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École Doctorale des Sciences de la Vie et de la Santé STRASBOURG

ÉCOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTÉ

INSERM UMR_S1255 Biologie et pharmacologie des plaquettes sanguines : hémostase, thrombose, transfusion

THÈSE présentée par :

Muhammad Usman AHMED

Soutenue le : **18 Décembre 2020**

Pour obtenir le grade de : **Docteur de l'université de Strasbourg**

Discipline/ Spécialité : Sciences pharmaceutiques-Pharmacologie-Pharmacocinétique

Rôle de l'interaction GPVI/fibrinogène dans l'activation plaquettaire et dans la formation et la stabilisation des thrombi

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This thesis is dedicated to my late brother,
Muhammad Farhan Ahmed.

We should not, by remaining ignorant and illiterate,
tarnish the image of our able elders.

Sir Syed Ahmed Khan (1817 – 1898)

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Table of Contents

List of Figures

List of Tables

Abbreviations

AC	adenylyl cyclase
ACS	acute coronary syndrome
ADAM	a disintegrin and metalloproteinase
ADAMTS-13	a disintegrin and metalloproteinase with thrombospondin type 1 repeat-13
ADAP	adhesion- and degranulation-promoting adapter protein
ADP	adenosine diphosphate
ATP	adenosine triphosphate
ApoE	Apolipoprotein E
ASA	Aspirin
Btk	Bruton agammaglobulinemia tyrosine kinase
CalDAG-GEFI	calcium and DAG-regulated guanine-nucleotide-exchange factor
cAMP	3',5'-cyclic adenosine monophosphate
CaM	Calmodulin
CD	cluster of differentiation
CLEC-2	C-type lectin-like receptor 2
CRP	collagen-related peptide
CTLD	C-type lectin-like domain
COX	Cyclooxygenase
COX-1	cyclooxygenase isoform 1
CYP450	cytochromes P450
DAG	Diacylglycerol
DAPT	dual antiplatelet treatment
DTS	dense tubular system
ECM	extracellular matrix
EEL	external elastic lamina
ET-1	endothelin-1
FACIT	Fibril-Associated Collagens with Interrupted Triple Helices
FAK	focal adhesion kinase
FLNa	filamin A
fpA	fibrinopeptide A
fpB	fibrinopeptides B
Gads	Grb2-related adapter downstream of Shc
GAG	Glycosaminoglycan
GP	Glycoprotein
GPCR	G protein-coupled receptor
GPO	glycine-proline-hydroxyproline
Grb2	growth factor receptor bound protein 2
GTP	guanosine diphosphate
I-EFG	integrin-epidermal growth factor
IEL	internal elastic lamina
IL4R	interleukin 4 receptor
IP_3	inositol 1,4,5-trisphosphate
ITAM	immunoreceptor tyrosine-based activation motif

kDa	kilo Dalton
LAIR	leukocyte-associated immunoglobulin-like receptor 1
LAT	linker for activation of T cells
LDL	low density lipoprotein
mAb	monoclonal antibody
MACIT	Membrane-Associated Collagens with Interrupted Triple Helices
MIDAS	metal ion-dependent adhesion site
MLC	myosin light chain
MLC-P	myosin light chain phosphorylation
Mm	Millimeter
µ	Micro
NO	nitric oxide
NSTMI	non-ST-elevation myocardial infarction
PAF	platelet-activating factor
PAI-1	plasminogen activator inhibitor-1
PAR	protease-activated receptor
PBS	phosphate buffered saline
PCI	percutaneous coronary intervention
PDGF	platelet-derived growth factor
PF4	platelet factor 4
PG	Proteoglycan
PGI2	Prostacyclin
PI3-K	phosphoinositide 3-kinase
PIP2	phosphatidylinositol 4',5'-bisphosphate
PIP3	phosphatidylinositol 3,4',5'-trisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PRD	proline-rich domain
PSI	plexin-semaphorin-integrin
PSGL-1	P-selectin glycoprotein ligand-1
Rap1b	Ras-related protein 1b
RIAM	Rap1-GTP-interacting adaptor molecule
ROCK	Rho- associated protein kinase
ROS	reactive oxygen species
SH2	Src homology 2
SH3	Src homology 3
SLP-76	Src-homology 2 domain-containing leukocyte-specific phosphoprotein of 76 kDa
STEMI	ST-segment elevation myocardial infarction
Syk	spleen tyrosine kinase
TIMI	thrombolysis in myocardial infarction
TF	tissue factor
TFPI-1	tissue factor pathway inhibitor-1
TGF-β	transforming growth factor β
TP	thromboxane/prostaglandin receptor
tPA	tissue plasminogen activator
TSP-1	thrombospondin-1
TTP	thrombotic thrombocytopenic purpura
TxA_2	thromboxane A2

TXS	TxA_2 synthase
ULVWF	ultra large von Willebrand factor
uPA	urokinase-type plasminogen activator
Vav	guanine nucleotide exchange factor
VCAM-1	vascular adhesion molecule-1
VEGF	vascular endothelial growth factors
vWF	von Willebrand factor
WSR	wall shear rate
WT	wild type

Résumé de thése

Les plaquettes sanguines sont de petites cellules anucléées provenant des mégacaryocytes de la moelle osseuse. Elles jouent un rôle central dans l'hémostase qui représente le processus physiologique conduisant à l'arrêt des saignements après une lésion vasculaire. La lésion de l'endothélium vasculaire expose de nombreuses protéines de la matrice sous-endothéliale, telles que le collagène, les laminines et la fibronectine. Dans des conditions de flux sanguin élevé, les plaquettes sont rapidement recrutées par l'engagement du complexe Ib-IX-V des récepteurs plaquettaires (GP) avec le facteur von Willebrand (vWF) immobilisé dans le sous-endothélium. L'adhérence stable est assurée par les intégrines de la famille β1 et β3, à savoir α2β1, α5β1, α6β1, αIIbβ3 et αvβ3 qui interagissent avec leurs ligands, le collagène, la fibronectine, les laminines, le fibrinogène et la vitronectine, respectivement. Cette étape facilite l'interaction de la glycoprotéine (GP) VI avec le collagène, qui déclenche des signaux intracellulaires conduisant à l'activation des plaquettes. Les plaquettes activées libèrent des agonistes solubles, dont le thromboxane (Tx)A2 et l'ADP qui agissent par l'intermédiaire de leurs récepteurs respectifs couplés à des protéines G pour potentialiser l'activation plaquettaire. Ce processus régule l'affinité de l'intégrine αIIbβ3 pour son principal ligand, le fibrinogène, afin de favoriser l'agrégation des plaquettes et de former un agrégat qui colmate la brèche. Parallèlement, une fraction des plaquettes hautement activées devient pro-coagulante, favorisant la génération de thrombine, qui clive le fibrinogène dans un réseau de fibrine insoluble qui stabilise le caillot.

Un processus similaire à l'hémostase peut avoir lieu dans une artère sténosée à la suite de la rupture d'une plaque d'athérome dans un vaisseau malade et cela est appelé thrombose artérielle. Étant donné que la géométrie de la plaque modifie profondément la rhéologie locale et que le matériau de la plaque exposé est très réactif, les plaquettes forment un thrombus qui peut devenir occlusif et entraîner des pathologies ischémiques mortelles telles que l'infarctus du myocarde et l'accident vasculaire cérébral. Au cours des 30 dernières années, plusieurs agents antiplaquettaires, dont l'aspirine et les bloqueurs P2Y12 ou αIIbβ3 ont été utilisés avec succès dans la prévention et le traitement des événements cardiovasculaires. Le principal inconvénient de ces médicaments est le risque accru d'hémorragie qui limite

leur utilisation. Par conséquent, l'identification d'un agent antiplaquettaire efficace présentant un risque d'hémorragie moindre représenterait une avancée majeure dans le traitement des événements cardiovasculaires. Récemment, il a été proposé qu'un récepteur spécifique des plaquettes, la GPVI, qui est le principal récepteur d'activation plaquettaire pour le collagène, pourrait représenter une cible antithrombotique potentiellement sûre car son absence ou son blocage empêche la thrombose expérimentale, sans impact sur l'hémostase.

Le GPVI appartient à la superfamille des immunoglobulines, dont l'expression est limitée aux plaquettes et aux mégacaryocytes. Elle est complexée avec la chaîne γ du récepteur Fc (FcR) qui est importante pour son expression de surface et pour transmettre son signal intracellulaire. Lors de la liaison du ligand, les récepteurs GPVI se regroupent, ce qui entraîne la phosphorylation du motif à base de tyrosine de l'immunorécepteur (ITAM) de la chaîne FcR γ par la kinase de la famille Src. Cela conduit au recrutement de Syk, qui initie une cascade de phosphorylation aboutissant à l'assemblage d'un grand complexe de signalisation constitué de protéines adaptatrices, dont LAT (Linker for Activation T-cells), Gads, SLP-76 (domaine SH2 contenant une phosphoprotéine spécifique des leucocytes de 76 kDa), PI3-kinases et phospholipase C (PLC γ2). L'activation de PLCγ2 provoque la libération de réserves internes de Ca^{2+} qui agit comme messager secondaire et favorise l'activation de l'intégrine αIIbβ3, un récepteur clé de l'agrégation plaquettaire. La signalisation GPVI favorise également la libération d'ADP et la génération de thromboxane A2, ce qui augmente encore plus le niveau d'activation de l'αIIbβ3.

Étant donné que les patients déficients en GPVI souffrent d'un syndrome hémorragique modéré et que les souris déficientes en GPVI ne présentent pas un temps de saignement de la queue prolongé, il est admis que le GPVI n'est pas absolument nécessaire pour assurer l'hémostase. Inversement, l'absence ou le blocage de la GPVI aboli la formation de thrombus in vitro et dans plusieurs modèles expérimentaux de thrombose. Pour ces raisons, le GPVI est considérée comme une cible anti-thrombotique potentiellement sûre. On a longtemps cru que le rôle de la GPVI dans la formation de thrombus était uniquement lié à sa capacité à activer les plaquettes après liaison au collagène sous-endothélial. Notre groupe a montré que le GPVI régule l'adhésion et l'activation des plaquettes sur le fibrinogène, ce qui suggère

un rôle dans la croissance du thrombus. On ignore si l'interaction du GPVI avec le fibrinogène est également importante pour la stabilité du thrombus.

L'objectif principal de mes études de doctorat a été de caractériser le rôle de l'interaction GPVI/fibrinogène dans la formation et la stabilisation des thrombi.

Les trois principaux axes de recherche de mes études de doctorat étaient les suivants

i. La caractérisation du rôle de la GPVI humaine dans la stabilité des thrombi en utilisant des essais in vitro basés sur le flux.
ii. Comparaison du rôle de la GPVI humaine et de la GPVI de souris dans la formation et la stabilité des thrombi.
iii. Comparaison du rôle de la GPVI et de FcγRIIA dans l'activation des plaquettes sur le fibrinogène et dans la formation et la stabilité des thrombi.

I) Caractérisation du rôle du GPVI dans la stabilité des thrombi

En utilisant une approche in silico, nous avons montré que la contrainte mécanique appliquée à un thrombus augmente de façon spectaculaire au fur et à mesure de sa croissance, et que de fortes interactions inter-plaquettaires sont essentielles pour maintenir sa stabilité. En utilisant un test basé sur le flux couplé à la vidéo-microscopie en temps réel, nous avons montré que le blocage de la GPVI avec les fragments Fab ACT017 ou 1G5 favorisait une désagrégation efficace des thrombi humains préformés sur le collagène ou sur le matériel de plaque d'athérosclérose humain. En revanche, lorsque les plaquettes sont traitées avec l'anti-GPVI Fab 3J24.2 non bloquant, il n'y a pas de désagrégation des thrombi préformés, ce qui suggère que c'est la capacité d'ACT017 et de 1G5 à altérer leur liaison au ligand de la GPVI qui explique la capacité à favoriser la désagrégation. Nous avons observé que la désagrégation induite par ACT017 était obtenue dans des conditions de flux sanguin artériel (300 s^{-1}), mais pas à flux faible, et que son effet augmente avec le taux de cisaillement de la paroi (>300 - 750 s^{-1}). Mes résultats ont indiqué que c'est la capacité de la GPVI à réguler l'activation des plaquettes dans un thrombus en croissance qui permet la stabilité du thrombus, comme le montrent : 1) une perte de contraction du thrombus lorsque le GPVI est bloqué ; 2) l'absence de l'effet de désagrégation d'un

agent anti-GPVI lorsque les thrombi sont entièrement activés par des agonistes solubles. Il est intéressant de noter que le blocage de 4 molécules clés de signalisation ITAM, les src-kinases, Syk, PI3Kβ ou la phospholipase C, a entraîné une cinétique de désagrégation du thrombus similaire à celle d'ACT017, confirmant que le rôle de la GPVI dans la stabilité du thrombus est effectivement lié à sa capacité à activer les plaquettes. L'incapacité d'ACT017 à favoriser la désagrégation des thrombi formés avec le sang de patients atteints d'afibrinogénémie indique que le ligand de la GPVI favorisant l'activation des plaquettes dans un thrombus est le fibrinogène. Enfin, mes travaux ont montré que la désagrégation plaquettaire de thrombi riches en fibrine était obtenue avec ACT017 lorsqu'elle était utilisée en combinaison avec le rtPA. En conclusion, ce travail met en évidence le rôle de la GPVI dans le maintien de la stabilité du thrombus et suggère que le ciblage du GPVI chez les patients pourrait favoriser la désagrégation du thrombus. Ce travail, qui a fait l'objet d'une publication sur laquelle je figure en premier auteur, a été accepté pour dans *ATVB* en juillet 2020.

II) Comparaison du rôle de la GPVI humaine et de souris dans la formation et la stabilité des thrombi.

Ma première publication a été réalisée avec du sang humain en utilisant une approche in vitro. Pour confirmer ces résultats in vivo, nous avons décidé de mener une étude avec du sang de souris. Pour étudier le rôle de la GPVI dans la formation de thrombi chez la souris, nous avons d'abord perfusé du sang de souris de type sauvage (WT) anticoagulé par de l'hirudine sur du collagène fibrillaire de type I immobilisé pour préformer des thrombi, puis nous avons perfusé du sang de souris WT ou déficientes en GPVI ($GPVI^{-/-}$). À notre surprise et contrairement à ce qui a été observé avec le sang humain, les images en 3D ont montré que les thrombi obtenu avec du sang WT et $GPVI^{-/-}$ étaient similaires. Cela a été confirmé en quantifiant le volume de la deuxième population à l'aide de la microscopie confocale, ce qui indique que la GPVI de souris, contrairement à la GPVI humaine, ne joue pas un rôle clé dans l'accumulation des thrombi. Nous avons ensuite évalué le rôle de la GPVI dans la stabilité des thrombi de souris dans des conditions de flux artériel. Nous avons donc perfusé du sang de souris $GPVI^{-/-}$ ou WT sur des agrégats formés avec du sang WT, avant de perfuser du PBS. Une observation par vidéo-microscopie en temps réel a

montré que les agrégats de plaquettes GPVI$^{-/-}$ étaient aussi stables que les thrombi WT, ce qui a été confirmé par une quantification. Ce résultat indique que la GPVI de souris n'est pas clé dans la stabilité des thrombi de souris. Cela signifie-t-il que la présence du GPVI chez l'homme augmente la stabilité des thrombi humains ? En effet, une comparaison de la stabilité des thrombi de souris et humains a indiqué que les thrombi humains étaient plus stables que les thrombi de souris, évalués dans une large gamme de conditions de flux. En outre, cette différence reposait en grande partie sur la GPVI, car le traitement du sang humain par ACT017 (blocage de la GPVI) a donné une courbe de stabilité proche de celle des souris. En conclusion, cette étude identifie une différence entre la souris et l'homme en termes de contribution de la GPVI dans la progression et la stabilité des thrombi. Ce travail, que j'ai signé en co-premier auteur, a été accepté pour publication sous forme de lettre à l'éditeur chez *Thrombosis and Haemostasis* en 2020.

III) Comparer le rôle de la GPVI et de FcγRIIa dans l'activation plaquettaire sur fibrinogène et dans la formation et la stabilité des thrombi.

Le principal récepteur plaquettaire du fibrinogène est l'intégrine αIIbβ3, qui permet l'adhérence des plaquettes au fibrinogène immobilisé et favorise l'activation des plaquettes par une signalisation appelée "outside-in". Le groupe de Peter Newman a rapporté que FcγRIIa joue un rôle important dans l'activation des plaquettes sur le fibrinogène grâce à sa capacité à réguler la signalisation "outside-in" d'αIIbβ3 et à réguler la croissance des thrombi. Les travaux effectués au laboratoire, y compris pendant mon doctorat, indiquent que la GPVI humaine joue un rôle clé dans la croissance et la stabilité des thrombi par une interaction avec le fibrinogène. À ce jour, le rôle respectif de GPVI et de FcγRIIa dans l'activation des plaquettes sur le fibrinogène ainsi que dans la formation et la stabilité des thrombi est inconnu. J'ai donc cherché à comparer la contribution de FcγRIIa et de GPVI dans la régulation de l'activation plaquettaire après l'adhérence au fibrinogène médiée par αIIbβ3 et les conséquences ultérieures sur la progression et la stabilité du thrombus. En utilisant des approches in vitro similaires à celles détaillées ci-dessus, j'ai observé que la GPVI, mais pas FcγRIIa, joue un rôle clé dans la stabilité des thrombi humains formés in vitro, puisque le blocage de GPVI avec le 1G5, mais pas celui de FcγRIIa avec le IV.3 inhibe la croissance des thrombi. En outre, j'ai également observé que le 1G5, mais

pas IV.3, favorisait une désagrégation efficace des thrombi in vitro, ce qui indique que la GPVI joue un rôle plus important que FcγRIIa dans la stabilité des thrombi. Ces travaux suggèrent que la GPVI est un régulateur de l'activation plaquettaire sur le fibrinogène plus important que FcγRIIa, et joue donc un rôle prépondérant dans la croissance et la stabilité des thrombi. Ce travail a été soumis pour publication.

General Introduction

Platelets Overview

Platelets are tiny anucleate circulating blood components playing a central role in hemostasis, which demonstrates as the physiological process resulting in the arrest of bleeding following vessel lesion. Platelets are the major actors in arterial thrombosis as well. After the rupture and/or erosion of an evolved atherosclerotic plaque in diseased arteries, they accumulate at site of lesion and can form an occlusive thrombus which results in ischemic pathologies for instance myocardial infarction and stroke. Moreover, platelets also exhibit non-hemostatic functions and regulate various physiological and pathological conditions viz; wound healing, embryogenesis, angiogenesis, tumor metastasis, and immune and inflammatory diseases including atherosclerosis, rheumatoid arthritis and sepsis.

Hemostasis

Hemostasis is a set of highly complex and tightly regulated physiological processes that cause arrest of bleeding at the site of vessel lesion to maintain a normal blood flow circulation. It is subdivided in three phases: primary hemostasis, coagulation and fibrinolysis. Primary hemostasis refers to initial vasoconstriction occurring after vessel lesion and with subsequent adhesion, activation and platelet aggregation allowing the formation of hemostatic plug. Coagulation is the activation of a cascade of enzymatic reactions which ultimately generates thrombin that cleaves fibrinogen into insoluble fibrin network. Fibrin forms a meshwork around the hemostatic plug which consolidates and stabilizes the clot. Even though, primary hemostasis occurs first, there is an overlap between primary hemostasis and coagulation. Some of platelets within the aggregate become pro-coagulant which contributes significantly in linking the two processes. Coagulation factors bind pro-coagulant platelets which helps to concentrate them and favors the enzymatic reaction to generate thrombin. Finally, at a later stage, the fibrinolytic system is activated which results in the dissolution of the blood clot during the wound healing process to restore a normal blood flow within the vessel.

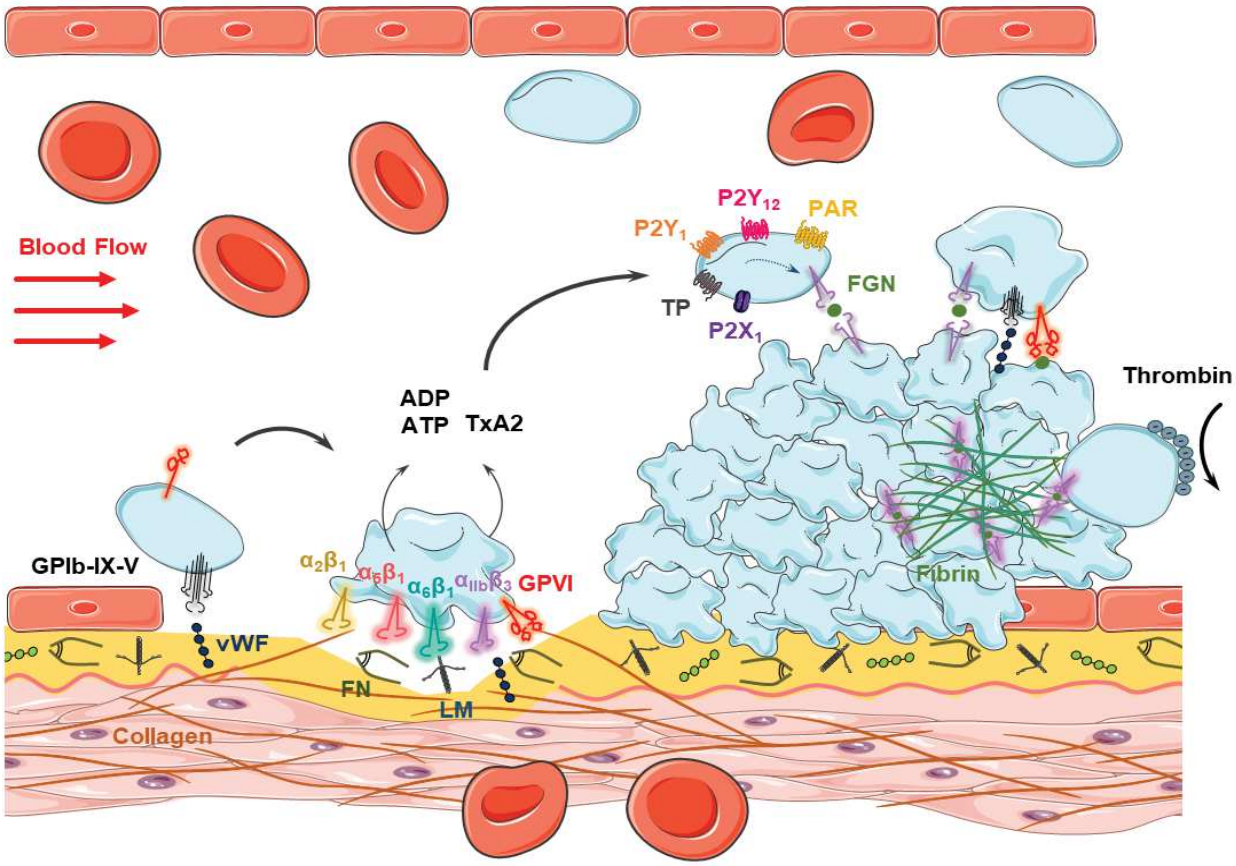

Figure 1. Role of platelets in hemostasis

ADP, adenosine diphosphate; ATP, adenosine triphosphate; FGN, fibrinogen; FN, fibronectin; GP, glycoprotein; LM, laminins; PAR, protease-activated receptor; TP, Thromboxane receptor; TxA_2, Thromboxane A2; vWF, von Willebrand Factor

The molecular mechanism of hemostasis is well defined. It follows the disruption of the endothelium that exposes numerous subendothelial matrix proteins, such as collagen, laminins or fibronectin. Under elevated blood flow condition, platelets are rapidly recruited through the interaction of a specific platelet cell-surface receptor GPIb-IX-V with vWF (Savage et al., 1996b). This ligand-receptor interaction has a rapid on-off rate that supports transient platelet adhesion with the vessel wall even under elevated shear conditions. Next, platelet integrins of the β1 and β3 family, namely α2β1, α5β1, α6β1, αIIbβ3 and αvβ3 ensure stable platelet adhesion through their ability to engage with the adhesive proteins collagen, fibronectin, laminin, fibrinogen and vitronectin, respectively (Bergmeier and Hynes, 2012). Concomitantly, these integrins along with the collagen receptor, GPVI, instigate intracellular signals leading to platelet activation (Zahid et al., 2012). Upon activation, platelets release soluble mediators, including TxA_2 and ADP which reinforce platelet activation ensuring firm adhesion, spreading and procoagulant activity. Platelet activation enhances the affinity of αIIbβ3 with its prime ligand, fibrinogen, allowing platelets to aggregate and to form a mural thrombus **(Figure 1)** (Versteeg et al., 2013). The thrombus composition is heterogeneous having a core comprising highly activated platelets and fibrin, and a shell having less-activated platelets (Welsh et al., 2014).

Once the hemostatic plug is formed, it is further strengthened by a fibrin meshwork formed as a result of the coagulation cascade. The coagulation cascade is initiated upon vessel wall injury which exposes tissue factor (TF). TF interacts with circulating FVII to form a TF-$FVII_a$ complex, initiating the ‘extrinsic' coagulation pathway whereas the polyphosphate (PolyP) released from platelets are proposed to activate FXII, initiating the ‘intrinsic’ pathway. These pathways lead to the generation of activated FX, which converts prothrombin to thrombin. The generated thrombin not merely stimulates the platelets strongly but also converts fibrinogen into fibrin to form a polymerized meshwork that stabilizes and consolidates the platelet thrombus. The formation of fibrin-rich platelet thrombus seals the breach and sustains the integrity of the vasculature until wound heals. The fibrinolytic system gets activated on the surface of fibrin-rich platelet thrombus that results in the dissolution of the thrombus and restores a normal blood flow within the vessel (Rau et al., 2007).

Arterial thrombosis

Platelets also contribute in arterial thrombosis, which occurs in a diseased artery following the rupture and/or erosion of an atheromatous plaque **(Figure 2)**. Since the plaque geometry profoundly modifies the local rheology and the exposed plaque material is highly reactive with TF and fibrillar collagen, the thrombus can become occlusive and result in life-threatening ischemic pathologies for instance myocardial infarction, ischemic stroke or peripheral artery diseases (McFadyen et al., 2018; Tendera et al., 2011). Arterial thrombosis results in more than fourteen million deaths per annum and is the most frequent cause of fatality and disability all around the globe. About 80% of the deaths due to arterial thrombosis occur in low- and middle-income countries and this is increasing excessively (Mensah et al., 2019). To prevent and/or treat arterial thrombosis anti-platelet agents were developed. The three main classes of anti-platelet agents comprise aspirin, $P2Y_{12}$ antagonists and $\alpha IIb\beta 3$ blockers. These agents have helped to significantly decrease the possibility of ischemic events. However, their administration carries heightened bleeding-risk which implies that the current standard of care composed of aspirin with a $P2Y_{12}$ antagonist, is not straight forward to use in grave risk individuals or in the setting of stroke.

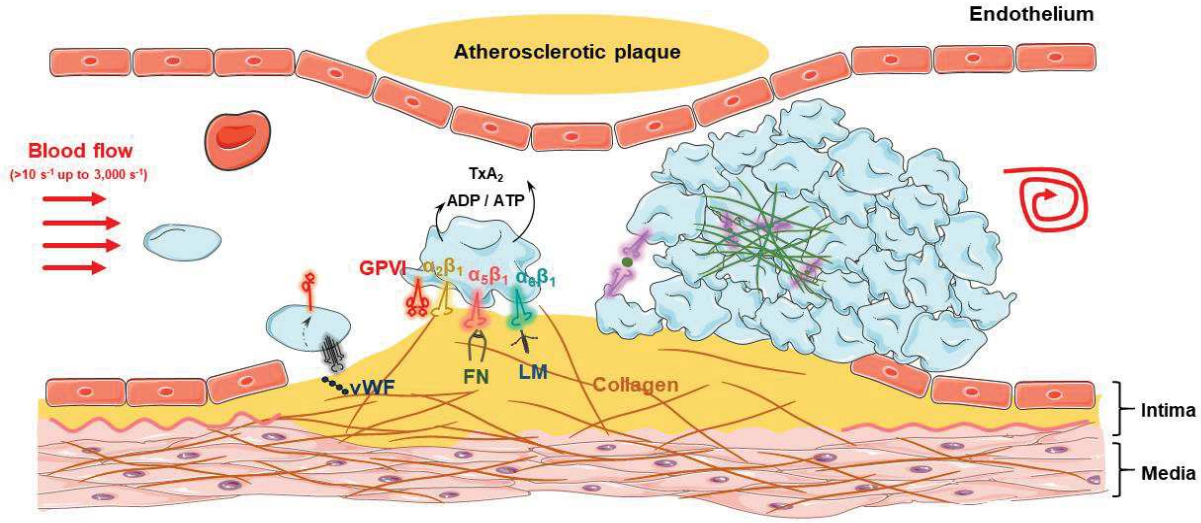

Figure 2. Role of platelets in arterial thrombosis

It occurs in a stenosed artery under elevated blood flow conditions when platelet rich thrombi are formed around ruptured atheromatous plaques and damaged endothelium

Context of the thesis

To further improve anti-platelet therapy, it seems ideal to develop an agent that impairs thrombus formation, with almost no impact on hemostasis to avoid bleeding complications. Such an agent is not easy to identify as the molecular mechanism of hemostasis and arterial thrombosis are closely related, and therefore when the thrombotic process is impaired an impact on hemostasis is observed. That is why the main drawback of clinically used anti-platelet therapies is the occurrence of bleeding events.

In the past 20 years, GPVI, initially identified as the main platelet activation receptor for collagen, has been extensively studied. It has been shown that the inhibition of this receptor impairs thrombus formation *in vitro*, when anticoagulated whole blood is perfused over collagen, proposing a role in thrombosis. This has been confirmed in some *in vivo* models of arterial thrombosis in which GPVI-knock out mice had the reduced thrombus formation (Zahid et al., 2012). In parallel, it has been reported that patients deficient for GPVI present only a mild bleeding diathesis (Jandrot-Perrus et al., 2019). Moreover, mice deficient for GPVI do not bleed at all in the broadly used tail-bleeding time assay. As a consequence, GPVI is viewed as a potential safe anti-thrombotic target. Agents targeting GPVI have been developed and two of them, Revacept, a dimeric GPVI, and Glenzocimab (ACT017), a blocking fab antibody have been successfully evaluated in phase 1 studies in which they did not prolong the bleeding time of healthy volunteers while impairing collagen-mediated platelet aggregation *ex vivo* (Ungerer et al., 2011; Voors-Pette et al., 2019). Both molecules are presently being assessed in phase 2 clinical trials in the settings of PCI or stroke.

My PhD work was focused on GPVI with the aim to further characterize it role in thrombus formation and stability. I have mostly studied his interplay with fibrinogen, a newly identified ligand of GPVI.

Review of Literature

1. Platelets

Platelets are tiny anucleate blood components originating from nucleated precursor cells, the megakaryocytes, mainly found within the bone marrow (Kaushansky, 2008; Mazzi et al., 2018). At rest, they are discoid in shape with a diameter ranging from 2 to 3 µm, a thickness of 0.5 µm and a volume of about 9 to 12 μm^3. In a healthy human adult, the number of circulating platelets ranges from 1.5 to 4.5 x 10^5 platelets/µL whereas, in mice it ranges from 1.1 to 1.3 x 10^6 platelets/µL. Usually, platelets have a lifespan of about 7 to 10 days in humans and of about 4 to 5 days in mice (Schmitt et al., 2001). They are eliminated by phagocytosis through macrophages in the spleen or Kupffer cells in the liver (Grozovsky et al., 2010).

1.1. Platelet morphology and ultrastructure

Microscopic analysis revealed that the ultrastructure of platelets is composed of three major components: plasma membrane, organelles, and the cytoskeleton.

1.1.1. Plasma Membrane

The plasma membrane of platelets is comprised of a phospholipid bilayer that includes cholesterol, glycolipids, proteoglycans and glycoproteins (Gremmel et al., 2016). The intrinsic leaflet of the cellular plasma membrane predominantly contains phosphatidylserine and phosphatidylinositol lipids whereas, the outer leaflet contains choline lipids (sphingolipids) and cholesterol. The plasma membrane is connected to an inner membrane system named the open canalicular sytem (OCS), with small ~20 nm size openings, which takes part in the release of platelet granules after activation and enables to increase the surface area while spreading (Gruba et al., 2015).

1.1.2. Cytoplasmic Organelles

Platelets contain cytoplasmic organelles including mitochondria, the dense tubular system and three major classes of platelet storage granules α, δ and λ, which are secreted following platelet activation. The dense tubular system (DTS) arises from the endoplasmic reticulum of the parent megakaryocyte (Daimon and Gotoh, 1982). It contains: i) a large pool of intracellular Ca^{2+}, which contributes in platelet activation and ii) enzymes which are involved in the prostaglandins synthesis (Gerrard et al., 1976). α granules are the most abundant (~40 per platelet) organelle (Eckly et al., 2016). They mainly contains adhesive proteins, coagulation factors, growth factors, chemokines, and clotting factors (Harrison and Cramer, 1993). δ granules, also called dense granules, are less in number about 4–8 per platelet (Eckly et al., 2016). They contain ADP, ATP, calcium, serotonin, polyphosphate and pyrophosphate (McNicol and Israels, 1999). Moreover, platelets have lysosomal granules (λ) which contain degradative enzymes including acid hydrolases and proteases. The importance of platelet granule release in hemostasis is evidenced by bleeding problems in patients presenting defects in the formation or content of the granules. The absence or reduction of α granules leads to Gray platelet syndrome (Gunay-Aygun et al., 2010) whereas, deficiency of dense granules leads to platelet storage pool deficiency (Dupuis et al., 2020).

α-granules	Dense granules (δ)	Lysosomes (λ)
Adhesive proteins (e.g. Fibrinogen, fibronectin, vWF, vitronectin, TSP-1) **Coagulation factors** (e.g. FV, XI, XIII, TF, kininogens) **Inhibitors of fibrinolysis** (e.g. α2-antiplasmin, PAI-1) **Growth factors and chemokines** (e.g. PF4, PDGF, VEGF, TGF-β,CXCL-7) Albumin P-selectin	ATP ADP Serotonin Polyphosphates Pyrophosphates Ca^{2+} Mg^{2+}	**Acid hydrolases** (e.g. β-galactosidase, cathepsins, aryl sulfatase, acid phosphatases and β-glucuronidase)

Table 1. Intra-platelet granules
(see list of abbreviations)

1.1.3. Platelet cytoskeleton

The platelet cytoskeleton is based on three main elements: microtubules, a spectrin based skeleton, and the actin filament. The microtubule coils encircle the whole platelet beneath the plasma membrane known as ‘platelet marginal band’, which retains the discoid morphology of the resting platelet. The actin filaments form a cytoskeletal framework throughout the cytoplasm adjacent to microtubules and membrane skeleton. During platelet activation, reorganization of platelet cytoskeleton leads to shape change, granule secretion and platelet spreading (Hartwig, 1992; Hartwig and DeSisto, 1991).

1.2. Platelet adhesion and activation receptors

Platelet adhesion, activation and aggregation on the exposed extracellular matrix are key events in hemostasis, but they can also lead to the formation of occlusive thrombi in diseased vessels (Nieswandt et al., 2011). These functions are regulated by multiple receptors present at the platelet plasma membrane. Platelet adhesion receptors can be categorized into different groups containing the leucine rich repeat family (viz. GPIb-IX-V receptor complex), the immunoglobulin superfamily (viz. GPVI and FcγRIIA), the integrins, and the C-type lectin receptor family (viz. CLEC-2). These receptors can either support platelet attachment, stable adhesion and/or initiate intracellular signalling including tyrosine kinases phosphorylation leading to platelet activation. This very first platelet activation step is further intensified by the release of soluble mediators including ADP, ATP, TxA_2, and thrombin, which act on their own G-protein coupled receptors.

1.2.1. The GPIb-IX-V Complex

The adherence of flowing platelets to the injured vessel wall is a critical step which initiates the process of hemostasis. Under conditions of elevated blood flow (>1,000 s^{-1}) (Savage et al., 1996b), the GPIb-IX-V complex is an even platelet receptor supporting platelet recruitment through its ability to bind subendothelial vWF or platelet-bound vWF.

1.2.1.1. Structure of GPIb-IX-V Complex

The GPIb-IX-V receptor complex is exclusively found on platelets and megakaryocytes at a high copy number (25,000 copies per platelet) (Modderman et al., 1992). It is composed of four glycoproteins namely α and β subunits of GPIb, GPIX and GPV (Cauwenberghs et al., 2000; George et al., 1984), all of them belonging to the leucine-rich repeat (LRR) protein superfamily, which is delineated by the presence of one or more leucine rich repeats (Berndt et al., 2001). Concerning the stoichiometry,

one GPIbα (120–140kDa) is linked through disulfide bonds with two GPIbβ (24kDa) to form GPIb (Luo et al., 2007b; Phillips and Agin, 1977), which associates non-covalently with GPIX (20kDa) to form a GPIb-IX complex. Two GPIb-IX complexes associate with one or two GPV (85kDa) (Du et al., 1987; Li and Emsley, 2013; Modderman et al., 1992).

1.2.1.2. Ligands of GPIb-IX-V complex

GPIbα is a major subunit of the complex as it interacts through its N-terminus domain with the majority of the ligands including vWF. It has been shown that shear regulates the GPIb-IX-V-vWF binding (Kroll et al., 1996). GPIbα-vWF binding has a rapid on-rate allowing vWF to capture flowing platelets through interaction with GPIb-IX-V complex, but as this bond has also a rapid off-rate, this interaction is unstable. As a result, platelets will form and let go many GPIb-vWF bonds when they are perfused on immobilized vWF resulting in platelet rolling over a wide range of blood flow conditions (Doggett et al., 2002). To be noted, such a behavior is observed under experimental conditions, and has no real or yet identified *in vivo* relevance.

GPIb also interacts with many other ligands that contribute in hemostasis, thrombosis and inflammation. Amongst them the adhesive protein thrombospondin-1 (TSP1) has been proposed to support adhesion of platelets at higher shear values up to 4,000 s^{-1} in the paucity of vWF *in vitro*, even though this remains controversial as TSP-1 could allow the adsorption of plasma vWF (Jurk et al., 2003). Thrombin also interacts with a high affinity to GPIb-IX-V. It is indicated that GPIbα-thrombin interaction facilitates platelet activation by acting as a co-factor for PAR-1 and PAR-4 but could also activate platelets directly by inducing GPIb-IX-V signaling (Celikel et al., 2003; De Candia et al., 2001). Other coagulation factors can bind GPIb including activated factor XIIa which may mediate the formation and function of the platelet procoagulant complex involving kininogen, FXI and thrombin. FXIIa linked to GPIb serves as a negative regulator of platelet activation by thrombin (Bradford et al., 2000). The high-molecular-weight kininogen (HK) also binds to GPIbα through its domain 3 and can inhibit the platelet activation by modulating thrombin binding (Bradford et al.,

1997). Contrarily, presence of HK may augment leukocyte-mediated platelet adhesion by facilitating the GPIbα/αMβ2 integrin interplay (Chavakis et al., 2003).

In 1999, P-selectin was reported as first cell-surface receptor for GPIb-IX-V (Romo et al., 1999). The interaction of GPIbα with P-selectin allows platelets to adhere either to activated endothelial cells or activated platelets expressing P-selectin which suggests a potential link between platelets and inflammatory diseases in which platelets could be recruited at site of inflammation and further enhance this process (Somers et al., 2000). The leukocyte integrin, αMβ2 (Mac-1), was also reported as another cell-surface receptor for the GPIb-IX-V complex. It was proposed to mediate adhesion and migration of leukocytes at sites of lesions in mice which is another potential mechanism by which platelets contribute to inflammation (Simon et al., 2000).

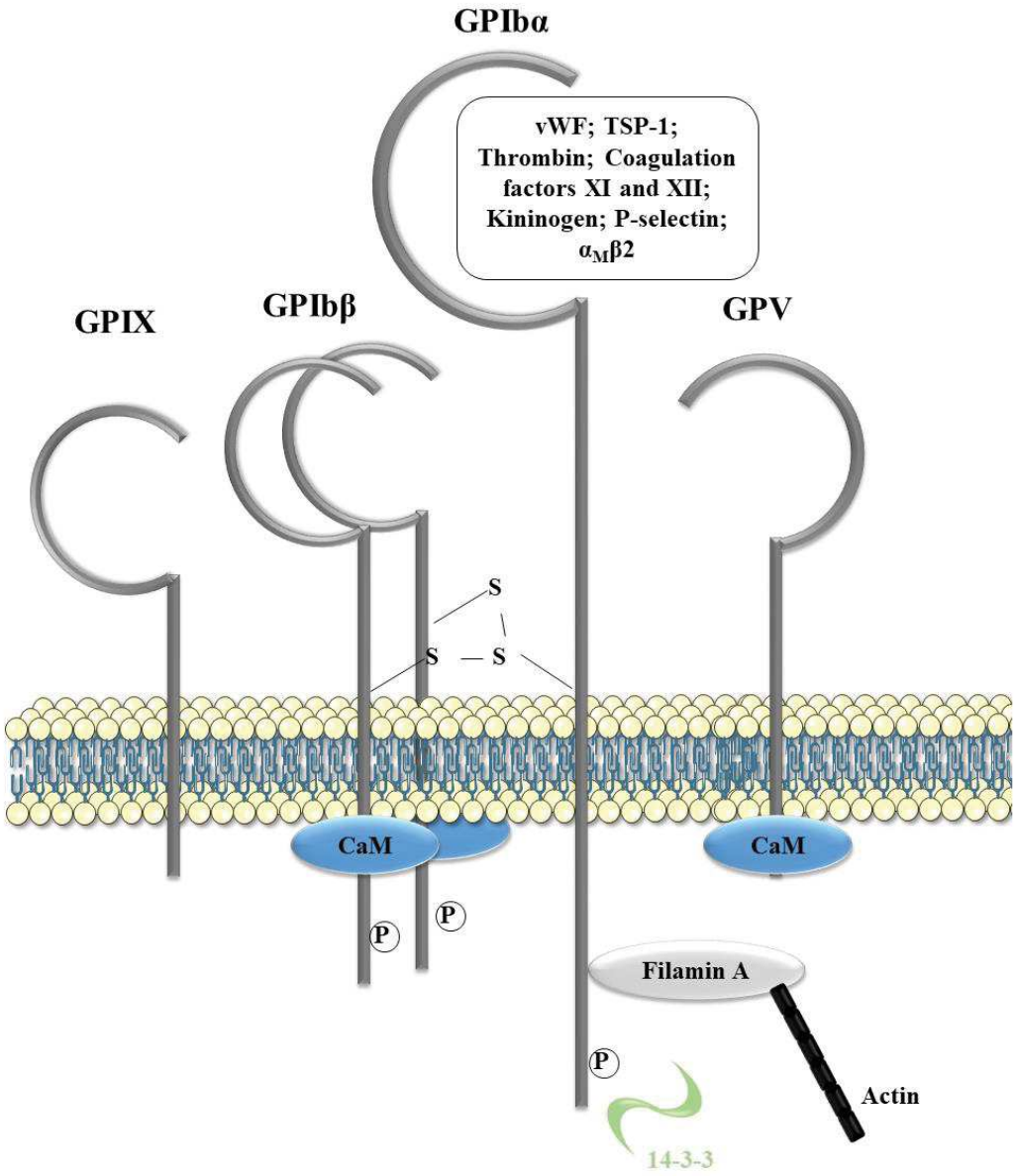

Figure 3. Structure of the GPIb-IX-V complex

14-3-3 ζ, adaptative protein 14-3-3; CaM, calmodulin; GP, glycoprotein; S, sulfite; TSP-1, Thrombospondin 1; vWF, von Willebrand Factor. Adapted from (Andrews et al., 2003)

1.2.1.3. GPIb-IX-V Complex Signaling

The GPIb-IX-V/vWF interaction generates a signaling cascade, involving Src family kinases and PLCγ2, the production of IP3 and intracellular Ca^{2+} mobilization (Mangin et al., 2003). Such an activation results in platelet shape change with filopodia extension. This weak signalling transduction has also been suggested to result in αIIbβ3 activation which allows stable adhesion and spreading of platelets, however, this is not what is observed when platelets roll on a vWF surface questioning the importance of GPIb signal in integrin activation (Lanza et al., 2008). The signaling function of GPIb-IX-V complex has been proposed to involve many other signaling molecules, however, this has often been performed under non-controlled experimental conditions where other pathways are activated and could be responsible for these observations.

The GPIb-IX-V cytoplasmic domain has no signaling motif but allows the interaction with several signaling molecules such as phosphoinositide 3-kinase (PI3K), adapter protein 14-3-3ζ (Mangin et al., 2009), calmodulin (Andrews et al., 2001) and actin cytoskeleton-associated protein filamin-A (Williamson et al., 2002). The 14-3-3ζ/GPIb interplay has also been proposed to promote vWF/GPIb-dependent signaling which could be linked to its ability to regulate the binding function of vWF with GPIbα (Dai et al., 2005; Yuan et al., 2009). Calmodulin directly binds to GPIbβ and GPV, while filamin-A ensures the anchoring of GPIb-IX-V to the actin cytoskeleton, regulating the adhesion of platelets to vWF and retaining platelet membrane integrity elevated wall shear rates (Cranmer et al., 2011). To date, the importance of GPIb signaling in platelet function is believed to be weak and its significance in hemostasis and thrombosis is not yet elucidated.

1.2.1.4. Role of GPIb-IX-V in hemostasis and arterial thrombosis

The GPIb-IX-V demonstrates a pivotal role in the first step of platelet attachment at a site of lesion, especially under elevated blood flow conditions, as those found in arterioles. In addition, this receptor also contributes in platelet aggregation

and thrombus growth, by allowing platelet attachment at the surface of activated platelets exposing vWF, within a growing thrombus (Kulkarni et al., 2000).

The significance of GPIb-IX-V receptor complex in hemostasis is evidenced by a congenital bleeding disorder named the Bernard-Soulier syndrome (BSS), that is the consequence of genetic alteration in either GPIbα, GPIbβ or GPIX (Bernard and Soulier, 1948; Lanza, 2006). This pathology is characterized by a macro-thrombocytopenia resulting in a bleeding diathesis which can vary between patients. This pathology could be reproduced in murine models showing macro-thrombocytopenia and increased tail-bleeding time when GPIbα or GPIbβ were mutated (Strassel et al., 2007).

Conversely, a transgenic mouse expressing the extracellular domain of the human IL4R instead of the ectodomain of GPIbα (with a mild macrothrombocytopenia) had a marked defect in thrombus formation in response to ferric chloride injury, highlighting a major role for GPIb in arterial thrombosis (Bergmeier et al., 2006). It has also been reported that the blockade or absence of GPIbα reduced (Lanza et al., 2008) the infarct volume within the brain with a strong protective effect in a tMCAO mice model (Kleinschnitz et al., 2007; Stoll et al., 2010). These results indicate that the GPIb-IX-V receptor complex could be a target for anti-thrombotic drugs. However, targeting such a receptor might not be devoid of bleeding risk.

1.2.2. GPVI

Glycoprotein VI (GPVI) was first identified as a 58-60 kDa glycosylated platelet membrane protein (Phillips and Agin, 1977). Several years later, this protein was proposed to be a receptor for collagen as it was missing in a patient presenting an auto-immune thrombocytopenia whose platelets showed no response to collagen (Sugiyama et al., 1987). Later on, GPVI was shown to be coupled to the γ-chain of Fc receptors (FcRγ) which is not only important for its surface expression but also to induce signaling (Gibbins et al., 1997; Tsuji et al., 1997). The identification and development of two specific GPVI agonists, the toxin convulxin and synthetic collagen related peptide were instrumental to better characterize the function of GPVI (Ezumi et al., 1998; Jandrot-Perrus et al., 1997; Kehrel et al., 1998; Morton et al., 1995; Polgár

et al., 1997; Tsuji et al., 1997). The gene of GPVI was simultaneously cloned by two separate groups using different approaches (Clemetson et al., 1999; Jandrot-Perrus et al., 2000) and GPVI expression was also shown to be restricted to platelets and megakaryocytes. Patients having congenital or acquired deficiencies in GPVI do not exhibit severe bleeding, a finding which has been used as evidence that GPVI is not absolutely required to ensure hemostasis (Dumont et al., 2009; Hermans et al., 2009; Matus et al., 2013). Conversely, findings in GPVI-deficient animal models point to its role in experimental thrombosis (Grüner et al., 2005; Lockyer et al., 2006; Nieswandt et al., 2001b).

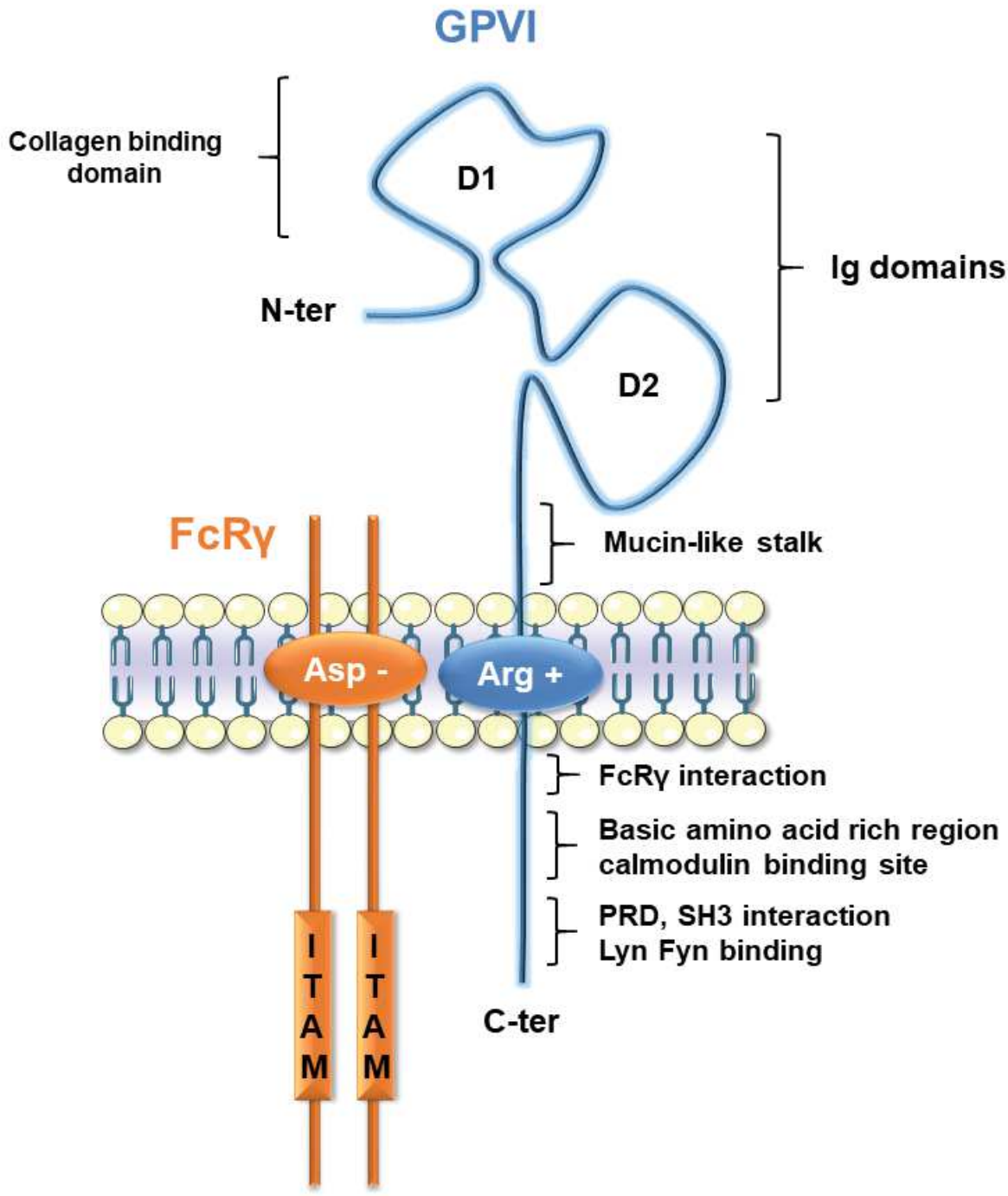

Figure 4. Structure of GPVI-FcRγ omplex

Arg, Arginine; Asp, Aspartate; D1, Domain; D2, Domain 2; GP, Glycoprotein; ITAM, immunoreceptor tyrosine-based activation motif; PRD, Proline-rich domain; SH3, Src homology 3

1.2.2.1. Structure of GPVI

Human GPVI has 339 amino acid residues. The GPVI gene, made up of eight exons, is located on chromosome 19q13.4 (Ezumi et al., 2000) which is mapped within the leukocyte receptor complex region (LCR). This region encodes for a variety of proteins that include leukocyte Ig-like receptors (LIRs), killer-cell Ig-like receptors (KIRs), leukocyte-associated Ig-like receptors (LAIRs) i.e. LAIR-1 and the receptor for IgA FcαRI (Jandrot-Perrus et al., 2000). It is worth mentioning, LAIR-1, which has structural resemblance to GPVI, also interacts with collagen but with a higher affinity (Brondijk et al., 2010). GPVI is an activation receptor on platelets and LAIR-1 act as an inhibitory receptor on immune cells. In humans, GPVI gene has two distinct alleles, GPVIa and GPVIb, evenly distributed on human platelets with an incidence of 0.85 and 0.13, respectively (Croft et al., 2001; Joutsi-Korhonen et al., 2003). These alleles differ from each other by 3 amino acids replacement in the glycosylated stem and 2 in the cytoplasmic domain. GPVI was identified as an immunoglobulin superfamily protein as it possesses two immunoglobulin-like extracellular domains formed by disulfide bonds (D1 and D2), a mucin-like stalk, a transmembrane region, and a cytoplasmic tail. A basic amino acid rich region is located in the intracytoplasmic domain near the transmembrane region which can bind calmodulin. This domain also contains a proline-rich domain (PRD) interacting with the Src homology 3 region of the SFKs, Lyn and Fyn.

Mouse GPVI is composed of 319 amino acids and shares 64.4% and 67.3% homology with human GPVI at the amino acid and nucleotide levels, respectively (Jandrot-Perrus et al., 2000). The cytoplasmic tail of human GPVI contains 51 amino acids whereas murine GPVI has only 27 amino acids residues and lacks the 24 amino acids residues of the proline-rich region. The difference in human and mouse GPVI sequence is shown in figure 19 (Page 150).

GPVI exists as a complex with the γ-chain of Fc receptors (FcRγ), which is not only crucial for its surface expression but also to induce signaling. Absence of the FcRγ-chain leads to the loss of collagen-mediated platelet activation (Nieswandt et al., 2000). The interface between GPVI and FcR γ-chain occurs through a salt bridge between an arginine and an aspartate within the transmembrane domains of these proteins (Berlanga et al., 2007; Clemetson et al., 1999). The FcRγ-chain is expressed

as homodimer; each monomer has two tyrosine residues in immunoreceptor tyrosine-based activation motif (ITAM). GPVI is expressed on the platelet surface ranging from 4,000 to 6,000 copies, mainly under a monomeric form (Berlanga et al., 2007). Following platelet activation, even weak signals as those induced by GPIb are sufficient to promote the dimerization of GPVI, which increases its affinity for collagen. The binding of collagen to GPVI dimers leads to oligomerization (also termed as clustering) which could initiate the recruitment of signaling molecules leading to platelet activation.

To date, two well-defined collagen binding domains on GPVI have been reported. The first and probably most important one is located in D1 domain (Lecut et al., 2004; Smethurst et al., 2004), and the second one is found in the D1–D2 hinge region, that is formed when GPVI clusters as back-to-back dimer (Jung et al., 2009). The binding sites of ligands such as collagen related peptide (CRP) and convulxin overlap and are also located in the D1 domain and D1-D2 hinge region of GPVI.

1.2.2.2. GPVI Signaling Pathway

The interaction of collagen with GPVI promotes its clustering bringing in close contact the Lyn and Fyn (SFKs) to the FcRγ-chain containing ITAM motif (Ezumi et al., 1998; Quek et al., 2000; Séverin et al., 2012). This event leads to the phosphorylation of the ITAM motif allowing the recruitment of the tyrosine protein kinase, Syk, via SH2 domains. The subsequent attachment of Syk with the phosphorylated ITAM motif results in its activation following phosphorylation by SFKs, conformational change and autophosphorylation (Mócsai et al., 2010). Filamin A is also required for the relocation of Syk to ITAM motif of the FcRγ-chain (Rosa et al., 2019). The relative importance of Syk in GPVI signaling is evidenced by the loss of tyrosine phosphorylation of numerous proteins upon collagen stimulation in the Syk deficient mouse platelets (Poole et al., 1997). The activation of Syk embarks the tyrosine phosphorylation cascade promoting the assembly of a large signaling complex comprising adapter and effector proteins. This signalosome is formed around the adapter protein linker for activation of T-cells (LAT), which localizes this complex to lipid rafts with nine-conserved tyrosine residues (Pasquet et al., 1999).

Phosphorylated LAT in turn leads to the recruitment of SH2 containing-adapter proteins Grb2 (growth factor receptor bound protein 2), Gads (Grb2 related adaptor protein downstream of Shc), and also SLP-76 (SH2 domain containing leukocyte protein of 76 kDa) (Asazuma et al., 2000; Hughes et al., 2008). LAT also allies with class 1 PI-3 kinases that catalyze the formation of PIP3 by phosphorylation of PIP2 (Gibbins et al., 1998). LAT, SLP76 and PIP3 recruit PLCγ2 and also induce its phosphorylation via effector protein kinases of the Tec family i.e. Btk (Bruton's tyrosine kinase) and Tec (Atkinson et al., 2003; Quek et al., 1998). Other effector proteins include the GTP exchange factor Vav1 and Vav3 (Pearce et al., 2004), the small GTPase Rac1 (Pleines et al., 2009; Stefanini et al., 2012) and activator of transcription 3 (STAT3) (Zhou et al., 2013) gather into the signaling complex.

These effector and adaptor proteins induce the activation of PLCγ2, resulting in the cleavage of PIP2 to generate the two secondary messengers, IP3 and DAG, leading to the release of Ca^{2+} from cytoplasmic storage pools and PKC activation, respectively. The increased cytosolic Ca^{2+} is sensed by CalDAG-GEFI which facilitates the activation of the GTPase Rap1 (Stefanini et al., 2009). The CalDAG-GEFI/Rap1 allows to respond to threshold levels of Ca^{2+} and prompts ADP release via Rac1 activation, ADP successively initiates the second wave of PKC/Rap1 activation. The activation of integrin, thromboxane A2 generation and granule secretion are promoted by Rap1, and altogether results in platelet aggregation (Moroi and Jung, 2004; Stefanini and Bergmeier, 2010).

GPVI signaling is regulated by several transmembrane proteins. Some are positive regulators such as Ephrin type-B receptor 2 (EPHB2) which has been described to contribute in GPVI activation as its deficiency altered GPVI signaling and platelet aggregation induced by convulxin (Berrou et al., 2018). Others are negative regulators including PECAM-1, as the platelets from PECAM-1-deficient mice exhibited a better platelet aggregation on collagen related peptide (CRP) and enhanced thrombus growth on an immobilized collagen matrix (Jones et al., 2001; Patil et al., 2001).

The role of lipid raft in regulating GPVI signaling has also been reported as the lipid raft disruption by removing cholestrol resulted in the inhibited platelet aggregation (Quinter et al., 2007).

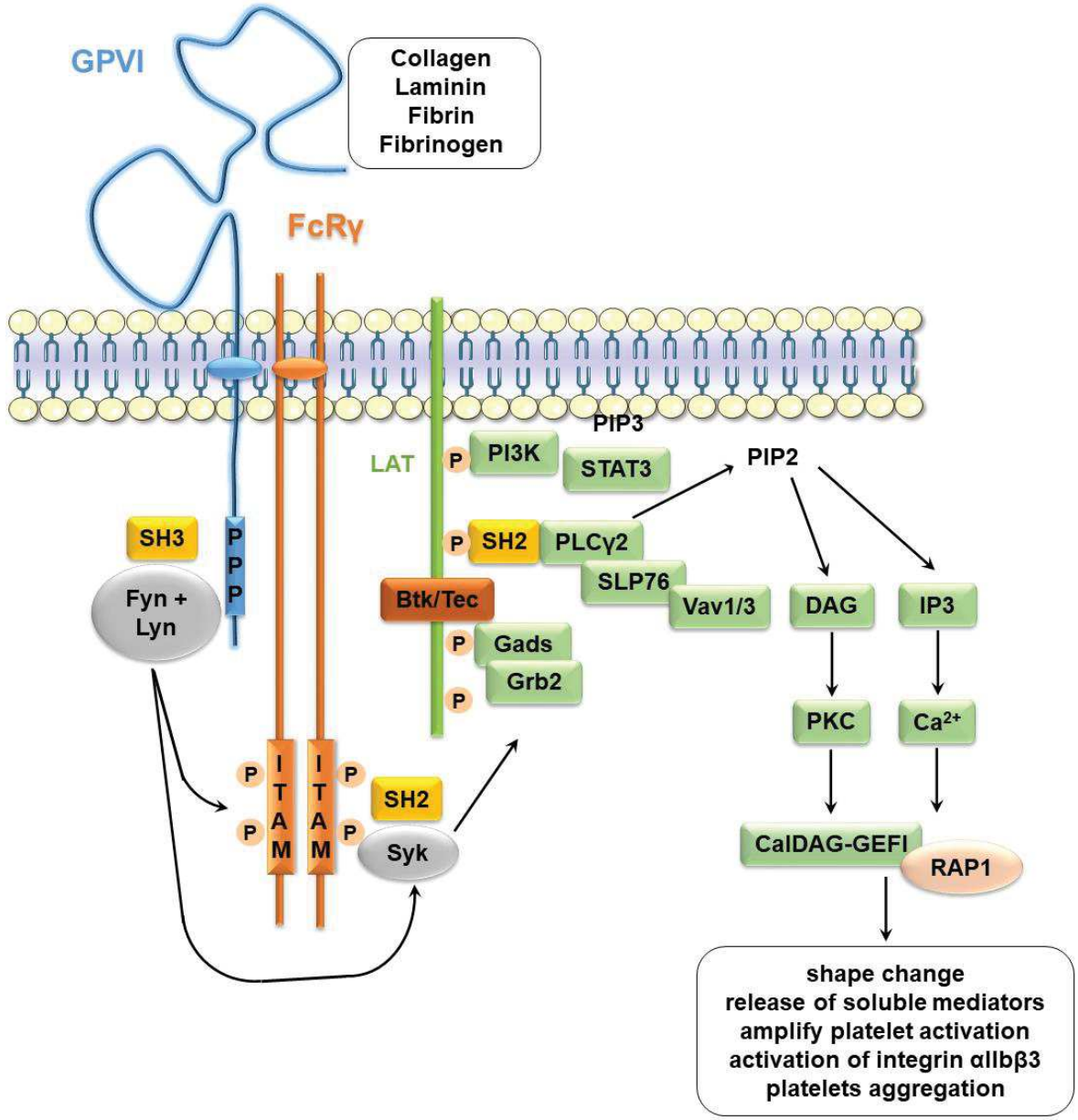

Figure 5. Structure of GPVI and its signaling pathway

Btk, Bruton tyrosine kinase; DAG, diacylglycerol; Fyn, tyrosine-protein kinase Fyn; Gads, Grb2-related adapter downstream of Shc;; Grb2, growth factor receptor bound protein 2; IP3, inositol 1,4,5-triphosphate; ITAM immunoreceptor tyrosine-based activation motif; LAT, linker for activation of T cells; Lyn, *Lck/Yes-related novel protein tyrosine kinase*; PI3K, phosphoinositide 3-kinase; PIP2, phosphatidylinositol 4',5'-bisphosphate; PIP3, phosphatidylinositol 3,4',5'-trisphosphate; PKC, protein kinase C; PLCγ2, phospholipase Cγ2; SH2, SRC homology 2 domain; SH3, SRC homology 3 domain; SLP76, SH2-domain-containing leukocyte protein of 76 kDa; Syk, spleen tyrosine kinase; Vav, guanine nucleotide exchange factor; CalDAG-GEFI, calcium-binding EF hands and DAG-binding C1 domains and RAP1, Ras-related protein 1. Adapted from (Rayes et al., 2019; Watson et al., 2005)

1.2.2.3. Ligands of GPVI

It is widely accepted that GPVI is the key receptor of platelet activation on collagen since platelets lacking GPVI poorly respond to this ligand (Nieswandt and Watson, 2003). GPVI binds efficiently to several forms of fibrillar and non-fibrillar collagen, but poorly with soluble monomeric collagen (Jung et al., 2008). It has been reported that GPVI directly interacts with gly-prol-hyp (GPO) triplet motifs of the types-I and III collagen fibers (Jarvis et al., 2008).

While integrin α6β1 is the key platelet receptor for laminins supporting both adhesion and activation of platelets, it has been shown that GPVI participates in platelet activation to laminins (Inoue et al., 2006). This observation was confirmed in a study from our laboratory proposing that the vascular α5-chain, but not α4-chain of laminins exhibit platelet activation via GPVI (Schaff et al., 2013).

Besides, Bultmann and co-authors proposed that GPVI can serve as a receptor for fibronectin (FN) and they suggested that this could contribute to platelet-triggered atheroprogression (Bültmann et al., 2010). These authors showed that adhesion of platelet on immobilized fibronectin was reduced by soluble dimeric GPVI-Fc and blocking anti-GPVI agent 5C4 under flow conditions. However, a study from our group reported that blockade or the deficiency of GPVI didn't alter the platelet adhesion and activation onto plasma or cellular FN immobilized in their soluble or fibrillar forms suggesting fibronectin is most likely not a ligand for GPVI (Maurer et al., 2015).

Fibrin has been reported as ligand for GPVI by two independent groups in 2015, but the physiological role of GPVI/fibrin interaction has been challenged (Ebrahim et al., 2018). The two initial studies identified GPVI as a functional receptor for polymerized fibrin with two major functions: a) to amplify thrombin generation and b) to recruit circulating platelets to a growing thrombus. These studies suggest that the GPVI-fibrin interaction could be important for thrombus formation (Alshehri et al., 2015a; Mammadova-Bach et al., 2015). Two additional studies confirmed that fibrin is a ligand for GPVI, and while one proposed that monomeric GPVI binds to the D-dimer region of fibrin, the other one suggested that it is the dimeric form of GPVI which allows the interaction (Induruwa et al., 2018; Onselaer et al., 2017). The group of Wolfgang Siess also confirmed an interaction between GPVI and fibrin formed by treating

plasma fibrinogen with thrombin, but they did not confirm its binding to fibrin formed under flow conditions when coagulation is active, challenging the relevance of the GPVI/fibrin interplay (Zhang et al., 2020). In summary, 4 independent groups reported that fibrin binds to GPVI with different experimental approaches, but one group challenged the physiological relevance of this interaction.

In a recent publication, our group reported that fibrinogen is also a ligand of GPVI (Mangin et al., 2018). This study showed that GPVI promotes platelet activation on immobilized fibrinogen since control platelets but not GPVI-deficient patient platelets spread on fibrinogen and this activation appears to be important in thrombus growth. Since I started my PhD, more publications on GPVI and fibrinogen where published, but I will discuss these later on in this manuscript.

Among other ligands, globular adiponectin (gAd) was identified as a ligand of GPVI which could induce platelet activation and aggregation (Riba et al., 2008). CD147 (EMMPRIN) was proposed as a counter-receptor of GPVI which could possibly mediate platelet/monocyte interactions (Schulz et al., 2011; Seizer et al., 2009). Alzheimer's disease (AD)-associated Amyloid beta (Aβ) 40 and 42 peptides are also found in platelet α-granules and might be a potential ligand for GPVI receptor, but the relevance of this interaction has to be determined (Elaskalani et al., 2018). Finally, it has been shown that diesel exhaust particles (DEP) could be ligands of GPVI as platelets from mice deficient of GPVI receptor do not aggregate in response to DEP (Alshehri et al., 2015b).

1.2.2.4. Down-regulation of GPVI

Receptor downregulation can occur via shedding of the ectodomains or/and internalization of the whole receptor (Hartmann et al., 2013; Murphy, 2009; van der Vorst et al., 2012). The proteolytic cleavage of the GPVI extracellular domain producing a soluble 55-kDa fragment of GPVI (sGPVI) which is believed to be a biomarker of platelet activation while, a 10-kDa remnant fragment is retained on to the surface. The receptor down-regulation can be caused by activation-dependent and independent pathways. For instance, the excessive activation GPVI induced by CRP, collagen or convulxin tends to activation-dependent shedding of GPVI causing its cleavage by the metalloproteinases (MMPs) namely ADAM17 and/or ADAM10 (Bender et al., 2010). Pathological shear rates and the coagulation FXa were also shown to cause down-regulation of GPVI by ADAM10-dependent and activation-independent mechanisms (Al-Tamimi et al., 2011; Al-Tamimi et al., 2012b). Anti-GPVI antibodies are also known to deplete platelet surface GPVI. The antibody-induced GPVI depletion mechanisms include internalization, shedding of GPVI and seem to be reliant on the antibody (Rabie et al., 2007). As an example, the injection of JAQ-1 caused a transient thrombocytopenia and a prolonged depletion of platelet surface GPVI (Nieswandt et al., 2001a).

Elevated plasma levels of sGPVI could represent a potential biomarker of various pathologies (Al-Tamimi et al., 2012a). Indeed, elevated sGPVI measurements have been identified in the plasma of patients with (i) disseminated intravascular coagulation (Al-Tamimi et al., 2011), (ii) coronary artery stenosis (Al-Tamimi et al., 2012b), and (iii) autoimmune anti- GPVI antibodies (Gardiner et al., 2008).

1.2.2.5. Role of GPVI in hemostasis and thrombosis

GPVI is differentially involved in arterial thrombosis and in hemostasis. To date, only a few cases of human hereditary GPVI deficiencies have been reported with most of them presenting only a mild bleeding tendency, mostly in the form of epistaxis, easy bruising, gum bleeding (Dumont et al., 2009; Hermans et al., 2009; Matus et al., 2013). There is a possibility that most GPVI deficiencies are under diagnosed due to mild or even absence of symptoms. The frequency of acquired GPVI deficiencies is higher. The majority of these patients with autoimmune thrombocytopenia exhibit autoantibody-induced GPVI shedding and/or internalization (Arthur et al., 2007). Overall, the mild bleeding tendency in GPVI deficient patients combined with the fact that GPVI-deficient mice do not show any sign of spontaneous bleeding and do not have an increased tail-bleeding time, strongly endorses that GPVI is not essential for hemostasis.

Unlike the minor role of GPVI in hemostasis, this receptor seems to be playing a central role in experimental models of arterial thrombosis. This was first demonstrated in mice injected with an immunodepleting anti-GPVI antibody JAQ1 which showed that such a treatment protected mice against thromboembolism induced upon injecting of collagen and adrenaline (Nieswandt et al., 2001b). Additional studies carried out in several mouse strains with no GPVI expression on platelets (GPVI knock outs, FcγR-chain knock outs or GPVI-immunodepleted WT mice) also showed that experimental thrombus formation was reduced in a number of arterial thrombosis models (Cheli et al., 2008; Grüner et al., 2005; Lockyer et al., 2006). However, it should be noted that such defects were not observed in other studies (Cheli et al., 2008; Mangin et al., 2006). A major limit of all these studies is that vascular injury is induced in healthy mouse vessels which is quite different from what takes place in a diseased vessel. The relative contribution of GPVI in thrombosis was further supported in the *in vivo* models closer to pathological conditions in virtue of rupture of atheromatous plaques in ApoE knock-out mice. The deficiency of GPVI in these mice significantly decreased thrombosis in response to ultrasound and mechanical plaque rupture with a needle (Hechler and Gachet, 2011; Kuijpers et al., 2009).

1.2.3. CLEC-2

C-type lectin-like receptor (CLEC) 2 is a type II transmembrane glycoprotein receptor that promotes activation of platelets. It is well-distributed on human platelets ranging from 2,000 to 4,000 copies (Gitz et al., 2014). It is also found in abundance on endothelial cells and Kupffer cells of the liver, and at low levels on monocytes, dendritic cells, granulocytes, natural killer cells and myeloid cells (Colonna et al., 2000; Tang et al., 2010). CLEC-2 has an extracellular C-type lectin domain (CTLD), which consists of a stalk region and a carbohydrate-like recognition domain that lacks Ca^{2+} binding site. The short cytoplasmic tail contains a conserved YxxL sequence known as hemITAM domain which presents similarities with the well described ITAMs (Hughes et al., 2013; Watson et al., 2007). Human and mouse CLEC-2 present a 62% amino acid sequence identity (Colonna et al., 2000).

Podoplanin, type I transmembrane glycoprotein, is the first identified ligand of CLEC-2 (Christou et al., 2008). Under normal conditions, podoplanin is expressed on lymphatic endothelial cells, renal podocytes, alveolar cells type I and the choroid plexus, but not in the vasculature (Breiteneder-Geleff et al., 1997; Rishi et al., 1995; Tomooka et al., 2013). However, podoplanin can be found in vessels after inflammation, notably in endothelial cells of the skin capillaries in patients with inflammatory diseases such as eczema or psoriasis (Gröger et al., 2007). The extracellular domain of podoplanin contains platelet aggregation–stimulating (PLAG) domains, an extensively *O*-glycosylated stalk, and a short cytoplasmic tail (Rayes et al., 2019). Based on data from knockout mice, it has been reported that activation of platelets via the interaction of podoplanin and CLEC-2 plays a central role in development to avoid blood filling of the lymphatic system (Bertozzi et al., 2010). This concept was recently challenged as the group of Steve Watson provided evidence that CLEC-2-mediated platelet function is not relentlessly needed to protect the lymphatic vasculature from blood in unchallenged adult mice (Haining et al., 2020). CLEC-2 has also been reported to bind to the snake venom toxin rhodocytin which was instrumental in its identification, diesel exhaust particles, and the dextran sulfate and fucoidan (Alshehri et al., 2015b; Manne et al., 2013).

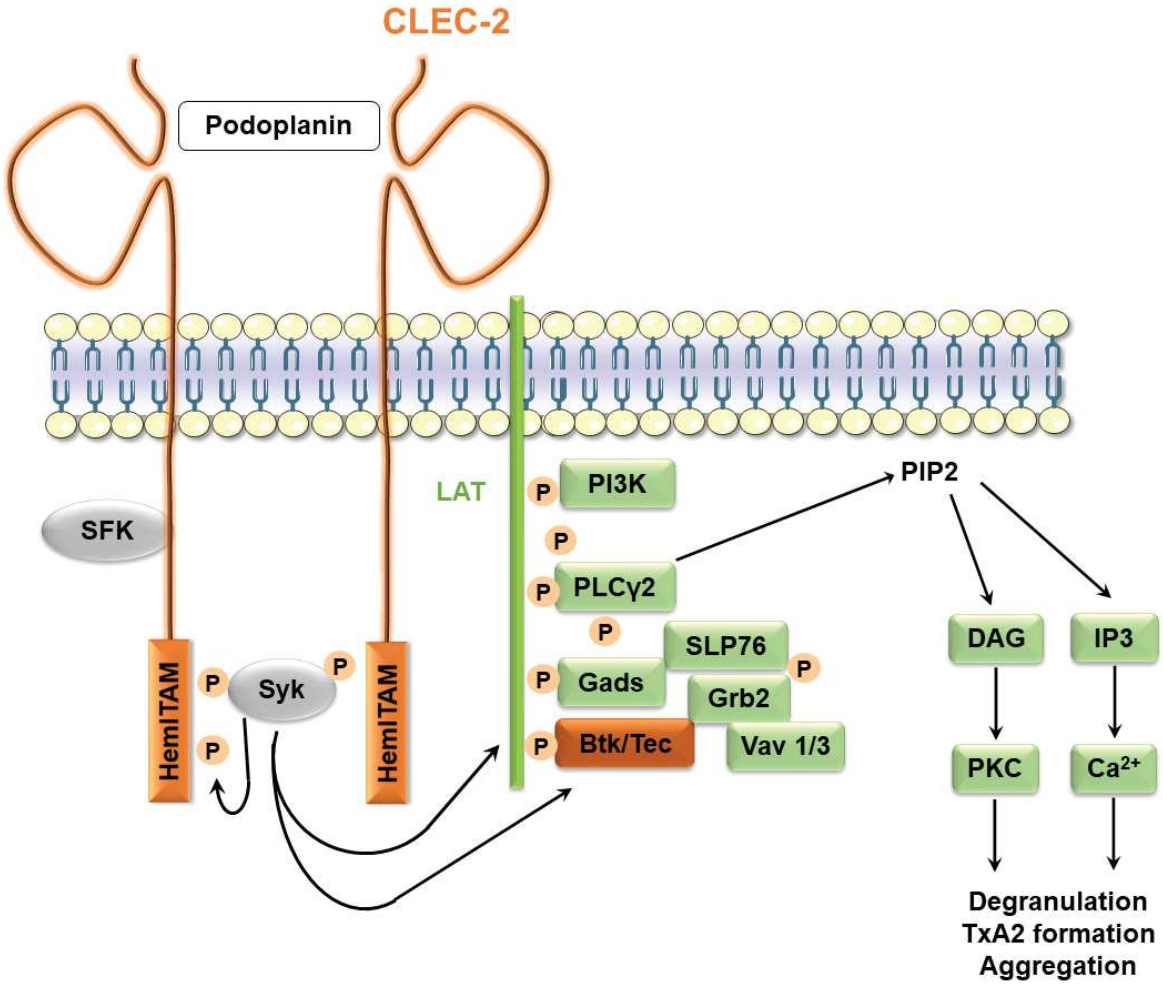

Figure 6. Structure of CLEC-2 and its signaling pathway

Btk, Bruton tyrosine kinase; Ca^{2+}, Calcium; CLEC-2, C-type lectin-like receptor-2; DAG, diacylglycerol; Gads, Grb2-related adapter downstream of Shc; Grb2, growth factor receptor bound protein 2; IP3, inositol 1,4,5-triphosphate; ITAM immunoreceptor tyrosine-based activation motif; LAT, linker for activation of T cells; PI3K, phosphoinositide 3-kinase; PIP2, phosphatidylinositol 4',5'-bisphosphate; PKC, protein kinase C; PLCγ2, phospholipase Cγ2; SFK, Src family kinases; SLP76, SH2-domain-containing leukocyte protein of 76 kDa; Syk, spleen tyrosine kinase; Vav, guanine nucleotide exchange factor. Adapted from (Ozaki et al., 2016; Rayes et al., 2019; Suzuki-Inoue et al., 2011)

Ligand binding promotes CLEC-2 dimerization which induces tyrosine residues contained within hemITAM motif to become phosphorylated on SH2 domains and recruit Syk, allowing the formation of the LAT signalosome. This leads to the activation of many effector proteins notably Grb-2, Gads, SLP-76, PI3-K, Btk and PLCγ2 (Fuller et al., 2007; Séverin et al., 2011; Spalton et al., 2009; Suzuki-Inoue et al., 2011). The activation of PLCγ2 results in the cleavage of PIP2 to generate the two secondary messengers, IP3 and DAG, leading to the release of Ca^{2+} from cytoplasmic storage pools and PKC activation, respectively. This signaling pathway then leads to platelet activation and aggregation, in particular via the secretion of ADP from platelet dense granules and the formation of TXA_2 (Pollitt et al., 2010). CLEC-2 does not seem to contribute in hemostasis since the bleeding time of mice with a platelet CLEC-2 deficiency is not prolonged (Haining et al., 2017; Hughes et al., 2010; Suzuki-Inoue et al., 2010). However, one study using CLEC-2 immunodepleted mice with INU1 (α-murine CLEC-2 antibody) presented an increased tail-bleeding time (May et al., 2009).

CLEC-2 has also been proposed to participate in arterial thrombosis. Since knocking out CLEC-2 is lethal, two approaches were used: i) implanting the fetal liver cells from CLEC-$2^{-/-}$ embryos into irradiated mice, thus obtaining mice which do not express CLEC-2 on platelets, which showed reduced thrombus formation after laser-injury of the mouse mesenteric artery (Inoue et al., 2015; Suzuki-Inoue et al., 2010); and ii) the administration of an anti-CLEC-2 antibody, INU1, which caused an immunodepletion of CLEC-2 on the platelet surface, which also prevented thrombosis in $FeCl_3$ injury model of mesenteric arterioles (Bender et al., 2013; Inoue et al., 2015; May et al., 2009). To date, the ligand to CLEC-2 which promotes its activation during thrombosis has not been identified. It is unlikely that podoplanin plays this role in experimental thrombosis since it is not present on platelets or on vascular endothelial cells in normal conditions. However, podoplanin is expressed in advanced atherosclerotic lesions (Inoue et al., 2015), suggesting a potential role of the podoplanin-CLEC-2 interaction in arterial thrombosis following plaque rupture. It has also been shown that CLEC-2 participates in arterial thrombosis independently of hemITAM signaling. Indeed, mice presenting the ectodomain of CLEC-2 receptor but without a functional signaling pathway were not protected against experimental thrombosis in a ferric chloride-induced injury model of mesenteric arteries, however injecting INU1 Fab to the same mice protected them against thrombosis after

mechanical lesion to the aorta or ferric chloride lesion to mesenteric artery, highlighting a role of ectodomain of CLEC-2 in arterial thrombosis but not hemITAM signaling pathway (Haining et al., 2017).

1.2.4. FcγRIIA

FcγRIIA (also called CD32a) is a transmembrane receptor that belongs to FcR subgroup that recognizes Fc-region of immunoglobulin (Ig) (Daëron, 1997). It is broadly expressed on immune cells notably monocytes, macrophages, neutrophils and dendritic cells (Arman and Krauel, 2015). It is also expressed on human platelets (Rosenfeld et al., 1985), where each platelet possesses 1,000-4,000 copies of this receptor (Tomiyama et al., 1992). It is the only FcγR expressed on human platelets. The expression of FcγRIIA protein is limited to higher primates and absent in the mouse genome. According to gene encoding, FCGR2 gene is located on chromosome 1 at the q23 locus (Qiao et al., 2015). FcγRIIA consist of two extracellular Ig-like domains D1 and D2 of which the second domain facilitates binding to IgG (Maxwell et al., 1999). There is also a transmembrane domain followed by a cytoplasmic tail containing an ITAM domain with two distinctive YXXL sequences. The ITAM sequence in FcγRIIA is unusual, with 12 amino acids between the dual YXXL amino acid sequences, rather than the usual six to eight amino acids (Arman and Krauel, 2015).

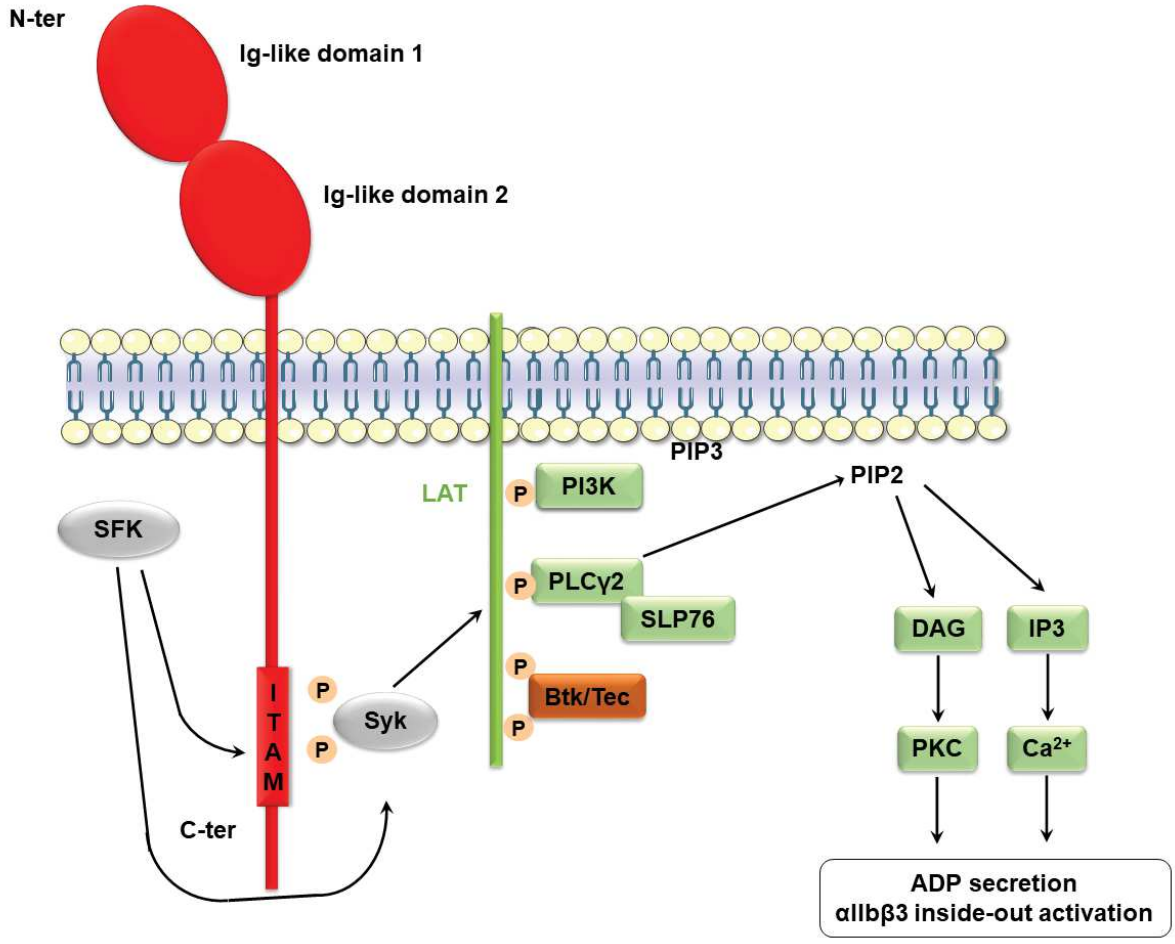

Figure 7.Structure of FcγRIIA and its signaling pathway

ADP, adenosine diphosphate; Btk, Bruton tyrosine kinase; DAG, diacylglycerol; PI3K, phosphoinositide 3-kinase; IP3, inositol 1,4,5-triphosphate; ITAM immunoreceptor tyrosine-based activation motif; LAT, linker for activation of T cells; PIP3, phosphatidylinositol 3,4',5'-trisphosphate; PIP2, phosphatidylinositol 4',5'-bisphosphate; Syk, spleen tyrosine kinase; PKC, protein kinase C; SLP76, SH2-domain-containing leukocyte protein of 76 kDa; PLCγ2, phospholipase Cγ2; Syk, spleen tyrosine kinase and SFK, Src family kinase. Adapted from (Arman and Krauel, 2015)

FcγRIIA is a low-affinity receptor for monomeric IgG that readily binds immune complexes (Karas et al., 1982) containing PF4, heparin and HIT antibodies. FcγRIIA has also been reported to bind to aggregated IgGs, IgG-opsonized pathogens and antiplatelet antibodies (Arman and Krauel, 2015). The ligand binding and clustering of the receptor results in phosphorylation of the ITAM domain of FcγRIIA by Src-family kinases (SFKs). The phosphorylated ITAM recruits Syk and forms a signalosome which activates PLCγ2 and hydrolyses PIP2 to generate IP3 and DAG. This prompts to the release of Ca^{2+} from cytoplasmic storage pools and to the PKC activation, which are essential for platelet secretion and integrin-dependent platelet aggregation (Arman and Krauel, 2015; Gratacap et al., 1998). Platelet FcγRIIA has been proposed to act as an amplifier of activation even in the absence of specific extracellular ligands. Indeed, it was stated that binding of fibrinogen with integrin αIIbβ3 could trigger ITAM phosphorylation of FcγRIIA which could result in Syk recruitment to transmit amplified signals into the platelet (Boylan et al., 2008). Similarly, an integrin/ITAM cooperation has been shown in FcγRIIA^{pos} transgenic mouse platelets which suggests that FcγRIIA enhances αIIbβ3-mediated outside-in integrin signaling (Zhi et al., 2013).

The role of FcγRIIA in hemostasis yet to be determined. Indeed, FcγRIIA seems to contribute in experimental thrombosis, as shown in studies evaluating FcγRIIA-transgenic mice in a laser-injury model of cremaster vessels and an electrolytic-induced vascular venous injury mice model as FcγRIIA^{pos} transgenic mice had significantly enhanced thrombus growth as compare to WT FcγRIIA^{neg} mice (Zhi et al., 2013). The potential role of FcγRIIA in thrombosis is in agreement with the fact that an increased expression of platelet FcγRIIA was identified in patients with an acute coronary or cerebrovascular event (Calverley et al., 2002; El-Shahawy et al., 2007; Schneider et al., 2018). Moreover, the polymorphism in FcγRIIA (the amino-acid substitution of arginine (R) with histidine (H) at position 131) is also associated with the heightened risk of cardio-vascular events (Chang et al., 2017; Clancy et al., 2019; Kroupis et al., 2010).

1.2.5. Integrins

Integrins compose a superfamily of cell-surface adhesion receptors that link the extracellular to the intracellular environment of cells. Integrins are heterodimeric transmembrane glycoproteins comprising two non-covalently-associated subunits, namely the α and β chains. Each of the subunit contains of a large N-terminal extracellular ectodomain that favors ligand interaction, a single-pass transmembrane (TM) spanning helix, and a tiny C-terminal cytoplasmic domain composed of nearly 20 ~ 70 amino acids which binds to the cytoskeleton and is involved in inducing intracellular signaling (Durrant et al., 2017; Huang et al., 2019).

Integrins are expressed on many different cell types and regulate a variety of cell functions including adhesion, spreading, migration, survival, differentiation, proliferation, and apoptosis. Therefore, they participate in many physiological processes including embryogenesis, wound healing, immunity and hemostasis. On the other hand, their aberrant response can lead to pathological consequences notably inflammatory diseases, cancer, bleeding, and thrombosis (Durrant et al., 2017; Huang et al., 2019).

Platelet Integrins

Integrins regulate different function of platelets including adhesion, activation and aggregation. Platelets express at least 5 different types of integrins at their surface which either belong to β1 or β3 family. The three β1 integrins are α2β1, α5β1, and α6β1, which mediate platelet adhesion to the extracellular matrix proteins collagen (Staatz et al., 1989), fibronectin (Piotrowicz et al., 1988), and laminins (Ill et al., 1984; Sonnenberg et al., 1988), respectively. Two β3 integrin subtypes on platelets are αvβ3 and αIIbβ3, which bind notably vitronectin and fibrinogen.

1.2.5.1. Integrin α2β1

Integrin α2β1 (also named GPIa-IIa, VLA-2 or CD49b/CD29) is a receptor for matrix collagen. It is found on platelets, fibroblasts, epithelial, and endothelial cells. On platelets, α2β1 is expressed at 2,000 to 8,000 copies and ensures stable platelet adhesion. α2β1 is composed of a 150-kDa α2-chain and a 130-kDa β1-chain. As all integrin α-subunits, the N-terminal domain of α2 contains a seven-bladed β-propeller structure. In addition, it also contains an inserted I domain, with a conserved metal coordination site termed metal ion-dependent adhesion site (MIDAS) with a clear preference for Mg^{2+}/Mn^{2+}, which is critical for collagen binding (Kamata et al., 1999). The β-subunit also contains cation-binding sites with a clear preference for Ca^{2+} (Emsley et al., 2000).

Integrin α2β1 has high affinity for collagen, notably for the GFOGER sequence present on the different collagen types I, II, III, IV and IX (Emsley et al., 2000) and on non-collagenous proteins notably collagen-like triple helical structures and proteoglycans (Madamanchi et al., 2014). Like other integrins, α2β1 has at least two conformations with different affinities for its ligands, including the low- “inactive” or “resting” form and the high- “active” or “stimulated” conformation. During platelet activation, an inside-out signaling is induced that results in a conformational change of α2β1 (Banno and Ginsberg, 2008; Margadant et al., 2011; Shattil et al., 2010). The ligand binding induces platelet activation obliquely by allowing GPVI-collagen interactions and directly by outside-in signaling through α2β1. The outside-in signaling pathway has multiple actors involving Src, Syk, SLP-76, PLCγ2, FAK, plasma membrane calcium ATPase, and results in Ca^{2+} mobilization, which reinforces integrin activation and therefore their role in adhesion and also promotes spreading on collagen (Inoue et al., 2003).

Two cases of genetic defects in α2β1 presenting a reduced platelet response to collagen with only a moderate bleeding tendency have been reported, suggesting that this integrin is not playing a key role in hemostasis (Kehrel et al., 1988; Nieuwenhuis et al., 1985). This is in agreement with the fact that α2β1-deficient mice do not display increased tail-bleeding time (Holtkötter et al., 2002). In contrast, this receptor might participate in experimental thrombosis *in vivo*, as it has been shown that α2 knock-out mice presented a delayed arterial occlusion in a carotid injury model

(He et al., 2003). In another study also based on α2-null mice, this integrin has been proposed to be involved in thrombus stability (Kuijpers et al., 2007). Of note, experiments performed in our laboratory with α2 knock-out mice did not observe any defect in two distinct thrombosis model, notably after laser-induced lesion to mesenteric vessels and mechanical lesion to the aorta, implying that this integrin is probably not an ideal target for anti-platelet agents.

1.2.5.2. Integrin α5β1

Integrin α5β1 is distributed on different types of cells notably endothelial cells, fibroblasts, lymphocytes, and monocytes. On platelets, α5β1 is expressed at 2,000 to 4,000 copies and is best known as a receptor for fibronectin. In a resting state, integrin α5β1 is in a bent and closed conformation, and after fibronectin binding it undergoes a conformational change which increases its affinity (Kasirer-Friede et al., 2007). The binding of α5β1 to fibronectin occurs through the recognition of an Arg-Gly-Asp (RGD) motif in the FIII10 domain of fibronectin, and a Pro-His-Ser-Arg-Asn (PHSRN) sequence located in the adjacent FIII9 domain (Altroff et al., 2001).

It was stated that α5β1 promotes platelet adhesion and activation on immobilized fibronectin under both static and shear flow conditions (Beumer et al., 1994; McCarty et al., 2004; Zaidi et al., 1996). My laboratory has reported that perfusion of platelets over cellular fibronectin in its fibrillar form facilitates platelet adhesion, activation, aggregation and procoagulant activity through its interplay with a series of receptors containing integrins α5β1 and αIIbβ3, the GPIb-IX-V complex, GPVI and Toll-like receptor 4 (Maurer et al., 2015). To date, the relative contribution of integrin α5β1 in hemostasis and thrombosis remains to be established.

1.2.5.3. Integrin α6β1

Integrin α6β1 is the key receptor of platelet activation on laminins, which are major components of the basement membrane (Hallmann et al., 2005). On platelets, α6β1 is expressed at around 11,500 copies (Burkhart et al., 2012) and ensures platelet adhesion to various laminins. Integrin α6β1 is widely expressed and in addition to platelets, it is found on neutrophils, eosinophils, endothelial cells, and pericytes (Bohnsack, 1992; Georas et al., 1993; Larrieu-Lahargue et al., 2011; Lathia et al., 2010; Reynolds et al., 2017; Wewer et al., 1997). It has been reported that α6β1 can promote adhesion of platelets on immobilized laminins under both static and flow conditions (Geberhiwot et al., 1999; Inoue et al., 2006; Nigatu et al., 2006). This adhesion to laminin is strongly supported by cations Mg^{2+} and Ca^{2+} (Hindriks et al., 1992).

Integrin α6β1 signaling pathway has not been extensively studied, but it has been shown that platelet adhesion to laminins promotes outside-in signaling including the tyrosine kinase Syk, PLC-γ2, Phosphoinositide 3-kinase (PI3K), and Cdc42, inducing the filopodia formation in human platelets (Chang et al., 2005; Ill et al., 1984; Inoue et al., 2006). α6β1 is not the only receptor supporting platelet activation on laminin as spreading has been reported to be dependent on GPVI (Inoue et al., 2006).

The studies carried out on α6β1-knock out mice confirmed that this integrin efficiently supports platelet adhesion and activation on laminin-411, laminin-511, and laminin-521 under a wide range of flow conditions. These mice do not display increased tail-bleeding time, ratifying that α6β1 is not essential for normal hemostasis. However, decreased thrombotic responses after the carotid, aorta, and mesenteric arterioles injuries were observed suggesting a potential contribution to arterial thrombosis (Schaff et al., 2013). These results indicate that α6β1 is an important functional integrin of platelets and could represent a potential target for anti-platelet agents.

1.2.5.4. Integrin αIIbβ3

Integrin αIIbβ3, also named the glycoprotein GPIIb-IIIa (CD41/CD61) complex, is the most important integrin found on platelets. Platelets express 80,000 copies of integrin αIIbβ3 on their surface, and can mobilize an additional pool of 30,000 receptors upon stimulation from platelet α-granule membranes. Integrin αIIbβ3 is crucial in hemostasis due to its central involvement in supporting stable platelet adhesion and platelet aggregation (Lefkovits et al., 1995).

1.2.5.4.1. Structure

Integrin αIIbβ3 is composed of a αIIb (1,008 amino acid) and β3 (762 amino acid) subunit. The extracellular domains of αIIbβ3 are comprised of a globular 'head' with two protruding 'stalks'. The globular 'head' of αIIb has a β-propeller domain and the 'stalk' of αIIb contains the thigh and two calf domains. The 'head' of the β3 subunit consists of a hybrid-domain, βA, and the 'stalk' is made up of a PSI domain and four EGF regions (Xiong et al., 2001). In the αIIb subunit, there is a 'genu'-bent knee-located in between the thigh and the calf-1 domains. It is also located between the hybrid, two EGF domains, and PSI domains on the β3 subunit (Campbell and Humphries, 2011; Ma et al., 2007; Xiong et al., 2001). The presence of a 'bent knee' facilitates the transition from a bent to an extended conformation, which is important for receptor activation (Figure of the integrin αIIbβ3 structure).

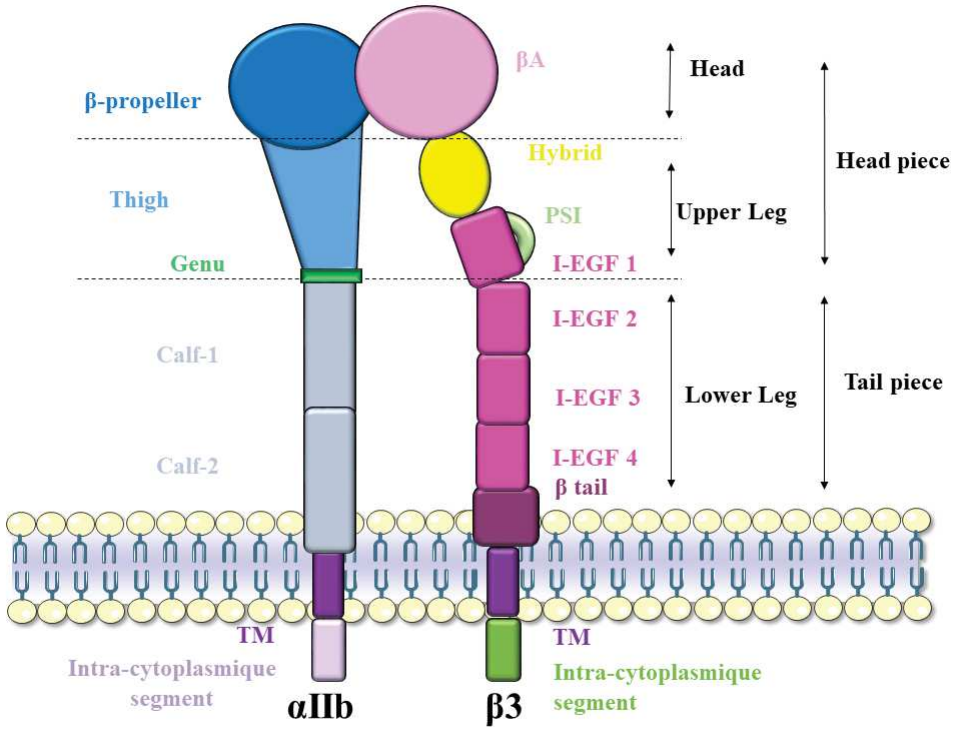

Figure 8. Structure of integrin αIIbβ3

I-EFG, integrin-epidermal growth factor; PSI, plexin-semaphorin-integrin; TM, transmembrane segment. Adapted from (Coller, 2015; Luo et al., 2007a; Xiao et al., 2004)

1.2.5.4.2. Ligands of integrin αIIbβ3

The main ligand of αIIbβ3 is fibrinogen, which supports platelet adhesion and aggregation. αIIbβ3 also binds several additional plasma proteins or extracellular matrix proteins including fibrin, vWF, fibronectin, vitronectin, and thrombospondin-1. The ligand binding to αIIbβ3 is ensured through the recognition of an Arg-Gly-Asp (RGD) motif (Springer et al., 2008). Concerning fibrinogen, αIIbβ3 recognizes an additional sequence KQAGDV located in the fibrinogen γ chain C terminus (Farrell et al., 1992; Litvinov et al., 2016).

1.2.5.4.3. Integrin αIIbβ3 activation

On quiescent platelets, αIIbβ3 is in an inactive conformation with a low affinity for fibrinogen. Following platelet activation, an inside-out signaling is induced that brings in a conformational change of αIIbβ3. This leads to a shift from low affinity (resting) to high affinity (active) state for ligand binding (Huang et al., 2019). Upon ligand interaction, αIIbβ3 induces an outside-in signaling which further contributes to platelet activation.

a. Integrin αIIbβ3 inside-out signalling

The inside-out signalling can be instigated by: i) soluble agonists, for instance ADP, TxA_2 or thrombin, which interact with G protein-coupled receptors (GPCRs) or by ii) adhesive proteins, for instance vWF or collagen, which primarily interact with receptors such as the GPIb-IX-V complex or GPVI, respectively (Huang et al., 2019). Activation through these two main pathways result in activation of phospholipase C (PLC) - generating IP3 and DAG, and subsequent release of Ca^{2+} from cytoplasmic storage pools and activation of PKC and CalDAG-GEFI (Cifuni et al., 2008; Crittenden et al., 2004). PKC and CalDAG-GEFI lead to the activation of the GTPase, Rap1b. The effector molecule of Rap1b is the small GTPase RIAM (Rap1-interacting adaptor molecule), which binds Talin-1 that causes integrin activation (Bromberger et al., 2018; Gingras et al., 2019). Talin is present in an auto-inhibited dimeric conformation, and following PKC-induced RIAM activation, it becomes activated (Han et al., 2006) and binds to the cytoplasmic domains of αIIbβ3 to promote its conformational change to a high affinity state for fibrinogen (Tadokoro et al., 2003). Talin coordinates its effect together with Kindlin-3 which also interacts with the cytoplasmic domains of the β3 subunits (Moser et al., 2008). Beside with talin and Kindlin-3, filamin A (FLNa) also regulates integrin activation by directly interacting with the β3 domain on resting platelets. During activation of platelets, FLNa undergoes a conformational change and detaches from β3 domain to let talin and kindlin-3 to bind β3 (Rosa et al., 2019).

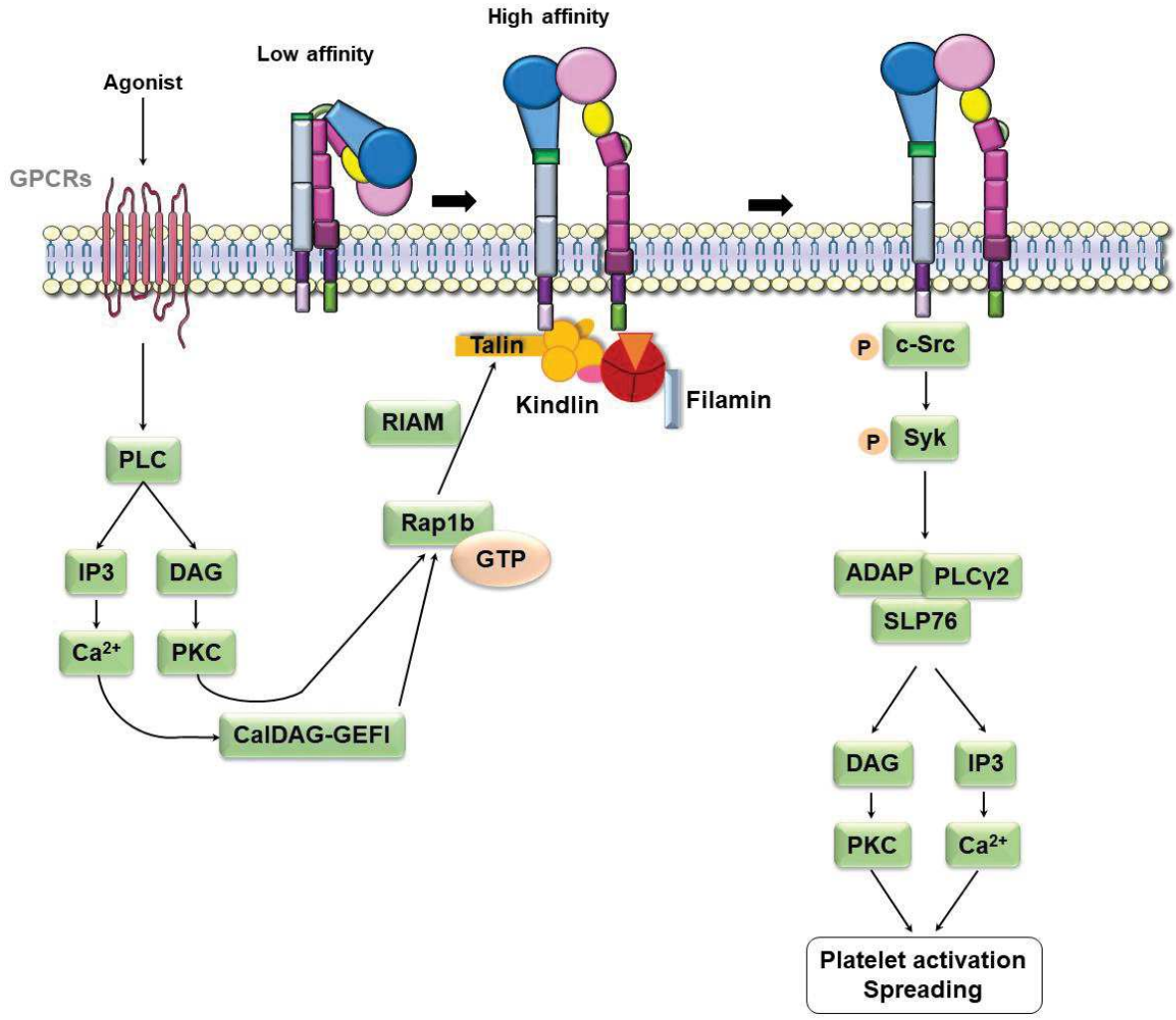

Figure 9. αIIbβ3 inside-out and outside-in signaling

ADAP, adhesion- and degranulation-promoting adapter protein; c-Src, pronto-oncogene Src protein; Ca^{2+} , Calcium; CalDAG-GEFI, calcium and DAG-regulated guanine-nucleotide-exchange factor; DAG, diacylglycerol; GP, glycoprotein; GTP, guanosine diphosphate; IP3, inositol 1,4,5-triphosphate; PKC, protein kinase C; PLC, phospholipase; PLCγ2, phospholipase Cγ2; Rap1b, Ras-related protein 1b; RIAM, Rap1-GTP-interacting adaptor molecule; SLP76, SH2-domain-containing leukocyte protein of 76 kDa; Syk, spleen tyrosine kinase. Adapted from (Huang et al., 2019; Moser et al., 2009)

b. Integrin αIIbβ3 outside-in signaling

The interaction of fibrinogen with integrin αIIbβ3 induces integrin clustering and the formation of a complex which initiates a signaling downstream of the integrin named, outside-in signaling (Arias-Salgado et al., 2003; Loftus and Albrecht, 1984). This signaling of αIIbβ3 requires interaction of cooperating proteins to the αIIbβ3 cytoplasmic tails, as this region does not contain any intrinsic signaling activity (Huang et al., 2019). Integrin outside-in signaling of αIIbβ3 promotes Src activation by autophosphorylation, which recruits Syk. Src and Syk may then phosphorylate the adapter proteins SLP-76 and ADAP, and PLCγ2 that promotes the mobilization of internal stores of calcium thus facilitating αIIbβ3-mediated platelet activation and spreading (Judd et al., 2000; Obergfell et al., 2001; Wonerow et al., 2003). This signaling cascade also results in cytoskeletal reorganization and platelet aggregation, resulting in thrombus growth.

1.2.5.4.4. Role of integrin αIIbβ3 in hemostasis and arterial thrombosis

αIIbβ3 plays a vital role in hemostasis mainly through its ability to support platelet aggregation and formation of a stable hemostatic plug (Bennett, 2005). The importance of integrin αIIbβ3 in hemostasis is evidenced by a bleeding disorder named Glanzmann Thrombasthenia, which is the consequence of congenital deficit or dysfunction of integrin αIIbβ3 in humans with a close to normal platelet count. This pathology is depicted by a severe bleeding event and inability of platelets to aggregate (Nurden et al., 2013). Likewise, mice deficient with either of the subunits αIIb or β3 have an impaired platelet aggregation, clot retraction and severe hemostatic defects as evidenced by a marked rise in tail-bleeding time (Hodivala-Dilke et al., 1999; Smyth et al., 2001; Tronik-Le Roux et al., 2000).

αIIbβ3 plays a very imperative role in arterial thrombosis. As a consequence, this integrin is a target for a class of very potent anti-platelet agents, the αIIbβ3 blockers which include abciximab, eptifibatide and tirofiban. The use of these agents

is limited to acute settings for instance during percutaneous coronary intervention (PCI) or acute coronary syndrome (ACS) due to the elevated bleeding complications experienced by patients injected with these drugs (details is section **3.2.4.**, page 78).

1.2.5.5. Integrin αvβ3

Integrin αvβ3 is expressed at around 200 copies and is best known as a functional receptor for vitronectin. Structurally, the sequence of αv is 36% identical to αIIb and can bind to several RGD containing adhesive proteins notably fibrinogen, fibronectin and vWF (Xiong et al., 2002). It has been stated that αvβ3 participates in platelet adhesion (Paul et al., 2003) and might take part in clot retraction (Mor-Cohen, 2016). The contribution of αvβ3 in hemostasis and thrombosis remains to be elucidated. Studies performed in our lab with tissue-specific αv-deficient animals did not identify a major role for this integrin in hemostasis, as reflected by a normal tail-bleeding time. Moreover, the absence of defect in experimental models of thrombosis does not favor a major role for this integrin in arterial thrombosis.

1.2.6. Receptors for soluble agonists

The receptors for soluble agonists either belong to seven-transmembrane domain GPCRs family or to the ligand-gated ion channel family. These receptors are activated by soluble mediators notably ADP, ATP, thrombin and TxA_2, which are released or generated by activated platelets. These receptors mainly promote or amplify platelet activation thereby promoting integrin activation or sustaining their activation level and supporting platelet aggregation and thrombus stability. For these reasons, the receptors for soluble agonists contribute both in the hemostatic and thrombotic responses.

1.2.6.1. The ADP receptor $P2Y_1$

$P2Y_1$ is a 42 kDa ADP receptor that contains 373 amino acid residues, and is broadly expressed in many tissues of the human body notably on endothelial cells, smooth muscle cells, immune cells including macrophages, eosinophils and lymphocytes (Abbracchio et al., 2006; Castaing-Berthou et al., 2017; Mishra et al., 2006; Zerr et al., 2011). It is also expressed on platelets and megakaryoblastic cells (Léon et al., 1997) with approximately 150 copies per platelet (Baurand et al., 2001; Ohlmann et al., 2010).

Activation of the $P2Y_1$ receptor by ADP leads to the G_q-mediated activation of β-isoforms of phospholipase C (PLCβ) and cleavage of PIP2 into IP3 and DAG, leading to the release of Ca^{2+} from cytoplasmic storage pools and protein kinase C (PKC) activation, respectively (Offermanns, 2006). This signaling pathway mediates reversible platelet shape change and platelet aggregation (Fabre et al., 1999; Hechler et al., 1998a; Hechler et al., 1998b; Jin et al., 1998; Jin et al., 2002; Léon et al., 1999; Savi et al., 1998; Turner et al., 2001). $P2Y_1$ also participates in the procoagulant function of platelets (Leon et al., 2004; Leon et al., 2003). The role of $P2Y_1$ in hemostasis has been assessed in different animal models showing a slightly increased tail-bleeding time when $P2Y_1$ receptor was deficient or blocked with selective antagonists (Fabre et al., 1999; Hechler et al., 2006; Lenain et al., 2003; Léon et al., 2001; Léon et al., 1999). Unlike the quite minor role of $P2Y_1$ in normal hemostasis, this receptor appears to contribute in experimental thrombosis, which has been demonstrated in a murine model of pulmonary thromboembolism induced by mixture of collagen and adrenaline (Fabre et al., 1999; Léon et al., 1999) or by tissue factor (Léon et al., 2001). It also contributes in localized thrombosis models, particularly in mouse mesenteric arteries after vascular lesion induced by ferric chloride or a laser beam (Hechler et al., 2006; Lenain et al., 2003). Selective inhibition of $P2Y_1$ with MRS2500 also reduced thrombus formation in vena cava or carotid artery thrombosis models (Bird et al., 2012; Wong et al., 2016). This role in thrombosis was also confirmed by the fact that overexpression of platelet $P2Y_1$ in transgenic mice induced an increase in arterial thrombosis (Hechler et al., 2003b). Altogether, these observations demonstrate that $P2Y_1$ represents an interesting potential target for antiplatelet agents.

1.2.6.2. The ADP receptor $P2Y_{12}$

$P2Y_{12}$ is an ADP receptor which contains 342 amino acid residues, and is expressed on platelets with approximately 425 copies per platelet (Ohlmann et al., 2013). $P2Y_{12}$ is also found on smooth muscle cells, dendritic cells, brain tissue, macrophages, microglial cells and some leukocytes (Ben Addi et al., 2010; Diehl et al., 2010; Hollopeter et al., 2001; Wang et al., 2004; Wihlborg et al., 2004). ADP binding to $P2Y_{12}$ leads to the G_i-mediated inhibition of adenylyl cyclase (AC) which prevents the formation of cyclic adenosine monophosphate (cAMP) and activation of cAMP-dependent protein kinase A (PKA) thereby enabling amplification of platelet activation (Jantzen et al., 2001). $P2Y_{12}$ also promotes activation of PI3Kγ and β, serine-threonine protein kinase B/Akt (PKB/Akt) and small GTPase Rap1b (Cosemans et al., 2006; Guidetti et al., 2008; Kim et al., 2004; Lova et al., 2002; Woulfe et al., 2002). These signaling events contribute in sustaining activation of integrins and platelet aggregation. $P2Y_{12}$ is also promoting the exposure of procoagulant phosphatidylserine at the surface of platelets (Leon et al., 2003). In addition, $P2Y_{12}$ potentiates platelet aggregation induced by other platelet agonists including collagen, thrombin, TxA_2 or ADP via $P2Y_1$ receptor (Dangelmaier et al., 2001; Nieswandt et al., 2001a; Nieswandt et al., 2002). As a consequence, $P2Y_{12}$ contributes in the stability of thrombi.

The relative importance of $P2Y_{12}$ in hemostasis is evidenced by a congenital $P2Y_{12}$ deficiency which is distinguished by an increased bleeding diathesis linked to reversible or sometimes impaired platelet aggregation to several agonists, further highlighting the ability of ADP to sustain integrin activation and participate in the stability of aggregates (Cattaneo et al., 2000; Cattaneo et al., 1992; Cattaneo et al., 2003; Lecchi et al., 2015; Nurden et al., 1995; Remijn et al., 2007; Shiraga et al., 2005). Likewise, $P2Y_{12}$ also contributes in arterial thrombosis, which is evidenced by the existence of a class of antithrombotic drugs targeting this receptor, which is discussed page **75**. Moreover, the importance of $P2Y_{12}$ in arterial thrombosis was also demonstrated experimentally by the reduction of experimental thrombosis when $P2Y_{12}$ receptor was knocked out or blocked with selective antagonists (Andre et al., 2003; van Gestel et al., 2003).

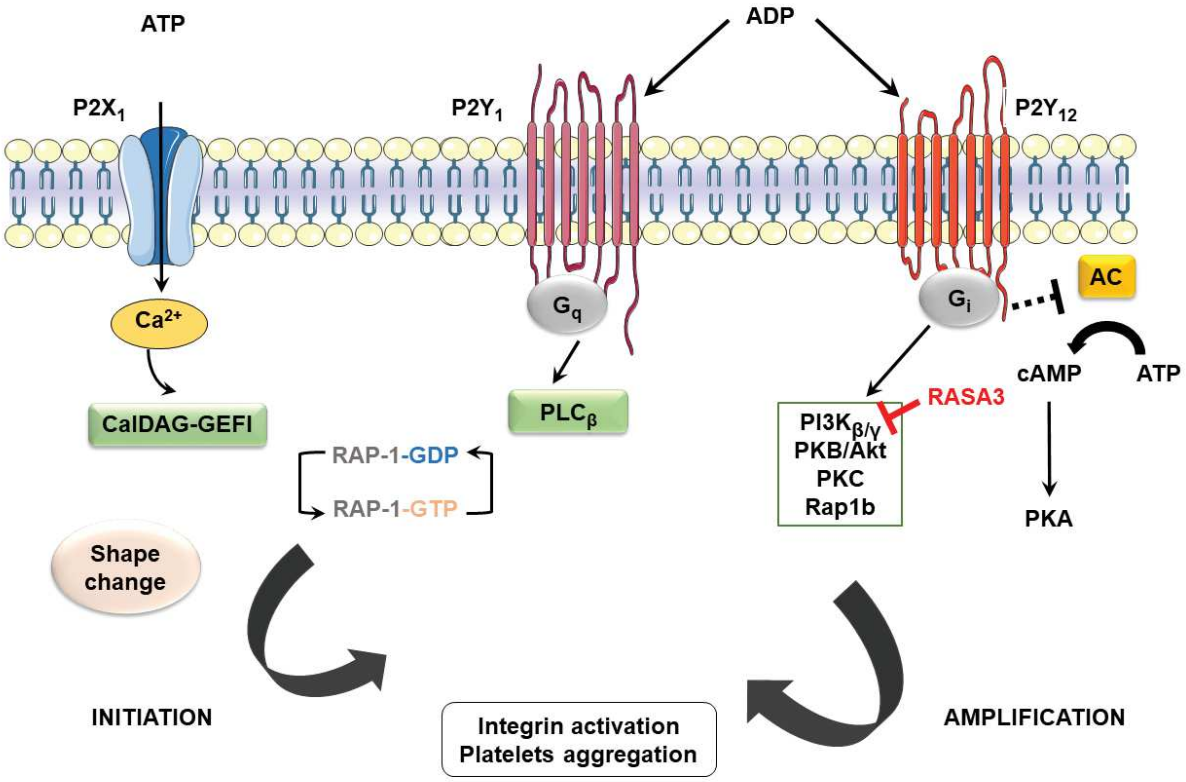

Figure 10. Schematic representation of the platelet activation induced by ATP and ADP

These agonists by acting on their respective receptors amplify platelet activation thereby promoting integrin activation or sustaining their activation level and supporting platelet aggregation. AC, adenylyl cyclase ; cAMP, 3',5'-cyclic adenosine monophosphate; PKA, protein kinase A

1.2.6.3. The ATP receptor $P2X_1$

The $P2X_1$ receptor is ligand-gated cation channel which is responsible for a fast calcium entry induced by ATP. The expression of $P2X_1$ on platelets ranges from 100 to 150 copies per platelet (MacKenzie et al., 1996). It is also expressed on smooth muscle cells, neutrophils, and macrophages (Hinze et al., 2013; Lecut et al., 2009; Sim et al., 2007). ATP binding leads to a rapid influx of Ca^{2+} from the external medium which causes membrane depolarization (MacKenzie et al., 1996; Mahaut-Smith et al., 1990) and induces myosin light chain phosphorylation (MLC-P), thereby resulting in platelet shape change (Mahaut-Smith et al., 2004; Rolf and Mahaut-Smith, 2002). $P2X_1$ also amplifies platelet aggregation induced by the agonists including collagen, ADP, thrombin, and TxA_2 (Erhardt et al., 2006; Fung et al., 2007; Jones et al., 2014; Oury et al., 2004; Oury et al., 2001; Toth-Zsamboki et al., 2003; Vial et al., 2002). Recently, $P2X_1$ has demonstrated to promote platelet aggregation in response to activation of the FcγRIIa receptor (Ilkan et al., 2018).

$P2X_1$ does not seem to contribute in hemostasis evidenced in knock out mouse models which exhibited no impact on the tail bleeding time (Hechler et al., 2003a; Hechler et al., 2005). In contrast, $P2X_1$ plays an important role in experimental thrombosis, in particular in models of collagen-adrenaline thromboembolism and after laser-induced injury of mesenteric vessels (Erhardt et al., 2006; Hechler et al., 2003a; Hechler et al., 2005). This role in thrombosis was also confirmed with an overexpression of the platelet $P2X_1$ receptor in transgenic mice which induces an increase in systemic thrombosis (Oury et al., 2003). Altogether, these observations support a role for the $P2X_1$ receptor as a potential target for safe antiplatelet drugs.

1.2.6.4. The TP Receptors (TxA_2)

The TP receptor is a 37 kDa protein which belongs to the G_q and $G_{12/13}$ subunits of G protein-coupled (GPCR) receptors. They are broadly expressed on a wide range of tissues and cells including smooth muscle cells, endothelial cells, monocytes, macrophages, lungs, kidneys, heart, thymus, and spleen (Davì et al., 2012; Norel, 2007). They are also present on platelets with approximately 1,500 copies per platelet (Habib et al., 1999; Halushka et al., 1986; Narumiya et al., 1986). TP receptors are mainly activated by thromboxane A_2 (TxA_2), which is synthesized during platelet activation.

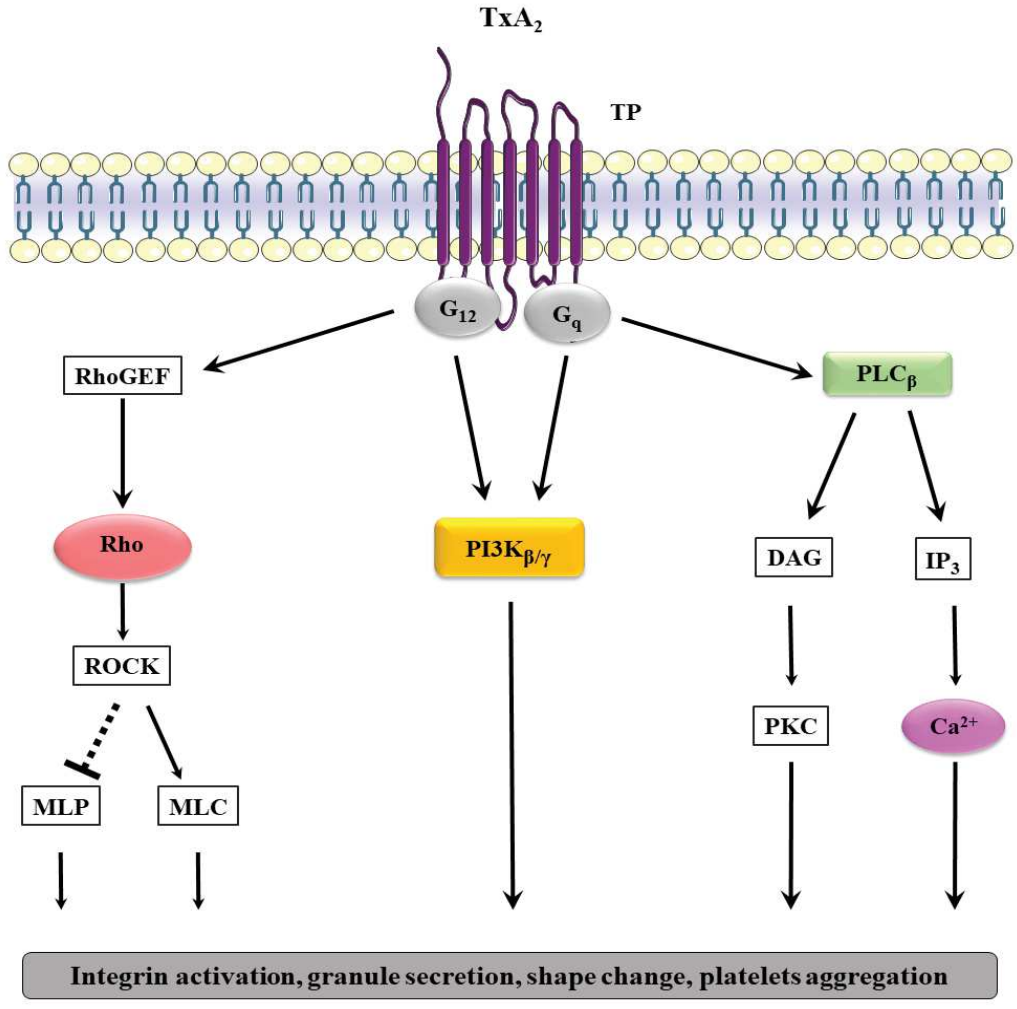

Figure 11. Schematic representation of the signaling pathway of TP receptor

DAG, diacyl- glycerol; GEF, guanine nucleo- tide exchange factor; IP3, inositol 1,4,5-trisphosphate; MLC, myosin light chain; MLCP, myosin light chain phosphatase; ROCK, Rho- associated protein kinase.

Phospholipase (PL) A_2 catalyzes the release of arachidonic acid (AA) by hydrolysis of membrane phospholipids which is then metabolized in unstable PGH2 by cyclooxygenase (COX) 1 and finally in TxA_2 by TxA_2 synthase (TXS) (O'Donnell et al., 2014). TxA_2 acts in an autocrine or paracrine manner following its diffusion through the platelet membrane due to its lipid soluble properties but has a very short half-life of around 30 seconds (Hamberg et al., 1975). The action of TxA_2 on the TP receptor mediates signaling via G_q causing activation of $PLC\beta$ which results in mobilizing intracellular Ca^{2+} and activating PKC (Shenker et al., 1991). This receptor also induces signaling via $G_{12/13}$ following the activation of Rho-GEF (Kozasa et al., 1998) and stimulate RhoA-associated kinase (ROCK) causing myosin light chain phosphorylation (Bauer et al., 1999). These signaling cascades lead to platelet shape change, granule secretion and platelet aggregation (Getz et al., 2010).

The patients having mutation in TP receptor or defect in TxA_2 signaling pathway experienced a modest increase in bleeding (Defreyn et al., 1981; Samama et al., 1981; Wu et al., 1981) and mice presented an increased tail-bleeding time (Cathcart et al., 2008; Thomas et al., 1998; Yu et al., 2004) demonstrating the role of this receptor in hemostasis. Likewise, TP receptor also contributes in arterial thrombosis, which is evidenced by the use of aspirin (details is section **3.2.1**, page 74) that targets COX-1 which is in the pathway of TxA_2 synthesis (DeWitt et al., 1990).

1.2.6.5. The Proteinase Activated Receptors (PARs)

Proteinase-activated receptors belong to the large family of G protein-coupled receptors that have a unique activation mechanism in which the N-terminus is proteolytically cleaved by thrombin that exposes a tethered ligand which binds to the receptor to activate it. It has been found that subtypes PAR1 and PAR4 are expressed on human platelets with approximately 1,500 to 2,000 copies per platelet (Kahn et al., 1999; Kahn et al., 1998) whereas PAR3 and PAR4 are expressed on mouse platelets. They are also expressed on several cell types including endothelial cells, smooth muscle cells, monocytes and astrocytes (Ossovskaya and Bunnett, 2004).

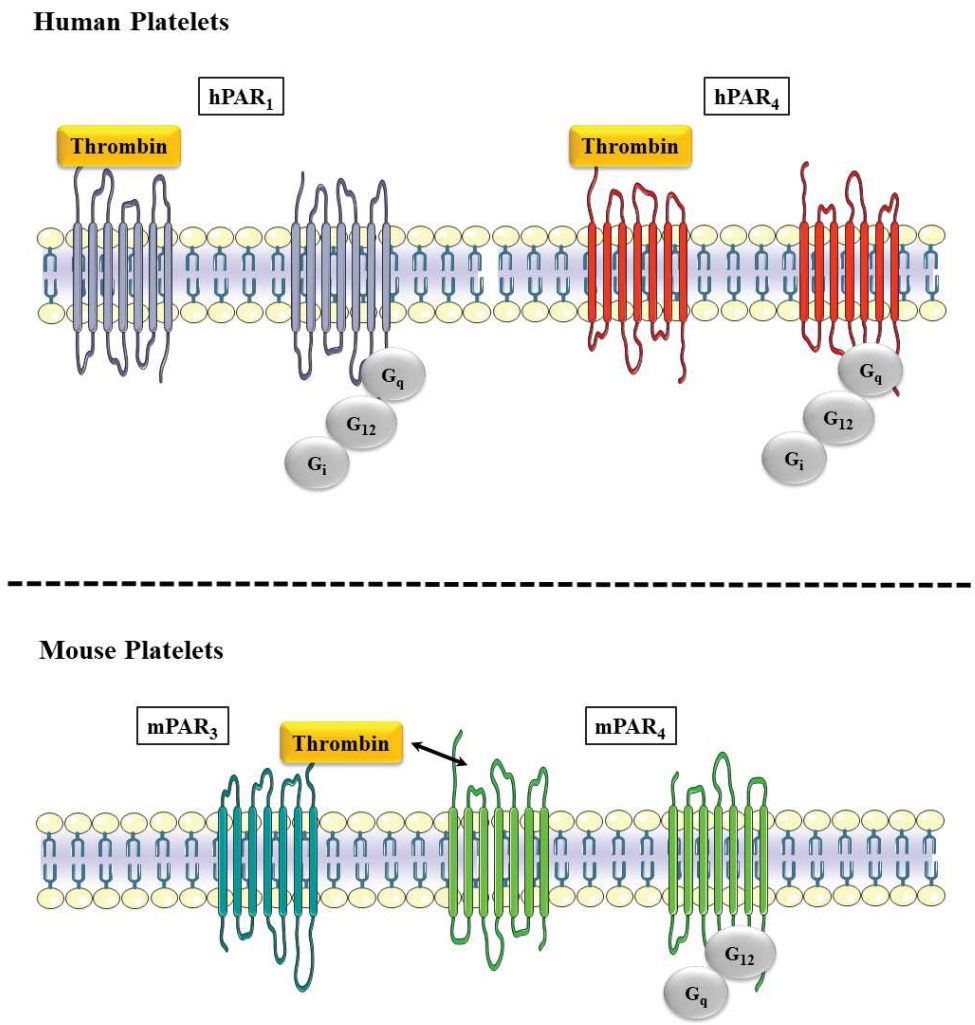

Figure 12. Schematic representation of activation of PARs by thrombin

The depicts thrombin signaling on human (upper panel) and mouse (lower panel) platelet

Thrombin, a serine protease considered to be the most potent platelet agonist, is the primary ligand for PAR receptors (Coughlin, 2005). PAR1 is activated by a small concentration of thrombin, due to the presence of a hirudin-like sequence facilitating the binding of thrombin (Hung et al., 1992; Ishihara et al., 1997), whereas the activation of PAR4 requires a higher concentration. In mouse platelets, in which no PAR1 is expressed, PAR3 functions as a co-receptor, which facilitates activation of PAR4 by increasing the local thrombin concentration. The activation of PAR1 and PAR4, is induced by thrombin which cleaves its N-terminal domain to unmask a new receptor amino terminus which then serves as a tethered peptide ligand that bounds back to the receptor and leads to G_q- and $G_{12/13}$-mediated signaling (Klages et al., 1999; Offermanns et al., 1994). Stimulation of platelets though either the PAR1 or PAR4 leads to shape change, granule secretion, release of TxA_2, activation of integrins and ultimately to platelet aggregation (Faruqi et al., 2000; Kahn et al., 1999; Vu et al., 1991). Moreover, these receptors also participate in the procoagulant activity of platelets (Andersen et al., 1999; French et al., 2016).

No impact on bleeding time was observed when PAR3 and PAR4 receptors were deficient or following inhibition of PAR1 by SCH 530348 (vorapaxar) in cynomolgus monkeys implying that PARs do not play a major role in hemostasis (Chintala et al., 2008; Coughlin, 2005). In contrast, these receptors contribute to thrombus formation, which has been demonstrated in PAR3 and PAR4 deficient mice which were protected against collagen-adrenaline thromboembolism, or after ferric chloride or laser injuries (Cornelissen et al., 2010; Hamilton et al., 2004; Sambrano et al., 2001; Vandendries et al., 2007; Weiss et al., 2002). The selective PAR1 inhibition, by RWJ-58259 in electrolytic injury to the carotid artery and by SCH 602539 in a Folts model in cynomolgus monkeys, has shown reduction in thrombus formation (Chintala et al., 2010; Derian et al., 2003). Moreover, vorapaxar, a competitive reversible PAR1 antagonist, has been approved in the United States and Europe for the secondary prevention of ischemic events following myocardial infarction or peripheral arterial disease, along with aspirin and clopidogrel (Moon et al., 2018; Scirica et al., 2012). However, it is contraindicated in patients having a history of stroke or cerebral hemorrhage due to heightened risk of intracerebral bleeding (Tricoci et al., 2012). The PAR4 inhibitors might have less side effects and are currently under development

(Mumaw et al., 2014; Wilson et al., 2018; Wong et al., 2017). Altogether, these observations demonstrate that the PARs represent interesting targets for antiplatelet agents.

2. The Vascular system

The cardio-vascular system is a complex network consisting of the heart and blood vessels, and its main purpose is to allow the delivery of essential fluids, electrolytes and oxygen to the different cells, organs and tissues and the removal of metabolic wastes. The arteries carry oxygenated blood from heart and distributes into the systemic circulation, while deoxygenated blood is carried to the lungs from pulmonary circulation, allowing to unload carbon dioxide and receive oxygen. The blood vessels are made up of the same cellular components but the concentric structure, organization and proportionality may vary depending upon the type of vessel.

2.1. The structure of blood vessel wall

The vessel wall consists of three layers called: the tunica intima, tunica media and tunica externa. The tunica intima (or innermost layer) is composed of a monolayer of endothelial cells lying on a basement membrane and provides a selective permeable barrier between blood and the vessel wall (Versari et al., 2007). The internal elastic lamina (IEL) formed by the elastic fibers separates the intima from the tunica media (Wagenseil and Mecham, 2009). The tunica media is composed of circularly arranged vascular smooth muscle cells, collagen fibers, elastins, proteoglycans and other components of the extracellular matrix. An external elastic lamina separates the media from the adventitia. The tunica externa or outermost layer is made up of fibroblasts, collagen, elastic fibers and perivascular nerves which regulate the vascular tone (Mazurek et al., 2017)

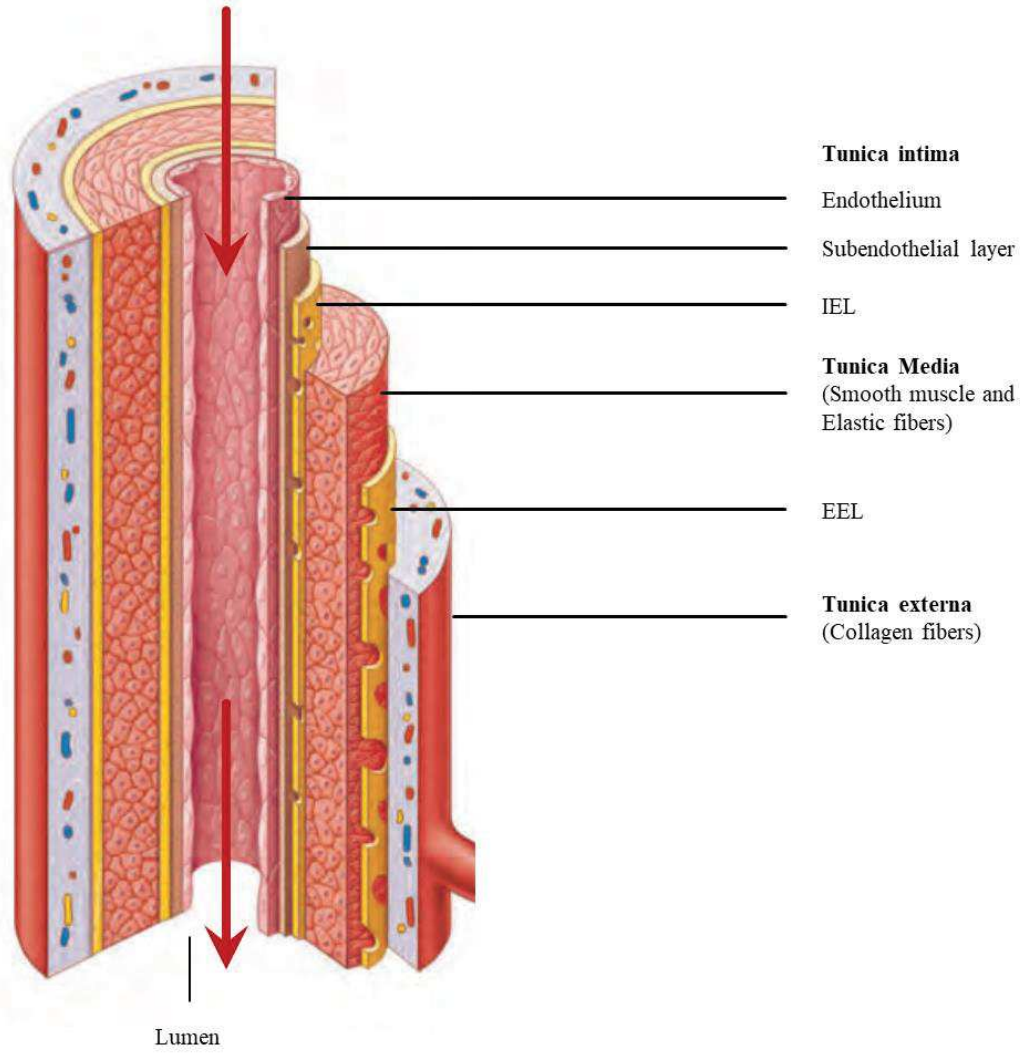

Figure 13. Schematic representation of structure of an artery

The arterial wall has smooth muscle, external elastic lamina (EEL), endothelium, internal elastic lamina (IEL), tunica externa, tunica media, and tunica intima. Adapted from (Tortora and Derrickson, 2018)

The blood vessels are generally divided into five main categories: i) arteries; ii) arterioles, iii) capillaries; iv) veins, and v) venules. The arteries come from the ventricles of the heart and distribute the blood throughout the body where required. The thick wall of arteries with a media rich in elastic fibers and smooth muscle cell maintains blood pressure by contracting the wall. The arteries connect to arterioles, which are smaller in diameter (less than 0.3 mm in humans). The arterioles are composed of one or two layers of smooth muscle cells, having no external elastic tissue and the adventitia is composed only of a layer of collagen and elastic fibers.

They are responsible for the peripheral resistance which makes it possible to reduce the blood pressure arriving in capillaries. The capillaries are small blood vessels of 5 to 10 μm in diameter, which are made up of endothelial cells surrounded by a basement membrane and pericytes. Due to their thin wall, they convey blood to the organs. The capillaries unite to form venules having diameter ranging from 8 μm to 100 μm in diameter. The venule wall either have smooth muscle cells or pericytes depending on their size. Finally, venules unite to form a vein, which has relatively few layers of smooth muscle cells in the tunica media, but a thick layer of collagen and smooth muscle cells in tunica externa (Tortora and Derrickson, 2018). Unlike arteries, the pressure in veins is low; veins are thin-walled and less elastic as compared to the arteries.

2.2. Composition of blood vessels

2.2.1. Endothelial Cells

Endothelial cells constitute the endothelium, a monolayer of cells lining the inner wall of the entire cardiovascular system. They are flattened and polarized cells oriented in the direction of blood flow. Endothelial cells are joined by tight and adherens junctions. These junctions reduce the vascular permeability by creating a semi-permeable organ that regulates the passage of fluids, macromolecules and leukocytes. The apical plasma membrane of endothelial cells contains the glycocalyx (GCX), which is a multilayer structure formed by glycoproteins, proteoglycans and glycosaminoglycans that provide the barrier function and prevent the activation of blood components (Kruger-Genge et al., 2019). Endothelial cells produce the components found in subendothelium and basement membrane including vWF, collagens, fibronectin and laminins, which are secreted by exocytosis from storage granules. Endothelial cells regulate the vascular tone, which is a balance between constriction and dilatation state of blood vessel, by producing various endogenous vasodilators including prostacyclin (PGI2), nitric oxide (NO) and endothelium-derived hyperpolarising factor (EDHF) (Arner and Högestätt, 1991; Huang et al., 1995; Loh et

al., 2018), and also vasoconstrictors including endothelin, reactive oxygen species (ROS) and angiotensin II (Arner and Högestätt, 1991; Sena et al., 2018).

Endothelial cells express both pro- and antithrombotic factors. Under normal vascular physiological conditions, anti-thrombotic factors keep the platelets and vessel wall in a quiescent state, thereby inhibiting endothelial activation and thrombosis.

2.2.1.1. Anti-thrombotic properties of the quiescent endothelium

The thromboresistance of the quiescent endothelium is due to passive properties of plasma membrane and can also be related to the action of anti-platelet, anti-coagulant, fibrinolytic factors and smooth muscle cells relaxation. The arrangement of phospholipids and glycoproteins and negatively charged surface of the endothelial plasma membrane prevents its interaction with platelets. The endothelium expresses the ecto-nucleotidases CD39 and CD73 which hydrolyze platelet agonists such as ADP and ATP, into adenosine stimulating the formation of cAMP which keeps platelets at rest (Marcus et al., 1997; Minor et al., 2019). This is also reinforced by prostacyclin (PGI2), adenosine and nitric oxide (NO) which also maintain platelets in a resting state and avoid interaction with endothelium and other platelets as well (Hamilos et al., 2018). In addition, endothelial cells also exhibit components with anticoagulant properties: i) proteoglycans, heparan sulfates, which potentiate anti-thrombin III and heparin cofactor II activation causing blockade of thrombin formation (Rosenberg, 1989; Tollefsen and Pestka, 1985); ii) thrombomodulin expressed on the lumen surface activates protein-C which also enforces anticoagulant activity by inactivating the coagulation cofactors Va and VIIIa (Loghmani and Conway, 2018); and iii) protease inhibitor, tissue factor pathway inhibitor (TFPI), present in plasma prevents activation of FVIIa-tissue factor complex resulting in decreased coagulation (Rapaport and Rao, 1992). Some of the fibrinolytic factors are also synthesized in endothelial cells in physiological settings notably tissue plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA) which transform plasminogen into plasmin resulting in fibrin degradation (Becker et al., 2000).

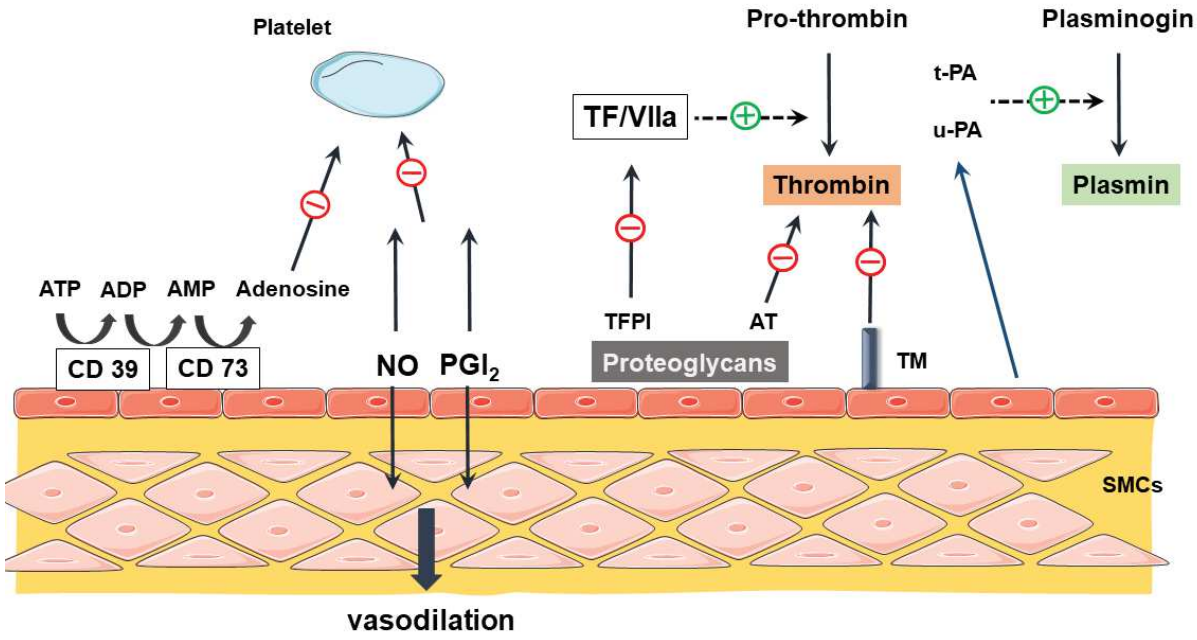

Figure 14. Anti-thrombotic properties of the quiescent endothelium

ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine 5'-monophosphate; AT, antithrombin; SMC, smooth muscle cell; TFPI, tissue factor pathway inhibitor; t-PA, tissue plasminogen activator; TM, thrombomodulin; u-PA, urokinase plasminogen activator

2.2.1.2. Pro-thrombotic properties of the activated endothelium

The loss of endothelium protective properties accompanied with the expression of pro-coagulant and pro-thrombotic factors and/or on the disruption of endothelium shifts the hemostatic balance toward a pro-thrombotic state. The activated endothelium can then express several adhesive proteins on its surface which ensure platelet adhesion or activation. Adhesive proteins include vWF and P-selectin released from Weibel-Palade granules by exocytosis (Cleator et al., 2006). The interactions of vWF and P-selectin via GPIbα and PSGL-1 (P-selectin glycoprotein ligand-1) promote adhesion of platelets followed by their rolling on the surface of the endothelium. The firm adhesion of platelets relies on the integrin αIIbβ3 that readily binds to fibrinogen as well as vWF. The activated endothelium can release thromboxane A2 and platelet-

activating factor (PAF), which increase platelet activation as well as endothelin-1 (ET-1) with long lasting vaso-constrictive effect. Platelet activation and vasoconstriction are increased, while nitric oxide production is reduced. Finally, exposure to tissue factor (TF) and anionic phospholipids promotes pro-coagulant state, while the expression of anticoagulant proteins such as thrombomodulin is reduced. Moreover, the activated endothelium secret plasminogen activator inhibitor type-1 (PAI-1) thereby reducing fibrinolytic activity.

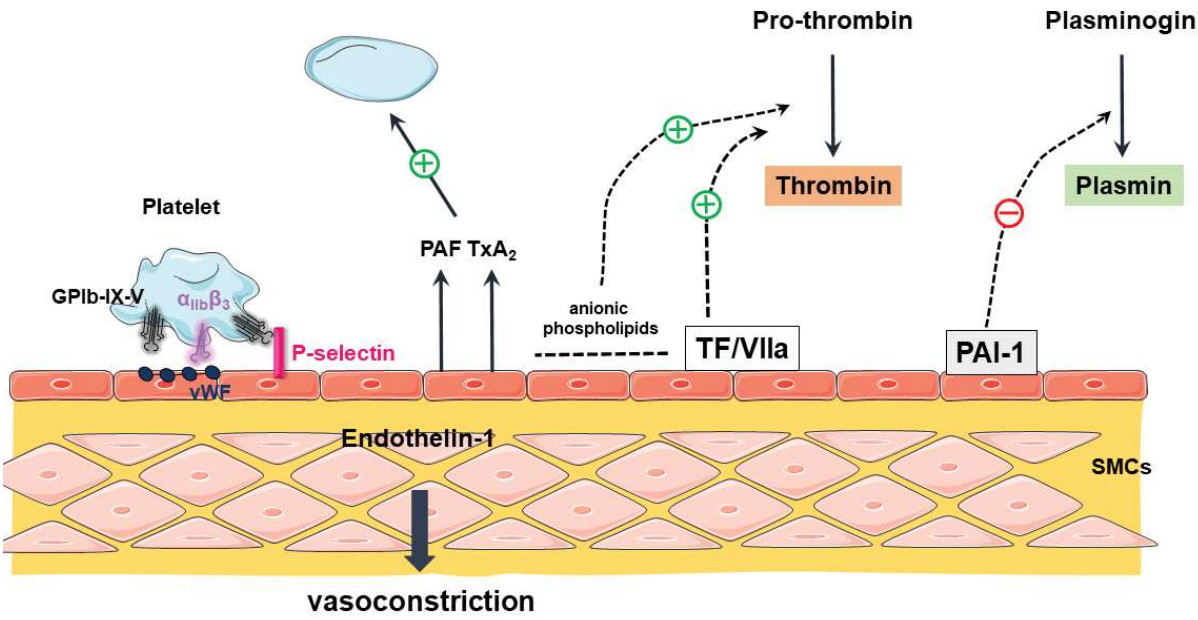

Figure 15. Pro-thrombotic properties of the activated endothelium

SMCs, smooth muscle cells; TF, tissue factor; PAF, platelet-activating factor; PAI-1, plasminogen activator inhibitor

2.2.2. Vascular smooth muscle cells, fibroblasts and pericytes

Vascular smooth muscle cells (VSMCs) are the predominant components of tunica media of blood vessels. They possess a spindle-like morphology and have a central nucleus. They not only contribute in the normal physiological functioning of blood vessels but also in pathological changes in them. Physiologically, their principal function is to regulate vascular tone, blood flow distribution, and blood pressure. In

healthy blood vessels, vascular smooth muscle cells exhibit a quiescent phenotype, often named as contractile phenotype. (Bacakova et al., 2018; Frismantiene et al., 2018). In response to vascular injury, inflammation, endothelium dysfunction, lipoprotein accumulation, they undergo phenotypic transition from a contractile to a synthetic phenotype (Alexander and Owens, 2012; Frismantiene et al., 2018), which is characterized by loss of contractile myofibrils and increased cell proliferation and migration.

Fibroblasts are cells which predominantly reside within adventitial layer of arteries and veins. They are flat, adherent, with a spindle-like morphology, and have oval nuclei. They are the main producers of the extracellular matrix (ECM) proteins such as collagen, elastin and fibronectin (Stenmark et al., 2013). Fibroblasts have a pivotal role in tissue repair due to their ability to degrade damaged tissue and regenerate matrix components (Coen et al., 2011; Stenmark et al., 2013). In pathological conditions, they are also involved in the development of atherosclerotic lesions through the formation of neo-intima (Shi et al., 1996; Shi et al., 1997) and in inflammatory responses through the production of cytokines, chemokines, growth factors, reactive oxygen species and proteases (Enzerink and Vaheri, 2011). Besides, they have other functions including wound healing, angiogenesis, and cancer progression (Kendall and Feghali-Bostwick, 2014).

Pericytes are mesenchymal cells which wrap around endothelial cells of capillaries walls and are closely associated with adventitia of vessel wall. They have a prominent nucleus as compared to endothelial cells. Pericytes maintain and regulate the permeability of the blood vessel wall by interacting with endothelial cells, particularly the blood–brain barrier (Brown et al., 2019). They are also involved in extracellular matrix synthesis, vessel contraction, tissue repair and angiogenesis (Thomas et al., 2017).

2.2.3. The Extracellular Matrix (ECM)

The extracellular matrix (ECM) is a three dimensional network composed of a large variety of macromolecules arranged in a cell/tissue-specific manner. The core constituents of the extracellular matrix include a network of hydrophilic glycosaminoglycans, proteoglycans, and fibrous proteins, such as collagens, elastin, fibronectin and laminins. The extracellular matrix provides a support for cells to form tissue, and is involved in the regulation of multiple cellular functions. Indeed, it is involved in cellular migration, proliferation, survival and differentiation of endothelial cells, smooth muscle cells and fibroblasts forming the vascular wall (Piperigkou et al., 2018). The extracellular matrix is a highly dynamic network that constantly undergoes remodelling both in physiological and pathological circumstances (Bonnans et al., 2014) to regulate the morphogenesis of the elastic fibers. At the vascular level, an injury to the wall exposes the extracellular matrix, which then permits platelet adhesion and activation.

2.2.3.1. The Basement Membrane

The basement membrane is a specialized form of extracellular matrix on which lies the endothelium. It is made up of collagen type IV, type XV and XVIII, laminins, nidogen, and proteoglycans like perlecan and agrin (LeBleu et al., 2007). The basement membrane consists of a network of laminins that allows adhesion of endothelial cells (Aumailley et al., 1989; LeBleu et al., 2007); and network of collagen type IV, which stabilizes the basement membrane structure (Kühn et al., 1981).

The basement membrane serves as a semi-permeable barrier due to its collagen composition and protein density (Rowe and Weiss, 2008) (Kottke and Walters, 2016). It has pro-angiogenic properties, since it stores and releases vascular endothelial growth factors (VEGF) that promotes the blood vessel formation as well as anti-angiogenic properties through its ability to release NC1-domains of collagen type IV (LeBleu et al., 2007). Upon vessel wall injury, the proteins from the basement membrane like vWF and fibronectin (Bergmeier and Hynes, 2012) as well as nidogen, collagen type IV and laminin allow platelet adhesion.

2.2.3.2. The Elastic Fibers

Elastic fibers are large extracellular matrix structures which are designed to experience repeating stretching forces allowing to stretch tissues such as blood vessels and then return to their original shape. They are composed of two distinct components: elastin, and microfibrils. Elastin is formed by cross-linking of its precursor, tropoelastin monomeric subunit, present in smooth muscle cells, endothelial cells and fibroblasts (Theocharis et al., 2016). Tropoelastin has intermittent hydrophobic and lysine-containing domains which cross-link to form insoluble aggregates (Muiznieks et al., 2010). Microfibrils are the complex composition of glycoproteins that form the outer layer of elastic fibers. The major glycoproteins of microfibrils are fibrillins and fibulins (Halper and Kjaer, 2014; Roark et al., 1995; Sakai et al., 1986). They not only assemble into the microfibrils but also interact directly with elastin and form mature elastic fibers (Ross and Bornstein, 1969; Sato et al., 2007).

The importance of the elastic fibers of the extracellular matrix is evidenced by a connective tissue disorder, the Marfan syndrome, which is characterized by the congenital mutation in fibrillin-1 gene leading to an abnormal aortic dilatation which can lead to aortic dissection (Pepe et al., 2016).

Concerning platelet function, it has been shown that fibrillin promotes platelet adhesion under low shear flow conditions (Ross et al., 1998) whereas, elastin-derived peptides decreased platelet aggregation and thrombus formation both *in vitro* and *in vivo* (Kawecki et al., 2014). These findings propose that fbrillin may modulate hemostasis and thrombosis.

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2.2.3.3. Proteoglycans (PGs)

PGs are macromolecules which provide structural basis for various biological functions. They are synthesized and secreted by vascular endothelial cells, smooth muscle cells and fibroblasts. The secreted proteoglycans include: i) large proteoglycans e.g. aggrecan and versican; ii) small leucine-rich proteoglycans e.g. decorin and lumican; and iii) basement membrane proteoglycans e.g. perlecan and agrin (Theocharis et al., 2016; Yue, 2014). These proteoglycans comprise a core protein onto which one or more glycosaminoglycan (GAGs) chains are covalently attached. GAGs are linear, anionic polysaccharides consisting of disaccharide repeats, such as chondroitin sulfate, keratan sulfate or heparan sulfate (Theocharis et al., 2019). Due to highly negative charge on GAGs, proteoglycans bind to water molecules and divalent cations thereby filling the intercellular spaces and playing lubrication functions (Buschmann and Grodzinsky, 1995; Karamanos et al., 2018).

PGs interact with cytokines, chemokines, growth factors thereby participating in different cellular functions including cell signaling, proliferation, migration, differentiation, adhesion and apoptosis (Hildebrand et al., 1994; Karamanos et al., 2018; Wight et al., 1992). Due to their ability to interact with ECM proteins, PGs are also important for the organization of the extracellular matrix (Karamanos et al., 2018; Merrilees et al., 2002). PGs can also modulate angiogenesis notably perlecan which has pro-angiogenic functions due to its N-terminus but also can be anti-angiogenic due to its V domain situated at the C-terminus (Gubbiotti et al., 2017). The importance of extracellular matrix proteoglycans is evidenced by genetic diseases (Järveläinen et al., 2009) such as dyssegmental dysplasia Silverman-Handmaker (DDSH) and Schwartz–Jampel syndrome (SJS), which are characterized by mutation in the gene encoding perlecan (Arikawa-Hirasawa et al., 2002; Arikawa-Hirasawa et al., 2001).

PGs also regulate hemostasis. It has been reported that chondroitin sulfate proteoglycan, versican, induces coagulation by suppressing anticoagulant feature of tissue factor pathway inhibitor-1 (TFPI-1) (Zheng et al., 2006). In contrast, the negatively charged surface of the GAG, heparan sulfate (HS), found to bind to FXIIa thereby initiates coagulation (Wujak et al., 2015). The studies on small leucine-rich proteoglycan, decorin, and basement membrane proteoglycan, perlecan, suggest that proteoglycans participate in the adhesion and activation of platelets via the integrin

α2β1 (Bix et al., 2007; Guidetti et al., 2002). It has also been shown that large proteoglycan, versican, promotes platelet adhesion under low shear flow conditions (Mazzucato et al., 2002). Finally, perlecan was reported to be the ligand of G6b-B (ITIM receptor critical for platelet production and activation) and inhibited collagen-mediated platelet aggregation in the presence of heparan sulfate thereby promoting anti-platelet activity (Vögtle et al., 2019).

2.2.3.4. Adhesive proteins

Adhesive proteins are glycoproteins which contain one or more glycan(s) covalently attached to amino acid side-chains. Many glycoproteins are found in the extracellular matrix of the vessel wall, including von Willebrand factor, collagen, fibrinogen, fibronectin, laminins, thrombospondins, etc.

2.2.3.4.1. von Willebrand Factor (vWF)

Von Willebrand factor is a large multivalent adhesive glycoprotein which is produced by endothelial cells and megakaryocytes and found in subendothelium, plasma (10 μg/mL), platelet α-granules and in Weibel–Palade bodies (WPB) of endothelial cells (Jaffe et al., 1974; Sporn et al., 1985). It is expressed in a pre-pro-vWF monomeric form in the endoplasmic reticulum which associates as a dimeric structure via disulfide bridges. The dimers can associate to form large multimers which can become very large forming the ultra large vWF (ULVWF) multimers of up to 20,000 kDa (Sadler, 2009; Wagner and Marder, 1984). VWF is composed of several different homologous domains that have distinct biological function: i) the A1 domain facilitates the vWF interaction with platelet GPIb-IX-V; ii) the A1/A3 domains allows its interplay with collagen I, III and IV; iii) the A2 domain has the cleavage site for the metalloprotease ADAMTS-13 (*A disintegrin and metalloproteinase with thrombospondin type 1 repeat-13*) that reduces the size and reactivity of vWF multimers; iv) the C1 domain contains the sequence Arg-Gly-Asp (RGD) that is recognized by integrin αIIbβ3 and the D'/D3 domain has a binding site for coagulation

cofactor, FVIII, which is important for the protection of this factor (Brehm, 2017; Mazzucato et al., 1999; Shiltagh et al., 2014; Zhou et al., 2012).

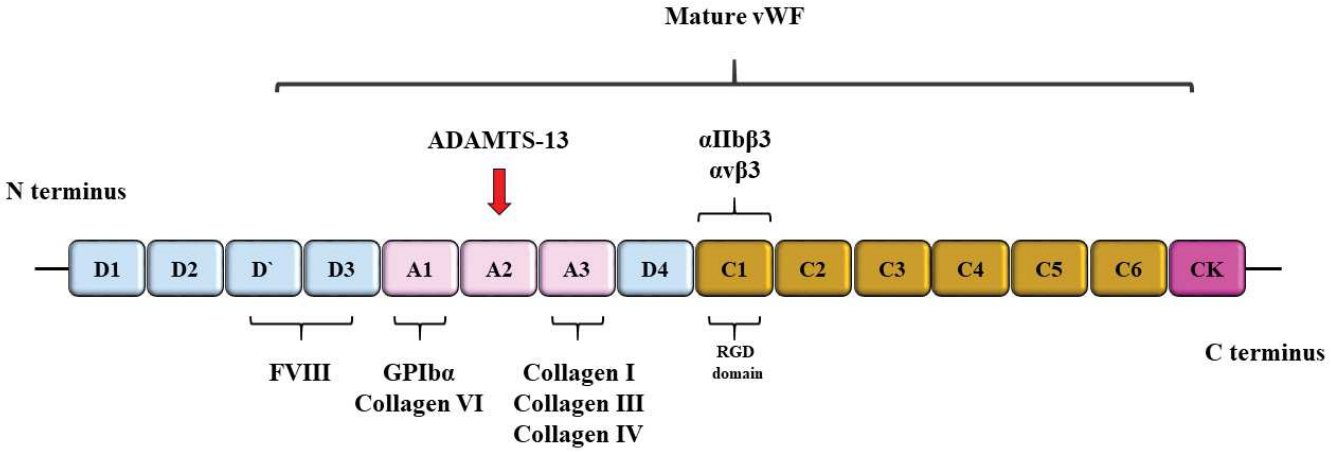

Figure 16. Schematic presentation of VWF

ADAMTS-13 (A disintegrin and metalloproteinase with thrombospondin type 1 repeat-13). Adapted from (Bergmeier and Hynes, 2012; Brehm, 2017)

At the site of vascular injury, vWF which can be present in the subendothelium can also become adsorbed on subendothelial collagen. It permits the adherence of flowing platelets. Immobilization and shear induce a conformational change of vWF exposing the A1 domains on vWF thus enabling its binding to GPIbα on platelets with increased affinity (Savage et al., 1996a; Siedlecki et al., 1996; Springer, 2014). This interaction has rapid association and dissociation rates, which means it is unstable and results in platelet rolling *in vitro*. VWF is the only adhesive protein found in the subendothelium allowing platelet capture at intermediate and high shear rates (>1,000 s^{-1}), and plays therefore a very important role in platelet adhesion. In addition, vWF does not only support platelet attachment to the subendothelium, but also to adherent platelets which expose vWF at their surface allowing the growth of thrombi (Kulkarni et al., 2000).

The role of vWF in hemostasis is highlighted by a congenital deficiency or defect in plasma vWF which results in a bleeding disorder named von Willebrand Disease

(vWD). This pathology is characterized by a defect in adhesion and aggregation of platelets resulting in mild to moderate bleeding diathesis notably epistaxis, easy bruising, menorrhagia, gingival bleeding, excessive posttraumatic bleeding, and postpartum bleedings (de Wee et al., 2012; Veltkamp and van Oosterom, 1973). There are three main types of von Willebrand disease: i) **Type-1 vWD**, the most common (70-80 %) and least serious, characterized by a partial quantitative defect in vWF. A decreased concentration of functionally normal vWF has been reported either because of increased clearance or because of decreased production/secretion; ii) **Type 2 vWD**, which represents 20 % of all cases of vWD and is characterized by a defect in vWF function. It is further divided into four sub-types: 2A, 2B, 2M, and 2N. In Type-2A vWD, there is reduction in high- and intermediate-weight vWF multimers resulting in reduced vWF driven platelet adhesion. Type-2B vWD is a gain-of-function mutation which is characterized by an increased affinity of vWF for GPIbα receptor. This results in a loss of high molecular weight vWF multimers and platelet clearance inducing the hemorrhagic phenotype. Type-2M vWD, is an autosomal dominant variant and is characterized by reduced binding of von Willebrand factor to GP Ib. Type-2N vWD is characterized by reduced affinity of vWF for FVIII (Leebeek and Atiq, 2019) and iii) **Type 3 vWD**, the rarest and most severe with less than 5% of cases, characterized by a total absence of circulating vWF (Leebeek and Eikenboom, 2016).

Along with congenital deficiency, acquired vWF deficiency named acquired von Willebrand Syndrome (AVWS) has also been reported. The pathological mechanisms may include reduced vWF synthesis, development of autoantibodies to vWF or proteolysis (Tiede, 2012).

The congenital or acquired deficiency in metalloprotease ADAMTS-13 can result in accumulation of vWF multimers in the circulation and leads to a rare severe disease Thrombotic thrombocytopenic purpura (TTP). This accumulation triggers formation of vWF-rich micro thrombi in vasculature followed by anemia, thrombocytopenia and organ dysfunction (Levy et al., 2001).

It has been shown in various *in vivo* mouse models that the absence of vWF has a major impact on platelet aggregation and thrombus formation, particularly in ferric chloride-injury to the mesenteric arteries and laser-induced injury models in mice (Dubois et al., 2007; Ni et al., 2000). Moreover, it has also been shown in different

ischemic stroke models that the absence or blockade of vWF/GPIbα protected mice from brain ischemia and prevented middle cerebral artery (MCA) thrombosis in guinea pigs (De Meyer et al., 2012; Momi et al., 2013). Together, these outcomes highlight the importance of vWF in arterial thrombosis and suggest that targeting vWF/GPIbα axis is a promising anti-thrombotic strategy.

Along with its role in arterial thrombosis, vWF has also been proposed to participate in venous thrombosis. In humans, histopathological analysis has shown that venous thrombi contain not only erythrocytes and fibrin but also have vWF thereby might participate in venous thromboembolism. Moreover, it has been demonstrated that $vWF^{-/-}$ mice had reduced venous thrombosis in the stenosis model of deep vein thrombosis (Brill et al., 2011). These results highlight that vWF might participate in venous thrombosis and imply that targeting vWF could potentially be an attractive strategy in the treatment of venous thrombosis (Bryckaert et al., 2015).

2.2.3.4.2. Collagen

Collagens are fibrous proteins and major components of the ECM. They are mainly synthesized by endothelial cells, smooth muscle cells and fibroblasts (Howard et al., 1976; Jaffe et al., 1976; Jones et al., 1979; Shi et al., 1997). There are more than 28 collagen types that are formed by at least 46 distinct polypeptide chains (also called α chains) in vertebrates (Ricard-Blum, 2011). Collagens can be classified into five main categories based on their structures (Table of Collagen). Many different collagen subtypes are found in the different layers of the vessel wall. Fibrillar collagen type I and III, but also collagen V are the major components of media and adventitia (Murata et al., 1986). The subendothelium mainly contains collagen type III and VI, to which collagen type-XII is associated. The collagen type IV and, a lesser amount of collagen type III, type IV and VII constitute the basement membrane (Farquharson and Robins, 1989; Fauvel-Lafeve, 1999). Other types of collagen can be found but are in minority.

Collagen is composed of three polypeptide chains called α-chains that form three left helices. The assembly of three left helices forms a right triple helix called procollagen, which is a soluble precursor of collagen secreted in the ECM (Bella et al., 1994; Sorushanova et al., 2019). The proteolytic cleavage of N- and C-termini converts

procollagen into tropocollagen, which is organized into an insoluble collagen polymer (Hojima et al., 1989; Hojima et al., 1985; Sorushanova et al., 2019). Every collagen has Gly-X-Y repetitive motifs where x and y are often a proline and hydroxyproline.

Classification	Collagen Types	Distribution
Fibrillar collagen	I	Bone, ligament, tendon, dermis
	II	Cartilage, vitrous
	III	Intestine, blood vessels, skin, hollow organs, Colocalized with collagen I
	V	Dermis, bone, placenta, cornea, Colocalized with collagen I
	XI	Colocalized with collagen II, intervertebral disk, cartilage
	XXIV	Cornea, bone
	XXVII	Embryonic and adult cartilage, dermis, cornea, retina, heart arteries
Network collagen	IV	Basement membranes
	VI	Cartilage, bone, cornea, basal lamina.
	VIII	Kidney, brain, heart, dermis, Descement's membrane
	X	Cartilage
Fibril-Associated Collagens with Interrupted Triple Helicies (FACIT)	IX	Colocalized with collagen II, cornea, cartilage, vitreous
	XII	Colocalized with collagen I, tendon, dermis
	XIV	Colocalized with collagen I, cartilage, bone dermis
	XVI	Kidney, dermis
	XIX	Basement membranes
	XX	Cornea
	XXI	Kidney, stomach
	XXII	Localized at tissue junctions
	XXVI	Testis, ovary
Membrane-Associated Collagens with Interrupted Triple Helices (MACIT)	XIII	Endothelium, dermis, eye, heart
	XVII	Hemidesmosomes in epithelia.
	XXIII	Heart, retina.
	XXV	Brain, heart, testis
Multiple Triple-Helix Domains and Interruptions (Multiplexin)	XV	Found in a wide range of tissues
	XVIII	Widespread in basement membranes.

Table 2. Collagen family classification and distribution

Fibrillar collagens are the most thrombogenic components of the vessel wall (Varga-Szabo et al., 2008). The perfusion of blood over immobilized collagen I or III leads to formation of platelet aggregates (Parsons et al., 1986; Ross et al., 1995; Saelman et al., 1994; Savage et al., 1998). This process involves at least three platelet receptors: i) GPIbα which recognizes plasma vWF adsorbed onto collagen, ii) α2β1 and iii) GPVI. GPVI is known to bind the glycine-proline-hydroxyproline (GPO) motif within fibrillar collagens, whereas α2β1 recognizes the GFOGER motif present in the sequence of collagen type I, II, III, IV and IX (Manon-Jensen et al., 2016).

The importance of collagen is highlighted by rare heterogeneous group of inherited disorders named Ehlers–Danlos syndrome (EDS), which is characterized by skin hyper extensibility, joint hypermobility, fragility of the skin, easy bruising.

2.2.3.4.3. Fibrinogen

Fibrinogen (factor I), is a glycoprotein well known to contribute in hemostasis. It is primarily synthesized by hepatocytes (Takeda, 1966; Weisel and Litvinov, 2017) and released in plasma where it circulates at concentrations of 2 to 4 g/L with a half-life of approximately 3 to 5 days (Collen et al., 1972; Pieters and Wolberg, 2019). Fibrinogen is a 340-kDa homodimer, made up of pair of three polypeptide chains designated Aα, Bβ, and γ. The individual polypeptide chains first assemble into Aα-γ and Bβ-γ heterodimeric complexes, then trimeric Aα/Bβ/γ half-molecules, and finally into hexameric complexes $(Aα/Bβ/γ)_2$ that are covalently attached via 29 disulfide bonds. All six chains are connected with their N-termini and form a central E-domain containing cleavage sites of fibrinopeptide A (fpA) and B (fpB). The C-termini of Bβ and γ chains extend outward to form two D-domains which are connected to the E-domains via a coiled-coil region (Kattula et al., 2017).

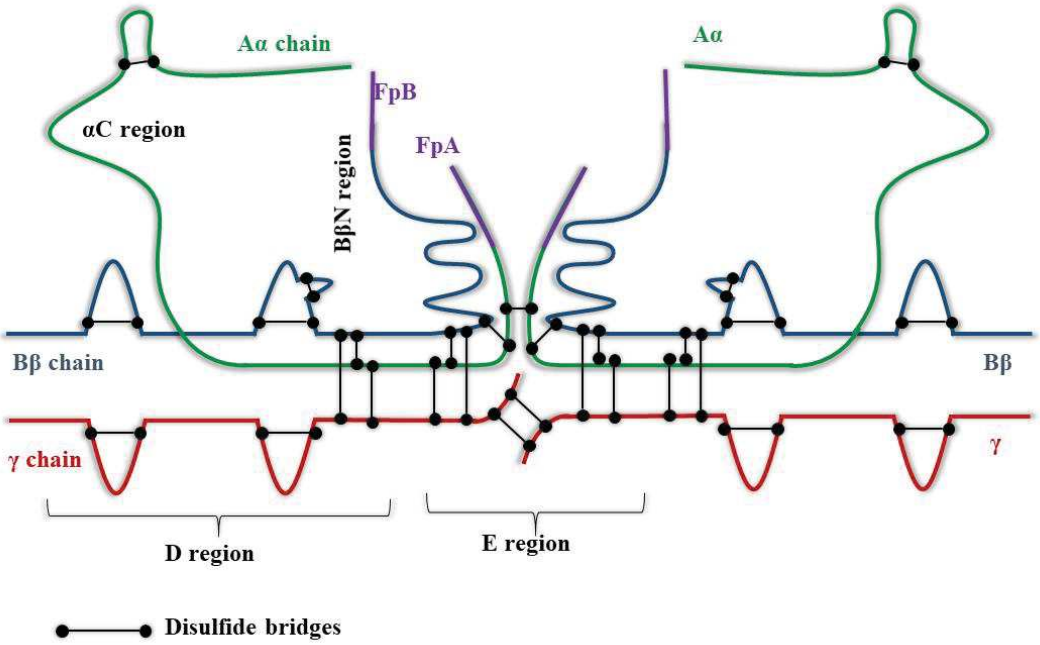

Figure 17. Structure of fibrinogen

The individual chains, Aα, Bβ and γ are green, blue and red, respectively; fibrinopeptides A and B (FpA and FpB) are purple; the disulfide bonds are shown by black bars triple arrows show proteolytic cleavages between the D and E regions. Adapted from (Medved and Weisel, 2009)

In solution, fibrinogen does not significantly interact with its main platelet receptor, integrin αIIbβ3, which is in a low affinity binding state. In contrast, under static conditions or low shear conditions, when fibrinogen is immobilized on a surface, platelets adhere to it and become activated, which probably means that immobilized fibrinogen exposes a cryptic site for αIIbβ3 (Polanowska-Grabowska et al., 1999; Savage et al., 1996b). Upon activation, the dimeric nature of fibrinogen allows to bridge adjacent platelets. There are 3 epitopes within fibrinogen: two RGD sequences on the α-chain and a 12 amino acid sequence on the C-terminus of the γ chain that binds the activated αIIbβ3 integrin, thus allowing platelet aggregation (Hawiger et al., 1989; Kloczewiak et al., 1984; Smith et al., 1990). The αIIbβ3/fibrinogen interaction allows platelets to interact and form an aggregate. In addition, once thrombin is

generated as the end-product of the coagulation cascade, it cleaves fpA and fpB which exposes polymerization sites facilitating the conversion of soluble fibrinogen into insoluble fibrin (Litvinov et al., 2005) which stabilizes the platelet aggregates (Sørensen et al., 2012). Recently, our group has proposed that fibrinogen by interacting with GPVI participates in thrombus growth (Mangin et al., 2018).

The importance of fibrinogen in hemostasis is evidenced by a congenital bleeding disorders named afibrinogenemia or hypofibrinogenemia, which are characterized by an absence of circulating fibrinogen and/or reduced levels of circulating fibrinogen, respectively (Casini et al., 2018), and increased bleeding diathesis. These pathologies are reproduced in the murine system with a reduction in platelet aggregation and an increase in bleeding time following an abnormality of fibrinogen (Holmback et al., 1996; Suh et al., 1995).

3. Role of platelets in arterial thrombosis

Arterial thrombosis can be referred as the formation of a blood clot, also named thrombus within an artery. Thrombus formation usually occurs following the rupture and/ or erosion of an evolved atheromatous plaque that has developed in a diseased artery. Because the plaque geometry profoundly modifies the local rheology and also because it exposes highly reactive material such as fibrillar collagen and TF, platelet adhesion, activation and aggregation are exacerbated and result in the formation of a thrombus that can become occlusive. As a consequence, this process hinders the delivery of blood to organs thereby resulting in their death. Arterial thrombosis can take place in different vessels notably: i) in coronary arteries leading to acute coronary syndrome, i.e. unstable angina and myocardial infarction; ii) in cerebral arteries resulting in ischemic stroke; or iii) in leg arteries leading to pathologies such as acute limb ischemia (McFadyen et al., 2018; Tendera et al., 2011).

3.1. Pathogenesis of atherosclerosis

The underlying cause of arterial thrombosis is atherosclerosis, which is a chronic inflammatory disease leading to narrowing of the artery due to the buildup of a fibrous and calcified plaque inside the lumen of medium- and large-sized arteries (Libby, 2002). Atherosclerosis is a silent process of months, years, and even decades that begins with accumulation of low density lipoprotein (LDL) particles in the inner most tunica intima through endocytosis, where they become oxidized (Libby et al., 2011; Lo and Plutzky, 2012). This activates the endothelial cells which express adhesion molecules for instance vascular cell adhesion molecule-1 (VCAM-1) and selectins allowing the recruitment of circulating immune cells and pro-inflammatory cells i.e. lymphocytes and monocytes which consequently, migrate via diapedesis into the intima (Galkina and Ley, 2007; Sakakura et al., 2013). Within the vessel wall, monocytes differentiate into macrophages (Qiao et al., 1997) whereas leukocytes become activated under the influence of inflammatory regulators i.e. cytokines and chemokines. The macrophages through scavenger receptors (e.g. A, B1, CD36 and CD68) take up the oxidized low density lipoprotein (OxLDL) yielding foam cells (also

called lipid-laden macrophages) (Hansson and Libby, 2006; Li and Glass, 2002). Macrophage foam cells eventually undergo apoptosis and buildup of cholesterol and cellular debris which contribute to formation of lipid rich necrotic core (Kyaw et al., 2013; Wolf and Ley, 2019). In advance lesions, smooth muscle cells (SMC) migrate from tunica media to the tunica intima and synthesize extracellular matrix macromolecule including interstitial collagen, which form a fibrous cap that initially limits the risk of plaque from rupture (Libby et al., 2011). At an advances stage, the activated immune cells produce cytokines which induce secretion of matrix metalloproteinase resulting in degradation of collagens and thinning of fibrous cap (Castellano et al., 2013; Libby et al., 2009). The atherosclerotic plaques with large lipid cores covered by weakened fibrous cap are then susceptible to the erosion and/or rupture, often termed as 'vulnerable plaques'. Whereas, the plaques with limited lipid accumulation covered by thicker fibrous cap are often termed as 'stable plaques'.

3.2. Antiplatelet agents

Antiplatelet agents are the cornerstone for the management of arterial thrombosis. These agents target either platelet activation by inhibiting cyclooxygenase-1 (COX-1) or the $P2Y_{12}$ receptor, or platelet aggregation by directly inhibiting integrin αIIbβ3. They are particularly indicated in patients with ACS or those undergoing PCI, and also for the secondary prevention after a first cardioembolic event.

3.2.1. Aspirin (ASA)

Aspirin (also known as acetyl salicylic acid) is a pro-drug, which is hydrolyzed into salicylic acid by esterases present in gastrointestinal mucosa, liver and plasma. Aspirin irreversibly inhibits platelet cyclooxygenase isoform 1 (COX-1), thereby suppressing the transformation of arachidonic acid into prostaglandin H_2 and synthesis of TXA_2, which is a strong inducer of platelet activation and aggregation, and also initiates vasoconstriction. The blockade of COX-1 leads to the suppression of platelet aggregation for about 7-10 days (average lifetime of platelets).

ASA is the first-line therapy for the management of arterial thrombosis. The ISIS-2 trial showed that aspirin reduced MI mortality risk by 23% after five week treatment (1988). In the context of secondary care of cardiovascular events, the meta-analysis of the Antithrombotic Trialists' Collaboration (ATC) demonstrated that aspirin decreased the risk of: i) CV events by 26% during the first year, ii) ACS recurrence by 34%, iii) stroke by 22% and iv) stroke recurrence by 60% at 6 weeks (Antithrombotic Trialists, 2002; Antithrombotic Trialists et al., 2009; Barash and Akhtar, 2010; Rothwell et al., 2016). Clinically, aspirin is recommended at low doses oscillating between 75–150 mg/day for the secondary care of ischemic events (Valgimigli et al., 2018). Higher doses of 325 mg/day have been reported to not confer incremental ischemic event protection but increase the probability of gastrointestinal bleeding (Mehta et al., 2010). The use of aspirin is indeed associated with the enhanced bleeding risk, notably gastrointestinal bleeding (Isles et al., 1999). Other adverse events include allergic reaction, which can lead to urticaria, asthma, angioedema and anaphylactic reactions (Berkes, 2003; Inomata, 2012).

In terms of primary prevention of CVDs, aspirin is indicated only when the risk of stroke is high and its use remains controversial because of an increased risk of bleeding (Authors/Task Force et al., 2016; Capodanno and Angiolillo, 2016; De Berardis et al., 2012).

3.2.2. $P2Y_{12}$ receptor antagonists

$P2Y_{12}$ receptor blockers suppress the amplification of ADP-induced platelet activation, resulting in potent antithrombotic effects. Amongst the $P2Y_{12}$ blockers belonging to the thienopyridine family one can find ticlopidine, clopidogrel and prasugrel; these compounds are oral pro-drug which are metabolized by hepatic enzymes such as cytochromes P450 (CYP450) and/or esterases into active metabolites that irreversibly and selectively inhibit $P2Y_{12}$. Other $P2Y_{12}$ antagonists belong to a class of adenine nucleotide derivatives such as cangrelor and ticagrelor.

Ticlopidine is the first generation derivative of thienopyridines and has been reported to be 8% more efficacious than ASA in preventing MI, stroke and mortality at one year according to the ATC study (1994). However, due to its rare but serious

adverse effects including neutropenia and thrombotic thrombocytopenic purpura, it is no longer recommended (Quinn and Fitzgerald, 1999; Valgimigli et al., 2018).

Clopidogrel is second generation derivative of thienopyridine and is still widely used in clinic. It is a powerful and effective anti-thrombotic agent, the benefit of which has been demonstrated in clinical trial which showed that clopidogrel in comparison with placebo diminished the risk of MI and recurrent ischemia (Yusuf et al., 2001). Similarly, clinical study of CAPRIE (Committee, 1996) exhibited that clopidogrel lowered the risk of ischemic events by 8.7% contrary to aspirin in patients. The use of clopidogrel is linked with an enhanced risk of bleeding and thrombocytopenia and is therefore administered at doses at which it does not completely block all $P2Y_{12}$ receptors (Peters et al., 2003; Zakarija et al., 2004).

Prasugrel is an oral, competitive, third generation $P2Y_{12}$ receptor blocker. It is an alternative option with a similar pharmacological property related to clopidogrel but with a quick onset of action. In phase 1 clinical trial, a greater suppression of ADP-mediated platelet aggregation was witnessed in healthy volunteers receiving prasugrel when compared with clopidogrel (Brandt et al., 2007). In the TRILOGY ACS trial, prasugrel did not improve the mortality-rate among the old patients (≥75 years) when compared with clopidogrel for the management of ACS (Roe et al., 2012). However, prasugrel lessened the risk of cardiovascular death, MI, or stroke in patients undergoing coronary angiography as compared to clopidogrel (Wiviott et al., 2013).

Ticagrelor is a reversible, direct acting, non-competitive and non-thienopyridine $P2Y_{12}$ receptor blockers. In individuals suffering with atherosclerosis, AZD6140 (ticagrelor) had a greater suppression of ADP-induced platelet aggregation than clopidogrel (Husted et al., 2006). In the PLATO trial of ACS patients, ticagrelor was found to be superior in reducing the risk of cardiovascular death and risk of post stenting thrombosis as compared to clopidogrel (Cannon et al., 2010). The use of ticagrelor is linked with the enhanced risk of major bleeding including intrapericardial bleeding and intracranial bleeding (Becker et al., 2011). Its use is also associated with side effect of mild to moderate intensity dyspnea, shortness of breath, which occurs in the first month of the treatment (Storey et al., 2011; Storey et al., 2010).

Cangrelor is a reversible, direct, competitive, adenosine triphosphate analog $P2Y_{12}$ receptor blocker. It has quick and greater suppression of ADP-induced platelet

aggregation as compared to cangrelor (Angiolillo et al., 2012). It is indicated for the patients undergoing PCI with stenting, in conjunction with aspirin without prior $P2Y_{12}$ antagonist treatment, to minimize risk of recurrence of thrombotic event (Gremmel et al., 2018). The administration of cangrelor in elderly patients (≥75 years) with PCI are at moderate/severe risk of bleeding (Cavender et al., 2017). Its use is also carries the risk of autoimmune reactions resulting in dyspnea (Serebruany et al., 2014).

Selatogrel is a novel potent, reversible and selective $P2Y_{12}$ receptor blocker with the particularity to be administered subcutaneously. It has a rapid onset of action when compared with clopidogrel. In a Phase 2 clinical trial, it showed a rapid and profound platelet inhibition in patients having acute myocardial infarction (AMI) without major bleeding complications (Sinnaeve et al., 2020). A phase 3 clinical trial for determining the efficacy and safety of selatogrel in patients of suspected AMI at risk of recurrent AMI will be initiated in the first half of 2021.

3.2.3. Dual Anti-Platelet Therapy (DAPT)

The standard of care to treat arterial thrombosis is based on dual anti-platelet treatment (DAPT), which combines aspirin and a $P2Y_{12}$ blocker. The CHARISMA study showed that combining aspirin with clopidogrel is more effective than aspirin or clopidogrel alone over a period of 28 months in secondary prevention, with an increased reduction in the risk of cardiovascular event by 7%, in myocardial infarction by 6% and stroke by 21% as compared to monotherapy (Bhatt et al., 2006).

Aspirin can also be combined to prasugrel and is indicated for acute coronary syndrome patients undergoing PCI with stenting (Gremmel et al., 2018). In the TRITON-TIMI 38 trial, the combination of prasugrel and aspirin was found to be more effective than clopidogrel and aspirin in lowering cardiovascular death but also carried a significantly greater rate of life-threatening bleeding including fatal bleeding as compared to clopidogrel (Wiviott et al., 2007).

Finally, it has been shown that aspirin can be combined with ticagrelor and this treatment is indicated for the management of ACS in combination with aspirin (Gremmel et al., 2018). In the PEGASUS-TIMI 54 trial, the combination of ticagrelor

and aspirin was found to be more effective than placebo and aspirin in lowering the cardiovascular death i.e. myocardial infarction or stroke but also carried an enhanced risk of major bleeding events including thrombolysis in myocardial infarction (TIMI) bleeding, bleeding leading to transfusion or discontinuation (Bonaca et al., 2015).

3.2.4. Integrin αIIbβ3 inhibitors

Integrin αIIbβ3 inhibitors prevent the interaction of integrin αIIbβ3 with fibrinogen and/or vWF, hindering platelet aggregation and thrombosis. The integrin αIIbβ3 antagonists used in clinic include: abciximab, tirofiban, and eptifibatide. Abciximab is a humanized Fab fragment of a mouse monoclonal antibody (mAb). Eptifibatide is a cyclic heptapeptide and tirofiban is a nonpeptidic small molecule. All αIIbβ3 blockers are ligand mimetic moieties that inhibit fibrinogen from binding to activated platelets and are administered intravenously. In unstable angina (UA)/non-ST-elevation MI (NSTMI) patients, a meta-data of the 12 clinical studies demonstrated that anti-αIIbβ3 agents had only a modest ischemic efficacy with an enhanced risk of major bleeding event (Tricoci et al., 2011). Similarly, in ST-segment elevation myocardial infarction (STEMI) patients anti-αIIbβ3 agents failed to confer incremental efficacy even on top of potent oral $P2Y_{12}$ receptor inhibitors (Di Mario, 2014). The clinical use of anti-αIIbβ3 agents is limited to patients with ACS having a high thrombus burden or no-reflow syndrome after PCI because of their heightened risk of life-threatening bleeding and thrombocytopenia (Buccheri et al., 2019; Webb et al., 2011). The development of oral active agents was halted due to an unexpected 30–35% increase in mortality, which is associate with the ability of these drugs to activate platelet integrins.

3.3. Future antiplatelet agents: targeting adhesion receptors

In recent years, advances in apprehending the role of platelets in hemostasis and thrombosis has led to the identification of several receptors as potential new anti-platelet targets. Some of these targets appear interesting particularly GPVI as its inhibition could impair thrombosis while preserving hemostasis and therefore represents a safe anti-thrombotic strategy.

3.3.1. Inhibitors of the GPIb–vWF axis

The GPIb-IX-V receptor complex/vWF interplay is a key step in platelet adhesion to the damaged vessel wall and thrombus progression. Experimental thrombosis models showed that blockade of the receptor, GPIb, or its ligand, vWF, efficiently prevents thrombus formation (see section **1.2.1.4.**). Various inhibitors of GPIb-vWF-axis are under therapeutic development.

Agents against vWF blocking GPIbα binding have been developed including ARC1779 and caplacizumab. ARC1779 is an oligonucleotide (aptamer) that has strong affinity for the vWF A1 domain. In a phase II study, ARC1779 has been shown to reduce cerebral thromboembolism in patients subjected to carotid endarterectomy. However, the trial was halted since two-thirds of patients administered ARC1779 experienced profoundly high bleeding complications compared to none of those given placebo and also due to lack of funding (Markus et al., 2011). Caplacizumab is a single-chain antibody that interacts with vWF on A1 domain. After promising results in phase I studies, caplacizumab was further investigated in conjunction with DAPT in patients suffering from ACS undergoing PCI. The findings revealed that the bleeding profile was comparable to that of DAPT plus abciximab (Bartunek et al., 2013). However, caplacizumab has emerged as a novel agent for the management of TTP, which rapidly normalized the platelet count, lowered the risk of TTP-related death and thromboembolic event (Peyvandi et al., 2016; Scully et al., 2019).

Anfibatide is a novel derivative of a snake-toxin and a direct anti-GPIb antagonist that also blocks the vWF interplay with GPIb. It has been shown that anfibatide suppresses platelet adhesion, aggregation and experimental thrombosis

without causing bleedings in mice and also exerted protective effects in ischemic stroke and reperfusion injury in mice (Lei et al., 2014; Li et al., 2015). A phase 2 clinical trial determining the safety and efficacy of anfibatide in patients having ST elevation MI before PCI is being carried out (NCT02495012).

3.3.2. Inhibitors of GPVI

Since patients having GPVI deficiency experience a mild bleeding if any and GPVI-deficient mice do not present prolonged tail-bleeding time, it is widely viewed that GPVI is not absolutely needed to ensure hemostasis. Moreover, absence or blockade of GPVI abolished thrombus formation *in vitro* and in several experimental thrombosis models. For these reasons, GPVI is recognized as a potentially safe anti-thrombotic target.

Revacept is a recombinant dimeric protein composed of two ectodomain of GPVI fused to the Fc region of the human immunoglobulin (IgG1) that competitively interacts with collagen and averts platelet adhesion and activation. In a phase 1 clinical findings, it was shown that revacept had no significant impact on the bleeding time of healthy volunteers, while inhibiting collagen-induced aggregation dose-dependently *ex vivo* (Ungerer et al., 2011). A phase 2 clinical trial evaluating the safety and efficacy of revacept when administered in conjunction with aspirin and/or clopidogrel, in patients having symptomatic stenosis of the internal carotid artery (NCT01645306, December 2019) has demonstrated that revacept reduced the number of new peri-interventional infarctions in the brain by 46% when compared with placebo. Another phase 2 clinical trials in patients with coronary artery disease undergoing PCI (NCT 03312855; EudraCT 2015-000686-32) is being carried out and the protocol has recently been published (Schupke et al., 2019).

Glenzocimab (ACT017) is a humanized antibody Fab fragment with high specificity and affinity to GPVI. The anti-human GPVI blocking Fab 9O12.2 (previous mouse antibody variant of ACT017) reduced the experimental thrombosis in humanized GPVI mice (Mangin et al., 2012). In non-human primates, ACT017 has been stated to inhibit collagen-induced platelet aggregation *ex vivo* without causing thrombocytopenia, GPVI shedding or bleeding events (Lebozec et al., 2017). In a

phase I trial, ACT017 had no significant impact on the bleeding time of healthy volunteers while dose-dependently prevented collagen-driven platelet aggregation (Voors-Pette et al., 2019). A phase II acute ischemic stroke interventional study (ACTIMIS) clinical trial for determining the safety and efficacy of glenzocimab in patients having acute ischemic stroke is currently being carried out (NCT03803007).

Experimental Approach and Results

Characterization of the role of GPVI in thrombus stability

Publication-1:

Pharmacological blockade of GPVI promotes platelet thrombus disaggregation

Muhammad Usman Ahmed, Valeria Kaneva, Stéphane Loyau, Dmitry Nechipurenko, Nicolas Receveur, Marion Le Bris, Emily Janus-Bell, Mélusine Didelot, Antoine Rauch, Sophie Susen, Nabil Chakfé, François Lanza, Elizabeth E. Gardiner, Robert K. Andrews, Mikhail Panteleev, Christian Gachet, Martine Jandrot-Perrus, Pierre H. Mangin

Introduction

GPVI is member of the immunoglobulin superfamily and is specifically expressed on platelets. It is recognized as a **promising safe antithrombotic target** as its absence or pharmacological blockade prevents thrombus formation *in vitro* and in several *in vivo* models of thrombosis, while patients with GPVI deficiency had a very mild bleeding tendency.

GPVI is coupled to FcRγ-chain which is important for its surface expression and to induce signaling resulting in the activation of platelets (Nieswandt et al., 2000). The role of GPVI in thrombus formation was for a long time believed to be solely linked to its ability to activate platelets following binding to subendothelial collagen (Nieswandt and Watson, 2003). Over the past few years, it has been showed that GPVI also binds to other adhesive proteins including laminin (Inoue et al., 2006) and fibrin (Alshehri et al., 2015a; Mammadova-Bach et al., 2015). More recently, our lab reported that fibrinogen is a ligand for GPVI. We provided evidence that GPVI promotes platelet activation on immobilized fibrinogen since control platelets but not GPVI-deficient platelets spread on fibrinogen and this activation appears to be important in thrombus build-up (Mangin et al., 2018). These findings imply that the activation induced following GPVI-fibrinogen interaction significantly contributes in thrombus growth. However, whether this activation is also important for thrombus stability remained unaddressed. Moreover, whether GPVI in general, has such a function during thrombosis was also not known.

The objective of my first research project was to characterize the **role of the GPVI/fibrinogen interaction in thrombus stability**. This collaborative work was performed together with the group of M Panteleev (Moscow state University), which developed a numerical model to study the importance of blood flow on thrombus stability. In parallel, I developed a new *in vitro* flow model to evaluate thrombus stability experimentally. This model consists in pre-forming platelet rich aggregates after perfusion of hirudinated human whole blood over immobilized fibrillar collagen and perfusing then buffer over these aggregates at a given flow rate. I evaluated the contribution of GPVI in thrombus stability in this model, by using: i) 2 monoclonal antibodies against human GPVI, ACT017 and 1G5; and ii) agents blocking the GPVI

signaling pathway. To evaluate the role of fibrinogen, I used blood of patients with congenital afibrinogenemia. Finally, I also assessed the effect of anti-GPVI agents alone or in combination with r-tPA on fibrin-rich thrombi.

This work on which I signed as a first author, was accepted for publication in *Arteriosclerosis, Thrombosis, and Vascular Biology* in July 2020.

Arteriosclerosis, Thrombosis, and Vascular Biology

BASIC SCIENCES

Pharmacological Blockade of Glycoprotein VI Promotes Thrombus Disaggregation in the Absence of Thrombin

Muhammad Usman Ahmed, Valeria Kaneva, Stéphane Loyau, Dmitry Nechipurenko, Nicolas Receveur, Marion Le Bris, Emily Janus-Bell, Mélusine Didelot, Antoine Rauch, Sophie Susen, Nabil Chakfé, François Lanza, Elizabeth E. Gardiner, Robert K. Andrews, Mikhail Panteleev, Christian Gachet, Martine Jandrot-Perrus, Pierre H. Mangin

OBJECTIVE: Atherothrombosis occurs upon rupture of an atherosclerotic plaque and leads to the formation of a mural thrombus. Computational fluid dynamics and numerical models indicated that the mechanical stress applied to a thrombus increases dramatically as a thrombus grows, and that strong inter-platelet interactions are essential to maintain its stability. We investigated whether GPVI (glycoprotein VI)-mediated platelet activation helps to maintain thrombus stability by using real-time video-microscopy.

APPROACH AND RESULTS: We showed that GPVI blockade with 2 distinct Fab fragments promoted efficient disaggregation of human thrombi preformed on collagen or on human atherosclerotic plaque material in the absence of thrombin. ACT017-induced disaggregation was achieved under arterial blood flow conditions, and its effect increased with wall shear rate. GPVI regulated platelet activation within a growing thrombus as evidenced by the loss in thrombus contraction when GPVI was blocked, and the absence of the disaggregating effect of an anti-GPVI agent when the thrombi were fully activated with soluble agonists. The GPVI-dependent thrombus stabilizing effect was further supported by the fact that inhibition of any of the 4 key immunoreceptor tyrosine-based motif signalling molecules, src-kinases, Syk, PI3Kβ, or phospholipase C, resulted in kinetics of thrombus disaggregation similar to ACT017. The absence of ACT017-induced disaggregation of thrombi from 2 afibrinogenemic patients suggests that the role of GPVI requires interaction with fibrinogen. Finally, platelet disaggregation of fibrin-rich thrombi was also promoted by ACT017 in combination with r-tPA (recombinant tissue plasminogen activator).

CONCLUSIONS: This work identifies an unrecognized role for GPVI in maintaining thrombus stability and suggests that targeting GPVI could dissolve platelet aggregates with a poor fibrin content.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: fibrinogen ■ hemostasis ■ platelet ■ rheology ■ thrombosis

Platelets play a central role in hemostasis and in arterial thrombosis. Upon vascular injury platelets adhere, become activated and aggregate to form a hemostatic plug in a healthy vessel and a mural thrombus that can become occlusive, in a diseased artery.[1] It has been reported that the thrombus is heterogeneous in composition with (1) a core, rich in fibrin composed of highly activated platelets located close to the site of injury, in which important amounts of thrombin are generated; (2) a shell, in which platelets are less activated and no significant amounts of thrombin are generated, as evidenced by the absence of fibrin.[2] From a therapeutic point of view, preventing the shell to grow or dissolving it appear as attractive strategies to prevent arterial occlusion and ischemic pathologies.

See accompanying editorial on page 1964

Platelets express a repertoire of adhesion receptors that engage with subendothelial adhesive matrix proteins

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Nonstandard Abbreviations and Acronyms

Fc	Fc receptor
GP	glycoprotein
PI3K	phosphoinositide 3-kinase
r-tPA	recombinant tissue plasminogen activator
VWF	von Willebrand factor
WSR	wall shear rate

Highlights

- GPVI-mediated platelet activation is important to maintain the stability of a thrombus.
- Blocking anti-GPVI Fab fragments, but not dimeric GPVI-Fc, promote platelet disaggregation in a shear-dependent manner.
- The use of anti-GPVI agents which directly target GPVI could promote disaggregation of platelet rich thrombi

after vessel wall injury. Under conditions of elevated blood flow found in arteries, the first step of platelet attachment is initiated by the GP (glycoprotein) Ib-IX-V complex interaction with VWF (von Willebrand factor) present in the subendothelium. Stable adhesion is supported by integrins of the β1 and β3 family, namely α2β1, α5β1, α6β1, αIIbβ3, and αvβ3 which bind to collagen, fibronectin, laminins, fibrinogen, and vitronectin, respectively. Concomitantly, these receptors together with the collagen receptor, GPVI (glycoprotein VI) initiate intracellular signals leading to platelet activation.[3] Activated platelets release soluble mediators, including thromboxane (Tx)A2 and ADP which reinforce platelet activation and increase the affinity of integrin αIIbβ3, for its main ligand, fibrinogen, allowing platelet aggregation.[4] The thrombus composition is heterogeneous with a core containing highly activated platelets and a shell with weakly and reversibly activated platelets.[2] The specific signaling mechanisms responsible for maintenance of platelets in this state is unclear, potential candidacies include GPIb (glycoprotein Ib)-dependent signalling,[5] soluble agonists,[2,6] and we recently proposed GPVI.[7]

GPVI is a member of immunoglobulin receptor family which is specifically expressed on megakaryocytes and platelets.[3] GPVI is considered as not being critical for hemostasis, based on the fact that GPVI-deficient subjects present a mild bleeding phenotype and GPVI-deficient mice show no spontaneous bleeding and no prolongation in tail-bleeding time.[8–10] In contrast, it has been reported that absence of GPVI impairs experimental thrombosis in several models of localized vascular injury.[11,12] Remarkably, GPVI-immunodepleted mice were also protected from thrombosis after ultrasound or mechanical injuries to atherosclerotic plaques in ApoE-deficient mice.[13,14] Together, these observations suggest that GPVI is a particularly promising and potentially safe antithrombotic target.[3]

GPVI physically associates with the FcR(Fc receptor) γ-chain and the Src family kinases Lyn and Fyn.[15] Ligand binding to GPVI promotes its clustering which results in phosphorylation of the immunoreceptor tyrosine-based motif of the FcR γ-chain by Src family kinases.[16] This leads to the recruitment of Syk and formation of signaling complexes which activate PLCγ2 (phospholipase Cγ2) resulting in mobilization of intracellular Ca^{2+} stores, a key second messenger of platelets. GPVI engagement efficiently results in the release of soluble agonists such as ADP and TxA2 which enhance platelet activation.[17] GPVI is best recognized as a platelet activation receptor for fibrillar collagen and supports efficient platelet aggregation in vitro.[17] We and others provided evidence that GPVI participates in platelet activation to laminins.[18,19] We also reported that GPVI plays an important role in platelet adhesion and activation on fibrin.[20,21] More recently, we proposed that human GPVI participates in platelet activation on immobilized fibrinogen, which could support a new role for GPVI in thrombus growth.[7] The primary aim of this study was to investigate the role of GPVI beyond the well-documented initial stages, on the process of thrombus stability. The outcome of this study was to assess the potential of targeting this receptor in the setting of a treatment, when a thrombus had already formed.

We showed that growing thrombi experience elevated mechanical stress, which represents a major challenge to maintain their stability. We developed a numerical model which highlighted the importance of maintaining strong inter-platelet bonds to resist to the increase in mechanical stress when a thrombus grows. Using an experimental approach, we identified a key role of GPVI-mediated platelet activation in maintaining thrombus stability in the absence of thrombin, through its interplay with fibrinogen. These results indicate that targeting GPVI when a platelet-rich aggregate had already formed promotes disaggregation, opening new avenues for the use of anti-GPVI agents to prevent vessel occlusion. In addition, an anti-GPVI agent can also be combined to a thrombolytic agent to dissolve a fibrin-rich thrombus.

MATERIALS AND METHODS

Patients

Two White patients suffering from congenital afibrinogenemia were collected for the study. The patients were related and expressed the same homozygous mutation c.1001 G>A in exon 5, resulting in a nonsense p.W334X mutation of FGA (fibrinogen A) gene, previously reported to lead to premature termination of translation of fibrinogen α chain.[22] For these 2 patients, plasma

fibrinogen was undetectable by antigen and functional assays with unclottable prothrombin, activated partial thromboplastin, thrombin time, and reptilase time tests. Both patients suffered from spontaneous and post-traumatic bleeds but also had a past history of arterial or venous thromboembolism (ischemic stroke in patient 1, 2 episodes of superficial venous thrombosis, and 1 episode of deep venous thrombosis in patient 2; the 3 venous thrombosis events in patient 2 occurred under replacement therapy with fibrinogen concentrate) as reported previously.[23] At the time of the study, the 2 patients were treated on demand with fibrinogen concentrates. No infusion of fibrinogen concentrate was performed in the previous 21 days before blood collection. The collection was approved by the French government under the number: DC-2008-642.

Computational Fluid Dynamics and Fluid-Structure Interaction Analysis

To assess the evolution of mechanical stress during thrombus growth, we used computational fluid dynamics combined with additional fluid-structure interaction analysis. Thrombi were represented as the hemi-spheres protruding into vessels of 600 µm diameter. Constant pressure boundary conditions were chosen for the blood flow corresponding to a wall shear rate (WSR) of 300 s^{-1}. Subendothelial matrix and thrombus were represented as isotropic elastic media with Young modulus value derived from thrombus nanoindentation analysis,[24] while blood was considered as a Newtonian fluid. A fluid-structure interaction module of Comsol Multiphysics was used for computation of flow and flow-induced mechanical stress throughout the system. See Figure I and Table I in the Data Supplement for more details.

Computational Model of Thrombus Formation

We developed a 2-dimensional model of thrombus formation with platelets represented by particles of different sizes.[25] The model considers 2 types of inter-platelet interaction: (1) a primary GPIb-VWF-mediated interaction, modelled by stochastically associating and disassociating springs between platelets; the model was validated using experiments addressing platelet interactions with VWF-coated surfaces[26]; (2) an αIIbβ3-fibrinogen-mediated interaction modelled by Morse potential. Parameters of potential were acquired using experimental data on forces between single platelets.[27] The adhesive force increases with the time platelets spend in the thrombus. To study thrombus dynamics during the initial phase of thrombus growth, we decreased integrin-mediated force between platelets by 10% to 80%. Blood was considered as an incompressible Newtonian fluid described with Navier-Stokes and the continuity equations. The flow field was computed considering the partial vessel blockade by the thrombus. Equations describing both the flow profile and hydrodynamical forces that act on platelets were solved using the Open-FOAM (field operation and manipulation) simpleFoam solver.[28] To infer how inter-platelet forces depend on both thrombus size and shear rate, the stationary configuration of tightly (hexagonally) packed equally sized disks under steady flow was analyzed. Disks, which represented platelets, were gathered in a semi-spherically shaped thrombus. To simplify the analysis, only Morse potential was used to describe platelet-platelet attraction. Injury was modelled as the array of adhesive interaction sites localized at the vessel wall.

In Vitro Flow-Based Adhesion Assay

Microfluidic flow chambers or glass microslides (for scanning electron microscopy experiments) were prepared as previously described.[29] For most experiments, human hirudinated blood (100 U/mL) was perfused through the microfluidic channels, with the exception of flows on plaque material for which we used nonrecalcified citrated blood. The human atherosclerotic plaque homogenates (1 mg/mL) prepared as previously described.[30] To form fibrin-rich clots, citrated blood incubated with $DIOC_6$ (0.5 µmol/L) to label platelets and with DyLight 650-conjugated anti-fibrin (10 µg/mL) was recalcified with $CaCl_2$ (12.5 mmol/L) and $MgCl_2$ (3.5 mmol/L) immediately before perfusing it through collagen coated microfluidic chambers at 1500 s^{-1}. Disaggregation of a thrombus was defined as the detachment of at least 3 platelets or more in a field.

Light Transmission Aggregometry

Human washed platelets were prepared from ACD-anticoagulated blood by sequential centrifugation as previously described.[31] The platelets were suspended at a concentration of 3×10^5/µL in Tyrode buffer containing 0.35% human serum albumin and 2 µL/mL apyrase. Platelet aggregation was measured turbidimetrically after addition of different agonists, in the presence of human fibrinogen (0.8 mg/mL).

Scanning Electron Microscopy

Adherent platelets in glass microslides were fixed for 45 minutes with 25 mg/mL glutaraldehyde in 0.1 mol/L cacodylate buffer containing 20 mg/mL sucrose (305 mOsm, pH 7.3) and scanning electron microscopy was performed as previously described.[5]

Statistical Analysis

Results are expressed as mean±SEM. All statistical analyses were performed with the GraphPad Prism program, version 5.0 (Prism, GraphPad, LaJolla, CA). Nonparametric Mann-Whitney *U* test was used when applicable, and χ^2 test with CI of 95% was used for categorical variables. One-way ANOVA or 2-way ANOVA followed with a post hoc Bonferroni was used for multiple comparisons. $P<0.05$ were considered statistically significant. Details are found in the figure legends.

RESULTS

Blood Flow-Induced Mechanical Stress Challenges the Stability of a Growing Thrombus

A mural thrombus in an injured artery experiences forces applied by the flowing blood. Computational fluid dynamics simulations indicated that surface shear stress was maximal at the thrombus apex and increases with thrombus height (Figure 1A through 1C). These external stress acting on the surface is redistributed throughout the thrombus and results in internal mechanical stress. Fluid-structure interaction analysis performed using the continuous 3-dimensional model indicated that internal mechanical stress (von Mises stress) was maximal at the

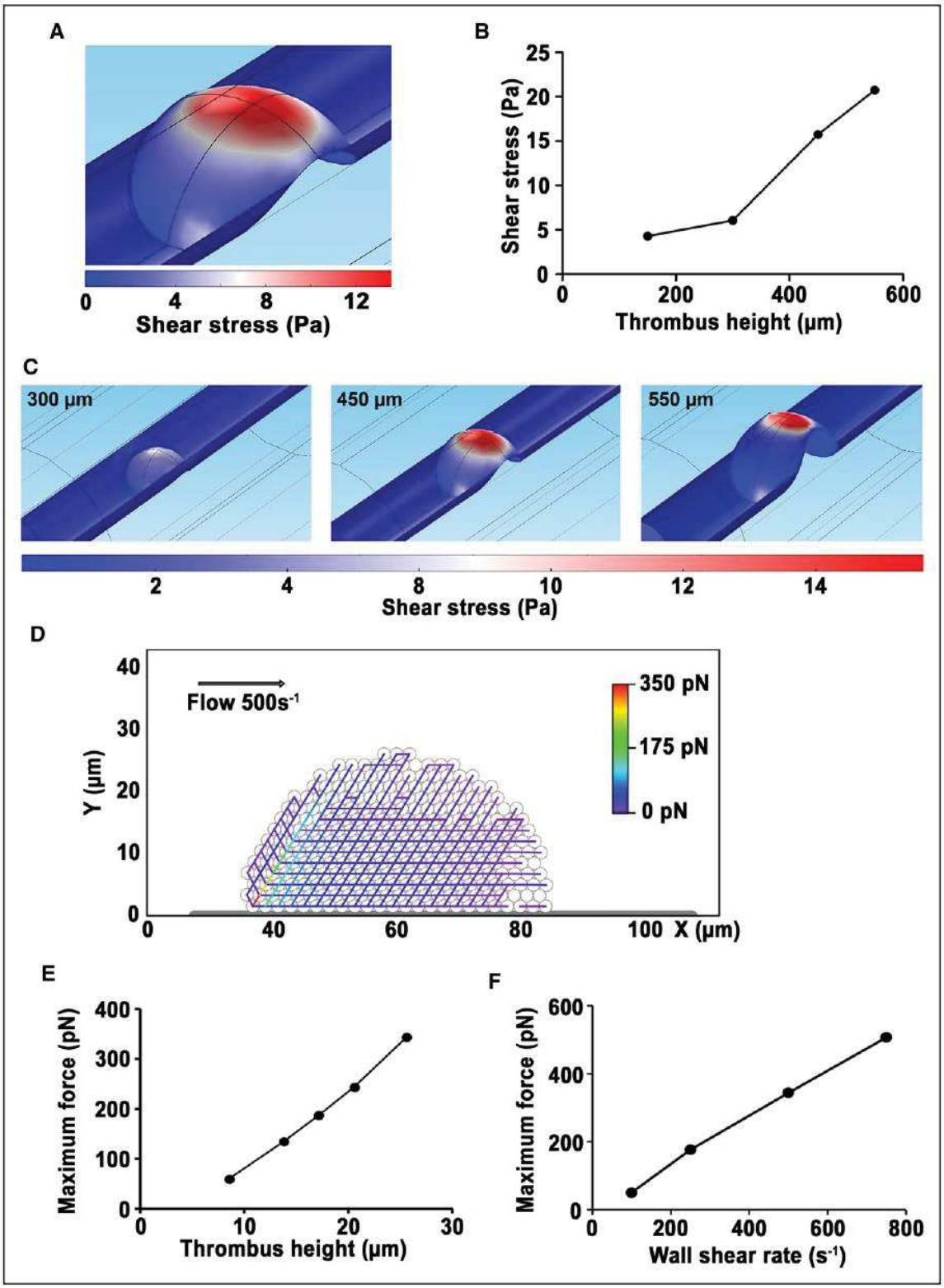

Figure 1. In silico evidence that sustained platelet activation is crucial for the stability of a growing thrombus.
A–C, Computational fluid dynamics simulations were performed to determine surface shear stress exerted on a semi-spherical thrombi of different sizes. **A**, Image representing the distribution of the shear stress on the surface of thrombi of 450 μm at flow conditions corresponding to wall shear rates of 300 s^{-1} (at sites distal to thrombus). **B**, Point-connecting line graph represents the shear stress as a function of thrombus height at constant pressure drop boundary conditions. **C**, Images represent the distribution of the shear stress on the surface of thrombi of 300 μm, 450 μm, and 550 μm height in a channel of 600 μm at constant pressure drop boundary conditions. **D**, Distribution of inter-platelet forces in a 2-dimensional (2D) thrombus subjected to the flow corresponding to 500 s^{-1}. (*Continued*)

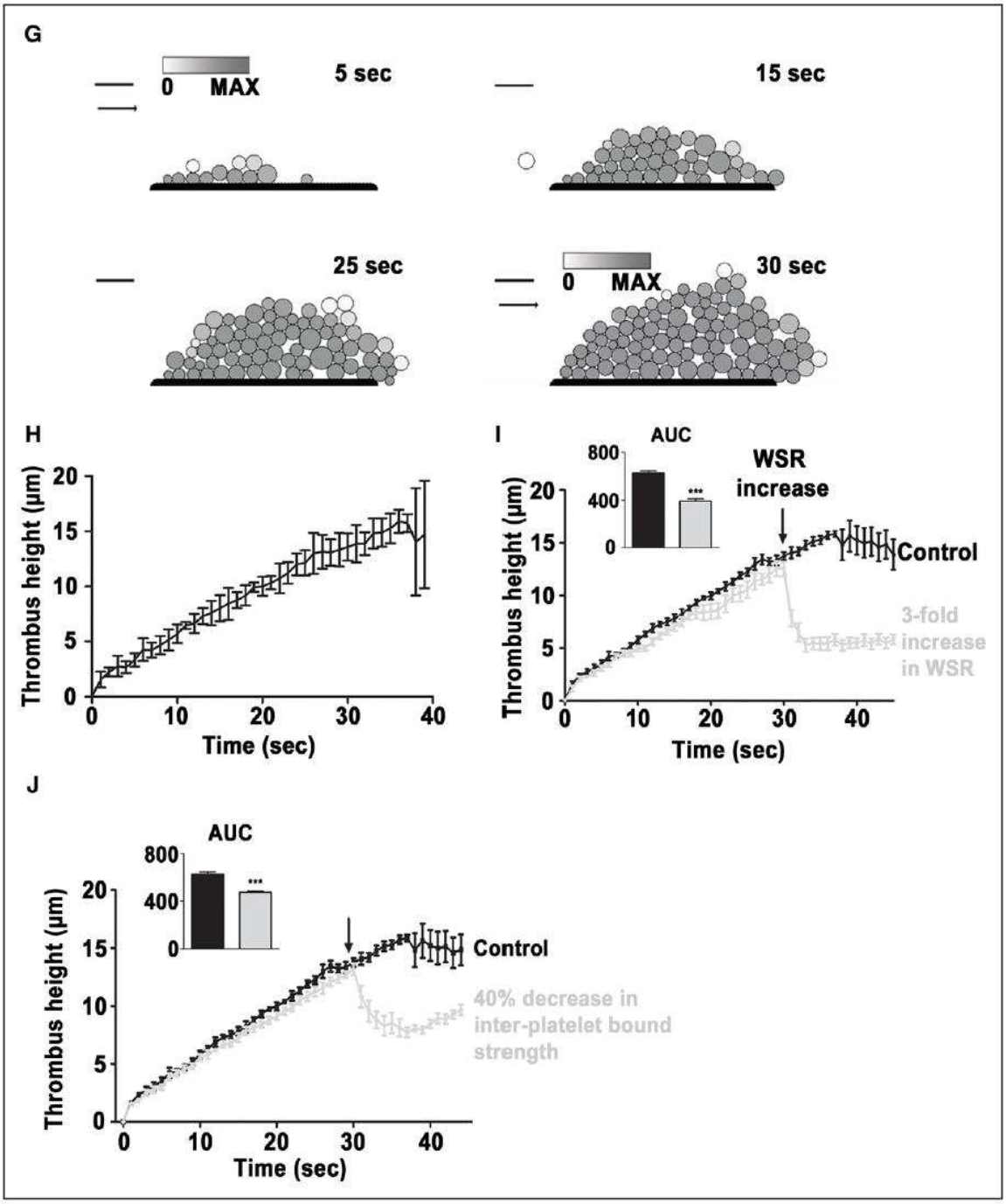

Figure 1 Continued. Each inter-platelet force is shown as a line colored corresponding to the force value. Only forces corresponding to extended inter-platelet bonds are shown. Platelets contours are shown in gray color. **E**, The dependence of maximum inter-platelet bond extension force on thrombus height obtained in a 2D model (wall shear rate [WSR] of 500 s^{-1}). **F**, The dependence of maximum inter-platelet bond extension force on a wall shear rate obtained for 2D thrombus of 26 μm height. **G**, Representative images of the dynamics of thrombus formation in silico. The injury region is shown in black; platelets are represented by circles of different colors (darkening color reflects the extent of platelet activation). WSR is 750 s^{-1}; arrow indicates flow direction. Scale bar represents 5 μm. **H–J**, The graphs plot thrombus height over time at 750 s^{-1}. **I**, The arrow represents the time at which a 3-fold increase in WSR was applied (**H** and **I**; n=6). **J**, The arrow represents the time at which the force between platelets was reduced by 40% (n=10). Bar graph represents area under curve (AUC). Nonparametric Mann-Whitney *U* Test; ***$P<0.001$.

thrombus base and was also increased with both thrombus height and blood flow velocities (Figure I in the Data Supplement). Similar results were obtained in a particle-based 2-dimensional model showing that inter-platelet forces within a thrombus were maximal at its base reaching elevated values of 350 pN for a thrombus of 26 μm in height and increased with thrombus height and WSR (Figure 1D through 1F). Together, these results indicate

that both flow-dependent surface forces and internal platelet-platelet forces increase as a mural thrombus is growing in the vessel lumen. To assess the impact of flow-induced mechanical forces during thrombus growth, we developed a numerical particle-based model of thrombus formation dynamics which reproduces rheology found in arteries (750 s^{-1}). This model recapitulates the main features of thrombosis with (1) platelet adhesion, (2) platelet activation depicted as they become darker (various levels of activation ranging from white (resting) to grey (activated), and (3) recruitment of circulating platelets which results in linear thrombus growth (Figure 1G and 1H; Movie I in the Data Supplement). The impact of elevated shear on thrombus stability was evidenced by the disruption of the thrombus occurring when a 3-fold increase in shear rate was applied at 30 seconds, (Figure 1I; Movie II in the Data Supplement). The importance of the inter-platelet bond strength in maintaining thrombus stability was shown by disaggregation taking place when the inter-platelet force was reduced by 40% at 30 seconds (Figure 1J; Movie III in the Data Supplement). This disaggregation highlighted the importance of a sustained platelet activation during thrombus growth to maintain its integrity. As GPVI is an activation receptor, we investigated whether it participates in maintaining thrombus ability to resist mechanical stress and whether its targeting could promote disaggregation.

Blockade of GPVI During Thrombus Growth Promotes Disaggregation in the Absence of Thrombin

Hirudinated human whole blood was flowed over collagen to preform aggregates followed by perfusion of 50 µg/mL of a blocking humanized anti-GPVI Fab fragment, ACT017, at an arterial WSR of 750 s^{-1}. Real-time video-microscopy showed that numerous individual platelets detached from the top of aggregates treated with ACT017 resulting in a clear disaggregation (Figure 2A; Movie IV in the Data Supplement). Quantification indicated a 4.9-fold increase in the number of platelet thrombi disaggregating after treatment with ACT017 when compared with control buffer (Figure 2B). ACT017 also lowered the time to initiation of disaggregation by 45% (Figure 2C). The diaggregating effect of ACT017 was also evidenced when we maintained whole blood perfusion throughout the experiment (Movie V in the Data Supplement) and was not limited to hirudin as an anticoagulant, as a similar result was obtained with heparin and citrate (data not shown). Similar results were obtained on human atherosclerotic plaque material, where ACT017 promoted efficient disaggregation of platelet thrombi that were formed with citrated blood (Figure 2D). This disaggregation obtained by blocking GPVI was not restricted to ACT017, as another GPVI blocker, the Fab fragment of 1G5 mAb exhibited similar effects (Figure 2E). In contrast, thrombi did not disaggregate when equivalent concentrations of Fab fragments of the nonblocking anti-GPVI antibody 3J24 were perfused, suggesting that the inhibitory effect of ACT017 and 1G5 is related to their ability to disrupt ligand binding to GPVI (Figure 2F and 2G). Finally, we observed that soluble GPVI-Fc, used at a concentration which inhibits collagen-induced platelet aggregation, did not disaggregate preformed aggregates (Figure 2F and 2G). Together, these results indicate that direct blockade of GPVI ligand binding, but not a competition approach, promotes disaggregation of preformed platelet thrombi, highlighting a novel role of GPVI in maintaining the stability of a thrombus during its build-up when thrombin generation was blocked. In the rest of our study, we have mainly used ACT017 as an agent that directly blocks GPVI.

Shear Forces Promote Disaggregation of Platelet Aggregates When GPVI Is Blocked

We next evaluated whether ACT017 promotes disaggregation in a Born-type aggregometer. Washed human platelets were stimulated with collagen, U46619 or ADP, and ACT017 was added to the suspension once aggregation reached maximal values. We did not observe any disaggregation upon the addition of ACT017 (Figure 3A). Because shear is low in an aggregometer (<100 s^{-1}), one hypothesis could be that ACT017-induced platelet disaggregation occurring in the blood perfusion assay depends on the presence of elevated shear forces. To examine that, we preformed aggregates in the flow system at 750 s^{-1} for 3 minutes and perfused ACT017 at WSR ranging from 150 s^{-1} up to 750 s^{-1}. In agreement with the result obtained in the light transmission aggregometer, ACT017 did not promote disaggregation of platelet thrombi under venous blood flow conditions (150 s^{-1}), (Figure 3B). In contrast, signs of disaggregation were observed at an arterial WSR of 300 s^{-1} with a maximal number of disaggregating thrombi being achieved at 750 s^{-1} (Figure 3B and 3C). Measuring the initial time of thrombus disaggregation further highlighted the importance of increasing blood flow conditions on ACT017-induced platelet thrombus disaggregation (Figure 3D). These results are in agreement with our observations in silico showing that elevated shear forces promote disaggregation when platelet activation is inhibited.

The Disaggregating Effect of GPVI Blockade Relies on the Inhibition of Platelet Activation

As GPVI is an activation receptor, we hypothesized that ACT017-induced disaggregation in the absence of thrombin, is linked to an impairment of GPVI-mediated platelet activation in the growing thrombus. To test this hypothesis,

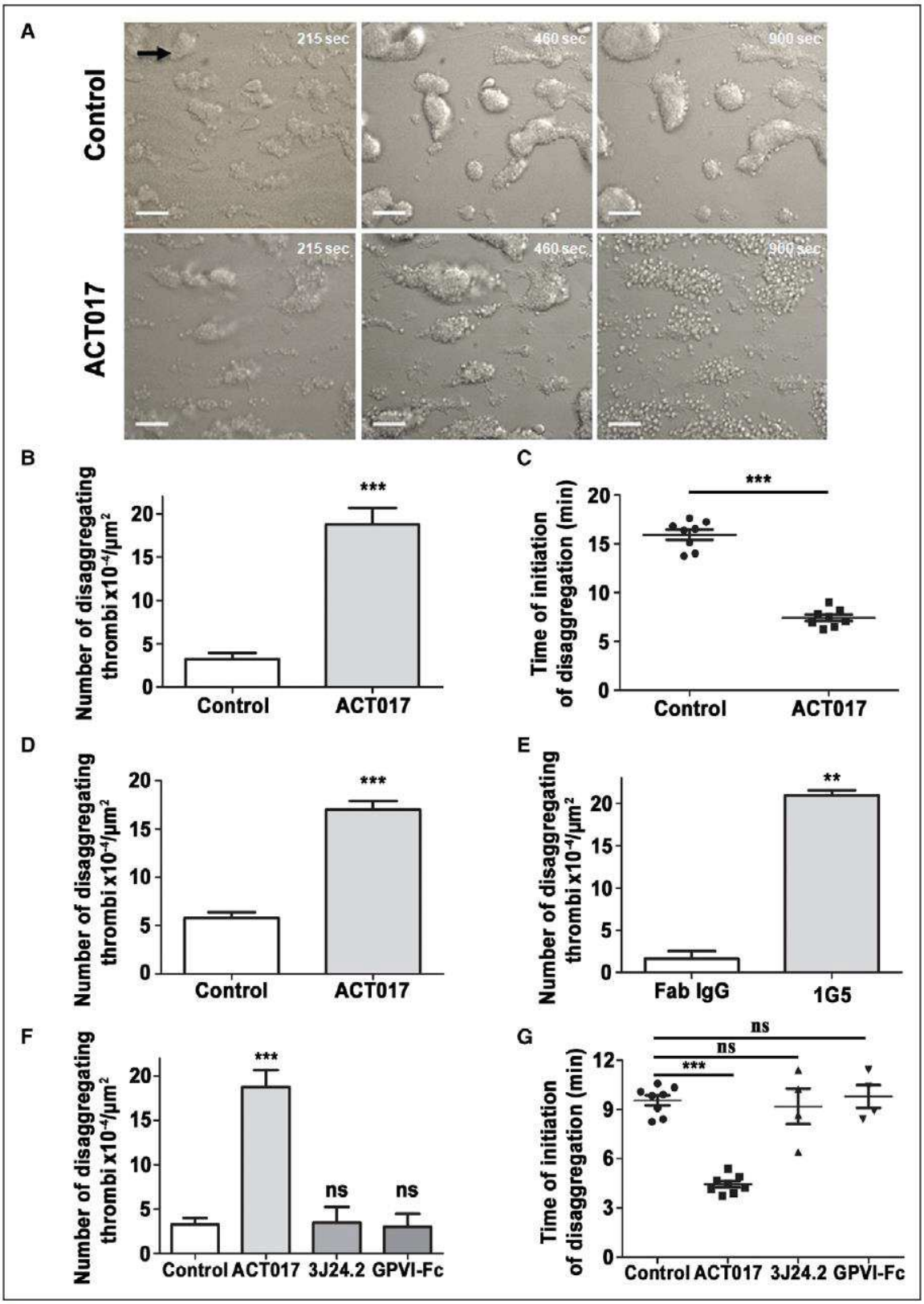

Figure 2. GPVI (glycoprotein VI) blockade promotes disaggregation of preformed thrombi in vitro.
A–C and **E–G**, Hirudinated (100 U/mL) human whole blood was perfused for 3 min at 750 s^{-1} through microfluidic flow chambers coated with a solution of type I fibrillar collagen (200 µg/mL) to preform aggregates. **A–C**, ACT017 (50 µg/mL) or control buffer was perfused over the preformed aggregates at 750 s^{-1} for 12 min. **A**, Representative differential interference contrast microscopy images of thrombi subjected to ACT017 or control buffer diluted in PBS. The black arrow indicates the flow direction. Scale bars represent 20 µm. (*Continued*)

we perfused elevated nonphysiological concentrations of soluble agonists (ADP or TRAP [thrombin receptor activator peptide]) over preformed aggregates to maximally activate platelets and overcome the GPVI activatory pathway, before injecting ACT017. We observed that under such conditions ACT017 was less efficient in promoting disaggregation and that the time of disaggregation was delayed, indicating that its effect is linked to the regulation of platelet activation (Figure 4A and 4B). In parallel, we examined thrombus contraction in real-time, as a readout of platelet activation. While thrombi were contracting and appeared more compact over time in the control, a clear loosening and surface expansion was observed when ACT017 was perfused over contracted thrombi (Figure 4C; Movie VI in the Data Supplement). This was confirmed by quantification which showed that the surface occupied by the ACT017-treated thrombi was significantly increased in comparison with untreated control (Figure 4D). Scanning electron microscopy images confirmed the loosening of the aggregates treated with ACT017 and showed that individual platelets were also less contracted when compared with control, confirming that ACT017 impairs platelet activation (Figure 4E). As GPVI promotes platelet activation through a signaling pathway that has been well characterized, we evaluated the effect of inhibitors of several key immunoreceptor tyrosine-based motif signaling proteins such as src-kinase (PP2), Syk (PRT060318), PI3Kβ (phosphoinositide 3-kinase; TGX-221), and PLCγ2 (U73122). The inhibitors increased the number of disaggregating thrombi by 3.6-fold, 2.6-fold, 2.2-fold, and 4.4-fold, respectively, to a similar level as the once achieved with ACT017 (Figure 4F). Moreover, the initial time of thrombus disaggregation also showed a significant decrease for all 4 inhibitors when compared with control which was very close to the one of ACT017 (Figure 4G). The number of disaggregating thrombi and time of initiation of disaggregation were very similar between ACT017 and 1G5 (Figure 2) as compared with inhibition of the 4 immunoreceptor tyrosine-based motif signaling molecules, suggesting that disaggregation occurring when GPVI is blocked, relies on an impairment of GPVI-mediated platelet activation.

Disaggregation Induced When GPVI Is Blocked Relies on the Presence of Fibrinogen

We recently proposed that the role of GPVI in thrombus build-up relies on its ability to promote platelet activation on fibrinogen.[7] To evaluate whether thrombus disaggregation occurring when GPVI is blocked, is linked to this property, we performed experiments with blood of 2 patients with congenital afibrinogenemia. We observed that perfusion of hirudinated whole blood of both patients over immobilized collagen resulted in the formation of thrombi at 750 s^{-1}, even though they appeared smaller than the control (Figure 5A). This was expected since platelet aggregation in the absence of significant levels of fibrinogen has been previously reported and shown to rely on VWF.[32] While ACT017-induced thrombus disaggregation in the control, little effect was observed on thrombi of either patient at 750 s^{-1} (Figure 5B). This result indicates that blockade of GPVI has no impact on thrombus disaggregation in the absence of normal levels of fibrinogen, even though these thrombi appeared looser than normal thrombi in agreement with a previous report.[33] Quantification confirmed the absence of ACT017-induced disaggregation with blood from the patients as compared with controls (Figure 5C). These results propose the existence of a link between fibrinogen and GPVI in allowing the stability of a growing thrombus.

Combination of ACT017 and r-tPA Promotes Disaggregation of Fibrin-Rich Clots In Vitro

So far, we observed that GPVI contributes to the stabilization of a platelet-rich thrombus which contains no fibrin, suggesting that anti-GPVI agents could remove the part of the thrombus corresponding to the shell.[34] We next evaluated whether blockade of GPVI has also an impact on a thrombus core in which thrombin is found and which is stabilized by fibrin. Initially, we formed fibrin-rich thrombi by perfusing recalcified citrated human blood over collagen at 1500 s^{-1}. The high fibrin content of the thrombi was evidenced on 3-dimensional reconstructed images obtained with a fluorescently conjugated anti-fibrin antibody (Figure 6A). ACT017 had a minor effect on thrombus disaggregation with detaching platelets being mainly localized

Figure 2 Continued. B, Bar graphs represent the number of disaggregating thrombi ($\times10^{-4}$)/μm²; control: $3.2\times10^{-4}\pm0.7\times10^{-4}$ disaggregating thrombi/μm²; ACT017: $18.7\times10^{-4}\pm1.9\times10^{-4}$ disaggregating thrombi/μm²; ***$P<0.001$, n=8. **C**, Scatter plots represent the time at which disaggregation was initiated, expressed in minutes; mean±SEM; nonparametric Mann-Whitney *U* Test; control: 9.6±0.3 min; ACT017: 4.4±0.1 min; ***$P<0.001$, n=8 (**D**) Citrated (3.2%) human whole blood was perfused for 4 min at 300 s^{-1} through glass microslides coated with a solution of human atherosclerotic plaque homogenates (1 mg/mL) to preform aggregates, before perfusing ACT017 (50 μg/mL) or control buffer in the same blood at 300 s^{-1} for 12 min. Bar graphs represent the number of disaggregating thrombi ($\times10^{-4}$)/μm² (n=4). mean±SEM; nonparametric Mann-Whitney *U* Test; ***$P<0.001$. **E**, 1G5 (50 μg/mL) or Fab IgG diluted in PBS was perfused over preform aggregates at 750 s^{-1} for 12 min. Bar graphs represent the number of disaggregating thrombi ($\times10^{-4}$)/μm² (n=3). Two-tailed paired *t* test; **$P<0.01$. **F**, ACT017 (50 μg/mL), 3J24.2 (50 μg/mL), GPVI-Fc (50 μg/mL), or control buffer diluted in PBS were perfused over the preformed aggregates at 750 s^{-1} for 12 min. Bar graphs represent the number of disaggregating thrombi ($\times10^{-4}$)/μm²; control: $3.7\times10^{-4}\pm0.9\times10^{-4}$ disaggregating thrombi/μm²; 3J24.2: $3.5\times10^{-4}\pm1.7\times10^{-4}$ disaggregating thrombi/μm²; ns, $P>0.5$, n=4–8. **G**, Scatter plots represent time to initiation of disaggregation, expressed in minutes; $3.0\times10^{-4}\pm1.4\times10^{-4}$ disaggregating thrombi/μm²; non significant (ns); $P>0.5$, n=4–8. Bar is the mean±SEM; Bonferroni multiple comparison test; ***$P<0.001$.

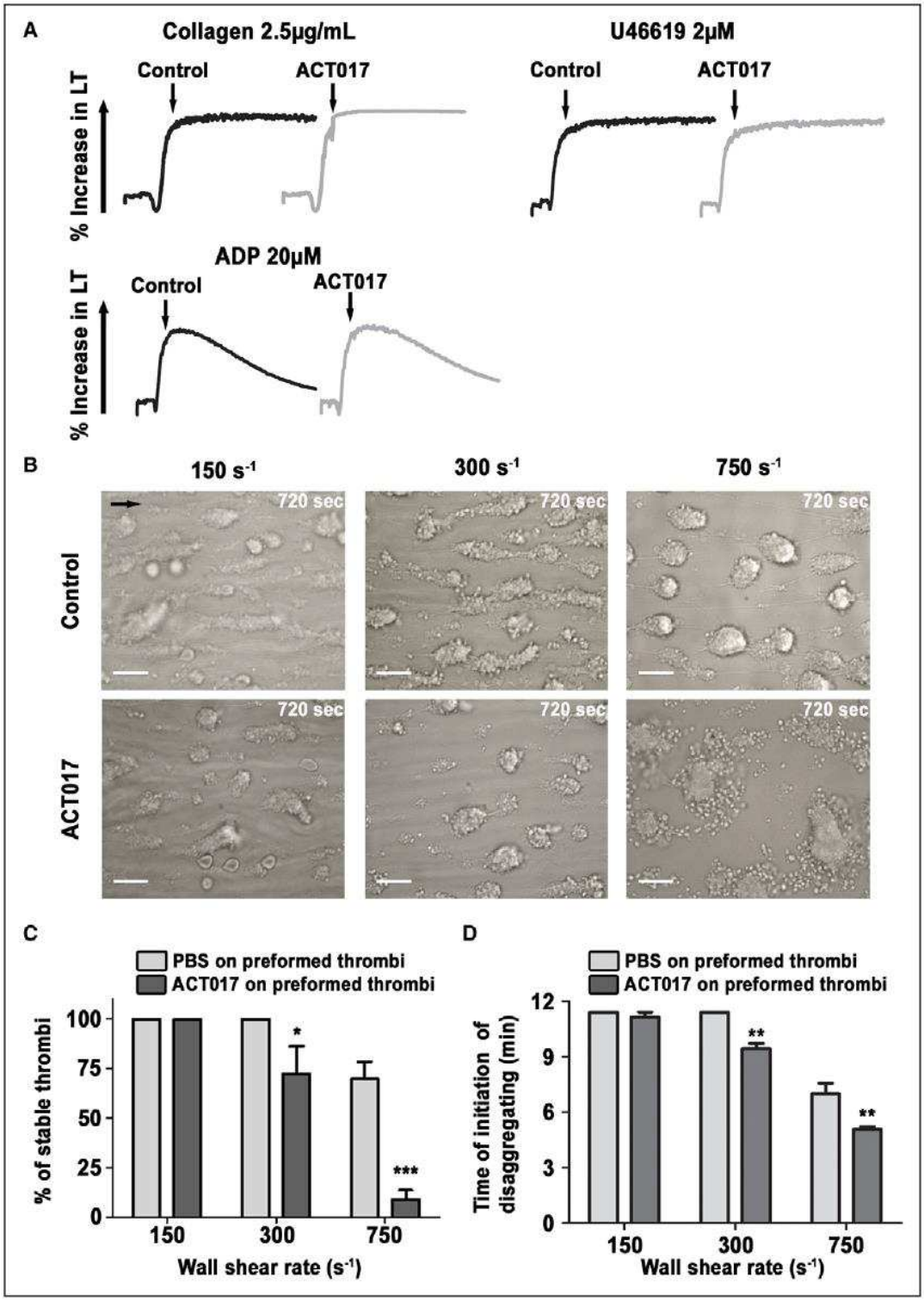

Figure 3. Shear regulates ACT017-induced disaggregation of preformed thrombi.
A, Human washed platelets (3.0×10^5/µL) were stimulated with collagen (2.5 µg/mL), U46619 (2 µmol/L) or ADP (20 µmol/L) in the presence of fibrinogen (64 µg/mL). ACT017 (50 µg/mL) or control buffer was added at maximal aggregation (indicated by an arrow). Aggregation profiles are representative of 3 separate experiments (n=3).**B–D**, Hirudinated (100 U/mL) human whole blood was perfused over immobilized collagen (200 µg/mL) for 3 min at 750 s^{-1} to preform aggregates. Then, ACT017 (50 µg/mL) diluted in PBS was perfused over preformed aggregates at different wall shear rate (WSR; 150 s^{-1}, 300 s^{-1}, and 750 s^{-1}) for 12 min. **B**, Representative differential interference contrast microscopy images of thrombi subjected to ACT017 or control buffer diluted in PBS at indicated WSR. The black arrow indicates the flow direction. Scale bars represent 20 µm. **C**, Bar graph represents the percentage of stable thrombi (n=3). χ^2 test with CI of 95%; *$P<0.05$ and ***$P<0.001$. **D**, Interleaved bars represent time to initiation of disaggregation after perfusion of ACT017 or control buffer diluted in PBS over human aggregates, expressed in minutes (n=3); mean±SEM. Bonferroni multiple comparison test; ***$P<0.001$.

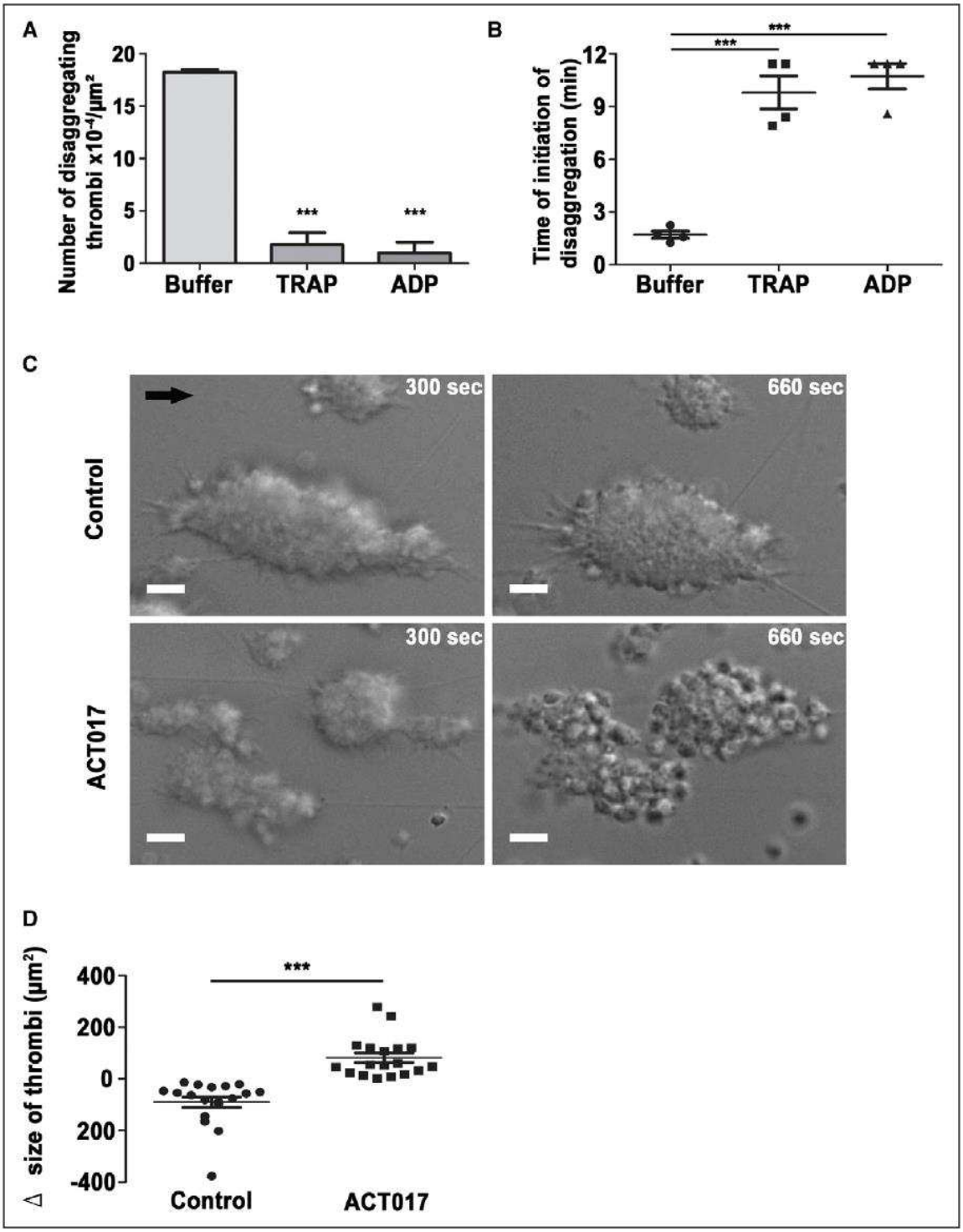

Figure 4. The disaggregating effect of ACT017 relies on impairment of GPVI (glycoprotein VI)-mediated platelet activation. **A–B**, Hirudinated (100 U/mL) human whole blood was perfused for 3 min at 750 s^{-1} through microfluidic flow chambers coated with a solution of type I fibrillar collagen (200 µg/mL) to preform aggregates, before perfusing buffer containing or not ADP (50 µmol/L) or TRAP (thrombin receptor activator peptide; 50 µmol/L) at 750 s^{-1} for 2 min and then at 150 s^{-1} for 3 min, followed by ACT017 (50 µg/mL) or control buffer diluted in PBS at 750 s^{-1} for 12 min. **A**, Bar graphs represent the number of disaggregating thrombi (×10^{-4})/µm² (n=4). **B**, Scatter plot represents the time at which disaggregation was initiated, expressed in minutes (n=4). mean±SEM; Bonferroni multiple comparison test; ***P<0.001. **C–D**, Hirudinated (100 U/mL) human whole blood was perfused for 3 min at 750 s^{-1} through microfluidic flow chambers coated with a solution of type I fibrillar collagen (200 µg/mL) to preform aggregates, before perfusing ACT017 (50 µg/mL) or control buffer diluted in PBS at 750 s^{-1} for 12 min. **C**, Representative differential interference contrast microscopy images of thrombi subjected to ACT017 or control buffer at 5 and 11 min. The black arrow indicates the flow direction. Scale bars represent 5 µm. **D**, Scatter plot represents the variation in thrombi size measured between 5 and 11 min (n=3). Mean±SEM; nonparametric Mann-Whitney U test; Δsize: control: −90.2±20.7 µm²; ACT017: 82.0±18.3 µm²; ***P<0.001; n=3. (*Continued*)

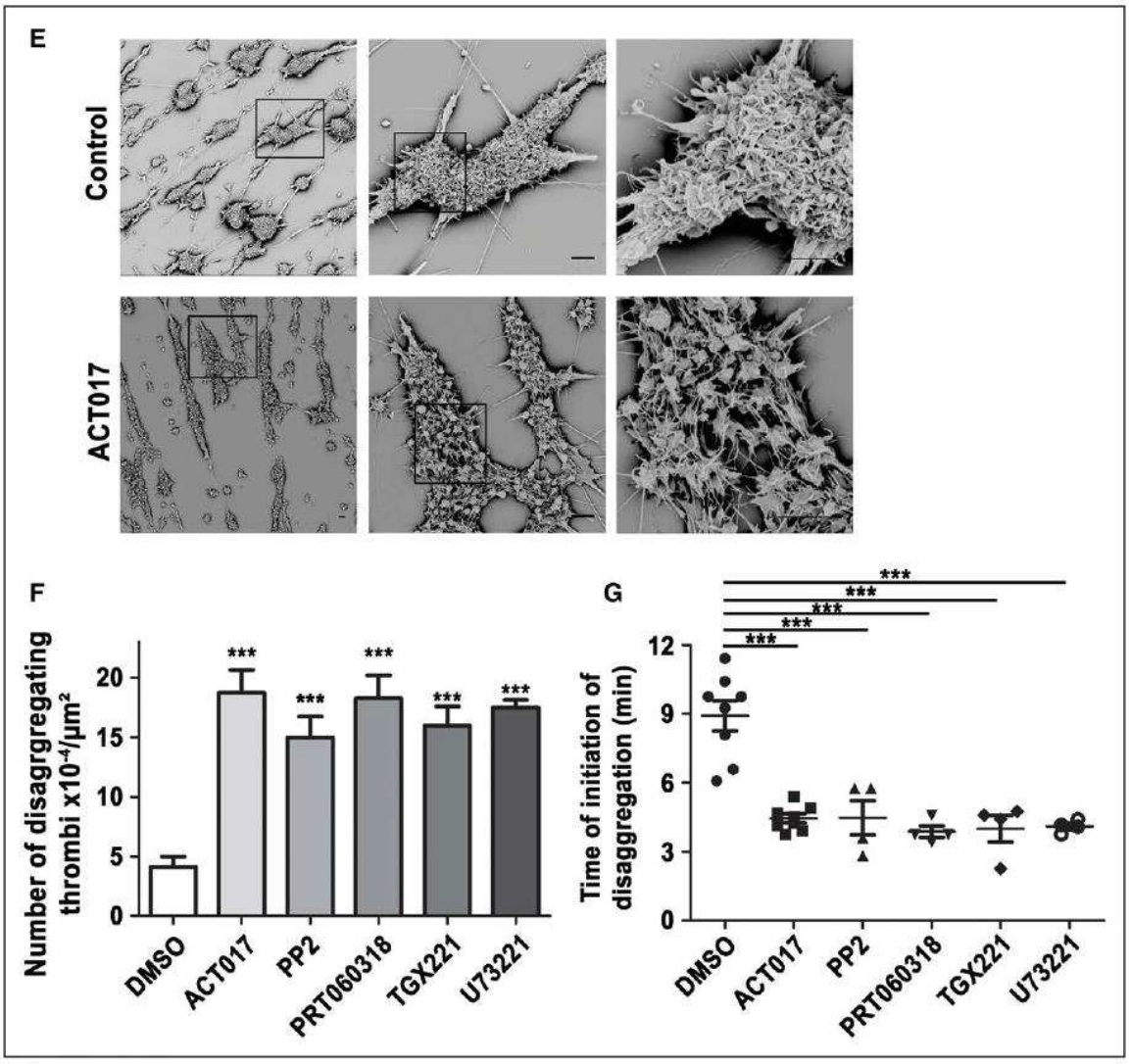

Figure 4 Continued. **E**, Representative scanning electron microscopy images of thrombi subjected to ACT017 or control buffer at 15 min. Scale bars represent 5 µm. **F–G**, Hirudinated (100 U/mL) human whole blood was perfused for 3 min at 750 s^{-1} through microfluidic flow chambers coated with a solution of type I fibrillar collagen (200 µg/mL) to preform aggregates, before perfusing ACT017 (50 µg/mL), PP2 (5 µmol/L), PRT060318 (5 µmol/L), TGX-221 (1 µmol/L), and U73122 (5 µmol/L) or DMSO diluted in PBS at 750 s^{-1} for 12 min. **F**, Bar graphs represent the number of disaggregating thrombi (×10^{-4})/µm²; control: 4.1×10^{-4}±0.8×10^{-4} disaggregating thrombi/µm²; ACT017: 18.7×10^{-4}±1.9×10^{-4} disaggregating thrombi/µm²; PP2: 15×10^{-4}±1.7×10^{-4} disaggregating thrombi/µm²; PRT060318: 18.2×10^{-4}±1.9×10^{-4} disaggregating thrombi/µm²; TGX-221: 16×10^{-4}±1.5×10^{-4} disaggregating thrombi/µm²; U73122: 17.5×10^{-4}±0.6×10^{-4} disaggregating thrombi/µm²; ***P<0.001; n=4–8. **G**, Scatter plot represents the time at which disaggregation was initiated, expressed in minutes (n=4–8). Mean±SEM; Bonferroni multiple comparison test; control: 8.9±0.6 min; PP2: 4.5±0.7 min; PRT060318: 3.8±0.2 min; TGX-221: 4.0±0.5 min; U73122: 4.1±0.1 min; ***P<0.001; n=4; ***P<0.001.

at the thrombus edge where less fibrin was present, which is explained by the ability of fibrin to entrap platelets and thereby stabilize the thrombus (Figure 6B; Movie VII in the Data Supplement). This result is in agreement with the view that fibrin stabilizes the clot. We next wondered whether ACT017 would cooperate with a thrombolytic approach based on r-tPA (recombinant tissue plasminogen activator) to enhance thrombus disaggregation. We showed that perfusion of r-tPA in platelet-poor plasma efficiently reduced the fibrin signal over time confirming its thrombolytic effect (Figure 6C and 6D). We observed that r-tPA alone promoted thrombus disaggregation which was probably linked to its ability to lyse fibrin and lower the stability of the thrombus (Figure 6E). Interestingly, combining r-tPA with ACT017 significantly increased thrombus disaggregation as compared with r-tPA alone, highlighting the potential of an anti-GPVI agent combined with a thrombolytic reagent to promote thrombus disaggregation (Figure 6E). Similar results were obtained when a combination of ACT017 and r-tPA was perfused over thrombi formed on a tissue factor-collagen coated surface with a clear loosening of platelet aggregates being evidenced by a marked increase in surface occupied by the thrombi (Figure 6F and 6 G). Together, these results suggest that

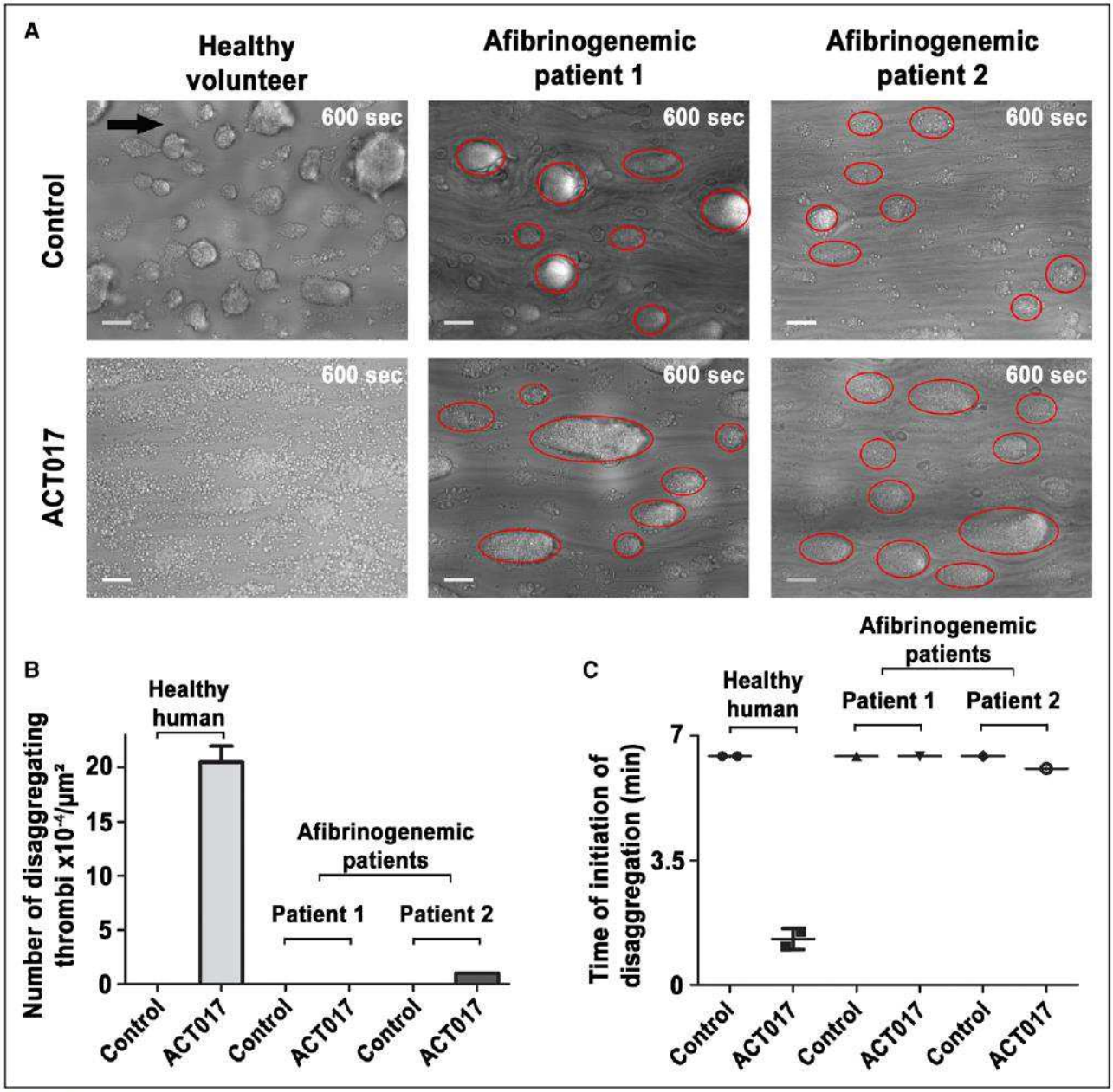

Figure 5. ACT017-induced thrombus disaggregation depends on fibrinogen.
Hirudinated (100 U/mL) human whole blood from healthy volunteers or patients suffering from afibrinogenemia was perfused for 3 min at 750 s^{-1} through microfluidic flow chambers coated with a solution of type I fibrillar collagen (200 µg/mL) to preform aggregates, before perfusing blood containing ACT017 (50 µg/mL) or control buffer for 7 min. **A**, Representative differential interference contrast microscopy images of thrombi subjected to ACT017 or control buffer. The black arrow indicates the flow direction. Scale bars represent 20 µm. **B**, Bar graphs represent the number of disaggregating thrombi ($\times 10^{-4}$)/µm² (n=2). **C**, Scatter plots represent the time at which disaggregation was initiated, expressed in minutes; ACT017: healthy human: $20.5\times10^{-4}\pm1.5\times10^{-4}$ disaggregating thrombi/µm²; afibrinogenemic patient-1 and 2: $0.0\times10^{-4}\pm0.0\times10^{-4}$ disaggregating thrombi/µm²; (n=2).

combining a thrombolytic strategy with a blockade of GPVI could dissolve mural thrombi lodged in the vascular bed.

DISCUSSION

In this study, based on the in silico model of thrombus dynamics, we propose that under conditions of arterial blood flow, thrombus dynamics is sensitive to platelet activation state which determines the critical platelet-platelet forces within the thrombus. A decrease in platelet activation level or an increase in the blood flow, result in platelet disaggregation in the numerical model. This in silico result is perfectly in line with the disaggregating effect of ACT017 observed in vitro, which is expected to lower platelet activation state within the thrombi and

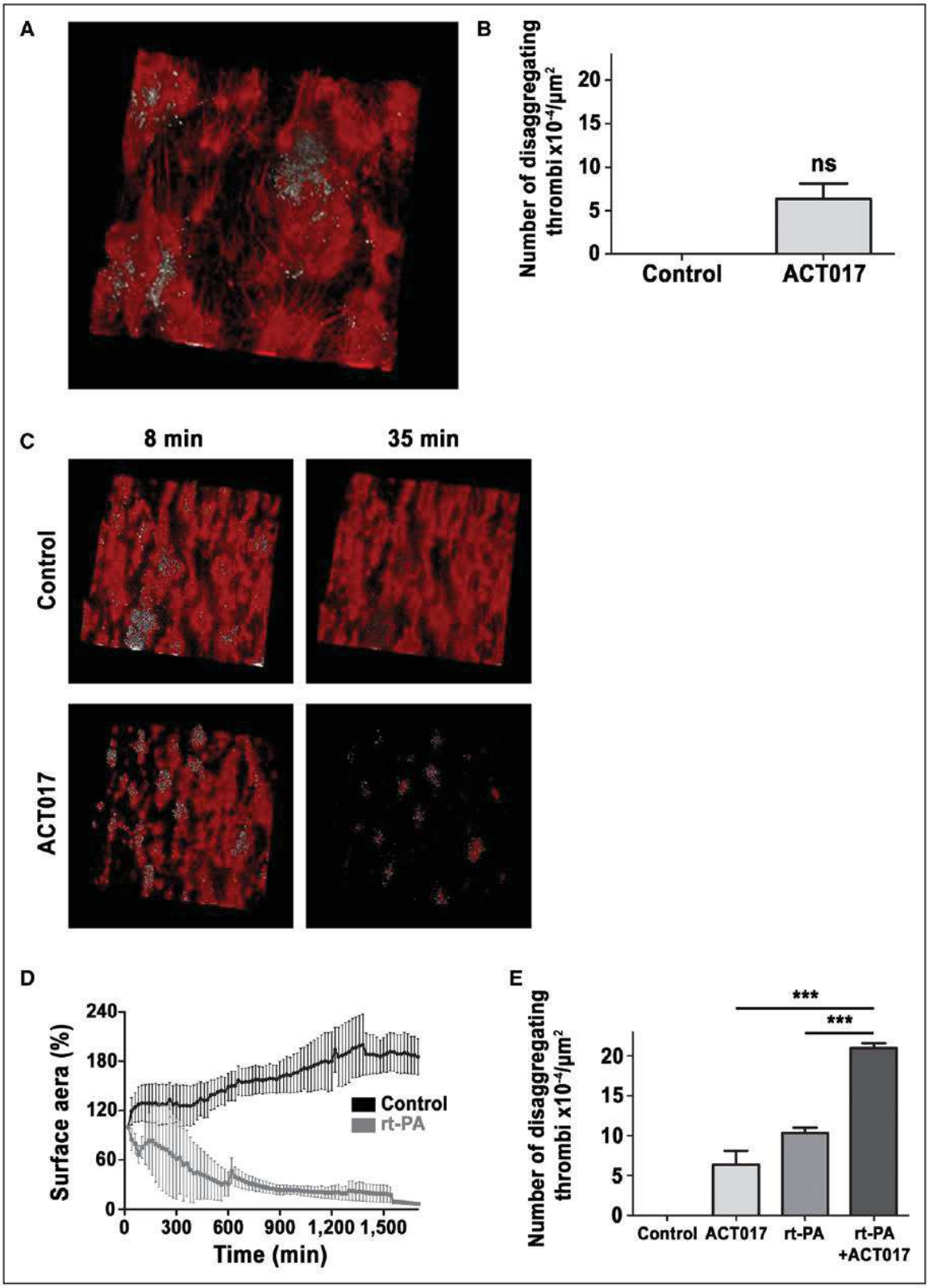

Figure 6. Effect of ACT017 and r-tPA (recombinant tissue plasminogen activator) on disaggregation of fibrin-rich clots. Citrated (3.2%) human whole blood was labelled with a DyLight 650-coupled anti-fibrin antibody (10 µg/mL) and was recalcified by adding $CaCl_2$ (12.5 mmol/L) and $MgCl_2$ (3.5 mmol/L) just before perfusing it through collagen coated microfluidic flow chambers at 1500 s^{-1}. Once platelet-rich thrombi were formed, a brief washing step was performed to rinse the thrombi before perfusing platelet-poor plasma (PPP) obtained from the same donor (hirudinated blood) in the presence of ACT017 (50 µg/mL) or r-tPA (50 µg/mL) alone or in combination, at 3000 s^{-1} for 35 min. **A**, Representative 3-dimensional (3D) reconstructed confocal microscopy image of thrombi. Fibrin is stained in red and platelets in gray. (*Continued*)

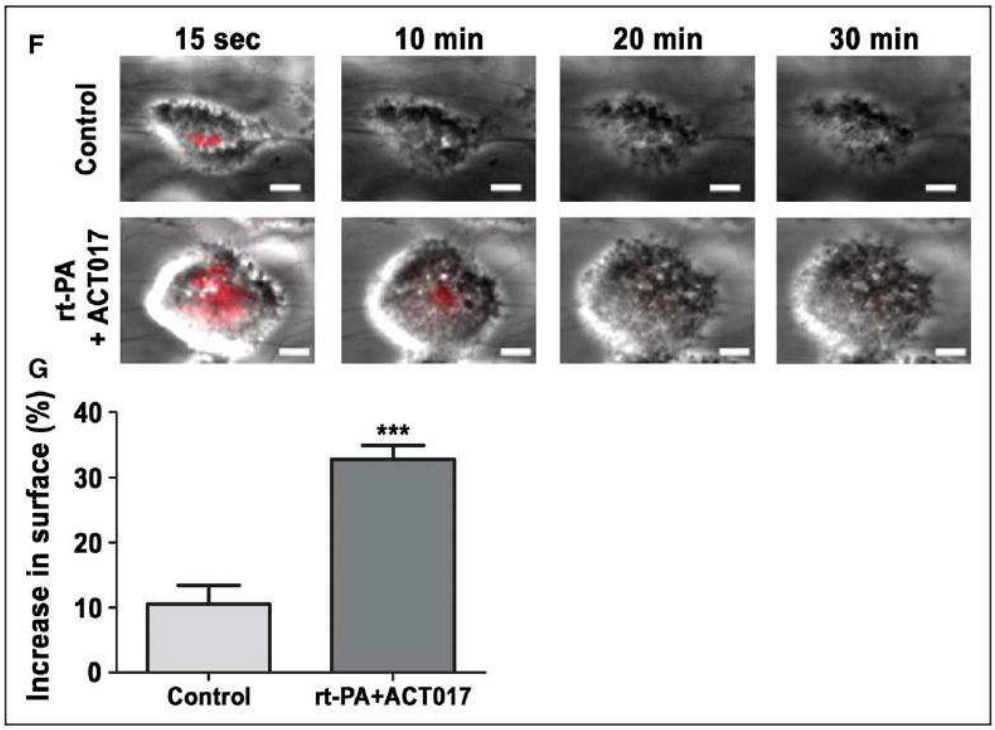

Figure 6 Continued. **B**, Bar graphs represent the number of disaggregating thrombi ($\times 10^{-4}$)/µm² (n=3). Mean±SEM; nonparametric Mann-Whitney *U* test; ns denotes not significant. **C**, Representative 3D reconstructed confocal microscopy image of thrombi subjected to r-tPA or control buffer diluted in PPP. **D**, Connecting line graphs represent the surface area coverage of fibrin (expressed as percentage of control) over time (n=3); mean±SEM. **E**, Bar graphs represent the number of disaggregating thrombi ($\times 10^{-4}$)/µm² (n=3). Mean±SEM; Bonferroni multiple comparison test; ***P<0.001. **F** and **G**, Citrated (3.2%) human whole blood was labelled with a DyLight 650-coupled anti-fibrin antibody (10 µg/mL) and was recalcified by adding $CaCl_2$ (12.5 mmol/L) and $MgCl_2$ (3.5 mmol/L) just before perfusing it through collagen and TF-coated microfluidic flow chambers at 1500 s^{-1}. **F**, Representative bright field/epifluorescence microscopy images of r-tPA pretreated thrombi subjected to ACT017 or control buffer diluted in PPP. Fibrin appears in red. **G**, Bar graphs represent increase in surface occupied (expressed in percentage) by the disaggregating thrombi (n=3). Mean±SEM; χ^2 test with CI of 95%; control: 10.5±2.9% increase in surface area; ACT017: 32.5±2.0% increase in surface area ***P<0.01; n=3.

with the fact that this disaggregating effect increases with the higher values of blood flow (WSR). This study demonstrates that platelet activation is not a short-lived process which aims only to activate integrins to allow bond formation, but represents a long-lasting process that maintains strong inter-platelet bonds.

Most of the experiments performed in this study have been based on perfusions of anticoagulated blood in flow chambers which represents a limitation, as no thrombin is generated under such conditions and, therefore, differs from an in vivo situation. On the contrary, such a situation could mimic the part of the thrombus named the shell, in which it has been reported that no significant amount of thrombin is found based on the absence of fibrin.

We have recently reported that blockade of GPVI impairs the growth of a thrombus that had already formed most likely by preventing platelet activation and, therefore, aggregation.[7] We proposed that this effect was linked to an inhibition of GPVI/fibrinogen-mediated platelet activation, as platelets from GPVI-deficient patients were unable to spread on fibrinogen. The importance of GPVI/fibrinogen interaction in thrombus formation is further evidenced in the present article through the use of blood from afibrinogenemic patients. First, we confirmed that perfusing fibrinogen-poor blood over collagen formed thrombi, through a mechanism proposed to rely on VWF/αIIbβ3 interaction.[32] We observed that while ACT017-induced disaggregation of control thrombi, it had little effect on thrombi formed with blood from 2 afibrinogenemic patients. Importantly, ACT017 exhibited almost no effect despite the fact that such thrombi were looser than control and, therefore, more prone to disaggregate.[33] This result suggests that the effect of GPVI blockade on thrombus disaggregation could be linked to its interplay with fibrinogen. Moreover, observation of thrombus disaggregation in real-time clearly shows that platelets detaching are the one on the top of the thrombus, that is, those interacting with fibrinogen, and never those attached to the surface through strong interaction with collagen.

It is well accepted that absence or blockade of GPVI prevents thrombus formation in vitro and in several in vivo models of experimental thrombosis.[11,12] These results underscore a potential beneficial role of anti-GPVI agents in preventing arterial thrombosis, notably during percutaneous coronary intervention. Indeed, this procedure leads

to exposure of highly reactive fibrillar collagen which initiates platelet activation and thrombus formation through GPVI. Therefore, blocking GPVI in such a setting now stands as a very promising antithrombotic strategy. Anti-GPVI agents could also be beneficial in patients with an ongoing thrombosis in addition to their use in prevention since we recently reported that an anti-GPVI blocking agent, ACT017, limits thrombus growth once a thrombus had already formed.[7] The results presented in this article extend these findings, showing that anti-GPVI agents promote disaggregation of thrombi, which was unexpected. This disaggregating effect was observed on fibrin-poor thrombi, which mimic the thrombus shell, representing the prominent part of the thrombus. This suggests that anti-GPVI agents could destabilize the shell, thereby preventing vessel occlusion. Additionally, we showed that thrombi rich in fibrin, a condition that mimics the core of the thrombus, are also destabilized by ACT017 when used in combination with r-tPA. The disaggregating effects of anti-GPVI agents used in combination with r-tPA on fibrin-rich thrombi opens new avenues for the use of such agents in clinical settings where a thrombus had already formed, such as during acute phases of myocardial infarction or stroke. This adds new arguments to previous observations that mice immunodepleted for GPVI were protected in a stroke model, in favor of a beneficial effect of anti-GPVI antibodies in stroke.[35]

This study presented the results obtained exclusively from experiments in vitro with human blood. In this model, human whole blood is perfused over fibrillar collagen and forms a mural thrombus which is likely to mimic a pathological situation encountered during arterial thrombosis. We aimed to confirm these observations in vivo. As ACT017 blocks human but not mouse GPVI, we used a mouse strain in which murine GPVI was genetically replaced by its human counterpart.[36] To our surprise, ACT017 did not promote disaggregation of preformed mouse platelet thrombi in vitro, indicating that GPVI is not important to maintain the integrity of mouse thrombi (article in preparation). For these reasons, evaluating the effect of blocking human GPVI on thrombus stability during the initial phase of thrombus growth could not be achieved in a mouse. This is not the only example of major differences between platelets of the 2 species, other being related to the thrombin receptors and the presence of the immunoreceptor tyrosine-based motif receptor, FcγRIIa (Fc gamma receptor IIA).

Dimeric GPVI-Fc, unlike the 2 distinct blocking anti-GPVI Fab fragments ACT017 and 1G5, did not promote thrombus disaggregation. There is strong experimental evidence that dimeric GPVI-Fc inhibits collagen-induced platelet aggregation in vitro and ex vivo. GPVI-Fc also reduces experimental thrombosis probably through impairing GPVI interaction with collagen exposed at site of injury.[37,38] The reason why GPVI-Fc has no effect on thrombus disaggregation could have 2 explanations. First, direct blockade of GPVI with an antibody has been proposed to be more efficient than using a competition approach based on GPVI-Fc in static and flow experiments.[34,35] Second, we reported that dimeric GPVI-Fc does not bind fibrinogen, which is the ligand of GPVI in a growing thrombus,[7] and therefore, it is expected that such an agent would have no effect. This observation has important clinical implications as ACT017 and GPVI-Fc have both successfully completed phase I and are currently being evaluated in phase II clinical trials.[39,40] Here we describe that direct blockade of GPVI with Fab fragments, but not with GPVI-Fc, promotes disaggregation of both fibrin-poor and fibrin-rich thrombi. Our data suggests that such agents should be evaluated in trials of patients with ischemic stroke treated with r-tPA. This is especially relevant since anti-GPVI agents do not appear to impact hemostasis and so carry a reduced bleeding risk when compared with other antiplatelet agents.[3]

ARTICLE INFORMATION

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Disclosures

M. Jandrot-Perrus is the founder of Acticor Biotech. C. Gachet is the cofounder of Acticor Biotech. The other authors report no conflicts.

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SUPPLEMENTAL MATERIALS

Pharmacological blockade of GPVI promotes platelet thrombus disaggregation in the absence of thrombin

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Running title: Role of GPVI in thrombus stability

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Figure count: 2 supplemental figures, and 2 supplemental table

References: 7

DOI [to be added]

I. Supplemental Methods

Material

Fatty acid-free human serum albumin (HSA) and fibrillar type-I collagen (ASC) from bovine achilles tendon were provided by Sigma-Aldrich (Lyon, France). Glutaraldehyde was from Electron Microscopy Sciences (Euromedex, Souffelweyersheim, France). PP2, PRT-060318, TGX-221 and U73122 were obtained from Sigma-Aldrich (Lyon, France), Merck Millipore (Ile-de-France), Adooq Biosciences (Nanterre, France) and Calbiochem (Darmstadt, Germany) respectively. Recombinant GPVI-Fc and Fab fragment of antibody to human GPVI 3J24.2 were obtained from Inserm U1148, Paris. ACT017 was provided by Acticor Biotech (Hôpital Bichat, Paris) [1]. The purified Fab fragment of 1G5 antibody against human GPVI was generated using standard laboratory methods as previously described [2]. Recombinant tissue plasminogen activator (r-tPA), Alteplase was from Boehringer Ingelheim (Ingelheim am Rhein, Germany); recombinant hirudin was from Transgene (Illkirch-Graffenstaden, France). The anti-fibrin antibody 59d8 was a generous gift from Dr. C. Dubois (Inserm, Marseille, France). We coupled it to DyLight™ 650 using a kit from Thermo scientific (Illkirch-Graffenstaden, France).

Computational fluid dynamics (CFD) and fluid-structure interaction analysis

In order to analyze distribution of the internal mechanical stress throughout the thrombi, von Mises stress was calculated. Von Mises stress is proportional to the square root of the second invariant of the deviatoric stress tensor:

$$\sigma_v = \sqrt{\frac{1}{2}[(\sigma_1 - \sigma_2)^2 - (\sigma_3 - \sigma_2)^2 - (\sigma_1 - \sigma_3)^2]},$$

where $\sigma_1, \sigma_2, \sigma_3$ are the principal stresses of the Cauchy stress tensor.
Von Mises yield criterion (implying that yielding occurs when local mechanical distortion energy reaches the critical value) is widely used for the analysis of the critical stress of the ductile materials and is also applied for characterization of biomechanical stress in vessel walls, including atherosclerotic plaques [3, 4].

In the fluid-structure interaction model, the absolute value of inlet pressure was chosen to provide the exact value of wall shear rate, given that the outlet pressure is zero. This simplification results in rather low values of mechanical stress in the vessel wall, which thus does not correspond to the artery, but rather to a vein.

Computational model of thrombus formation: additional information

We have built a 2-dimentional particle-based model of thrombus formation, where each platelet is represented as a round-shaped particle with radius R = 1μm. The dynamics of platelets satisfy the equations of motion:

1) $m\vec{a} = \vec{F}$ - second law of motion (m – platelet mass, $\vec{a}$ – platelet acceleration, $\vec{F}$ – total force acting on a platelet)

DOI [to be added]

2) $\dot{L}_z = J_z \frac{d\omega_z}{dt} = M_z$ ($\vec{L}$ – angular momentum (L_z – z-component of $\vec{L}$), $J_z = \frac{1}{2} mR^2$ – moment of inertia taken about z axis, ω_z – rate of the platelet rotation, $\vec{M}$ (M_z – z-component of $\vec{M}$) – torque, R – platelet radius)

We take into account platelet interaction with the flow, platelet-platelet interaction, platelet-injury interaction. The injury is modelled as the sequence of already adhered platelets.

Let $\vec{F}_{ij}$ be the force between i- and j- particles (acting on the i-particle), $\vec{r}_i$ - platelet radius-vector, $\vec{r}_{ij} = \vec{r}_i - \vec{r}_j$ – vector between platelet centers. The state of the i-platelet is also characterized by the variable α_i – the extent of activation, which is described below. The model takes into account platelet interaction via GPIb-VWF and αIIbβ3-fibrinogen.

GPIb – mediated interaction is illustrated in supplemental figure IIA. It is modelled by stochastic springs of initial length L_0.

Each pair of platelets has a probability to create a bond within the current time step – dt (Supplemental Figure IIA, 1, 2):

$$p_{as} = \begin{cases} dt * k_{0\,as}(\alpha_i, \alpha_j), \left(|\vec{r}_{ij}| < 2R + L_0\right) \\ dt * k_{0\,as}(\alpha_i, \alpha_j) e^{-c_{as}\left(|\vec{r}_{ij}| - 2R - L_0\right)^2}, \left(|\vec{r}_{ij}| \geq 2R + L_0\right) \end{cases}$$

Here $k_{0\,as}$ is a bond creation rate, c_{as} – constant, which describes the dependence of bond creating probability on the distance between platelets. Bond creation rate depends on extents of platelets activation - α_i, α_j. The more activated they are, the greater is a probability to create the bond.

Once the bond is created, its ends are fixed on platelets surfaces (Supplemental Figure IIA, 2, 3). The bond is described as a Hookean spring: it can stretch, so that the force between platelets is proportional to the bond elongation:

$$\vec{F}_{ij}^{stoch.} = \vec{F}_{ij}^{GPIb} = k_{ST}\left(\left|\vec{l}_{ij}\right| - L_0\right)\frac{\vec{l}_{ij}}{\left|\vec{l}_{ij}\right|}$$

where k_{ST} – spring constant, $\left|\vec{l}_{ij}\right|$ – length of the elongated spring.

Each spring can also break (Supplemental Figure IIA, 3, 4). Probability of the spring rupture within the current time step (dt) is described by Bell's law:

$$p_{dis} = \begin{cases} dt * k_{0\,dis}, |\vec{l}_{ij}| \leq L_0 \\ dt * k_{0\,dis} e^{\beta |\vec{F}_{ij}^{GPIb}|}, |\vec{l}_{ij}| > L_0 \end{cases}$$

where β is a constant that describes the dependence of bond rupture rate on the tensile force. Stochastic springs model parameters were inferred using experiments addressing platelet interaction with vWF–coated surface [5]. We obtained average platelet rolling velocity and mean stop and go times dependencies on shear rate consistent with experiment (Kaneva et al., submitted to Biophysical Journal; under revision).

The $\alpha_{IIb}\beta_3$ – mediated interaction is described by short-ranged Morse potential:

$\vec{F}_{ij}^{\alpha_{IIb}\beta_3} = \alpha_i \alpha_j \vec{F}_{ij}^{M}(|\vec{r}_{ij}|)$,

DOI [to be added]

where α_i, α_j – extents of activation,

$$\vec{F}_{ij}^{M}(|\vec{r}_{ij}|) = F_{max} = A_M e^{-\lambda_M(|\vec{r}_{ij}| - 2R)}\left(e^{-\lambda_M(|\vec{r}_{ij}| - 2R)} - 1\right)\frac{\vec{r}_{ij}}{|\vec{r}_{ij}|}$$

where λ_M A_M – constants (Supplemental Figure IIB)

Constants were chosen so that the force acted only at the small distances (< 1 μm – characteristic platelet size). The maximum force equals 2-3 nN. The value of the maximal force was estimated based on the data of atomic force microscopy inferred for strongly activated platelets [6].

In our model, activation is a time-dependent process: the more time a platelet has spent in the thrombus, the more activated it is. As we mentioned above, the state of the *i*th-platelet is described by the variable α_i – the extent of activation:

$\alpha_i = \frac{\alpha_{max}}{(\frac{t_{act}}{t_i})^2+1}$,

where t_{act} – is an activation time, t_i- time that *i*th-platelet has spent in the thrombus, α_{max} – maximum level of platelet activation in the thrombus shell (Supplemental Figure IIC). The value of α_{max} is less than 1, meaning that we describe only initial stages of thrombus formation at which platelets are only slightly activated.

Parameters are shown in supplemental Table 2. The model of interplatelet interaction is described in more details in (Kaneva et al., submitted to Biophysical Journal; under revision).

Platelet-flow interaction has been described previously [7]. Briefly, if platelet dynamics results in the change of the thrombus structure (e.g. attachment or detachment of the new platelets, significant shape change), then the CFD module is initialized. All platelets of the thrombus are considered as solid impermeable barriers with no-slip boundary condition on their contours. To account aggregate permeability for the fluid, the effective hydrodynamic radius of these platelets is considered two times smaller than the interaction radius (R=1 μm, mentioned above). The continuity equation and Navier–Stokes equations is solved numerically using the Open-FOAM simpleFoam solver.

Model restrictions are the following:

1) We are using a 2-dimensional model. Computational domain may be interpreted as a longitudinal section of the flow chamber or a blood vessel – in a two-dimensional case there is no difference in terms of geometry.

2) We only consider platelets. Platelet margination is modelled by a non-uniform platelet distribution at the inflow of the computational area. The direct influence of the red blood cells is not taken into account, so we can only model small arterial platelet-rich thrombi.

3) The platelet activation results only in the change of inter-platelet force: both resting and activated platelets are round-shaped in the model. The model does not take into account platelet degranulation or thrombus contraction. We only consider thrombus shell, where platelets are weakly activated and their shape is changed to a small degree.

4) Platelet activation is a time-dependent process – the more time platelet has spent in the thrombus, the higher are the forces acting between platelets. All the platelets become

DOI [to be added]

activated identically, regardless of their distance from the activator. It is valid to model small thrombi with low platelet packing density (like in the shell), in which the diffusion is not hindered, and agonist distribution is relatively homogeneous.
5) We do not consider fibrin formation, which takes place in the thrombus core.
6) We do not model the attachment of the first layer of platelets, but assume them to be adhered from the very beginning of the simulation. Therefore, we ignore some effects in the first layers of the thrombus.

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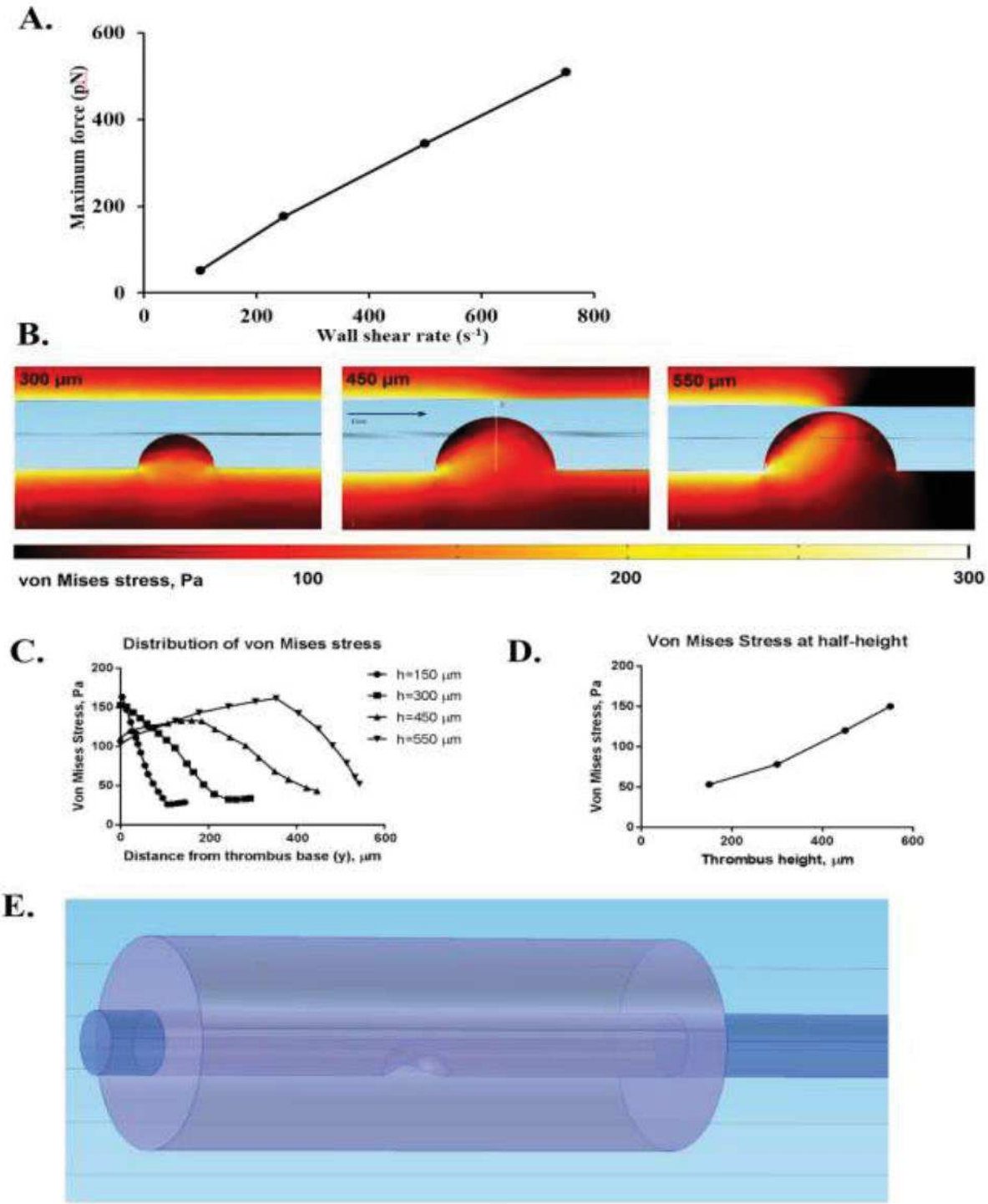
DOI [to be added]

II. Supplemental Figures

Supplemental Figure I

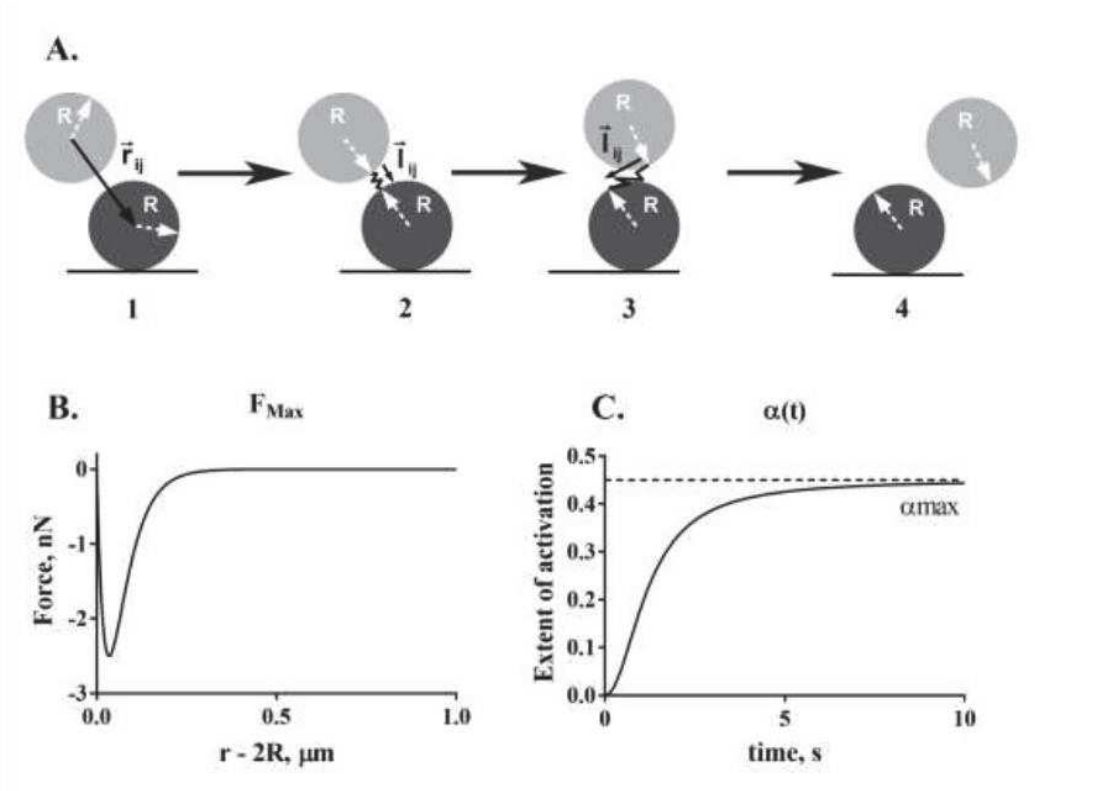


DOI [to be added]

Supplemental Figure I. *In silico* data on inter-platelet interaction forces in a 2D model and internal mechanical stress in a 3D model. (A) The dependence of maximum inter-platelet bond extension force on wall shear rate in a 2D model. The maximum force was reached at the thrombus base and plotted as a function of wall shear rate for a thrombus of 26 μm in height (15 platelet layers, see Figure 1D). (B) Von Mises stress distribution throughout the central section of the 3D thrombus. (C) Distribution of von Mises stress throughout the central axis of thrombus. The four connecting curves correspond to three thrombi of 150, 300, 450 and 550 μm height. (D) Dependence of the von Mises stress in the middle of the thrombus on thrombus height. (E) Schematic representation of the 3D model. Outer cylinder representing subendothelium media (R= 1,000 μm) surrounds smaller cylinder representing inner part of the vessel (r=300 μm). The length of the external cylinder was 5 mm, while the length of the inner vessel was 30 mm. The side surface of the external cylinder was considered fixed during simulation (zero displacement boundary conditions). Thrombus was considered as hemi-spherical protrusion into the vessel (the center of the sphere was located on the surface of the inner cylinder). Both external cylinder and hemisphere were considered as isotropic elastic material with the same parameters (see Supplemental Table 1).

DOI [to be added]

Supplemental Figure II



Supplemental Figure II. Platelet interaction in the *in silico* model of thrombus formation. (A) GPIb-mediated interaction is described by stochastic spring that form when platelets are close to each other (1, 2). The ends of the spring are fixed on the surfaces of the interacting platelets, the force is proportional to the bond elongation (2, 3). The bond can be broken, and the probability of bond rupture depends on interplatelet force as described in the text above. (B) The curve describes the dependence of the integrins-mediated force on the distance between platelets (Morse potential) (C) The dependence of the extent of platelet activation on the time that platelet has spent in the thrombus.

DOI [to be added]

III. Supplemental Tables

Supplemental Table I. Parameters of the CFD and fluid-structure interaction models.

Expression	Value	Units	Description
R	1000	μm	External cylinder radius
r	300	μm	Internal cylinder radius
L	5000	μm	External cylinder length
l	30 000	μm	Internal cylinder length
μ	3.4	mPa * s	Dynamic viscosity of blood
P	300	Pa	Pressure at the inlet (outlet pressure was zero)
E	$2 *10^5$	Pa	Young modulus of external media and thrombus
η	0.45		Poisson ratio
ρ	1000	kg/m^3	Density of blood and elastic material

DOI [to be added]

Supplemental Table II. Parameters of platelet interaction model.

Parameter	Value
m – platelet mass	$4 * 10^{-15}$ kg
R – platelet radius	1 μm
H – vessel height (height of computational domain)	35 μm
X – length of computational domain	200 μm
k_{ST} – stochastic spring constant	30 $\frac{pN}{\mu m}$
L_0 – spring equilibrium length	0.05 μm
c_{as} – constant, characterizing the dependence of the stochastic spring creation probability on the distance between platelets	10 μm^{-2}
k_0 – maximal rate of bond creation (fully activated platelets)	9 ms^{-1}
$k_{0\,dis}$ – rate of bond dissociation with no force applied	17.1 s^{-1}
β – constant, characterizing the dependence of the bond rupture probability on the applied force	1.358 nN^{-1}
λ_M – constant, characterizing the dependence of integrins-mediated force on the distance between platelets	20 μm^{-1}
A_M – constant, characterizing the maximum integrins-mediated force between activated platelets	10 nN
α_{max}– maximum level of platelet activation in the thrombus shell	0.45
t_{act} – activation time	1.2 s

DOI [to be added]

Conclusion

The *in silico* modeling showed that the mechanical stress applied to a thrombus increases dramatically as a thrombus grows, and that strong inter-platelet interactions are essential to maintain its stability. Using a flow-based assay coupled to real-time video-microscopy, I demonstrated that pharmacological blockade of GPVI with the Fab fragments ACT017 or 1G5 promoted efficient disaggregation of human thrombi pre-formed on fibrillar collagen or on human atheromatous plaque material. We observed that ACT017-induced disaggregation was achieved under arterial blood flow conditions (300 s^{-1}), but not under low flow, and that its effect increased with wall shear rate (>300 - 750 s^{-1}). My results indicated that thrombus disaggregation might be linked to the impairment of GPVI-mediated platelet activation, as evidenced by 1) a loss in thrombus contraction when GPVI was blocked; 2) the absence of the disaggregating effect of an anti-GPVI agent when the thrombi were fully activated with soluble agonists. We also observed an absence of disaggregation upon blocking GPVI of afibrinogenemic patients' thrombi, which form independently of fibrinogen through αIIbβ3 bonds with vWF. This observation is in line with the view that thrombus disaggregation induced by ACT017 relies on GPVI interaction with fibrinogen.

From a pharmacological standpoint, I provided evidence that dimeric GPVI-Fc, unlike the 2 distinct blocking anti-GPVI Fab fragments ACT017 and 1G5, did not promote thrombus disaggregation *in vitro* which indicates that direct blockade of the receptor, but not a competition approach, promotes disaggregation of pre-formed platelet thrombi. Finally, my work showed that platelet disaggregation of fibrin-rich thrombi was achieved with ACT017 when used in combination with rtPA and that it was superior to rtPA used alone. This effect of combining an anti-GPVI agent along with rtPA opens new avenues for the use of such agents in the clinical manifestation of ischemic stroke in which rtPA is already used. In conclusion, this work highlights a role for GPVI/fibrinogen interplay in maintaining thrombus stability and refers that targeting GPVI in patients could promote thrombus disaggregation.

Comparison of the role of human and mouse GPVI in thrombus build-up and stability

Publication-2:

Differential role of GPVI in mouse and human thrombus progression and stability

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* Equal contribution

Introduction

In 2018, my research laboratory reported that the GPVI/fibrinogen interaction promotes platelet activation which participates in thrombus growth (Mangin et al., 2018). In this manuscript, we also observed that human GPVI, but not mouse GPVI promotes platelet activation on fibrinogen in a static adhesion assay. I have then reported in a publication in 2020, that this GPVI/fibrinogen-mediated activation is also important for thrombus stability. This work has been based on human blood used in an *in vitro* assay. In the frame of this work, we planned to perform *in vivo* experiments but had first to confirm with mouse blood, the observations we made *in vitro* using human blood.

For this study, we used the same *in vitro* system as the one described in my first publication (Ahmed et al., 2020), to study the role of mouse GPVI in thrombus stability. In addition, we also evaluated the role of mouse GPVI in thrombus build-up. For that, we used an assay in which mouse blood was perfused over immobilized collagen and used confocal microscopy to measure thrombus volume. While this work was supposed to confirm the contribution of mouse GPVI in thrombus growth and stability and generate the data necessary to start *in vivo* experiments, we identified a species difference related to GPVI function.

This work, on which I signed as a co-first author, was accepted as a letter to the Editor in *Thrombosis and Haemostasis* in September 2020.

Differential Role of Glycoprotein VI in Mouse and Human Thrombus Progression and Stability

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Thromb Haemost

Introduction

Platelet glycoprotein (GP) VI is a promising, safe, antithrombotic target as its absence or blockade prevents in vitro thrombus formation and experimental thrombosis in various animal models without impacting the tail-bleeding time.[1] In addition, patients with a mutation in the GPVI gene exhibit only a mild bleeding diathesis,[2] further suggesting that GPVI does not play a critical role in hemostasis. GPVI is the main platelet activation receptor for collagen and viewed as being important in the initiation of thrombus formation.[1] In addition to collagen, GPVI interacts physically or functionally with other adhesive proteins including laminins, fibrin, and fibrinogen.[3–7] We have shown that human GPVI activates platelets on immobilized fibrinogen and that this process is key for the progression and stability of human thrombi.[7,8] In sharp contrast, we observed that mouse GPVI does not promote such an activation, as platelets deposited on fibrinogen do not fully spread.[7] In this study, we investigated the consequence of absence of GPVI/fibrinogen-mediated platelet activation in mice on the regulation of thrombosis in comparison to the human system. It is important to appreciate species difference between humans and mice as the latter represent the most broadly used animal model to study experimental thrombosis and that a significant part of our current understanding of the molecular mechanism of thrombosis relies on experiments performed with these animals.

Results and Discussion

To investigate the role of GPVI in mouse thrombus build-up, beyond its role as a collagen receptor, we used a flow-based assay. We preformed thrombi by perfusing hirudinated whole blood of wild-type (WT) mice over immobilized type-I fibrillar collagen for 1 minute at 300 s^{-1} and stained platelet aggregates with $DiOC_6$ (green). We next perfused hirudinated blood from WT or $GPVI^{-/-}$ mice for 6 minutes at 300 s^{-1} which were stained with the anti-GPIbβ antibody RAM.1-A647 (red) to visualize thrombus progression. Three-dimensional reconstructed confocal microscopy images showed that the second population of red platelets formed thrombi on the top of the first population of green aggregates similarly with $GPVI^{-/-}$ and WT blood (►**Fig. 1A**). This was confirmed by measuring thrombus volume, which indicated that the aggregates formed with $GPVI^{-/-}$ mouse blood presented a similar volume to WT thrombi (WT: $5.7 \pm 0.9\,\mu m^3/\mu m^2$; $GPVI^{-/-}$: $5.1 \pm 0.6\,\mu m^3/\mu m^2$, $p > 0.05$; ►**Fig. 1B**). This result demonstrates that mouse GPVI does not play a critical role in thrombus build-up beyond its role as an initial collagen receptor and is in sharp contrast with the key role played by human GPVI.[7] Moreover, this result also implies that murine thrombus progression in the absence of thrombin in mice primarily occurs via GPVI-independent activation pathways, notably those relying on soluble agonists such as thromboxane A2 and adenosine diphosphate (ADP; ►**Fig. 1C**). These in vitro observations are consistent with in vivo results showing that following $FeCl_3$ injury of the carotid artery, which does not expose subendothelial proteins such as collagen, thrombus build-up is largely independent of GPVI.[9]

* *These authors contributed equally to this work.*

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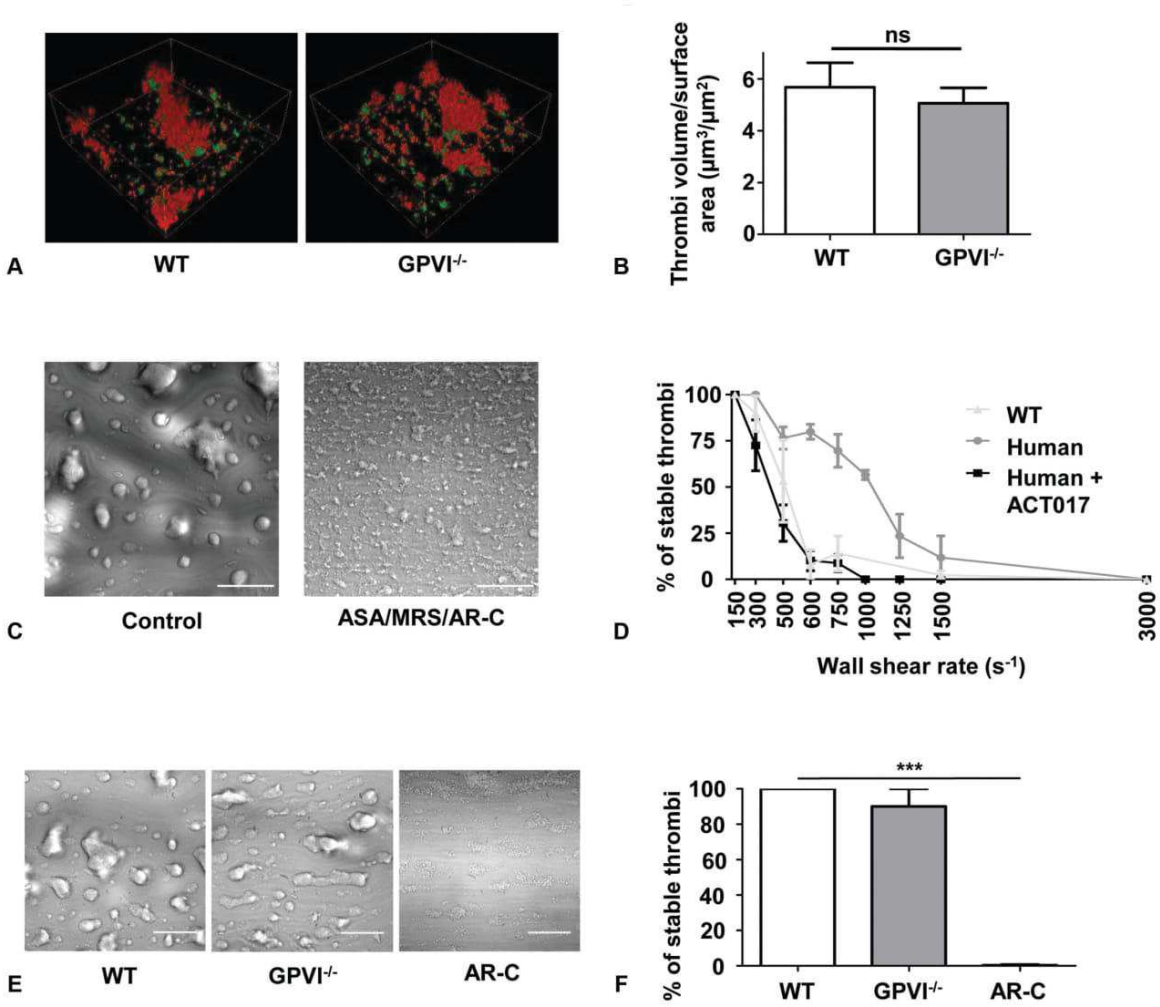

Fig. 1 GPVI plays a key role in human but not in mouse thrombus progression and stability. (**A**) Representative 3D reconstructed confocal images of wild-type (WT) and GPVI-deficient ($GPVI^{-/-}$) mouse thrombi obtained after perfusing hirudinated whole blood stained with RAM.1-A647 2 µg/mL (in *red*) over WT aggregates for 6 minutes at 300 s^{-1}. The preformed thrombi were obtained by perfusing WT mice hirudinated whole blood stained with $DiOC_6$ 1 µmol/L (in *green*) over fibrillar collagen for 1 minute at 300 s^{-1}. (**B**) Quantification of WT ($n = 6$) and $GPVI^{-/-}$ ($n = 7$) thrombus volume by confocal microscopy. (**C**) Representative DIC images obtained after perfusing WT mice hirudinated whole blood for 5 minutes at 300 s^{-1} and washed with PBS for 3 minutes at 300 s^{-1}. For the ASA/MRS/ARC69931MX (AR-C) condition, WT whole blood was treated with 1 mM of aspirin, 10 µM of MRS 2500, and 10 µM of AR-C, 10 minutes before perfusion. Scale bar represents 50 µm. (**D**) The curves represent the percentage of stable thrombi obtained 8 minutes after perfusion of PBS over WT mouse ($n = 4$) and human ($n = 3$) aggregates at indicated wall shear rates. Thrombi were preformed by perfusing human or WT mouse hirudinated whole blood over collagen. For the ACT017 condition, 50 µg/mL of ACT017 was perfused over human thrombi at indicated wall shear rates. (**E**) Representative DIC images obtained 8 minutes after perfusion of PBS at 300 s^{-1} over preformed aggregates. Thrombi were preformed by perfusing WT mice hirudinated whole blood over collagen, followed by the perfusion of WT or $GPVI^{-/-}$ whole blood. For the ARC69931MX (AR-C) condition, 10 µM of AR-C in PBS was perfused over preformed WT aggregates. Scale bar represents 50 µm. (**F**) Quantification of the percentage of stable thrombi of WT ($n = 5$), $GPVI^{-/-}$ ($n = 5$) ,and WT + AR-C ($n = 5$) after perfusing PBS with or without AR-C over preformed aggregates for 8 minutes at 300 s^{-1}. All the experiments were done with PDMS flow chambers coated with type I fibrillar collagen at 200 µg/mL. Results are presented as mean ± standard error of the mean (SEM). Ns $p > 0.05$, $^{***}p < 0.001$. Data were compared using Mann–Whitney and chi-square test. 3D, three-dimensional; DIC, differential interference contrast; GPVI, glycoprotein VI; PBS, phosphate-buffered saline.

We next studied the role of GPVI in thrombus stability and compared this process in humans and mice. Therefore, we preformed aggregates in a similar way to that described for ►**Fig. 1(A, B)**, before testing the thrombus stability by perfusing PBS over the surface. We observed that mouse platelet aggregates were much less stable when compared to human thrombi (►**Fig. 1D**). Indeed, a reduction in thrombus stability in mouse versus human platelet thrombi was already observed at wall shear rates of 300 s^{-1}. This difference became very obvious at 600 s^{-1} where almost all mouse thrombi disaggregated while more than 80% of human platelet aggregates were still stable (►**Fig. 1D**). These results indicate that mouse platelet aggregates are much less stable than their human counterpart in such an experimental setting. In parallel, we observed that the blocking anti-GPVI agent ACT017 reduced the stability of human platelet aggregates to the level of mouse thrombi (►**Fig. 1D**). This result confirms that GPVI-fibrinogen mediated platelet activation in humans markedly increases thrombus stability. In contrast, when we perfused $GPVI^{-/-}$ or WT blood over aggregates formed with WT blood as described

Table 1 Comparision of mouse and human system with focus on GPVI

	Human GPVI	Mouse GPVI
Platelet count	300 ± 150 ($\times 10^3$) platelets/μL	956 ± 38 ($\times 10^3$) platelets/μL
Copy number per platelet	3,000–4,000	4,500–5,500
Structure	• Human and mouse GPVI share a 64% homology • Mouse GPVI cytoplasmic tail lacks the 24 C-terminal residues of human GPVI	
Physiological ligands	Fibrillar collagen Fibrinogen Fibrin Laminin	Fibrillar collagen Fibrin Laminin
Nonphysiological ligands	Convulxin CRP	Convulxin CRP (slightly less reactive)
GPVI shedding	ADAMs family members (ADAMs 10 and 17), physiological and nonphysiological ligands	ADAMs family members and nonphysiological ligands
Signaling pathway	No major differences identified to date (FcRγ chain, Src kinases, Syk, PI3 kinases, LAT, SPLP76, and calmodulin all involved in human and mouse GPVI function)	
Dependency on soluble mediators	Human and mouse GPVI-initiated platelet activation relies on soluble agonists ADP and TxA2 for full activation especially with low collagen concentrations	
GPVI/collagen interaction	Human and mouse GPVI activate platelets through collagen which promotes thrombus formation	
GPVI/fibrinogen interplay	Human GPVI interaction with fibrinogen supports thrombus growth and stability	Mouse GPVI does not interact with fibrinogen
Arterial thrombosis	Under evaluation in phase II studies	• Reduced experimental thrombosis in several models when GPVI is absent/blocked • Protection in an experimental stroke model in GPVI-immunodepleted mice
Hemostasis	Patients with GPVI deficiencies (acquired or genetic) have a modest/no bleeding diathesis. No spontaneous bleeding and no prolonged bleeding time in healthy volunteers treated with anti-GPVI agents (phase I studies)	GPVI- or FcγR-deficient mice • No spontaneous bleeding • No bleeding during surgery • No prolonged tail-bleeding time and normal volume of blood loss when GPVI is absent or blocked

Abbreviations: ADP, adenosine diphosphate; CRP, C-reactive protein; GPVI, glycoprotein VI.

for the data in ►**Fig. 1(A, B)**, we observed that GPVI$^{-/-}$ platelet aggregates were as stable as WT platelet thrombi (►**Fig. 1E**). Quantification confirmed no difference in the number of stable platelet aggregates between GPVI$^{-/-}$ and WT thrombi (WT: $100 \pm 0\%$; GPVI$^{-/-}$: $90 \pm 10\%$; ►**Fig. 1F**). As a positive control, we used ARC69931MX (10 μM), an antagonist of the ADP receptor $P2Y_{12}$, which efficiently destabilized mouse platelet aggregates (►**Fig. 1E, F**). Together, these results indicate that mouse platelet thrombi are less stable than human thrombi and that their stability at low shear does not critically rely on GPVI, whereas GPVI is critical for the stability of human platelet thrombi. We propose that this species difference does limit the relevance of using standard mouse models to study the thrombus stabilizing action of GPVI and limits extrapolation of data obtained on the progression and stability of thrombi in mice to the human system with regard to GPVI.

In conclusion, this study highlights a significant species difference between the human and mouse hemostatic systems with regard to the contribution of GPVI in thrombus progression and stability beyond GPVI's role as a collagen receptor (►**Table 1**). The reason why mouse GPVI does not contribute to thrombus build-up and stability most likely results from an inability to promote platelet activation on fibrinogen. This adhesive protein, found in every layer of a growing thrombus, is very well known to support platelet aggregation through its interaction with integrin αIIbβ3, but also through its interplay with human GPVI to promote and maintain platelet activation. This species difference is critical when judging the importance of GPVI in thrombosis as most of our knowledge is acquired from in vivo experiments performed in mice. This study suggests that the importance of GPVI in cardiovascular events in humans might be more important than previously anticipated based on experiments in mice. This is particularly important to highlight since anti-GPVI agents are currently under evaluation in phase II studies in the setting of acute coronary syndromes and ischemic stroke.

Authors' Contributions

E.J.-B., M.U.A., and N.R. acquired, analyzed, and interpreted the data, and wrote the manuscript; C.M. acquired and analyzed the data; B.N. provided essential tools and contributed to the writing of the manuscript; C.G. and E.E. G. contributed to the writing of the manuscript; M.J.-P. and P.H.M. conceived and designed the research, interpreted the data, wrote the manuscript, and handled funding and supervision.

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Conflict of Interest

M.J.-P.: founder of Acticor Biotech. All other authors have declared that they have no conflict of interest.

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Conclusion

Using a blood flow assay, we perfused hirudinated-treated wild-type (WT) mouse blood over immobilized fibrillar collagen to pre-form thrombi before perfusing blood of WT or GPVI-deficient ($GPVI^{-/-}$) mice. Unexpectedly, 3D reconstructed images indicated no major difference in the size of the thrombi for both conditions. Quantification of thrombus volume of 2^{nd} population confirmed this observation, indicating that mouse GPVI, unlike human GPVI, does not play a key role in thrombus build-up. We next evaluated the role of GPVI in the stability of mouse thrombi under arterial shear conditions. We used a similar experimental design by preforming thrombi and perfusing PBS buffer. We observed that $GPVI^{-/-}$ platelet aggregates were as stable as WT platelet thrombi, which was further confirmed quantitatively by image analysis. This indicates that mouse GPVI is also not playing a major role in the stability of mouse thrombi.

In parallel, we compared the stability of mouse and human thrombi as a function of wall shear rate and observed that human platelet aggregates were more stable than mouse platelet aggregates. When human thrombi were treated with ACT017 to block GPVI, I observed that the thrombi became unstable and reached the stability of mouse thrombi. This results indicates that human GPVI is responsible for the increased stability of human versus mouse thrombi. In conclusion, this study points out species difference between mouse and human in terms of the contribution of GPVI in thrombus progression and stability.

Comparing the role of GPVI and FcγRIIA in platelet activation on fibrinogen and in thrombus build-up and stability

Publication-3:

GPVI, but not FcγRIIA, regulates αIIbβ3-mediated platelet activation on fibrinogen, thrombus build-up and stability

Muhammad Usman Ahmed[1*], Nicolas Receveur[1*], Emily Janus-Bell[1], Clarisse Mouriaux[1], Christian Gachet[1], Martine Jandrot-Perrus[2], Elizabeth E. Gardiner[3], Pierre H. Mangin[1]

(In Preparation)

[*] MUA and NR contributed equally

Introduction

The main platelet receptor for fibrinogen is integrin αIIbβ3, which allows platelet adhesion to immobilized fibrinogen, mediates their activation through outside-in signaling, and promotes platelet aggregation through fibrinogen bonds. The group of Peter Newman reported that when platelets adhere to fibrinogen, αIIbβ3 uses the FcγRIIA signaling pathway to reinforce platelet activation. He also showed that FcγRIIA is important for thrombus growth (Zhi et al., 2013).

As we reported that GPVI regulates platelet activation on fibrinogen as well as thrombus growth and stability, we wondered about the relative importance of GPVI versus FcγRIIA in these processes. The objective of this study was to compare the contribution of FcγRIIA and GPVI in regulating platelet activation events post αIIbβ3-mediated adhesion to fibrinogen and subsequent consequences on thrombus progression and stability.

For this purpose, similar *in vitro* approaches as those described for the 2 previous studies were used. At first, I compared the contribution of GPVI and FcγRIIA by using selective inhibitors in the progression of human thrombi using confocal microscopy to measure thrombi formed *in vitro*. Secondly, I evaluated the differential role of GPVI and FcγRIIA in the stability of human thrombi under arterial shear conditions. Finally, the respective roles of GPVI and FcγRIIA in platelet spreading on fibrinogen were studied by using blocking agents and genetically engineered mice. I have prepared a manuscript on this work.

GPVI, but not FcγRIIA, regulates αIIbβ3-mediated platelet activation on fibrinogen, thrombus build-up and stability

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Running header: Role of FcγRIIA and GPVI in thrombus growth and stability

INTRODUCTION

Upon vessel wall injury, platelets adhere to the lesion site through the GPIb-IX complex and integrins of the β1 and β3 family (1, 2). Activation is enhanced by the action of soluble agonists such as ADP, thromboxane A2 and thrombin and platelet aggregates then form a hemostatic plug which seals the breach (3). Integrin αIIbβ3 plays a central role in platelet aggregation through its ability to bind fibrinogen, allowing the bridging of adjacent platelets. This integrin engagement leads to 'outside-in' signaling which on one hand up-regulates the affinity of resting αIIbβ3 integrins on the same platelet and on the other hand, maintains the molecules already activated in an elevated affinity state for fibrinogen-binding. This process is important to promote thrombus build-up and to maintain the stability of the hemostatic plug (4). Activation of αIIbβ3 through outside-in signaling is known to be less robust compared to that induced by soluble agonists. In addition it has been reported that αIIbβ3 activation is reinforced by the signaling of immunoreceptor tyrosine-based activation motif (ITAM) containing receptors, notably FcγRIIA (5, 6) and more recently GPVI/Fcγ (7, 8). FcγRIIA is a low affinity receptor for IgG found on human but not mouse platelets (9). FcγRIIA appears to contribute to platelet activation on immobilized fibrinogen as well as in thrombus growth (5, 6). While the mechanism remains unclear, it has been proposed that FcγRIIA participates through its signaling cascade and is most likely independent of a direct ligand binding, but as FcγRIIA associates with GPIb-IX and αIIbβ3, the activation might also be the result of a clustering effect. The human collagen receptor GPVI, through its interplay with fibrinogen, has a very similar function and we provided evidence that it ensures both thrombus build-up and stability (7, 8). To date, the relative contribution of these two ITAM-bearing receptors to platelet activation on fibrinogen has never been assessed. In addition, the importance of FcγRIIA and GPVI in the process of the build-up and stability of a thrombus, where fibrinogen is abundant but no other GPVI and FcγRIIA ligand is found, has also never been compared. The aim of this study was to assess the respective contributions of GPVI and FcγRIIA to the growth and stability of human thrombi using an *in vitro* flow based approach and compare respective abilities of these receptors to support platelet activation on fibrinogen. Identification of a receptor that plays a prominent role in the regulation of platelet activation post-integrin αIIbβ3 engagement with fibrinogen within

a thrombus could appear particularly important from a pharmacological standpoint to regulate thrombus growth and stability.

RESULTS AND DISCUSSION

To investigate the respective roles of human platelet FcγRIIA and GPVI to thrombus build-up once an aggregate had been initiated, we used an *in vitro* flow-based assay. We pre-formed aggregates by perfusing hirudinated whole blood from healthy donors over immobilized type-I fibrillar collagen for 90 sec at an arterial wall shear rate of 750 s^{-1} and stained adherent platelets with $DiOC_6$ (green). We then perfused autologous blood treated with the anti-GPIbβ antibody RAM.1-A647 to label platelets (red) in the presence of function-blocking monoclonal antibodies against either FcγRIIA (IV.3) or GPVI (1G5 Fab) for 6 min at 750 s^{-1} to visualize real-time thrombus progression and compare the growth to a control IgG or Fab fragments. 3D reconstructed confocal microscopy images indicated that the second (red) population of platelets formed thrombi over the top of the first population of green aggregates with no difference between blood treated with either IV.3 or a control IgG (**Figure 1A)**. In contrast, blood treated with 1G5 Fab resulted in the formation of smaller thrombi (**Figure 1A)**. This result was confirmed by measuring thrombus volume which indicated that the aggregate volumes were similar in the presence of IV.3 and control, and markedly reduced when 1G5 was compared with a Fab Control (IgG Control: 1.1±0.57 x 10^5 μm^3; IV.3: 1.1±0.9 x 10^5 μm^3, 1G5: 0.6±0.3 x 10^5 μm^3, Fab control: 1.6±0.6 x10^5 μm^3; P<0.05), (**Figure 1B**). Of note, both antibodies were used at concentrations where they efficiently inhibited platelet aggregation in response to an anti-CD9 antibody (activating via Fc engagement of FcγRIIA) for IV.3 or to collagen for 1G5 (**data not shown**). These results suggest that human GPVI plays a more important role in thrombus build-up beyond its role as a collagen receptor, than FcγRIIA.

We next compared the role of FcγRIIA and GPVI in thrombus stability in an *in vitro* perfusion assay. We pre-formed aggregates by flowing human hirudinated blood over collagen for 3 min at 750 s^{-1}, before perfusing PBS for 12 min at 750 s^{-1} over the thrombi in the presence of IV.3 or 1G5. We observed that IV.3 had no effect on human platelet aggregate stability when compared to a control IgG (**Figure 1C**). This

observation was confirmed by a quantification showing that the number of disaggregating aggregates was indistinguishable between thrombi treated with a control IgG and those treated with IV.3 (IgG Control: 2.3±0.6 x 10^{-4} disaggregating thrombi/μm^2; IV.3: 2.3±1.1 x10^{-4} disaggregating thrombi/μm^2; ns, *P*>0.5), (**Figure 1D**). Moreover, the time of initiation of disaggregation was also similar between both conditions (IgG Control: 9.0±0.6 min; IV.3: 9.5±1 min; ns, *P*>0.5), (**Figure 1E**). In sharp contrast, we observed that perfusing PBS containing 1G5 resulted in a marked disaggregation when compared to IV.3 and control Fabs (**Figure 1C**). Quantification confirmed that the number of disaggregating thrombi was significantly elevated with 1G5 and the time taken to disaggregate was shortened when compared to IV.3 and control (Fab Control: 2.3±0.6 x 10^{-4} disaggregating thrombi/μm^2 and 10.1±0.2 min, 1G5: 10.9±0.7 x 10^{-4} disaggregating thrombi/μm^2 and 4.7 ± 0.1 min; IgG Control: 2.3±0.6 x 10^{-4} disaggregating thrombi/μm^2 and 9.0±0.6 min; IV.3: 2.3±1.1 x 10^{-4} disaggregating thrombi/μm^2 and 9.5±1 min; ***P<0.001; **P<0.01), (**Figure 1D and E**). These results indicate that blockade of GPVI, but not of FcγRIIA, promoted efficient and rapid disaggregation of human platelet aggregates.

In parallel, we compared the ability of FcγRIIA and GPVI to support platelet activation on fibrinogen. Human washed platelets were deposited on immobilized fibrinogen (100 μg/mL) in the presence of the blocking agents, IV.3 or 1G5, for 40 min. As expected, epifluorescence images indicated that ReoPro (40 μg/mL), an antibody which functionally blocks integrin αIIbβ3, significantly reduced platelet adhesion (68%), while IV.3 had no effect and 1G5 presented a non-significant tendency of reduced platelet adhesion (**Figure 1F, G**). We observed that 1G5, but not IV.3, markedly inhibited platelet spreading on fibrinogen (IgG Control: 2.4±0.5 x 10^3 platelets/mm^2; IV.3: 3.1±0.5 x 10^3 platelets/mm^2, 1G5: 1.1±0.3 x 10^3 platelets/mm^2, Fab control: 3.9±0.8 x 10^3 platelets/mm^2, Reopro: 0.1±0.2 x 10^3 platelets/mm^2; **P<0.01, ***P<0.001) (**Figure 1F, H**). These results are in agreement with experiments performed with washed mouse platelets expressing human GPVI which were able to spread on fibrinogen, while this was not the case for mouse platelets expressing human FcγRIIA (WT: 0.14±0.05 x 10^3 platelets/mm^2; hGPVI: 5.7±0.5 x 10^3 platelets/mm^2, GPVI$^{-/-}$: 0.034±0.012 x 10^3 platelets/mm^2, FcγRIIA: 0.6±0.1 x 10^3 platelets/mm^2; ***P<0.001) (**Figure 1I-K**). In parallel, we observed that β3-deficient platelets but also wild-type platelets did also not spread on fibrinogen (data not

shown), which is in agreement with the fact that human, but not mouse GPVI activates platelets on fibrinogen (8). Together, these results confirm the key role played by αIIbβ3 in platelet adhesion and activation on fibrinogen and indicate that human GPVI but not human FcγRIIA supports platelet activation and spreading on this surface.

In summary, this study shows that GPVI is more important than FcγRIIA in promoting human platelet activation and spreading onto immobilized fibrinogen. As a consequence, GPVI plays a prominent role compared to FcγRIIA in supporting both thrombus growth and stability, beyond its role as a collagen receptor. The reason why we did not observe an inhibitory effect with IV.3 on platelet spreading as previously reported (5, 6) is unclear. This cannot be linked to the use of a suboptimal concentration as we used a 2-time higher concentration as the one reported. In addition, we demonstrated the inhibitory effect of our IV.3 batch by its ability to inhibit anti-CD9-induced aggregation, known to be mediated via FcγRIIA. Another evidence for a role of FcγRIIA in platelet spreading on fibrinogen in the publication of Boylan et al. (5), came from the use of platelets from a patient presenting a decrease in FcγRIIA levels. Interestingly this patient was also deficient for GPVI, which most likely explains the reduced platelet spreading on fibrinogen, in agreement with our observations. In conclusion, from a pharmacological standpoint, our work suggests that it might be more effective to target GPVI than FcγRIIA, either to prevent thrombus growth or to destabilize an existing thrombus and thereby prevent vessel occlusion.

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AUTHORSHIP CONTRIBUTIONS

M.U.A., N.R., E.J.B. and C.M. acquired and analyzed the data and participated in the writing of the manuscript; C.G. and M.J.P. contributed to the writing of the manuscript; P.H.M. and E.E.G. conceived and designed the research, interpreted the data, wrote the manuscript and handled funding and supervision.

DISCLOSURE OF CONFLICTS OF INTEREST

All other authors have declared that no conflicts of interest exist.

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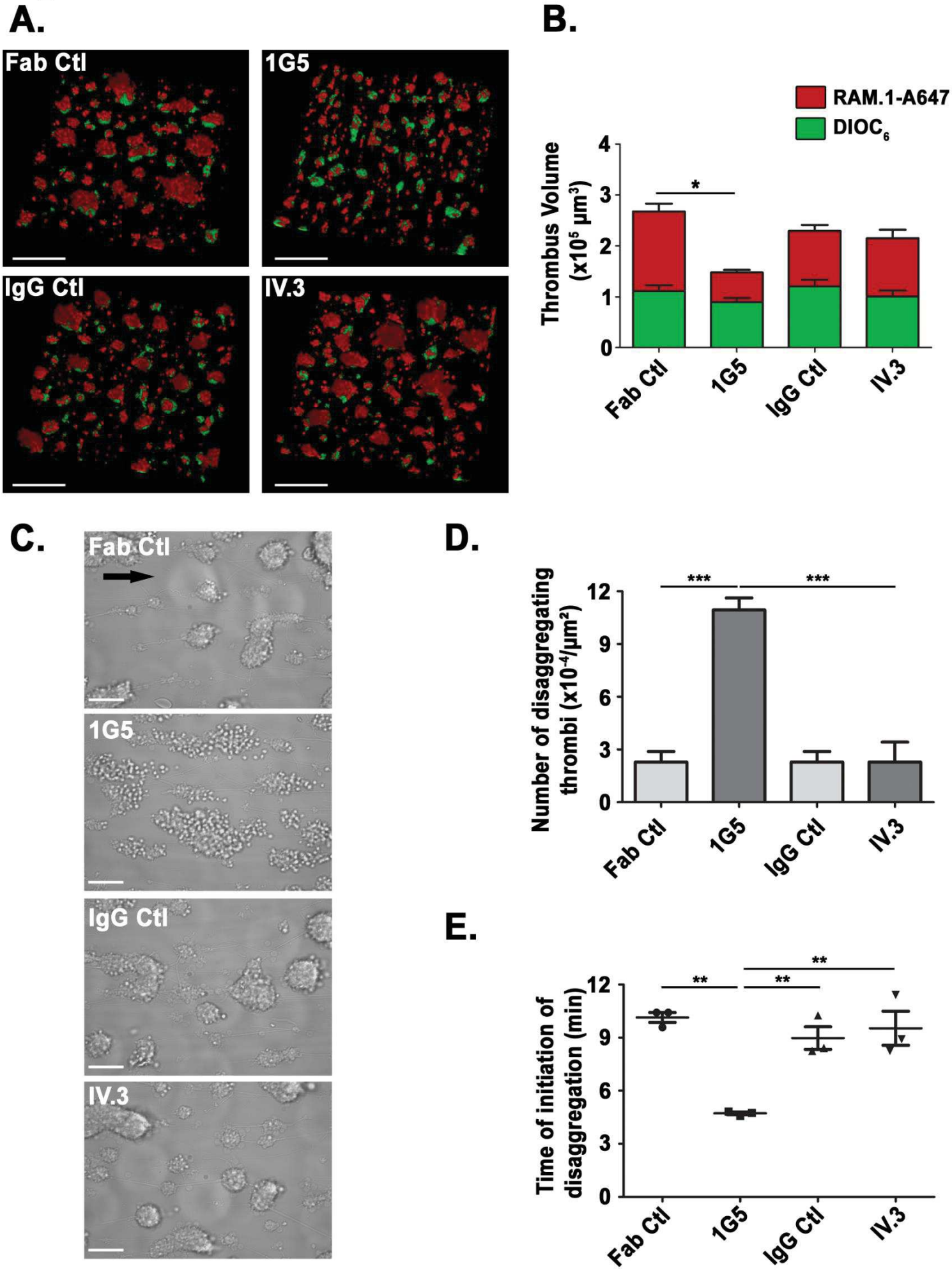
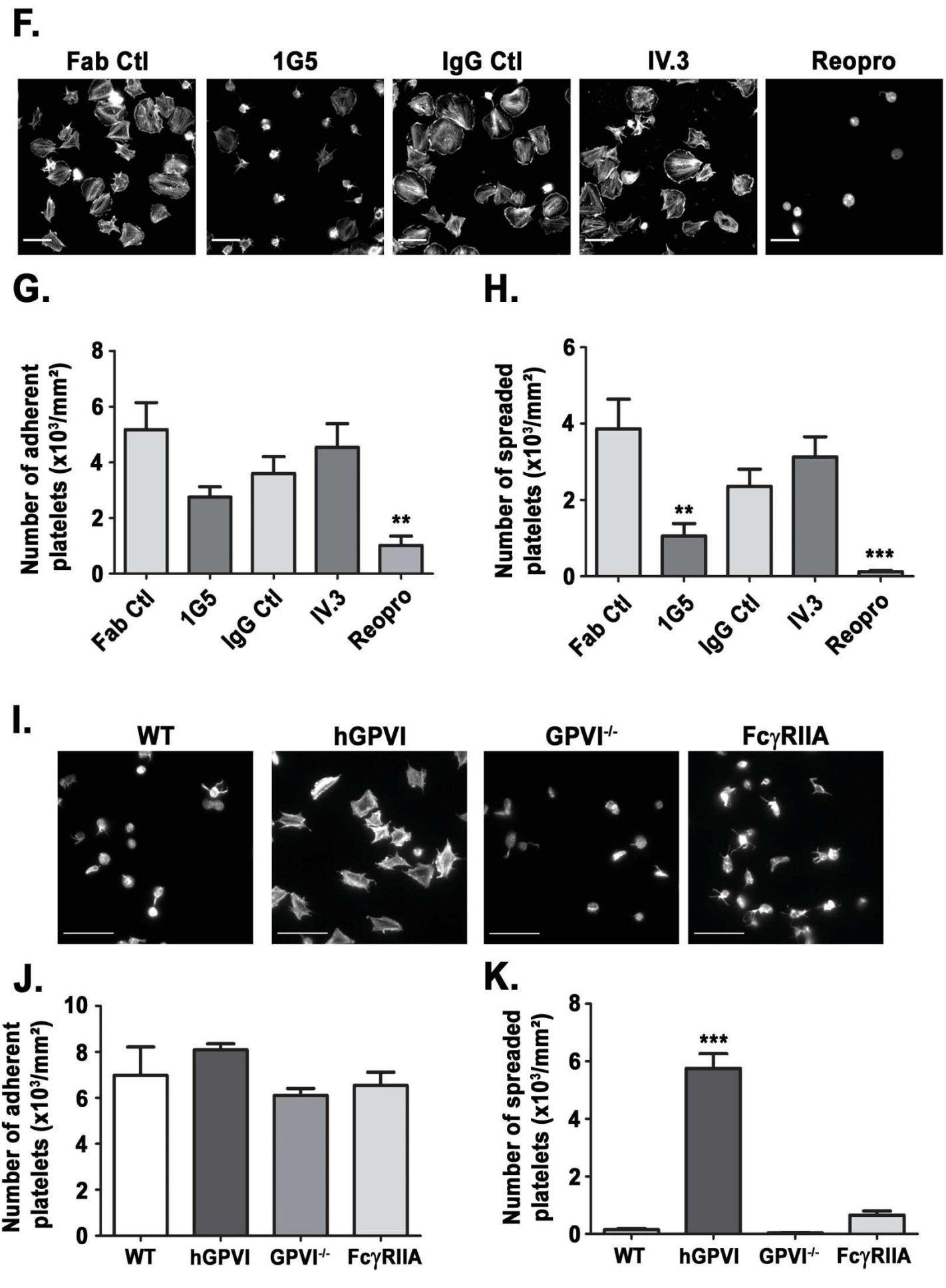
FIGURE LEGEND

Figure 1: GPVI but not FcγRIIA promotes platelet activation on fibrinogen and plays a key role in thrombus progression and stability. A, Representative 3D reconstructed confocal images of human thrombi treated with anti-GPVI or anti-FcγRIIA agents obtained after perfusing hirudinated whole blood stained with RAM.1-A647 2 µg/mL (in red) over human aggregates during 6 min at 750 s^{-1}. The performed thrombi were obtained by perfusing human hirudinated whole blood stained with $DiOC_6$ 1 µmol/L (in green) over fibrillar collagen during 1 min 30 sec at 750 s^{-1}. Scale bar represent 50 µm. **B,** Quantification of human (n = 6) thrombus volume after treating with anti-GPVI or anti-FcγRIIA agent by confocal microscopy. Kruskal-Wallis test and post-hoc Dunn multiple comparison test, **P*<0.05, n=5. **C-E**, Hirudinated (100 U/mL) human whole blood was perfused for 3 min at 750 s^{-1} through microfluidic flow chambers coated with a solution of type I fibrillar collagen (200 µg/mL) to preform aggregates. **C,** Representative differential interference contrast microscopy images of thrombi subjected to anti-GPVI or anti-FcγRIIA agent or IgG control(s) diluted in PBS. The black arrow indicates the flow direction. Scale bars represent 20 µm. **D,** Bar graphs represent the number of disaggregating thrombi ($\times 10^{-4}$)/μm^2; Bonferroni multiple comparison test; ****P*<0.001, n=3. **E,** Scatter plots represent the time at which disaggregation was initiated, expressed in minutes; Bonferroni multiple comparison test; ***P*<0.01, n=3. **F-H,** Washed platelets from healthy human donors treated with anti-αIIbβ3 or anti-FcγRIIA or anti-GPVI agents were allowed to adhere to human fibrinogen for 40 min, and fixed with PFA and stained with Phalloidin-TRITC (2 µg/mL). **F,** Representative epifluorescence images of washed platelets adhering to fibrinogen. Scale bars represent 10 µm. **G,** Bar graph representing the number of platelets adhering to immobilized fibrinogen per mm². Adhesion is expressed as mean±SEM in 8 random fields, in 6 separate experiments (One-way ANOVA, Bonferroni post-hoc test: **P*<0.05, ***P*<0.001, n=6). **H,** Bar graph representing the number of platelets spreading over immobilized fibrinogen per mm². Spreading is expressed as mean±SEM in 8 random fields, in 6 separate experiments (One-way ANOVA, Bonferroni post-hoc test: ***P*<0.001, ****P*<0.001, n=6). **I-K,** Washed platelets from wild-type mice (WT mice) or mice expressing human FcγRIIA (hFcγRIIA mice) or GPVI-deficient mice ($GPVI^{-/-}$ mice) or mice expressing human GPVI (hGPVI mice)

were allowed to adhere to human fibrinogen for 40 min, and fixed with PFA and stained with Phalloidin-TRITC (2 µg/mL). **I,** Representative epifluorescence images of washed platelets adhering to fibrinogen. Scale bars represent 10 µm. **J,** Bar graph representing the number of platelets adhering to immobilized fibrinogen per mm². Adhesion is expressed as mean±SEM in 8 random fields, in 4 separate experiments (one-way ANOVA, Bonferroni post-hoc test: $P>0.05$). **K,** Bar graph representing the number of platelets spreading on immobilized fibrinogen per mm². Spreading is expressed as the mean±SEM in 8 random fields, in 4 separate experiments. Significance was attained using a one-way ANOVA, Bonferroni post-hoc test: ***$P<0.001$.

Figure 1


A.
Fab Ctl
1G5
IgG Ctl
IV.3
B.
RAM.1-A647
DIOC6
Thrombus Volume (x10^5 µm^3)
*
Fab Ctl
1G5
IgG Ctl
IV.3
C.
Fab Ctl
1G5
IgG Ctl
IV.3
D.
Number of disaggregating thrombi (x10^-4/µm^2)
***
***
Fab Ctl
1G5
IgG Ctl
IV.3
E.
Time of initiation of disaggregation (min)
**
**
**
Fab Ctl
1G5
IgG Ctl
IV.3


F.
Fab Ctl
1G5
IgG Ctl
IV.3
Reopro
G.
Number of adherent platelets (x10³/mm²)
0
2
4
6
8
**
Fab Ctl
1G5
IgG Ctl
IV.3
Reopro
H.
Number of spreaded platelets (x10³/mm²)
0
2
4
6
**
***
Fab Ctl
1G5
IgG Ctl
IV.3
Reopro
I.
WT
hGPVI
GPVI-/-
FcγRIIA
J.
Number of adherent platelets (x10³/mm²)
0
2
4
6
8
10
WT
hGPVI
GPVI-/-
FcγRIIA
K.
Number of spreaded platelets (x10³/mm²)
0
2
4
6
8
***
WT
hGPVI
GPVI-/-
FcγRIIA

Conclusion

Using similar *in vitro* approaches as those detailed previously, I observed that GPVI, but not FcγRIIA plays a key role in the growth of human thrombi formed *in vitro*, since blockade of GPVI with 1G5, but not of FcγRIIA with IV.3 blocked thrombus growth as measured by confocal microscopy. Moreover, I also observed that 1G5, but not IV.3 promoted efficient thrombus disaggregation *in vitro*, indicating that GPVI plays a more important role than FcγRIIA in thrombus stability. These results are in agreement with my observation that platelet spreading on fibrinogen, which is a marker of platelet activation, was more inhibited with 1G5 than with IV.3. This work suggests that GPVI is a more important regulator of platelet activation on fibrinogen than FcγRIIA, and thereby plays a prominent role in thrombus growth and stability.

It is challenging to provide a clear explanation for the differences in the role of FcγRIIA in regulating αIIbβ3 function and thrombosis between our studies and those published by the group of Peter Newman. These differences can probably not be explained by the tools as they are the same in the two studies: firstly, IV.3 to block FcγRIIA and secondly the hFcγRIIA mice. In addition, we ensured that the absence of effect of IV.3 was not explained by a non-functional batch as we observed that this antibody inhibited aggregation induced by an anti-CD9 antibody, known to activate platelets through FcγRIIA. Moreover, we used IV.3 concentrations up to 2 times higher in our adhesion assay as those used by Peter Newman. One strong argument of Peter Newman to involve FcγRIIA in platelet activation on fibrinogen was the use of a patient platelets which presented a marked reduction in the number of FcγRIIA copies on platelets and which failed to spread on fibrinogen. Interestingly, this patient was first identified with auto-antibodies cleaving GPVI, which is likely to explain the defect in platelet spreading on fibrinogen, in line with our observations. Altogether, these findings suggest that GPVI, but not FcγRIIA, is an important regulator of platelet activation on fibrinogen, thrombus growth and stability.

General Discussion and Future Directions

Antiplatelet agents are the cornerstone for the management of arterial thrombosis. They are particularly indicated in patients having ACS, those undergoing PCI and they are also extensively used in the secondary prevention after a first cardio-embolic event. The current standard of care of arterial thrombosis is based on the combination of aspirin with a $P2Y_{12}$ blocker, named as DAPT. This therapeutic approach has significantly lowered the risk of cardiovascular events, myocardial infarction or stroke, as well as the risk of post-stenting thrombosis and recurrent ischemia. The major limitation of current antiplatelet medication is the associated risk of bleeding, which is a concern in the setting of stroke, in which DAPT is not recommended to avoid intracranial hemorrhage. For this reason, investigators in the field of hemostasis and arterial thrombosis are in search of a safe antiplatelet agent which would impair thrombus formation, with almost no impact on hemostasis. The platelet specific receptor GPVI, which is the principal platelet activation receptor for collagen matrix protein, has emerged as a promising safe antithrombotic target. Firstly, because patients having GPVI deficiency experience a mild bleeding if any and GPVI-deficient mice do not present prolonged tail-bleeding time, indicating that GPVI is not absolutely needed to ensure hemostasis. Secondly, because the absence or blockade of GPVI reduces thrombus formation *in vitro* and in several experimental thrombosis models.

The work presented in this thesis is focused on GPVI and aims to further characterize its role in thrombus formation. I have mostly studied the interplay between GPVI and fibrinogen, a newly identified ligand of GPVI. The results led to a publication in *Arteriosclerosis, Thrombosis, and Vascular Biology*, a letter in *Thrombosis and Haemostasis* and another letter that we plan to submit in the upcoming weeks. The main results I obtained are summarized below:

i) Firstly, I showed that pharmacological blockade of GPVI promotes efficient disaggregation of human thrombi pre-formed on collagen or on human atherosclerotic plaque material *in vitro*, identifying an unrecognized role of GPVI in thrombus stability.

ii) Secondly, I identified a species difference between the human and mouse system related to the contribution of GPVI interplay with fibrinogen in thrombus progression and stability.

iii) Finally, I provided evidence that GPVI is a more important than FcγRIIA in regulating platelet activation events post integrin αIIbβ3-mediated adhesion to fibrinogen, and thereby plays a prominent role in thrombus growth and stability.

GPVI has been identified more than 2 decades ago as the principal platelet activation receptor for matrix protein collagen. The historical view is that the GPVI/collagen interaction plays an important role in thrombus formation, mainly based on the fact that the blockade or absence of GPVI prevents experimental thrombosis in several *in vivo* models. However, it has since been reported that GPVI also interacts with additional adhesion proteins such as laminins, fibrin and fibrinogen which could challenge the idea that collagen is the major ligand responsible for GPVI-mediated thrombosis. Today, the relative contribution of collagen versus the other ligands of GPVI in thrombus formation is not known and would require the development of specific inhibitors. However, it is tempting to speculate about the contribution of each of these agonists based on information found in the literature. Concerning fibrillar collagen, there is no doubt that this is the most potent GPVI agonist which supports the formation of large aggregates when whole blood is perfused over it. However, within a context of thrombus formation, fibrillar collagen is mainly found in the deepest layers of the vessel wall and would therefore be exposed only if the vessel injury is profound. Non-fibrillar collagen are found in the first layers of the vessel wall, but such collagens, like collagen type IV, promote a weak activation of platelets through GPVI. Moreover, collagen binds exclusively to the first layer of platelets and therefore only directly activates a very limited number of platelets, even though, as it promotes the release of soluble mediators, it has also an impact on the upper layers. Concerning laminins, they are found in the basement membrane and have been shown to support platelet adhesion and activation through integrin α6β1 with GPVI contributing modestly to the activation step (Inoue et al., 2006). Laminins is a much less potent platelet activator compared to collagen, and one could hypothesize that it could contribute to thrombus formation in the scenario of a weak injury when the endothelial cell layer is removed. Concerning fibrin, *in vitro* studies have shown that it promotes enough activation in a flow model to support aggregation, even though it is clearly not as potent as collagen. Importantly, intravital microscopy showed that fibrin appears in the core of a growing thrombus after several minutes, which means that the GPVI/fibrin

interaction is very unlikely to support thrombus growth, but might be important in thrombus stability (Alshehri et al., 2015a; Mammadova-Bach et al., 2015), however, this has to be demonstrated. My work was focused on the GPVI/fibrinogen interplay. It seems clear that this interaction supports a modest level of platelet activation promoting platelet spreading on a fibrinogen surface. However, from a perspective of thrombus, it is very tempting to speculate that this interaction is important as a thrombus is full of fibrinogen and that therefore a very elevated number of GPVI/fibrinogen bonds can form and sustain a significant signal. In agreement with this view, my lab has shown that the GPVI/fibrinogen interplay is key in thrombus growth (Mangin et al., 2018) and my PhD work has identified a role for this interaction in thrombus stability. Therefore, I would speculate that the GPVI/fibrinogen interaction is important in thrombosis. However, to discriminate its importance relatively to the other ligands, it is of crucial importance to develop specific agents.

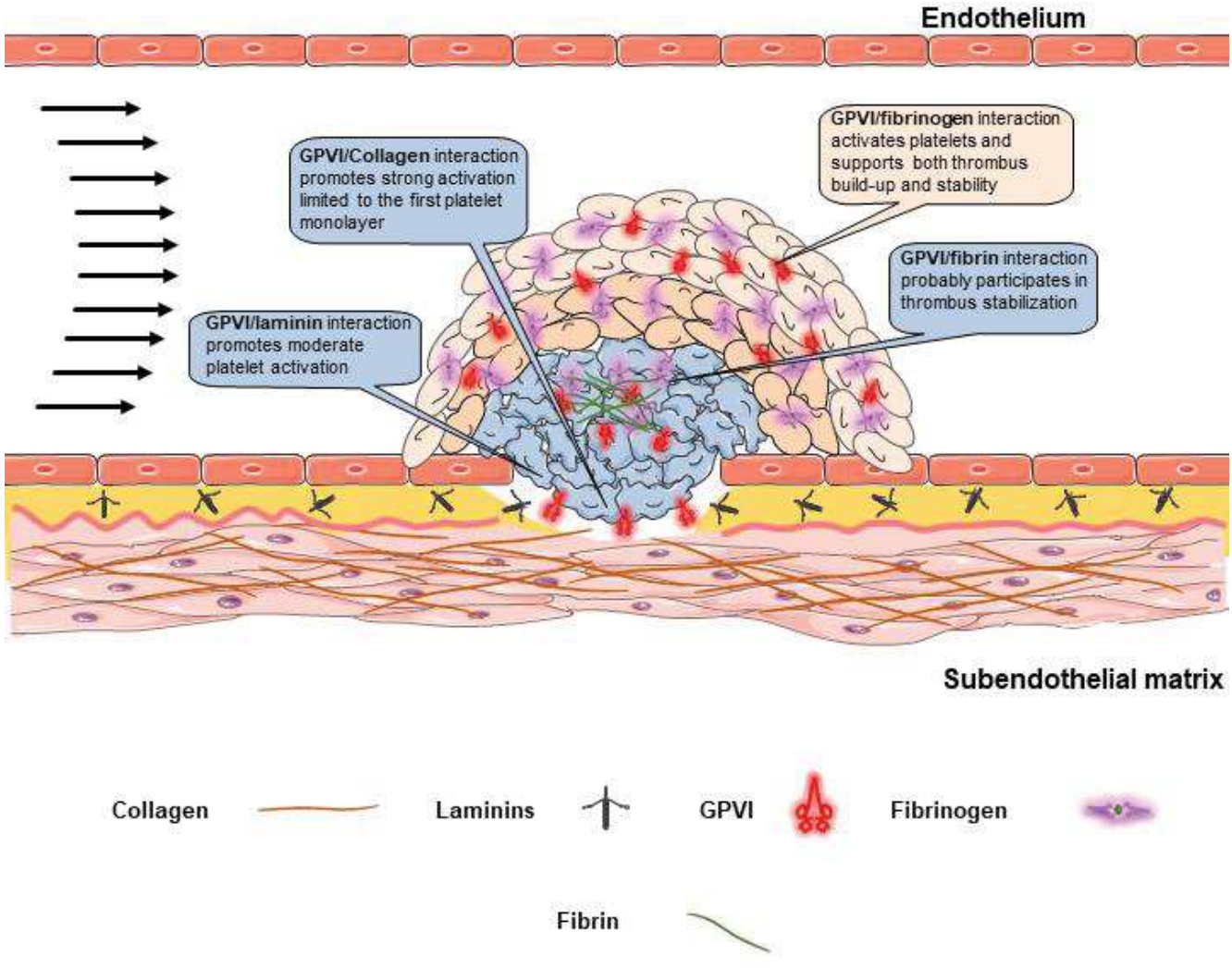


Figure 18. Schematic representation of the interplay of GPVI with different adhesive proteins in regulating thrombosis

Two pharmacological strategies aiming to block GPVI function in clinic have been developed in the last decade. The first is based on a GPVI mimetic and the second on a blocking anti-GPVI antibody. The GPVI mimetic, called Revacept developed by AdvanceCOR, is a soluble molecule composed of two GPVI ectodomains fused to the Fc portion of an IgG (GPVI-Fc). Concerning GPVI blockade, a humanized version of a Fab (ACT017, now called Glenzocimab) with high specificity and affinity to GPVI was developed by Acticor Biotech. Both Revacept and Glenzocimab have successfully passed a phase 1 clinical trial with no significant impact on the bleeding time of healthy volunteers (Ungerer et al., 2011; Voors-Pette et al., 2019) and are currently being evaluated in phase 2 clinical trials in patients with coronary artery disease undergoing PCI or acute ischemic stroke. My PhD work indicated that the blockade of GPVI with Glenzocimab (ACT017), but not the use of GPVI-Fc, promoted efficient disaggregation, which is most likely the result of an impairment of GPVI/fibrinogen interplay. This disparity is probably explained by the fact that Glenzocimab inhibits GPVI/fibrinogen binding, while dimeric GPVI-Fc does not. Indeed, it has been reported that only the monomeric GPVI, but not the dimeric form of GPVI-Fc binds to fibrinogen (Mammadova-Bach et al., 2015). To test this hypothesis in the future, we have established a collaboration with Professor Roberts Ariëns (University of Leeds, UK), who is currently producing an important amount of monomeric GPVI, that I will be testing in the flow assay. Another explanation of the differential effect of Glenzocimab versus GPVI-Fc is that an approach with a blocking antibody is more potent than a competition approach with a soluble molecule. This is demonstrated in two distinct publications showing that dimeric GPVI-Fc prevented less efficiently atherosclerotic plaque-induced platelet aggregation under static and flow conditions and had no effect in preventing platelet adhesion and thrombus formation at the injured arterial wall as compared to anti-GPVI antibodies (Grüner et al., 2005; Jamasbi et al., 2015). In summary, my work indicated that Glenzocimab, but not GPVI-Fc, promotes disaggregation by shutting down activation mediated by the GPVI/fibrinogen interplay and this might represent an advantage of Glenzocimab over Revacept from a therapeutic point of view. The ongoing phase 2 clinical trials might provide some initial clues on that.

One outcome of my PhD thesis was to highlight a species difference related to the contribution of GPVI in the regulation of thrombus formation and stability in humans versus mice. Indeed, my results obtained *in vitro*, showed that mouse platelet thrombi are less stable than human thrombi and that human GPVI was responsible for this difference as its blockade reduced the stability of human thrombi to the level of mouse thrombi. The most likely explanation for this difference relates to an inability of mouse platelets to promote platelet activation on fibrinogen through GPVI. This highlights a new difference between the hemostatic systems of the two species and adds it to a long list including: i) platelet size and count, ii) surface expression level of some receptors and their down-regulation through shedding, iii) heterogeneity in granule content, and iv) differential responses to some agonists notably ADP (Schmitt et al., 2001; Ware, 2004). As mouse models are extensively utilized to investigate the role of platelets in hemostasis and thrombosis, it is important to be aware of these species differences and be cautious when extrapolating results obtained with mice to the human system. Concerning my work, we propose that results of thrombus formation and stability in mouse models underestimate the role of GPVI in these processes as, mouse GPVI, unless human GPVI does not promote platelet activation on fibrinogen.

During my PhD studies I investigated the differential role of GPVI and FcγRIIA in promoting platelet activation on fibrinogen and the consequences on thrombus growth and stability. I demonstrated that the blockade of GPVI, but not of FcγRIIA, profoundly inhibits the platelet activation on fibrinogen and also limits thrombus growth and stability *in vitro*. We were surprised not to observe any inhibition of platelet spreading on fibrinogen when FcγRIIA was absent or blocked, which is conflicting with the work of Prof. Peter Newman's group (Boylan et al., 2008). The absence of effect of IV.3 is not explained by the use of a non-functional batch, as we ensured that it was inhibiting platelet aggregation mediated via FcγRIIA. Moreover, this was also not explained by the use of a suboptimal concentration as we used a twice higher concentration compared to Boylan et al. (Boylan et al., 2008). Therefore, we have a difficult time to explain the differences we observed with IV.3 on platelet spreading to fibrinogen compared to those of Boylan's publication. One additional evidence from Peter Newman's publication supporting a role of FcγRIIA in regulating αIIbβ3 activation came from patient platelets deficient for this ITAM receptor which failed to spread on

fibrinogen. Interestingly, this patient was also deficient in GPVI, which explains the phenotype and makes it impossible to conclude that FcγRIIA is important in the regulation of αIIbβ3-mediated platelet activation. In summary, even though, we cannot fully explain the difference we observed with Peter Newman's publications, our results propose that GPVI is a much more important regulator of platelet activation on fibrinogen compared to FcγRIIA.

Amongst the two anti-GPVI agents currently evaluated in phase 2 clinical trials, Revacept has already been shown to reduce the number of new peri-interventional infarctions in the brain by 46% as compared to placebo in patients with symptomatic stenosis of the internal carotid artery (NCT01645306, December 2019). It is currently evaluated in another phase 2 clinical study in patients with coronary artery disease undergoing PCI (NCT 03312855; EudraCT 2015-000686-32). Concerning Glenzocimab, it is evaluated in an acute ischemic stroke interventional study (ACTIMIS) in patients with acute ischemic stroke (NCT03803007). One could speculate whether these indications are optimal for anti-GPVI agents or whether they would be more efficient in other settings. Pre-clinical studies in flow chambers and *in vivo* thrombosis models have shown that the anti-thrombotic effect of anti-GPVI agents might not be as efficient as other agents, notably αIIbβ3 or $P2Y_{12}$ blockers. My PhD work demonstrated that GPVI blockade had a limited effect on disaggregating a fibrin-rich thrombus compared to a fibrin-poor thrombus. One could therefore speculate that an anti-GPVI agent would be more efficient on a "fresh" thrombus as those formed on stents after angioplasty. While the DAPT prevents efficiently stent thrombosis, it is associated with an increased risk of bleeding which is problematic in the setting of stroke. One would therefore propose that using an anti-GPVI agent to prevent carotid stent thrombosis appears as an exciting indication which would need to be tested experimentally before launching clinical trials.

In conclusion, my work highlighted the significant contribution of the interplay of GPVI/fibrinogen in thrombus formation and stability. This interaction could profoundly contribute in thrombosis and represent a novel anti-thrombotic strategy which would help to disaggregate a thrombus already formed in an artery and prevent the formation

of new thrombi. The relative importance to the GPVI/fibrinogen interplay in platelet activation in a growing thrombus versus other pathways is still not fully understood and to further characterize it, specific antagonists should be developed in the future.

Supplementary Data

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              10         20         30         40         50
Human MSPSPTALFC LGLC.LGRVP AQSGPLPKPS LQALPSSLVP LEKPVTLRCQ
Mouse MSPASPTFFC IGLCVLQVIQ TQSGPLPKPS LQAQPSSLVP LGQSVILRCQ

              60         70         80         90        100
Human GPPGVDLYRL EKLSSSRYQD QAVLFIPAMK RSLAGRYRCS YQNGSLWSLP
Mouse GPPDVDLYRL EKLKPEKYED QDFLFIPTME RSNAGRYRCS YQNGSHWSLP

             110        120        130        140        150
Human SDQLELVATG VFAKPSLSAQ PGPAVSSGGD VTLQCQTRYG FDQFALYKEG
Mouse SDQLELVITG VYAKPSLSAH PSSAVPQGRD VTLKCQSPYS FDEFVLYKEG

             160        170        180        190        200
Human DPAPYKNPER WYRASFPIIT VTAAHSGTYR CYSFSSRDPY LWSAPSDPLE
Mouse DTGPYKRPEK WYRANFPIIT VTAAHSGTYR CYSFSSSSPY LWSAPSDPLV

             210        220        230        240        250
Human LVVTGTSVTP SRLPTEPPSS VAEFSEATAE LTVSFTNKVF TTETSRSITT
Mouse LVVTGLSATP SQVPTEESFP VTESSRRPSI LP...TNEIS TTEKPMNITA

             260        270        280        290        300
Human SPKESDSPAG PARQYYTKGN LVRICLGAVI LIILAGFLAE DWHSRRKRLR
Mouse SPKGLSPPIG FAHQHYAKGN LVRICLGATI IIILLGLLAE DWHSRKKCLQ

             310        320        330
Human HRGRAVQRPL PPLPPLPQTR KSHGGQDGGR QDVHSRGLCS*
Mouse HRMRALQRPL PPLPLA*
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Figure 19. Amino acid sequence of human and mouse GPVI

The amino acid sequences of human and mouse GPVI share 64.4% identity. The difference of mouse GPVI amino acid sequence from human is highlighted in red. Adopted from (Jandrot-Perrus et al., 2000)

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Rôle de l'interaction GPVI/fibrinogène dans l'activation plaquettaire et dans la formation et la stabilisation des thrombi

Résumé

La GPVI, un membre de la superfamille des immunoglobulines, est exprimée uniquement dans les plaquettes et les mégacaryocytes. Comme les patients déficients en GPVI souffrent d'une diathèse hémorragique très légère et que les souris déficientes en GPVI ne présentent pas un temps de saignement de la queue prolongé, la GPVI n'est pas considérée comme absolument essentielle pour l'hémostase. À l'inverse, l'absence ou le blocage du GPVI inhibe la formation de thrombi in vitro et dans plusieurs modèles expérimentaux de thrombose. Pour ces raisons, la GPVI est considérée comme une cible anti-thrombotique potentiellement sûre. L'objectif de mon travail de doctorat était de mieux caractériser le rôle de l'interaction GPVI/fibrinogène dans la formation d'un thrombus. Les principaux résultats que j'ai obtenus sont les suivants : i) le blocage pharmacologique de la GPVI favorise une désagrégation efficace des thrombi humains préformés sur du collagène ou sur du matériel de plaques d'athérome humain *in vitro*, ce qui a permis d'identifier un rôle non reconnu de la GPVI dans la stabilité des thrombi, ii) l'identification d'une différence d'espèce entre l'homme et la souris liée à la contribution de l'interaction de GPVI avec le fibrinogène dans la progression et la stabilité des thrombi et iii) enfin, j'ai fourni des preuves que la GPVI est plus important que FcγRIIA dans la régulation de l''activation plaquettaire sur le fibrinogène, et joue ainsi un rôle prépondérant dans la croissance et la stabilité des thrombi.

Mots clés : Plaquettes, hémostase, thrombose, GPVI

Résumé en anglais

GPVI belongs to the immunoglobulin superfamily. It is specifically expressed on platelets and megakaryocytes. Since GPVI-deficient patients suffer from a very mild bleeding diathesis and GPVI-deficient mice do not present a prolonged tail-bleeding time, GPVI is not absolutely required for hemostasis. Conversely, absence or blockade of GPVI inhibits thrombus formation in vitro and in several experimental thrombosis models. For these reasons, GPVI is considered as a potentially safe anti-thrombotic target. The aim of my PhD work was to characterize the role of the GPVI/fibrinogen interplay in thrombus formation. The main results I obtained are: i) pharmacological blockade of GPVI promotes efficient disaggregation of human thrombi pre-formed on collagen or on human atherosclerotic plaque material in vitro, identifying an unrecognized role of GPVI in thrombus stability, ii) identified a species difference between the human and mouse system related to the contribution of GPVI interplay with fibrinogen in thrombus progression and stability and iii) Finally, provided evidence that GPVI is more important than FcγRIIA in regulating platelet activation events post integrin αIIbβ3-mediated adhesion to fibrinogen, and thereby plays a prominent role in thrombus growth and stability.

Keywords: platelet, hemostasis, thrombosis, GPVI