is primarily responsible for the GPVI cleavage secondary to

conditions including receptor activation, high shear stress, or exposure to Ca^{2+}-ionophore or factor Xa.[81,83-85] In contrast to the GPVI shedding, ADAM17-induced cleavage of GPIbα appears to be a constitutive process upon platelet aging, which occurs independently of GPVI ligands.[86] Because GPVI is selectively expressed on platelets and megakaryocytes, the presence in the circulation of sGPVI (soluble GPVI) has been postulated as a biomarker reflecting platelet activation in vivo. Elevated plasma levels of sGPVI are reported in circumstances with a prothrombotic propensity, such as acute ischemic stroke, thrombotic microangiopathy, rheumatoid arthritis, disseminated intravascular coagulation, atrial fibrillation, and DVT (Table 2).[56-58,61] Along the same line, elevated sGPVI accompanied the major bleeding events in patients with heparin-induced thrombocytopenia, that is, a condition relying on prior platelet activation through the low-affinity Fc receptor, FcγRIIA.[60] Higher levels of circulating sGPVI are also seen during sepsis progression, in worse-outcome trauma patients, or at gout flares.[59,62]

Although the majority of studies on cleaved GPVI relate to arterial thrombotic conditions, elevated sGPVI was also observed in patients developing DVT postoperatively, suggesting that the cleaved fragment can act as a biomarker in venous thrombosis.[58] In support of this, GPVI shedding can be induced by fibrin clots in sepsis or trauma.[62]

Taken together, these data suggest that the presence of soluble GPVI reflects in situ platelet activation in cases of multiple longer-term cardiovascular-related pathologies. Other diagnostic markers of platelet activation, such as β-thromboglobulin and platelet factor 4,[87] or of coagulant activity, such as thrombin-antithrombin complexes and D-dimers,[88] may rather detect more acute thrombotic events. A future well-structured study to compare these various platelet activation-dependent biomarkers may help to better understand the pathologies of distinct thrombotic diseases.

Table 2. *Changes in circulating sGPVI observed in thrombotic and related diseases*

Disease	sGPVI level	Effect	References
Acute ischemic stroke	↑	increased in cohort of patients	Al-Tamimi et al[56]
Atrial fibrillation	↑	increased in cohort of patients	Bigalke et al[57]
Deep venous thrombosis (DVT)	↑	increased postoperatively	Aota et al[58]
Disseminated intravascular coagulation	↑	increased in cohort of patients	Al-Tamimi et al[56]
Gout	↑	correlated with gout flares	Pishko et al[60]
Heparin-induced thrombocytopenia	↑	correlated with bleeding events	Conway et al[59]
Rheumatoid arthritis	↑	increased in seropositive arthritis	Stack et al[61]
Sepsis	↑	correlated with sepsis progression	Montague et al[62]
Stable angina pectoris	↑	increased in cohort of patients	Bigalke et al[57]
Thrombotic microangiopathy	↑	correlated with thrombotic events	Yamashita et al[63]
Trauma	↑	correlated with mortality	Montague et al[62]
DVT indicates deep venous thrombosis; and sGPVI, soluble glycoprotein VI.			

Platelet GPVI in the wider context of thromboinflammatory conditions

The term thromboinflammation is used to describe a condition where thrombotic and inflammatory events are pathological and lead to organ damage. It also implies a role of platelets in the vascular-related inflammation, often occurring in the microcirculation. Thrombo-inflammation was first described as a pathological process in sepsis and in ischemia-reperfusion injury.[9] The thromboinflammatory process is

supposed to be driven by coagulation activation, with anticoagulant therapies being effective although with bleeding side effects.[89] Mechanistically, is not well understood how platelets contribute to a vascular inflammatory potential, but they likely support this by the release of chemokines and cytokines.[90] An attractive model here is that the activated endothelial cells of the inflamed vessel wall express P-selectin and VWF, which triggers leukocyte and platelet adhesion, after which leukocyte-expressed tissue factor induces the formation of a venous clot.[44] Support for a platelet secretory role in the clotting process comes from the use of mice with platelet secretion defects.[47] In mouse models of nonsterile thromboinflammation, platelets were found to stimulate neutrophil granular release in a GPVI-dependent manner.[91] In pneumonia-induced sepsis, GPVI appeared regulate the formation of platelet-leukocyte complexes.[92] However, based on this limited knowledge, it appears that at the inflamed vascular beds GPVI can contribute to the inflammation process, besides its involvement in thrombus formation. Platelets furthermore help to maintain vascular integrity and prevent blood loss from leaky vessels in inflammatory conditions. The few published mouse studies on inflammatory hemostasis indicate that platelet GPVI plays a role in the regulation of vascular integrity albeit in an organ- and stimulus-dependent manner, alongside the receptors CLEC2 (C-type lectin 2) and GPIb-IX-V.[93-96] However, there are no reports on inflammatory hemostasis or bleeding in GPVI-deficient individuals. Inflammation-propagating effects of platelets have also been examined in the context of coronavirus disease 2019 (COVID-19). An infection by severe acute respiratory syndrome coronavirus-2 can lead to a wide range of clinical manifestations, varying from absence of symptoms to severe pneumonia which can progress into an acute respiratory distress syndrome and sepsis. In subjects at risk (elderly, patients with comorbidities), massive vascular inflammation is frequently observed, culminating in disseminated intravascular coagulopathy, arterial or venous thrombosis, and pulmonary

thromboembolism.[97,98] The underlying condition is characterized as (pulmonary) endothelialitis and thromboinflammation.[9] An assumption is that platelets, likely by interacting with leukocytes, promote the thromboinflammatory activity in SARS-CoV-2 infections.[99] Reports are, however, inconsistent regarding alterations in platelet responses in severely diseased patients with COVID-19, ranging from negative priming[100,101] to positive priming.[102,103] Because low platelet counts are uncommon,[97,104] it is unlikely that platelet activation is a disease trigger. A clinical study on the effect of GPVI antagonist glenzocimab in severe acute respiratory syndrome coronavirus-2 syndrome is in current progress (https://www.clinicaltrials.gov; NCT04659109).

Role of GPVI in cancer-induced thrombosis

The relation between cancers and (venous) thrombosis was already discovered in the nineteenth century.[105] Nowadays, it appears that (venous) thrombotic events are the second leading cause of death in cohorts of cancer patients, whereas conversely, a history of idiopathic venous thrombosis increases the susceptibility for developing cancer.[106] A high risk score for VTE is a predictive variable for earlier mortality in treated cancer patients.[107] In spite of the fact that cancers are different in origin, development, and fate, there is increasing evidence that platelet interaction with a tumor exposed to the circulation promotes cancer dissemination and metastasis.[108] Platelets can influence tumor cells by several mechanisms, including (i) the release of granular growth factors, matrix proteins, and inflammatory mediators, (ii) the expression of P-selectin and other cell-adhesive receptors, and (iii) the fibrin-mediated interaction of immune cells with a tumor.[108-110]

The few available mouse studies point to a role of platelet GPVI especially in tumor metastasis. Platelet GPVI deficiency resulted in less metastatic foci after the implantation of tumor cells into mice.[75,111] A recently suggested mechanism is that GPVI mediates platelet interaction with cancer cell-derived galectin.[75] We speculate that GPVI can also promote

metastasis via the binding to fibrin clots, which are formed around tumor cells expressing tissue factor.

Clinical possibilities for GPVI antagonism and agonists

Selected inhibitors of platelet GPVI are close to enter the clinic for treatment of thrombosis. A clinical study is under way with a GPVI-blocking Fab (ACT017, glenzocimab), aiming to treat acute ischemic stroke.[112] In transgenic mice carrying the human *GP6* gene, glenzocimab was found to be effective in thrombus suppression, without impacting GPVI-dependent inflammatory hemostasis.[113] However, multiple inhibitors of protein tyrosine kinases downstream of GPVI, that is, Syk and Btk (Bruton tyrosine kinase), have extensively been evaluated in clinical trials and are currently prescribed for the treatment of B cell malignancies.[114-117] This type of chemotherapy is mostly well tolerated in diseased patients while causing only limited side effects such as bleeding, nausea, vomiting, or diarrhea.[118,119] GPVI-dependent antiplatelet effects can be expected from the use of these drugs as reported for the Btk inhibitor ibrutinib, prescribed to target B cells in chronic lymphocytic leukemia, along with effects on GPIb-mediated platelet responses.[120] With the introduction into the clinic of more tyrosine kinase inhibitors, it will be interesting to see if the antithrombotic effects eventually reported will link to a suppression of GPVI activation in platelets, such as already shown for ibrutinib in the setting of deep vein.[119,121]

GPVI-fibrin-thrombin loop as a novel target

Given the recent evidence for a role of GPVI in fibrin(ogen)-dependent thrombus formation and stability,[28,35] we speculate that in the venous thrombosis setting especially fibrin may act as a relevant GPVI agonist. Although the signaling strength of fibrin to GPVI is only low,[31] we note that venous thrombus formation is usually a slow process, in comparison to arteries. This raises the possibility that a weak but prolonged GPVI signal might be pathophysiologically relevant. Because in the venous

setting, GPVI will act on platelets in conjunction with thrombin, anti-GPVI treatment is likely to enforce the antithrombotic effect of anticoagulant drugs in venous thrombotic diseases. However, trials still need to be performed to support this idea.

Conclusive model and perspective

Collectively the studies in this review support the concept of VTE as a disease process, where platelet GPVI may play a role at different stages, although multiple questions remain (Table 3). A mechanistical question is whether fibrin is indeed the principal GPVI agonist under conditions of venous thrombosis and embolism. Assuming that the answer is yes, we propose that the formation of a venous thrombus is steered by a slow but continuous GPVI-thrombin-fibrin feedforward loop, in which fibrin-bound platelets expose low levels of phosphatidylserine in a GPVI-dependent manner, after which the activity of locally generated thrombin is dampened by its binding to fibrin. Under flow conditions in vitro, we have observed that GPVI consolidates rather than propagates the formation of a thrombus.[31] However, once stasis is reached, this loop can still allow the gradual growing of a clot over time, balanced in vivo by endothelial activity. Future elucidation of the crystal structure resolving the binding sites of collagen and fibrin(ogen) to GPVI (Figure [C]), and development of selective inhibitors based on the fibrin binding site, will help to resolve the importance of this feedforward loop. So far, it is also known that binding to fibrinogen relies on the avidity of GPVI-GPVI interactions.[122] At the inflamed and then less antithrombotic endothelium, we propose that GPVI can influence thrombus formation in multiple ways. The endothelial activation leads to VWF release and P-selectin expression, which results in capturing and activation of both platelets and neutrophils in part via GPVI.[44] In this setting, GPVI can increase the thromboinflammatory status by promoting neutrophil granular release[91] and support the thrombus formation through fibrin binding.[28,31] The GPVI-activated and pro-coagulant platelets will furthermore contribute to the generation of

thrombin and fibrin. At present, it is unclear how the interindividual variation in platelet GPVI levels fits in this scenario. However, in analogy to the other genetic risk factors of venous thrombosis (defects in the anticoagulants protein C, protein S, antithrombin), one can assume that GPVI expression level determines the activity of these interactions as in the proposed GPVI-thrombin-fibrin loop. All these intriguing aspects together make GPVI an attractive receptor for further studies on VTE and for deeper understanding of the sets of mouse and human data (Table 3).

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The authors report that no relevant conflicts of interest exist.

Nonstandard abbreviations and acronyms

ADAM	a disintegrin and metalloprotease
Btk	Bruton tyrosine kinase
COVID-19	coronavirus disease 2019
DVT	deep venous thrombosis
FcR γ	Fc receptor γ
GPVI	glycoprotein VI
sGPVI	soluble GPVI
Syk	spleen tyrosine kinase
VTE	venous thromboembolism
VWF	von Willebrand factor

Table 3. *A Summary of what is currently known and unknown regarding platelet GPVI, murine VT, and VTE.*

What is known?	Open questions	Authors' opinions
1. Platelets via GPVI are drivers of arterial thrombosis by collagen on plaque.	1. Is fibrin the agonist for GPVI in venous thrombosis?	1. Fibrin likely acts as early GPVI agonist in the VTE setting. Rationale: although fibrin is a weak GPVI agonist, its effects are enhanced by tissue factor and thrombin.
2. Crystal structure of GPVI binding to collagen peptide. Avidity interaction of GPVI binding to fibrin required.	2. How do the crystal structures of GPVI-collagen peptide and GPVI-fibrin peptide compare?	2. Resolving both crystal structures will improve understanding the relative importance of GPVI in venous thrombosis and thromboinflammation.
3. In vitro flow models describe a role of human GPVI at low shear rates.	3. Is there a thresh-old of coagulant activity that restricts the role of GPVI in venous thrombus propagation and stability?	3. Assuming that fibrin is the GPVI agonist in VT, fibrin will limit thrombin activity by acting as a sink. This provides a coagulation-dampening effect.
4. Platelet granule secretion and P-selectin expression support experimental VT in mice.	4. Does agonist-GPVI interaction cause relevant granule secretion in VT?	4. In VTE, thrombin may promote platelet secretion and P-selectin expression. At least in mouse models, GPVI has a (non)redundant role herein.
5. Thromboinflammation links VTE to inflammation.	5. Is GPVI contributing to VTE by driving inflam-mation or by altering vascular integrity?	5. In thromboinflammation, the role of GPVI may be dual: *(i)* platelet adhesion to collagen in the extracellular matrix preserving vascular integrity; *(ii)* platelet adhesion at an activated endothelium to induce leukocyte stimulation.
6. In vivo mouse models demonstrate a role of platelet GPVI in VT.	6. Can these mouse models be translated to human disease?	6. To link mouse and human data sets we propose the following research lines: *(i)* fine-tuning of mouse VT models to approximate the human situation; *(ii)* dose-response evaluation of effects of GPVI antagonism in mouse VT models; *(iii)* analysis of current clinical trials with GPVI antagonists for (un-anticipated) changes in VTE or PE.

GPVI indicates glycoprotein VI; PE, pulmonary thromboembolism; VT, venous thrombosis; and VTE, venous thromboembolism.

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Chapter 8

General discussion

The suggested minor role of human glycoprotein VI (GPVI) in haemostasis versus thrombosis, alongside the finding that GPVI is a receptor for fibrin and fibrinogen, raised questions on the physiological significance of these interactions and on whether GPVI can be targeted in arterial thrombosis without causing bleeding complications. As a general hypothesis for this thesis, I proposed that GPVI is a relevant signalling receptor for fibrin and fibrinogen in thrombus propagation and stability. To investigate this, my aims were: *i)* to further explore the platelet phenotype in individuals with a GPVI-deficiency, in order to better understand the roles of this receptor in thrombosis and haemostasis; *ii)* to compare the processes of GPVI-dependent platelet activation and thrombus formation in response to collagen, fibrin and fibrinogen; *iii)* to explore whether targeting signalling pathways downstream GPVI could affect thrombus propagation and stability; and *iv)* to examine the role of GPVI in venous thrombotic complications, analysing its usefulness as a target in the clinical setup. Overall, these studies demonstrate that human GPVI is critical for platelet aggregation and phosphatidylserine exposure on collagen and non-collagen surfaces, but not for adhesion under arterial flow conditions. In addition, they indicate that binding of fibrinogen and fibrin to GPVI supports thrombus growth and stability, with a partial overlap with integrin αIIbβ3. All together, these findings indicate that GPVI is an interesting platelet receptor with great potential in the clinic. Table 1 provides a summary of major findings of this thesis and their contribution to the field.

1. Platelet GPVI in thrombus formation and haemostasis

The most direct evidence for a role of platelet GPVI in arterial thrombosis came from mouse models, where pharmacological blockage or deficiency in GPVI suppressed thrombus formation after injury of a healthy or atherosclerotic artery.[1] The interest in this receptor as a possible anti-thrombotic target was further raised by the observation that individuals with an inherited deficiency in GPVI have a mild bleeding diathesis,[2]

alongside with the findings that GPVI is exclusively expressed on platelets and megakaryocytes and that it supports thrombus formation on atherosclerotic plaques.[3,4] However, there remained lack of information on the phenotype and functional response of platelets from patients with genetic loss of GPVI. To explore this, in *Chapter 3,* I studied the process of thrombus formation using blood from GPVI-deficient individuals. As of today, nine individuals from eight unrelated families with a GPVI deficiency, have been identified worldwide. They carry an adenine insertion in the *GP6* gene that prevents surface expression of the receptor. For this thesis, I used platelets from three heterozygous and four homozygous individuals with this mutation. The experiments were performed under coagulating and non-coagulating conditions, in order to compare GPVI activation in the presence and absence of thrombin. Using the Maastricht flow chamber, I showed that GPVI supports the formation of platelet aggregates on both collagen and non-collagen surfaces, and that it regulates phosphatidylserine exposure on collagen, regardless the presence of thrombin. I also showed that platelet adhesion to collagen is relatively unaffected by lack of GPVI, a result in contrast to mice, where absence of the receptor suppresses both platelet adhesion and thrombus formation on collagen.[5]

Together, these results add new insight to the accepted model of arterial thrombosis, which proposes that GPVI with integrin α2β1 supports the initial interaction with collagen.[5,6] The initial platelet adhesion through the integrin α2β1 may explain the mild bleeding diathesis of the homozygous GPVI-deficient individuals, suggesting that in these patients, platelet adhesion and aggregation through integrin α2β1 and αIIbβ3 respectively, along with tissue factor-driven thrombin generation provide for the lack of GPVI. The hypothesis of a compensatory mechanism is supported by the calculated high frequency of the *GP6* c.711_712insA mutation in the Chilean population, which indicated that approximately 4000 asymptomatic individuals homozygous for this mutation are

currently living in Chile without showing bleeding symptoms (*Chapter 3*). On the other side, on a site of rupture of an atherosclerotic plaque, GPVI may be critical for thrombus formation, as the very high shear might causes the rapid dilution of agonists making collagen-induced activation of platelets via GPVI more significant. Interestingly, many components of atherosclerotic plaques have a charged surface.[7,8] In *Chapter 3*, we showed that GPVI can be crosslinked by charge interactions as spreading on VWF and glass surface, two structural different surfaces, was abolished with GPVI-deficient platelets. Together, this work supports the conclusion that GPVI could be selectively targeted to prevent arterial thrombosis with limited effects on haemostasis.

2. Ligands of GPVI and integrin αIIbβ3 in thrombus formation

In 2015, fibrin and later fibrinogen were discovered as novel ligands for GPVI. However, the mechanistic details and the physiological significance of this interaction have remained unclear. In order to investigate these, in the present thesis I used two approaches. At first, I re-examined the controversies existing in literature concerning the binding of recombinant GPVI constructs to fibrin and fibrinogen (*Chapter 2*). Secondly, I explored the involvement of GPVI in response to fibrin- and fibrinogen-dependent thrombus formation under arterial shear rate (*Chapter 4*).

The evidence that GPVI monomers bind to fibrin was surprising, as it was in contrast with the idea at the time that platelet stimulation by soluble agonists induces GPVI dimerization, thereby priming platelets for activation.[9] It was also unclear how monomeric GPVI could induce platelet signalling responses. Although functional data consistently demonstrated a critical role for GPVI in mediating platelet activation by fibrin and fibrinogen, controversies arose about the structural and mechanistic details of this interaction (*Chapter 1*). To fully understand this, in *Chapter 2,* I critically analysed the monomeric and dimeric GPVI constructs used by the various groups to study fibrin or fibrinogen binding, alongside the techniques and reagents employed. This extensive analysis revealed some

of the limitations of the studies, including lack of knowledge on the recombinant GPVI proteins used. As GPVI has domains with high charges, we hypothesised that even small changes in the GPVI constructs may have implied diverse dynamics and three-dimensional orientation of the proteins. In addition, the use of constructs formed by only the extracellular region of GPVI makes unclear whether the transmembrane and intracellular regions also take part to the activation. An answer to the debate came from a recent work showing that fibrin and fibrinogen can bind to both monomeric and dimeric GPVI, with higher affinity for the dimer due to an increased avidity.[10] Furthermore, a separate study has shown that GPVI dimerization is not critical for GPVI-collagen adhesion.[11] In conclusion, it seems that solving the whole structures of GPVI with collagen, fibrin and fibrinogen is needed to identify the specific binding sites. When this is solved, it can guide the design of new therapeutics that effectively target GPVI interaction with one ligand or another.

In this work we also proposed the use of D-dimer as standard reagent for ELISA assays. D-dimer is a fibrin degradation product and GPVI binding site on fibrin. D-Dimer has a simpler structure than the three-dimensional network of fibrin fibers and therefore could be more suitable for coating than immobilized fibrinogen or fibrin. Interestingly, D-dimer has been shown to weakly inhibit GPVI binding to a fibrin clot, as well to inhibit platelet aggregation in response to collagen[12], perhaps suggesting that this fragment could represent a potential anti-thrombotic treatment. In line with this, high levels of D-dimer have been associated with impaired platelet signalling during trauma.[13] Furthermore, platelet have been shown to uptake D-dimer from plasma of trauma patients, thus becoming unable to aggregate in response to various agonist including GPVI ligands and ADP.[13] Given that D-dimer does not induce platelet aggregation by itselfs,[12] it might be used as platelet inhibitor. Further studies are needed to assess the potential bleeding risk of this therapeutic strategy that could be arise from the inhibition of the integrin inside-out signalling.

The finding that GPVI is a receptor for fibrin(ogen), next to the well-known integrin αIIbβ3, raised questions as how these two receptors contribute to fibrin-dependent platelet activation. In *Chapter 4*, I observed that under coagulating conditions, GPVI induces low-level platelet aggregation and thrombus formation on immobilised fibrin and fibrinogen at arterial shear rate. I also observed that GPVI is not required for platelet adhesion to fibrin, which instead depends on the integrin αIIbβ3 as shown with blood of Glanzmann patients. Inhibition of Syk, which signals downstream both GPVI and integrin αIIbβ3, more drastically impaired platelet aggregation, leading me to conclude that thrombus formation on fibrin and fibrinogen occurs by complementary and non-redundant roles of GPVI and integrin αIIbβ3. Inhibition of GPIb-V-IX, which may also signal via Syk, had a minor impact on platelet activation and mostly affected adhesion. I also observed that platelet aggregates formed on fibrin and fibrinogen were smaller than those on collagen, and that they expressed P-selectin with low phosphatidylserine exposure. Given that GPVI governs platelet phosphatidylserine exposure on both collagen and non-collagen surfaces (*Chapter 3*), I concluded that fibrin and fibrinogen surfaces elicit a weak GPVI activation. The low phosphatidylserine level under flow condition is in contrast with a previous work, measuring GPVI-fibrin dependent thrombin generation in platelet-rich plasma.[14] One explanation is that, under flow, thrombin remains bound to the fibrin network,[15,16] becoming unable to activate platelets via PAR receptors (*Chapter 3*). More work is needed to validate this observation. A cooperation of GPVI and αIIbβ3 was also observed in *Chapter 6*, where both receptors appeared to contribute to fibrinogen-dependent stability of a preformed thrombus.

Together, these results support a model of thrombus whereby collagen induces platelet activation via GPVI while fibrin and fibrinogen support continued platelet adhesion and low-grade platelet activation to stabilize the thrombus, in agreement with the commonly accepted core-shell

model of mice thrombi. How these findings relate to the *in vivo* formation of human arterial thrombi is difficult to say as, the latter have a complex structure with patches of platelets, red blood cells and fibrin. However, an important aspect to take into account is the age of the thrombi as, the core-shell organisation described in mice, depicts thrombi in early stages of formation and up to 1 hour post-injury.[17] Instead, thrombi extracted from patients can be 2 to 4 days old.[18,19] In light of these observations, the use of GPVI-fibrin(ogen) antagonists might contribute to increase thrombus dissolution during the earliest stages of thrombus formation and to increase permeability of thrombi rich in fibrin in combination with anti-fibrinolytic agents. This is the rational behind the current phase II trial using glenzocimab with recombinant tissue plasminogen activator (tPA) in patients who have had an ischaemic stroke (NCT03803007).

3. Targeting platelet GPVI and its signalling pathway in arterial thrombosis

The distinguishing feature between the thrombus core and shell regions is the presence of fibrin and the grade of platelet activation, which is high in the former and low in the latter.[17] The diffusion of secondary mediators, ADP and TxA_2, from the core to the shell mediates inside-out activation of the integrin αIIbβ3 which binds to fibrinogen holding the platelets together. The release of these mediators from the core region diminishes over time, raising question to whether alternative pathways exist that can support integrin activation in the shell. In light of the finding that platelet activation by fibrin(ogen) is driven by the interplay of integrin αIIbβ3 and GPVI (*Chapter 4*), I hypothesized that binding of both receptors to fibrinogen contributes to the stability of the platelet aggregates, providing a feedback pathway that is additive to that of ADP and TxA_2 in reinforcing integrin activation. This hypothesis was supported by the observation of Ahmed *et al.* that the GPVI antagonist, ACT017, destabilises preformed thrombi by interfering with fibrinogen binding,[20] and by the work of Andre *et al.* showing that the Syk inhibitor, PRT-

060318, caused a disaggregation of thrombi.[21] My results showed that the combined action of GPVI and outside-in signalling by integrin αIIbβ3 supports aggregates stability on collagen-containing surfaces, in the absence of fibrin (*Chapter 6)*. Further, they show that inhibition of Syk destabilises platelet aggregates to the same extent as inhibition of ADP and TxA_2. A similar aggregates instability was observed upon inhibition of Src kinase (downstream of GPVI and αIIbβ3), but not Btk (downstream of only GPVI). Together, these results demonstrated a critical role of Src and Syk tyrosine kinases in supporting thrombus stability, in concert with the signals from ADP and TxA_2.

GPVI has long been recognised as a promising anti-thrombotic target, but as of today glenzocimab is the only anti-GPVI Fab in phase II trial and results are eagerly awaited. In contrast, phase II trial with revacept has been suspended as the compound did not prevent myocardial infarction. However, this result should not dampen the enthusiasm for anti-GPVI medications in coronary artery disease as revacept is designed to shield collagen from platelets[22] and does not block GPVI activation, which can be triggered by multiple ligands, including fibrin(ogen).[12,23,24] One of the limitations of anti-GPVI Fab fragments or GPVI recombinant proteins is that they lack oral bioavailability and therefore, are not suitable for self-administration. In contrast, small-molecule inhibitors are orally bio-available and are less expensive to produce, thus offering a suitable therapeutic alternative. For long, small molecule inhibitors of tyrosine kinases have been recognised as potential drugs.[25] After the first approval of fasudil in 1995, further 71 drugs have been approved by the United States Food and Drug Administration (May 2021). Several of these small molecules are used in the clinic especially for temporary treatment of patients with haematological malignancies, solid tumours or immune thrombocytopenia.[26] Recent findings provide evidence for a decreased risk of thrombosis without bleeding in patients with immune thrombocytopenia using the Syk tyrosine kinase inhibitor, fostamatinib.[27]

Minor side effects were also observed in patients treated with second generation Syk inhibitors, such as cevidoplenib, entospletinib, lanraplenib and MK-8457, which are in clinical trials for haematological malignancies or inflammatory diseases.[26,28-30] In addition, Lanraplenib, a third generation Syk inhibitor, was shown to inhibit GPVI activation in platelets, again without causing bleeding.[28] Alongside Syk, also several Src kinase inhibitors have reached the clinic for treatment of blood cancers, although these lack enzyme selectivity.[26] Inhibitors of Btk have recently gained interest as promising anti-thrombotic drugs due to their ability to affect thrombus formation on plaque material.[31] However, in *Chapter 6*, I showed that unlike Src or Syk inhibition, Btk inhibition did not affect thrombus stability, leading to the conclusion that targeting Btk is less efficient in inducing thrombus breakdown. Considering the risk of bleeding caused by inhibitors of secondary mediators (aspirin, $P2Y_{12}$ inhibitors), and the observation that they enhance thrombus dissolution similarly to Syk inhibition *(Chapter 6)*, I speculate that Syk or Src inhibitors may be an effective anti-thrombotic treatment with minor bleeding risk. Targeted clinical trials will be needed to explore this speculation.

Several small-molecule compounds have been reported to act as GPVI inhibitors and among these, losartan and honokiol. Losartan is a prodrug and a selective non-peptide angiotensin AT_1 receptor antagonist, while honokiol is a natural bioactive product isolated from *Magnolia* species.[32] Although both molecules inhibit the aggregation of platelets induced by collagen, we found them to lack specificity for the GPVI receptor and we could not reproduce the sub-nanomolar potency of honokiol.

In conclusion, targeting GPVI could represent a useful antiplatelet therapy aiming to prevent thrombus growth and to induce instability of the less activated platelets in the outer thrombus region. The observation that genetic loss of GPVI in humans is associated with only mild bleeding diathesis indicates that GPVI is not critical for haemostasis. Tailoring inhibitors to be specific for GPVI ligands (collagen, fibrinogen, fibrin) may

provide a new class of anti-thrombotic drugs and minimise off-target effects. Whether GPVI inhibition will lead to a lower risk of bleeding compared to the current antiplatelet medications is difficult to say, as of today glenzocimab is the only anti-GPVI agent in a phase II trial. In the meantime, tyrosine kinase inhibitors, especially directed against Syk, seem a promising medication in the prophylaxis of arterial thrombosis and a safe long-term therapy orally available. Given that many of these compounds have already entered the clinics, I believe that clinical trials should be initiated in order to explore this possibility.

Table 1. *A summary of major findings of this thesis and their contribution to the field.*

Findings	Contribution to the field	Authors' opinions
1. The analysis of the controversies concerning the binding of monomeric or dimeric GPVI to fibrin(ogen) suggested some of the aspects to consider solving them.	The binding assays and the reagents used to understand GPVI-fibrin(ogen) interaction need to be standardised. Better knowledge on the GPVI constructs used will help explaining the discrepant results existing in literature.	1. As some of GPVI domains are highly charged, the use of different monomeric and dimeric GPVI constructs may have generated intramolecular charges causing diverse orientations of the protein. The use of a GPVI construct consisting of the transmembrane and cytoplasmatic domains will be a more powerful tool to study GPVI bining to fibrin(ogen) in the future.
2. GPVI regulates the level of phosphatidylserine exposure on collagen, independently of thrombin. There are approximately 4000 unidentified GPVI-deficient individuals currently living in Chile, asymptomatic for the mutation.	2. GPVI is not essential to haemostasis. In its absence, thrombus formation is preserved with the integrin α2β1, which mediates platlet adhesion to collagen, and tissue factor-driven thrombin generation.	2. The mild bleeding phenotype of GPVI-def individuals, along with the estimated number of these individuals currently living in Chile, make GPVI a promising anti-thrombotic target. The binding of the receptor to collagen may be a key target against thrombus formation on a site of rupture of an atherosclerotic plaque, where the very high shear might cause the rapid dilution of other platelet agonists making collagen-GPVI interaction crucial.
3. Under flow conditions, fibrin elicits only moderate GPVI activation which relies on integrin αIIbβ3.	3. At arterial shear rate, GPVI is not required for platelet adhesion to fibrin, which instead depends on the integrin αIIbβ3. On fibrin(ogen) surfaces, GPVI cooperates with the integrin αIIbβ3 to form platelet aggregates which are smaller than those on collagen, and express high level of P-selectin but have low phosphatidylserine exposure.	3. Fibrin and fibrinogen elicit a weak GPVI activation, shown by the low level of phosphatidylserine exposure. This GPVI activation supports the stability of the thrombus but does not promote massive platelet activation.
4. The interplay of integrin αIIbβ3 and GPVI contributes to the stability of platelet aggregates via fibrinogen binding.	4. Binding of αIIbβ3 and GPVI to fibrinogen provides a feedback pathway that is additive to that of ADP and TxA_2 in reinforcing integrin activation in the shell region, which is devoid of fibrin. The inhibition of Src and Syk, causes thrombus disaggregation at same extent as secondary mediator inhibitors.	4. Tyrosine kinase inhibitors, especially directed against Syk, could be a promising antithrombotic drugs carrying lower risk of life-threatening bleeding. They could be combined with anti-fibrinolytic agents to dissolve the clot or alone, for the primary and secondary prevention of arterial thrombosis.

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Chapter 9

Samenvatting

Summary

Riassunto

Impact

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Publications

Acknowledgements

Ringraziamenti

Samenvatting

Glycoproteïne VI (GPVI) op bloedplaatjes staat bekend als de belangrijkste signaalreceptor voor collageen. Studies met muizen hebben een rol aangetoond van GPVI bij de arteriële trombusvorming met een niet meer dan beperkte bijdrage aan de hemostase. Personen met een erfelijke deficiëntie in GPVI hebben dan ook slechts een milde bloedingsdiathese. Tesamen suggereert dit dat een op GPVI gerichte interventie de arteriële trombose kan onderdrukken, terwijl de hemostase behouden blijft. De recente ontdekking dat GPVI ook nog werkt als een receptor voor fibrinogeen en fibrine, suggereert een functie die verder reikt dan initiatie van het proces van trombusvorming. Dit werpt ook de vraag op over de functionele betekenis van deze nieuwe GPVI-interacties. **Hoofdstuk 1** geeft een algemene inleiding op het werk van dit proefschrift met een nadruk op de rol van bloedplaatjes bij trombose en hemostase, relevante plaatjesreceptoren en liganden, en de architectuur van arteriële trombi. De algemene hypothese voor dit proefschrift is dat GPVI werkt als een belangrijke signalerende receptor voor fibrine en fibrinogeen bij het in stand houden van de propagatie en stabiliteit van een trombus. Daarmee heb ik de relatieve rol van collageen, fibrinogeen en fibrine aan de groei en stabiliteit van een trombus onderzocht door het gebruik van remmers van GPVI zelf en van signaleringspaden onder GPVI. In **Hoofdstuk 2** is de achtergrond onderzocht van tegenstrijdige waarnemingen in de literatuur, over de vraag of fibrine en fibrinogeen binden aan monomere danwel dimere vormen van GPVI. Geanalyseerd werden de verschillen tussen de (dimere) GPVI-constructen, die door de onderzoekers werden gebruikt, de gebruikte bindingsassays en de manier waarop fibrine werd gegenereerd. Uit de analyse bleek het belang van de moleculaire structuur van de verschillende GPVI-constructen en de rol van ladingsinteracties voor de ligandbinding. Aangezien sommige GPVI-domeinen sterk geladen zijn, is geconcludeerd dat intramoleculaire ladingen bepalend zijn voor de preciese oriëntatie van de verschillende GPVI-constructen, waardoor de discrepantie in bindingsstudies verklaard

kan worden. Daarnaast kunnen verschillen in gebruikte reagentia en technieken de tegenstrijdige literatuurresultaten verklaren.

In **Hoofdstuk 3** onderzochten we de rol van bloedplaatjes GPVI in de stromingsafhankelijke trombusvorming op zowel collageen- als niet-collageenoppervlakken in de aan- of afwezigheid van stolling. Voor deze studies was er bloed beschikbaar van vier homozygote en drie heterozygote individuen, afkomstig van drie niet-verwante families, met een erfelijke mutatie van het *GP6*-gen, dat de GPVI-expressie op bloedplaatjes uitschakelt. De resultaten tonen aan dat GPVI van cruciaal belang is voor de plaatjesaggregatie onder stromingscondities, alsmede voor de fosfatidylserine-expositie zowel bij collageen- als niet-collageenoppervlakken. Anderszing blijkt GPVI niet vereist voor de plaatjesadhesie. Daarnaast konden we bevestigen dat GPVI kan worden geactiveerd door ladingsinteracties; dit omdat de spreiding van bloedplaatjes op collageen, VWF en negatief geladen glasoppervlakken werd verminderd of verhinderd in geval van GPVI-deficiëntie. Gezien het grote aantal bekende GPVI-deficiënte individuen in de Chileense populatie, hebben we het *GP6*-exon 6 gesequenced in 1212 verschillende DNA-monsters, representatief voor de Chileense populatie, en berekend dat er zo'n 4079 personen met de *GP6*-mutatie in Chili zijn, zonder genetische diagnose of bekend bloedingsfenotype.

In **Hoofdstuk 4** is de rol van GPVI bestudeerd bij het ondersteunen van bloedplaatjesadhesie, activering en aggregatie in bloed stromend over een reeks van fibrine- en fibrinogeenoppervlakken, dit in vergelijking met een collageenoppervlak. In de studie werden de GPVI-afhankelijke plaatjesresponsen vergeleken met de responses via integrine αIIbβ3 en GPIb-V-IX. Door gebruik te maken van Fab 9O12 (blokt GPVI), PRT-060318 (remt Syk tyrosine kinase) en bloed van Glanzmann-patiënten zonder integrine αIIbβ3, concludeerden we dat αIIbβ3 bepalend is voor de hechting van bloedplaatjes aan fibrine en fibrinogeen, en dat het integrine hierin samenwerkt met GPVI in het opwekken van de plaatjesactivering. Verder bleek dat de trombi gevormd op fibrine of fibrinogeen relatief

klein zijn in vergelijking met die op collagen, waarbij de bloedplaatjes licht geactiveerd zijn middels P-selectine-expressie, kortstondige Ca^{2+}-verhogingen en een lage fosfatidylserine-expositie. In **Hoofdstuk 5** hebben wij het effect onderzocht van twee kleine moleculen, losartan en honokiol, waarvan gerapporteerd is dat deze werken als GPVI-antagonisten. Beide verbindingen verminderden inderdaad de trombusvorming op collageen, maar dit effect bleek niet specifiek voor GPVI, aangezien ze ook de door CLEC-2-agonisten geïnduceerde plaatjesactivering verslechterden.

Hoofdstuk 6 beschouwt in hoeverre de tyrosinekinase-cascade opgewekt via GPVI en integrine αIIbβ3 betrokken is bij het proces van trombusstabilisering, en of hierin mogelijkheden liggen voor het induceren van desaggregatie van bloedplaatjes. Om zulks te doen, werden proeven onder stromingscondities uitgevoerd met geïmmobiliseerd collageen en humaan plaquemateriaal onder niet-stollende omstandigheden, waardoor de vorming van fibrine voorkomen werd. De stabiliteit van gevormde trombi werd getest door een postperfusie met Syk-, Src- of Btk-remmers. Bovendien werden de effecten van deze inhibitoren vergeleken met remming van de secundaire mediatoren van plaatjesactering, namelijk ADP en tromboxaan A_2 (TxA_2). We vonden dat remming van Syk en Src de desaggregatie van bloedplaatjes in trombi versterkte, zowel op collageen als op plaquemateriaal, en wel in een vergelijkbare mate als de gecombineerde remming van ADP en TxA_2. We konden geen additief effect waarnemen wanneer de tyrosinekinaseremmers werden gecombineerd met ADP- en TxA_2-blokkers, hetgeen suggereert dat deze twee routes in synergie werkten om de trombus in stand te houden.

Het overzichts **hoofdstuk 7** geeft een inventarisatie van de literatuuraanwijzingen dat er een rol van bloedplaatjes GPVI is bij veneus trombo-embolische aandoeningen, een en ander gebaseerd op bevindingen bij mensen en muizen. De review omvat onderzoeken die GPVI in verband brengen met experimentele trombose, pulmonaire

trombo-embolieën en ontstekings-gerelateerde tromboses. Bij mensen blijkt een veel voorkomende variant rs1613662 van het *GP6*-gen gekoppeld aan het vóórkomen van veneuze trombo-embolie. Over het algemeen laat de literatuur consistent bewijs zien dat bloeplaatjes en daarmee GPVI werkzaam mijn in het veneuze deel van de bloedsomloop. Dit betreft aandoeningen uiteenlopend van leukocyte-afhankelijke ontstekingsprocessen, veneuze trombo-embolie, trombose in de longen en metastase van kwaadaardige gezwellen. Ook hebben we een kritische geanalyse uitgevoerd of fibrine de verantwoordelijke GPVI-agonist kan zijn onder veneuze trombose-omstandigheden. Een algemene conclusie is dat bloedplaatjes GPVI een geschikte co-target kan zijn bij de preventie van veneuze trombose vanwege diens rol in de trombusconsolidatie en de vorming van bloedplaatjes-leukocytencomplexen. **Hoofdstuk 8** bediscussieert de bevindingen van dit proefschrift, waarbij gewezen is op het belang van een antagonist die specifiek interfereert in de GPVI-interactie met fibrine en fibrinogeen, dit mede gezien de duidelijkere rol van de GPVI-opgewekte signalering bij trombotische complicaties.

Summary

Glycoprotein VI (GPVI) is the major signalling receptor for collagen. Mouse studies have shown that GPVI is critical for arterial thrombus formation but has a limited contribution to haemostasis. Individuals with an inherited deficiency in GPVI also have only a mild bleeding diathesis. Together, these observations suggest that targeting GPVI may suppress arterial thrombosis while preserving haemostasis. The recent discovery that GPVI is also a receptor for fibrinogen and fibrin suggests that its role extends beyond the onset of thrombus formation. In addition, it raised question about the functional significance of these new interactions. **Chapter 1** provides a general introduction to the work performed with emphasis on the roles of platelets in thrombosis and haemostasis, relevant platelet receptors and ligands, and the architecture of arterial thrombi. As an overarching hypothesis, I proposed that GPVI acts as a relevant signalling receptor for fibrin and fibrinogen in thrombus propagation and stability. Therefore, I investigated the relative contribution of collagen, fibrinogen and fibrin to thrombus growth and stability using inhibitors of GPVI and downstream tyrosine kinases. In **Chapter 2**, I examined the reasons for conflicting results in the literature on whether fibrin and fibrinogen bind to monomeric or dimeric GPVI. I analysed the differences between the various (dimeric and monomeric) GPVI constructs used by the groups involved in the contradictory results, the binding assays employed, and the methods used for fibrin generation. This analysis highlighted the importance of knowing the precise molecular structure of the various GPVI constructs and the role of charge interactions in the ligand binding. As some of the GPVI domains are highly charged, it was concluded that the intramolecular charges gave rise to diverse orientations of the various GPVI constructs, thereby accounting for the discrepant results. Hence, the different forms of GPVI constructs, along with a different use of reagents and techniques could explain the discrepant results.

In **Chapter 3**, I explored the role of platelet GPVI in flow-dependent thrombus formation on collagen and non-collagen surfaces in the

presence or absence of coagulation. For the studies, I used blood from four homozygous and three heterozygous individuals, from three unrelated families, with an inherited mutation in the *GP6* gene, which prevents GPVI expression on platelets. The results show that GPVI is critical for flow-dependent platelet aggregation and phosphatidylserine exposure on both collagen and non-collagen surfaces, whereas it is not required for platelet adhesion. In addition, I confirmed that GPVI can be activated through charge interactions as spreading of platelets on collagen, VWF and negatively charged glass was reduced or abolished with platelets lacking expression of GPVI. Given the unique presentation of GPVI-deficient individuals in the Chilean population, we sequenced the *GP6* exon 6 in 1212 DNA samples, representative of the Chilean population, and calculated that about 4079 individuals carrying the *GP6* mutation may be living in Chile without genetic diagnosis or recognised bleeding phenotype.

In **Chapter 4**, I studied the role of GPVI in supporting platelet adhesion, activation, and aggregation under flow conditions on a range of fibrin and fibrinogen surfaces, in comparison to a collagen surface. In the study platelet responses elicited by GPVI were compared to those relying on integrin αIIbβ3 and GPIb-V-IX. Using Fab 9O12 (blocking GPVI), PRT-060318 (inhibiting Syk tyrosine kinase) and blood from Glanzmann patients lacking integrin αIIbβ3, I concluded that αIIbβ3 is key for platelet adhesion to fibrin and fibrinogen and that it synergises with GPVI in provoking adhesion-dependent platelet activation. Furthermore, I showed that thrombi formed on fibrin and fibrinogen are relatively small when compared to collagen, with platelets showing P-selectin expression, transient cytosolic Ca^{2+} rises and a low phosphatidylserine exposure. In **Chapter 5** I investigated the effect of two small molecules, losartan and honokiol, reported as GPVI antagonists, on platelet responses. Both compounds indeed decreased thrombus formation on collagen, but they were not specific for GPVI, as they also impaired platelet activation induced by CLEC-2 agonists.

Chapter 6 observes whether the tyrosine kinase cascade downstream GPVI and integrin αIIbβ3 is involved in the process of thrombus stabilization, and whether this can be targeted to cause disaggregation of platelets. To do that, flow studies were performed on immobilised collagen and human plaque material under non-coagulating conditions, thus preventing the formation of fibrin. Aggregate stability was challenged by post-perfusion of Syk, Src or Btk inhibitors. In addition, the effects of these inhibitors were compared to antagonism of the secondary mediators of platelet activation, ADP and thromboxane A_2 (TxA_2). We found that inhibition of Syk and Src increased platelet disaggregation of preformed thrombi, both on collagen and plaque material, to a similar extent as blockage of ADP and TxA_2. No additive effect was seen when tyrosine kinase inhibitors were combined with ADP and TxA_2 blockers, suggesting that the pathways act in synergy to maintain thrombus stability. In contrast, selective inhibition of Btk did not significantly impair the stability of the preformed aggregates. We concluded that targeting Syk might be promising to disrupt the thrombus shell, a region of the thrombus which is devoid of fibrin and consists of loosely aggregated platelets.

The review of **Chapter 7** provides an inventory of the literature evidence supporting a role of platelet GPVI in venous thromboembolism, based on findings with humans and mice. This concerned studies that related GPVI to experimental thrombosis, pulmonary thromboembolism and thrombo-inflammation. In humans, a common variant rs1613662 in the *GP6* gene is linked to venous thromboembolism. Overall, the literature shows emerging evidence for platelet GPVI acting in the venous part of the circulation, including leukocyte-dependent thrombo-inflammation, venous thromboembolism, pulmonary thromboembolism and cancer metastasis. We critically evaluated whether fibrin might be the responsible GPVI agonist under venous thrombosis conditions. A general conclusion was that platelet GPVI may be a suitable co-target in the prevention of venous thrombosis due to its role in thrombus

consolidation and platelet-leukocyte complex formation. **Chapter 8** discusses the findings of this thesis, noting the importance of having an antagonist specific for GPVI interaction with fibrin and fibrinogen given the prospective of targeting GPVI and its signalling in thrombotic complications.

Riassunto

La glicoproteina VI (GPVI) è il principale recettore di segnalazione per il collagene. Studi *in vivo* hanno dimostrato che GPVI è essenziale per la formazione della trombosi arteriosa mentre contribuisce solo limitatamente al processo emostatico. In aggiunta è stato osservato che l'assenza di GPVI, in individui portatori di una specifica mutazione nel gene *GP6*, comporta solamente una lieve diatesi emorragica. Collettivamente questi studi hanno suscitato interesse in GPVI come target anti-trombotico, in quanto terapie basate sull'inibizione di questo recettore porebbero rivelarsi efficaci nel combattere la trombosi arteriora preservando il processo emostatico.

Di recente è stato scoperto che GPVI è anche un recettore per il fibrinogeno e la fibrina; risultato che suggerisce che il ruolo di GPVI non è solamente limitato alle prime fasi di formazione del trombo ma che si estende anche a quelle successive sollevando pertanto, diverse domande sul significato funzionale di queste interazioni.

Il **1° Capitolo** offre una panoramica sul lavoro realizzato in questa tesi, con particolare enfasi sul ruolo delle piastrine nei processi di trombosi ed emostasi. L'ipotesi proposta è che GPVI, agendo da ricettore di segnalazione per la fibrina e il fibrinogeno, contribuisce alla propagazione e alla stabilità del trombo.

Al fine di dimostrare questa ipotesi, in questo lavoro ho esaminato il ruolo assunto da GPVI nella formazione e stabilità del trombo in relazione al collagene, il fibrinogeno e la fibrina, tramite l'utilizzo di inibitori specifici di GPVI e le tirosine chinasi coinvolte nella sua attivazione. Nel **2° Capitolo**, ho esaminato i vari aspetti alla base della disputa esistente in litteratura riguardanti la conformazione (monomerica o dimerica) assunta da GPVI nel legame con la fibrina ed il fibrinogeno. Ho quindi analizzato le differenze esistenti tra i costrutti (monomeri e dimeri) sintetici di GPVI utilizzati dai gruppi coinvolti nel dibattito, insieme ad altri fattori quali i saggi di legame adoperati e i protocolli adottati per la generazione della fibrina. Quest'analisi ha evidenziato l'importanza di conoscere la precisa struttura molecolare dei costrutti di GPVI utilizzati e di considerare il ruolo

svolto dalle interazione di carica nel legame tra recettore e ligando. Data la presenza in GPVI di domini altamente carichi, ho concluso che l'utilizzo di construtti diversi abbia comportato la presenza di forze intramolecolari differenti, causando quindi l'adozione da parte dei costrutti di diverse orientazioni nello spazio e conseguentemente, risultati discrepanti.

Nel **3º Capitolo**, tramite l'utlizzo di una camera microfluidica, ho studiato il ruolo di GPVI nell'aggregazione piastrinica in risposta al collagene e ad altre superfici proteiche, in presenza ed in assenza della coagulazione. Per questo studio, ho utilizzato campioni di sangue prelevati da quattro individui omozigoti e tre eterozigoti, discendenti da tre famiglie diverse, per una mutazione ereditaria nel gene *GP6* che comporta la mancata espressione di GPVI sulle piastrine. I risultati ottenuti da questo studio hanno dimostrato che GPVI è indispensabile per l'aggregazione piastrinica e per l'espressione della fosfatidilserina ma non per l'adesione piastrinica, in risposta al collagene ed altre superfici proteiche. In aggiunta, questo studio mi ha consentito di confermare che l'attivazione di GPVI avviene anche attraverso interazioni di carica, conclusione a cui sono giunta osservando che la mancata espressione di GPVI sulle piastrine non solo comporta la riduzione dell'attivazione piastrinica in seguito all'esposizione al collagene, come previsto, ma anche al VWF e ad un vetrino carico negativamente. Inoltre, in questo studio abbiamo sequenziato l'esone 6 del gene *GP6* in 1212 campioni di DNA provenienti dalla popolazione Cilena, dato che ad oggi, individui portatori della suddetta mutazione sono stati identificati solo in Cile. Quest'analisi ci ha concesso di stimare che circa 4079 individui portatori di questa mutazione vivono attualmente in Cile. Tuttavia l'assenza di un fenotipo emorragico non ne ha consentito la diagnosi.

Sempre attraverso l'utilizzo di una camera microfluidica, nel **4º Capitolo**, ho studiato il ruolo di GPVI nell'adesione, attivazione e aggregazione piastrinica in risposta alla fibrina e al fibrinogeno, paragonandolo a quello in risposta al collagene. In aggiunta, ho confrontato il ruolo svolto da GPVI con quello dell'integrina αIIbβ3 e GPIb-V-IX. Utilizzando il Fab 9O12

(inibitore di GPVI), PRT-060318 (inibitore della tirosina chinasi Syk) e campiondi di sangue prelevati da pazienti Glanzmann che non esprimono l'integrina αIIbβ3, ho concluso che quest'ultima è fondamentale per l'adesione della piastrine sulla fibrina e il fibrinogeno e che inoltre sinergizza con GPVI nell'attivazione piastrinica. Ho inoltre dimonstrato che gli aggregati priastrinici formati in riposta alla fibrina ed al fibrinogeno sono relativamente piccoli rispetto a quelli formati in risposta al collagene e che le piastrine da cui sono composti presentano un'adeguata secrezione di P-selectina, un transitorio aumento del Ca^{2+} citoplasmatico ma una bassa espressione della fosfatidilserina.

Nel **5º Capitolo**, ho esaminato l'effetto di due piccole molecole, losartan e honokiol, riportate per essere degli inibitori di GPVI. Con questo lavoro abbiamo concluso che entrambi questi composti riducono l'attivazione piastrinica in risposta al collagene, ma che non sono inibitori specifici di GPVI, dato chec un'inibizione piastrinica simile è stata riscontrata anche a seguito dell'utilizzo di agonisti di un altro recettore piastrinico, CLEC-2.

Nel **6º Capitolo** mi sono chiesta se le tirosine chinasi coinvolte nell'attivazione di GPVI e dell'integrina αIIbβ3 prendessero parte alla stabilità del trombo e laddove potessero essere utilizzati come target terapeutici al fine di indure la disaggregazione dello stesso. Pertanto, con l'utilizzo di una camera microfludica, ho condotto esperimenti volti ad esaminare il ruolo di queste tirosine chinasi nella formazione del trombo su superfici come il collagene e placche aterosclerotiche. Questi esperimenti sono stati condotti in assenza della coagulazione e pertanto, inibendo la generazione della fibrina. Al fine di osservare i meccanismi coinvolti nella stabilità degli aggregati piastrinici generati sulle superfici di cui sopra, quest'ultimi sono stati post-perfusi con inibitori delle tirosine chinasi Syk, Src e Btk. In aggiunta, gli effetti causati da questi inibitori sono stati paragonati a quelli provocati da inibitori di due mediatori secondari dell'aggregatione piastrinica: adenosina difosfato (ADP) e il trombossano A_2 (TxA_2). Con questo lavoro ho dimostrato che l'inibizione di Syk and Src induce la disaggregazione di trombi preformati sia sul collagene che su

placche aterosclerotiche, con un effettto simile a quello indotto dall'inbizione di ADP and TxA_2. In aggiunta, siccome l'azione congiunta di entrambi gli inibitori non ha comportato una maggiore perdita della stabilità degli aggregati, ho concluso che Syk e Src agiscono in sinergia con ADP e TxA_2. Dato che l'inibizione di Btk non ha avuto alcun effetto significativo, ho concluso che questa tirosina chinasi non contribuisce a preservare la stabilità degli aggregati. Concludendo, con questo studio, ho dimostrato che Syk potrebbe essere un interessante target terapeutico da utilizzare al fine di dissolvere il cosidetto guscio trombotico, una regione della struttura del trombo caratterizzate dall'assenza di fibrina e da piastrine debolmente attive.

Nel **7° Capitolo** ho raccolto vari studi, condotti su modelli animali e sull'uomo, a supporto di un coinvolgimento di GPVI nella trombosi venosa. Tra questi, la scoperta della variante rs1613662 nel gene *GP6* associata ad un maggior rischio di venosi tromboembolica. La letteratura riportata in questo capitolo dimostra che GPVI potrebbe prender parte alla attivazione piastrinica nella circolazione venosa contribuendo a svariati meccanismi come ad esempio quelli trombo-infiammatori, per poi passare alla venosi tromboembolica fino alla fomarzione di metastasi tumorali. A fronte degli studi riportati in questo capitolo, abbiamo concluso che GPVI potrebbe essere utilizzato come co-target nella prevenzione delle trombosi venosa dato il suo possible coinvolgimento nell'attivazione piastrinica e nella formazione di complessi pastrinici-leucocitosi.

Infine, nell' **8° Capitolo,** i risultati di questa tesi sono discussi, auspicando per il futuro lo sviluppo di inibitori specifici per l'interazione tra GPVI e la fibrina o il fibrinogeno, al fine di esplorare ancor meglio questo legame che potrebbe rappresentare un nuovo target per la prevenzione e il trattamento di eventi trombotici.

Impact

The role of the haemostatic system is to prevent excessive blood loss in case of vessel injury. Platelets are the main regulators of haemostasis alongside with the coagulation system. The endothelium lining the blood vessels prevents platelet activation by separating platelets from thrombogenic factors present in the subendothelium. Damage to the endothelium exposes extracellular matrix proteins such as collagen fibres, which triggers platelet activation, plug formation and vascular occlusion of the site of bleeding. On the other hand, the same platelet and coagulation activation processes, at a site of rupture of an atherosclerotic plaque can lead to formation of occlusive thrombi, and life-threatening complications such as heart attack and stroke. Patients at risk of arterial thrombosis are treated prophylactically with anti-platelet agents, such as aspirin and $P2Y_{12}$ ADP receptor antagonists, but such medications have the risk of life-threatening bleeding. There is hence an urgent need for novel anti-platelet agents that selectively target arterial thrombosis over haemostasis. GPVI is the principal collagen signalling receptor on platelets. The fact that GPVI-deficient individuals have an only mild bleeding diathesis suggests that this receptor is not crucial in haemostasis. Further, the recent observation that fibrinogen and fibrin also bind to GPVI indicates a role for this receptor in collagen-independent thrombus formation, alongside the roles of ADP and TxA_2. In this thesis, I investigated the role of GPVI in platelet activation, thrombus growth and stability with the aim to further elucidate the benefits of targeting this receptor in patients with arterial cardiovascular disease.

In 2015, the finding that fibrin and fibrinogen are ligands for GPVI, raised questions about the functional significance of these two interactions. At that time, one of the most debated questions was whether GPVI binds to fibrin or fibrinogen as monomer or dimer. Moreover, the observation that fibrin and fibrinogen bind to monomeric GPVI was surprising, as in contrast with the accepted theory at the time that GPVI dimerises following collagen binding. In order to understand the reasons accounting for the published discrepancies, we critically analysed the GPVI constructs

used by the various groups, alongside other key reagents and the techniques employed (***Chapter 2***). This analysis highlighted the importance of the structures of the GPVI constructs and the role of charge interactions in ligand binding. As GPVI contains domains that are highly charged, it was concluded that the charges may give rise to diverse orientations of the various GPVI constructs, thereby accounting for the discrepancies. In line with this observation, I also showed that GPVI can be crosslinked by charge interactions, as platelet spreading on VWF or glass surface, is abolished in GPVI-deficient platelets (***Chapter 3***). Solving the whole structures of GPVI with collagen and fibrin(ogen) will have important implications in understanding the role of these interactions in various disease and in designing therapeutics that effectively target GPVI interaction with one ligand or another.

As a follow-up, I investigated the mechanism of fibrin- and fibrinogen-induced platelet activation via GPVI under arterial shear rate (***Chapter 4***). As integrin αIIbβ3 is a well-known fibrin and fibrinogen receptor, I asked how the two receptors contribute to platelet activation. I concluded that platelet activation via GPVI in response to either fibrin or fibrinogen is supported by integrin αIIbβ3 outside-in signalling. Furthermore, I showed that binding of fibrinogen to GPVI is important for stability of platelet aggregates. Considering these observations, antagonists specific for fibrinogen- and fibrin-GPVI interaction, could be effective in the primary and secondary prevention of cardiovascular events as they may contribute to inhibit the formation of occlusive thrombi during the first stages of thrombus formation. Moreover, inhibition of GPVI may help to increase permeability of thrombi rich in fibrin in combination with anti-fibrinolytic agents.

The evidence from animal experiments that GPVI can be targeted to suppress arterial thrombosis with limited effects on haemostasis has raised wide interest towards this receptor but, there remained concern over whether anti-GPVI agents would cause excessive bleeding. One way

to explore this aspect is by studying the clinical history and functional response of platelets from patients with the genetic loss of GPVI (***Chapter 3***). By using the Maastricht flow chamber, I showed that GPVI signalling regulates platelet phosphatidylserine exposure, and that it supports platelet aggregation on collagen and non-collagen surfaces. Considering the mild bleeding diathesis of individuals with GPVI deficiency, this finding suggests that, in GPVI-deficient individuals, a compensatory mechanism provides for the lack of GPVI. The mild bleeding phenotype is further supported by the calculation that about 4079 individuals, homozygous for the mutation, may be living in Chile without known bleeding disorder. In light of these observations and the finding that GPVI is key in thrombus formation on atherosclerotic plaques,[1, 2] these results strengthen the idea that anti-GPVI agents can selectively target arterial thrombosis over haemostasis. As of today, glenzocimab is the only one anti-GPVI antibody undergoing phase II trial in patients with acute ischemic stroke (NCT03803007) and resulted are awaited. In the previous phase I clinical trial, it was shown to be well tolerated, with inhibition lasting up to 24 h for the highest dose of 2,000 mg.[3] Moreover, inhibition of GPVI might be beneficial in different scenarios where the use of classic anti-platelet medications can be either ineffective or unsafe. For instance, in patients undergoing percutaneous injury interventions whereby the use of inhibitors of the integrin αIIbβ3 carries the risk of excessive bleeding. Interestingly, these patients experience high incidence of microvascular obstruction despite receiving dual antiplatelet therapy consisting of inhibitors of secondary mediators,[4, 5] therefore indicating that novel alternative therapies that target different signalling pathways are needed to reduce this risk.

Another therapeutic approach for targeting GPVI is represented by small-molecule inhibitors either directly affecting GPVI or interferring with its downstream signalling. The advantages of these compounds are the oral bioavailability and low production costs. Several small-molecule GPVI

inhibitors have been reported, and we investigated the effects of two of these, namely losartan and honokiol (***Chapter 5***). Although both compounds decreased thrombus formation on collagen, they showed a lack of specificity for GPVI, as they also impaired platelet activation induced by CLEC-2. Further, a sub-nanomolar potency of honokiol, reported in the literature, could not be reproduced. A sub-class of small-molecule inhibitors is represented by tyrosine kinase inhibitors. Advantages of several of these compounds are that they have already reached the clinics, are well tolerated and are orally available. Furthermore, there is evidence of a decreased risk of thrombosis in some patients on anti-tyrosine kinase treatment, however bleeding complications appear in these patients.

In light of these observations and the finding that platelet activation by fibrin(ogen) is driven by the interplay of integrin αIIbβ3 and GPVI, we investigated whether the inhibition of Syk, which signals dowstream of the two receptors, could affect the formation of an occlusive thrombus (***Chapter 6***). I concluded that fibrinogen regulates the stability of the thrombus shell region by binding to GPVI and by maintaining the integrin αIIbβ3 in an active state. I found that blockage of Syk increases instability of the platelet aggregates at arterial shear rate and that this caused a similar extent of disaggregation, as seen with inhibitors of the secondary mediators, ADP and TxA_2. Overall, this work indicates that targeting GPVI and the downstream signalling pathway could be used to prevent thrombus growth or to induce instability on a newly formed aggregate. The risk of bleeding related to signalling inhibitors should be further examined, however encouraging data comes from fostamitinib, an approved anti-Syk inhibitor currently used for chronic immune thrombocytopenia which has shown low bleeding risk.[6] Finally, we explored whether binding of fibrin to GPVI could contribute to venous thrombosis and discussed evidence supporting an inflammatory role of the receptor at venous shear conditions (***Chapter 7***). Despite these

observations, limited studies have been performed in investigating GPVI as a possible anti-thrombotic target in the venous setup. To better understand the role of GPVI in venous thromboembolism, new studies need to be performed in comparison with the currently used antithrombotic regiment.

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Curriculum vitae

Curriculum Vitae

Gina Perrella was born on October 25th 1990 in Naples, Italy. In 2014 she graduated in Biological Sciences at the University of Naples Federico II. After the bachelor, she moved to Tuscany where she started a master program in Medical Biotechnologies, at the University of Siena, Italy. During this period, she completed an internship at the University Medical Centre in Utrecht (The Netherlands) based on an Erasmus bursary for a traineeship scholarship. During the Erasmus training she performed research on the expression of micro-RNAs in the nipple fluid as potential diagnostic tool in early breast cancer detection, in the laboratory of Prof. Paul van Diest. Upon completion of the Erasmus period, she returned to Siena and in 2016 she obtained the master's degree with honour. Successively, she did an additional traineeship in the laboratory of Prof. Lorenzo Leoncini in Siena, Italy. In 2018, she started the PhD project, which was part of the joint doctoral programme between the University of Birmingham (United Kingdom) and the University of Maastricht (The Netherlands), under the joint supervision of Prof. Steve Watson, Prof. Johan Heemskerk, Dr. Mark Thomas and Dr. Magdolna Nagy. In 2018-2021, she performed research in the field of experimental thrombosis and haemostasis, as described in this thesis. During her PhD period, she visited the laboratory of Prof. Diego Mezzano (Santiago, Chile), where she had the opportunity to perform the work on the GPVI-deficient individuals presented in this thesis. She also visited the laboratories of Prof. Bernhard Nieswandt (Würzburg, Germany) and Prof. Paolo Simioni (Padua, Italy). She currently works as a post-doctoral researcher in the group of Dr. Julie Rayes at the University of Birmingham.

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Symposium presentations

1. **Perrella G.** Monomeric GPVI and D-dimer: the variables to consider for the solution of a dilemma. 4th European Platelet Conference. Bruges, Belgium. September 2018.

2. **Perrella G.** Non-redundant roles of platelet glycoprotein VI and IIb/IIIa via Syk kinase in moderate fibrin-dependent thrombus formation under flow. Platelet Society Meeting. Cambridge, United Kingdom. September 2019.

3. **Perrella G.** Exploiting the role of GPVI and Syk inhibition in thrombus stability. BSHT Virtual Scientific Meeting, United Kingdom. March 2021.

4. **Perrella G.** Exploiting the role of GPVI and Syk inhibition in thrombus stability. Platelet Society Meeting. Keele, United Kingdom. March 2021.

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