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Understanding the role of damage-associated molecular patterns Heme and S100A8/A9 in platelet function

By

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Abstract

Platelets are small blood cell fragments, derived from the megakaryocytes, essential for adequate haemostasis at the site of vascular injury, but also drivers of pathological processes such as thrombosis. In the context of both sterile and infection-driven inflammation, platelets interact with immune and endothelial cells supporting immunothrombosis which can subsequently lead to thromboinflammation and organ damage. The contribution of platelets is not solely dependent on their classical activation and aggregation, but also relies on the development of different platelet subpopulations including procoagulant platelets. Those drive different pathways fuelling thromboinflammation. The mechanisms leading to platelet activation and the formation of procoagulant platelets under pathogenic conditions remains poorly understood.

Damage-associated molecular patterns (DAMPs) are danger molecules released by injured and/or activated host cells which bind and activate pattern recognition receptors on different cells. Once secreted extracellularly, DAMPs drive both immunothrombosis and thromboinflammation. Moreover, multiple DAMPs have been proposed as biomarkers of immunothrombosis and their plasma levels correlate with disease severity. In this thesis, we propose that the two DAMPs, S100A8/A9 and heme, which are primarily released from neutrophils and damaged erythrocytes respectively, lead to direct platelet activation and support immunothrombosis and thromboinflammation.

We identify S100A8/A9-GPIb axis as a novel pathway mediating platelet activation. We show that S100A8/A9 levels are elevated in the plasma of patients with COVID-19

and sustained high levels correlate with adverse outcome. We demonstrate that S100A8/A9 induces non-classical platelet activation and the formation of procoagulant platelets accelerating fibrin generation at venous shear. However, S100A8/A9 does not induce platelet aggregation. The effect of S100A8/A9 on platelets is regulated by the platelet adhesion receptor GPIbα with a supportive role for CD36. These data shed light on a new immune-driven mechanism of thrombosis.

We next identify heme as a novel agonist leading to platelet activation. We describe that the mechanism driving platelet aggregation is regulated by the levels of free heme whereby low concentrations induce classical aggregation whilst high concentrations induce platelet death and agglutination. At low concentrations, as observed in patients with haemolytic diseases, heme induces platelet activation and aggregation through the receptor CLEC-2, independent of heme-driven oxidative stress. These data identify a new mechanism by which haemolysis induces platelet activation and aggregation through CLEC-2. We demonstrate that both S100A8/A9 and heme-mediated platelet activation are resistant to classic anti-platelet drugs targeting cyclo-oxygenase and P2Y12 and raise the need to develop new therapies to limit the effect of these DAMPs on platelets.

Taken together, these results highlight the importance of heme and S100A8/A9 in platelet activation and immunothrombosis. These data underline the importance of finding novel molecules or repurposing known compounds to complement current anti-platelet therapies and address the immune- and damage-driven thrombotic pathways regulating immunothrombosis.

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Dedicated to Me.

For all the times I felt behind and wrong,

For all the times I had to justify myself, my decisions, my actions,

For all the times a smile was hiding unseen need.

Stop fighting against myself was the way to get back to myself

Grateful for every lesson this journey has brought to my life.

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“There is a crack in everything, that’s how the light gets in” **— Leonard Cohen**

“Words are windows, or they’re walls, They sentence us, or set us free”

— Ruth Bebermeyer- Marshall B. Rosenberg

“Above all, don’t fear difficult moments. The best comes from them”

Rita Levi-Montalcini

“She wasn't looking for a knight. She was looking for a sword”


It's an Immunology thing, you wouldn't understand
Succinates Treat is Soooo Goooood!
HELLO I'M A SCIENTIST
Dr Scientist Martina's Cellular Biology Lab

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Abbreviations

ACD - Acid citrate-dextrose

ADP - Adenosine 5’-diphosphate

AMI - Acute Myocardial Infarction

ANOVA - Analysis of variance

AA - Arachidonic Acid

ARDS - acute respiratory distress syndrome

ATP - Adenosine 5’-triphosphate

AUC - Area under the curve

BAPS ballooned and procoagulant spread

BAPTA-1 AM1,2-bis(o-amino phenoxy)ethane-N,N,N′,N′-tetra acetic acid

BMDMs - Bone marrow-derived macrophages

BNS platelets, ballooned non-spread platelets

BPs - ballooned platelets

BSA - Bovine serum albumin

BSS - Bernard Soulier Syndrome

BTK - Bruton tyrosine kinase

Ca2+ - Calcium

cAMP - Cyclic adenosine monophosphate

CD62P - P-selectin

cGMP - Cyclic guanosine monophosphate

CLEC-2 - C-type lectin-like receptor 2

COX - Cyclooxygenase

CVD - cardiovascular diseases

Cy5 - Cyanine-5

DAG - 1, 2-diacylglycerol

DAMPs - Damage-associated molecular patterns

DIC - Disseminated intravascular coagulation

DMSO - Dimethyl sulfoxide

DNA - Deoxyribonucleic acid

DVT - Deep vein thrombosis

E.G. – exempli gratia = for example

EDTA - Ethylenediaminetetraacetic acid

ELISA - Enzyme-linked immunosorbent assay

EMMPRIN - Extracellular Matrix Metalloproteinase Inducer

EF hand - helix–loop–helix

Fab - Fragment antigen-binding

FBS - Foetal bovine serum

FcR - Fc receptor

FeDP - Fe (III) Deuteroporphyrin IX chloride

FeDME - Fe (III) Protoporphyrin IX dimethyl ester chloride

FeMP - Fe (III) Mesoporphyrin IX chloride

FIB-CAP fibrinogen capped platelets

FITC - Fluorescein isothiocyanate

GFP - Green fluorescent protein

GM-CSF - Granulocyte-macrophage colony stimulating factor

GP - Glycoprotein

GPCR - G-protein coupled receptor

G-protein - Guanine nucleotide-binding proteins

GPVI - Glycoprotein VI

HEPES - N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

hFc-CLEC-2 - Recombinant human CLEC-2-Fc

HIV - Human immunodeficiency virus

HO-1 - Haem-oxygenase 1

HRP - Horseradish peroxidase

HUVECs - Human umbilical vein endothelial cells

ICAM - Intercellular adhesion molecule 1

ICF - Immunocytofluorescent

IFNg - Interferon gamma

IgG - Immunoglobulin G

IHF - immunohistofluorescent

IL - Interleukin

IP3 - Inositol 1, 4, 5-triphosphate

ITIM - Immunoreceptor tyrosine- based inhibitory motif

ITAM - Immunoreceptor tyrosine-based activation motif

IVC - Inferior vena cava

JAK-STAT - Janus kinase-signal transducer and activator of transcription

kDa - Kilo-Dalton

LDL - Low density lipoprotein

LPS - Lipopolysaccharide

LRR - Leucine-rich repeats receptors

MAC-1 (or Mac-1) - Macrophage antigen-1

MAPK - Mitogen-activated protein kinase

M-CSF - Macrophage colony stimulating factor

MFI - Mean fluorescence intensity

MMP – Metalloproteinase

MSD – mechanosensitive domain

MRP-8 and MRP-14 - Myeloid-related protein-8 and 14

MtDNA – mitochondrial DNA

NETs - Neutrophil extracellular traps

NO - Nitric oxide

NOS - Nitric oxide synthase

NSAIDS - Non-steroidal anti-inflammatory drugs

OCS - Open canalicular system

OCT - Optical cutting temperature

OxLDL - Oxidised low-density lipoproteins

PAMPs - Pathogen-associated molecular patterns

PAR - Protease activating receptor

PBS - Phosphate buffered saline

PBS-T - Phosphate buffered saline with 0.1 % Tween 20

PDGF - Platelet-derived growth factor

PECAM-1 - Platelet endothelial cell adhesion molecule-1

PF4 - Platelet factor 4

PGI2 – Prostacyclin

PP IX cobalt - Protoporphyrin IX cobalt chloride

PI3K - Phosphoinositide 3-kinase

PIP3 - Phosphatidylinositol-3, 4, 5-trisphosphate

PKA - Protein kinase A

PKG - Protein kinase G

PLC - Phospholipase C

PPACK - D-Phenylalanyl-L-prolyl-L-arginine chloromethyl ketone

PPP - Platelet poor plasma

PRP - Platelet rich plasma

PS - Phosphatidylserine

PTK - Protein tyrosine kinase

P2Y1/12 - Purinergic receptor P2Y G protein-coupled 1/12

qPCR - Quantitative polymerase chain reaction

RAGE - Receptor for Advanced Glycation End products

rCLEC-2-Fc - Recombinant mouse CLEC-2-Fc

RBC - Red blood cell

REF - Reference

REFs - References

RNA - Ribonucleic acid

ROS - Reactive oxygen species

rpm - Rotations per minute

RPMI - Roswell Park Memorial Institute Medium

RT - Room temperature

SARS - severe acute respiratory syndrome

SCD - Sickle Cell Disease

SCIP - sustained calcium-induced platelets

SD - Standard deviation

SDS-PAGE - Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SEM - Standard error of the mean

SFK - Src family kinase

SH - Src homology 2 domain-containing protein-tyrosine phosphatase-1

SIM - Structured illumination microscopy

SIRS - Systemic inflammatory response syndrome

SLP-76 - SH2 domain-containing leukocyte phosphoprotein of 76 kDa

Syk - Spleen tyrosine kinase

TBS - Tris buffered saline

TBS-T - Tris buffered saline with 0.1 % Tween 20

TF - Tissue factor

TGF-b - Transforming growth factor-β

TLR4 - Toll-like receptor 4

TLR9 - Toll-like receptor 9

TNF-a - Tumour necrosis factor-α

TP - Thromboxane receptors

TxA2 - Thromboxane A2

VCAM - Vascular cell adhesion molecule

VEGF - Vascular endothelial growth factor

VOC - Vaso-occlusive crisis

VWD – Von Willebrand Disease

vWF - von Willebrand Factor

WB - Western blotting

WPB - Weibel-Palade bodies

WT - Wild type

α2A - α2A-adrenergic receptor

CHAPTER 1

General Introduction

In this introduction, an overview about platelet structure and function is given. There will be initially a focus on the two major platelet receptors, GPIb and CLEC-2, as these ones are the cornerstones of Chapter 3 and Chapter 4. Therefore, the concepts of thrombosis and haemostasis will be described including platelet activation, the formation of procoagulant platelets and the mechanisms involved in the coagulation cascade and fibrinolysis. Finally, the concept of thromboinflammation will be introduced including the release of Damage-Associated Molecular Patterns (DAMPs) and their relevance in thrombosis.

1.1 Thrombosis and Haemostasis

The maintenance of blood circulation and fluidity within the vascular system is one of the most important processes in the human body. “Haemostasis” refers to the physiological mechanism leading to the arrest of bleeding at the site of vascular injury. “Thrombosis” is the pathological clot formation that results when the haemostatic mechanism is excessively, inappropriately activated and/or uncontrolled (Rasche, 2001). In 1856, Rudolf Virchow, a renowned Berlin scientist and physician, identified three factors as predisposing causes to venous thrombosis (Virchow’s triad). These factors include alteration in blood flow, vessel wall injury/endothelial damage and blood hypercoagulability (Esmon, 2009) (**Figure 1.1**).

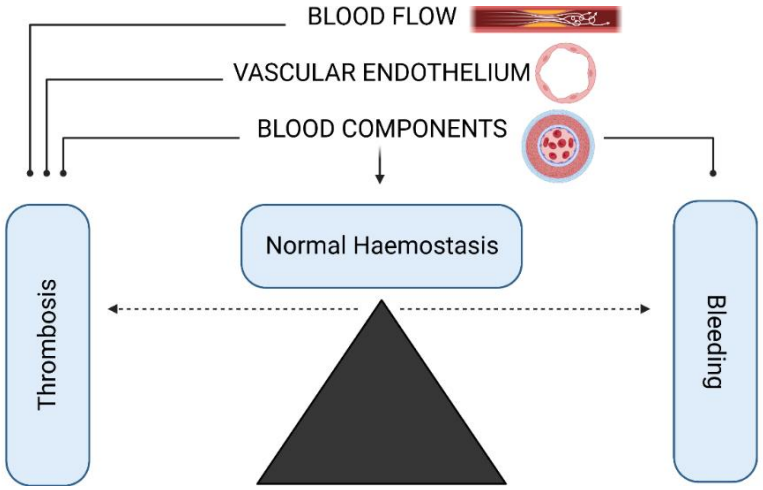


Figure 1.1 – The balance of haemostasis. Hypercoagulable state and thrombosis are induced by alteration in the blood flow such as stasis or turbulences, defects in the vascular endothelium or changes in the blood constituents. Maintaining the equilibrium between a physiological blood flow, healthy vascular endothelium and blood components is essential to prevent hypercoagulable or hypo-coagulable states and to maintain a balanced homeostatic equilibrium. Modified from Ref (Rasche, 2001). Created with BioRender.com.

The endothelium, the cellular monolayer that lines the entire vascular system, is strategically located between the blood and the deeper layers of the vessel wall. Under physiological conditions, platelets circulate in the bloodstream without adhering to endothelial cells as a result of released anti-coagulant and anti-platelet molecules such as CD39, nitric oxide (NO) and prostacyclin (PGI2), which are involved in maintaining vascular homeostasis (Wu and Thiagarajan, 1996). Moreover, the glycocalyx, a layer of negatively charged proteoglycans and glycoproteins covering the endothelium, limits the interaction with platelets (Britten et al., 2021). Pathological processes or vessel injury, lead to the shedding of the glycocalyx, dysregulate the release of NO and PGI2 and drive the formation of a pro-thrombotic environment (Patterson et al., 2022). Endothelial injury also leads to the secretion of Weibel-Palade bodies (WPB), the release of prothrombotic molecules such as von Willebrand Factor (vWF) and the exposure of the highly prothrombotic subendothelial extracellular matrix. These mechanisms, along with the downregulation of anti-thrombotic molecules, induce platelet recruitment, activation of the coagulation cascade and subsequent clot formation (Rasche, 2001).

The haemostatic process is an orchestrated, tightly regulated mechanism, conventionally subdivided into three sequential interconnected processes: primary haemostasis (platelet plug formation), secondary haemostasis (formation of a stable fibrin cloth), and tertiary haemostasis (formation of plasmin for the breakdown of fibrin via fibrinolysis) (**Figure 1.2**). Platelets participate and contribute to each of these stages. In the following paragraphs a detailed description of platelet function, activation and clot formation is provided.

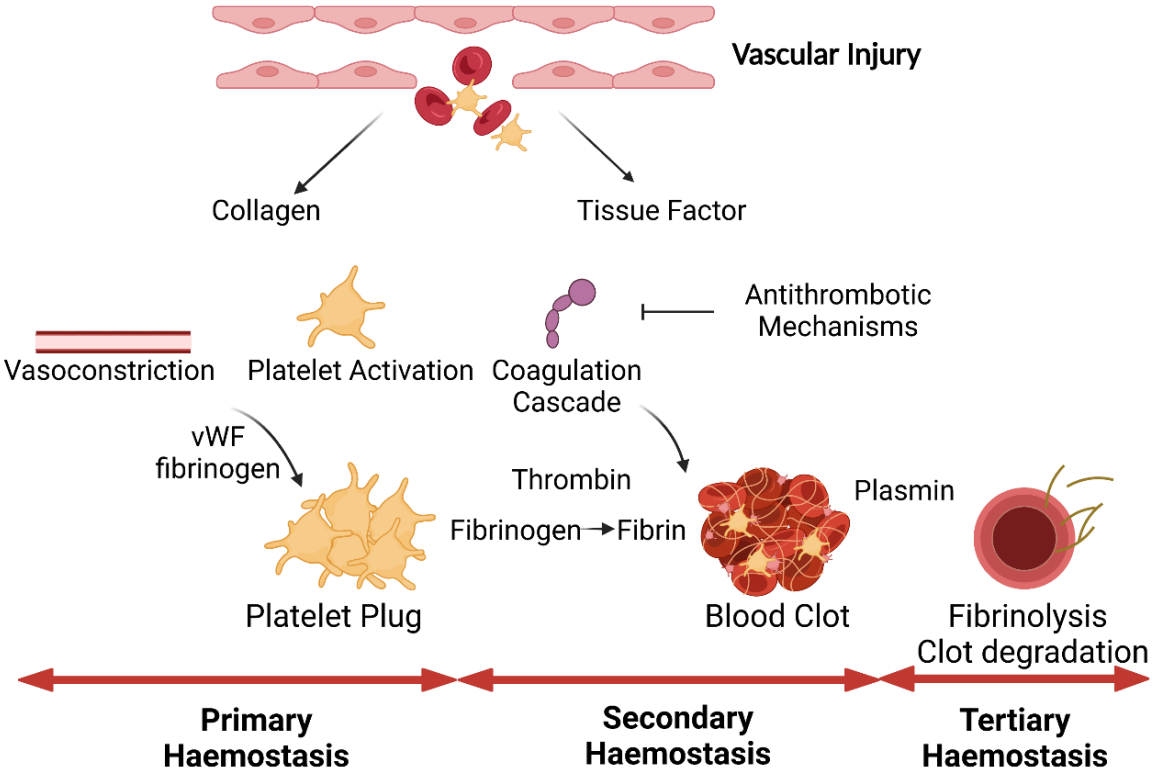


Figure 1.2 – Major components of haemostasis. Primary haemostasis refers to vasoconstriction and platelet aggregation leading to unstable platelet plug formation. Secondary haemostasis refers to the deposition of insoluble fibrin, which is generated by the proteolytic cleavage of the coagulation proteins leading to thrombin generation, cleavage of fibrinogen and formation of fibrin. This insoluble fibrin strengthens and stabilises the blood clot. The fibrinolytic pathway constitutes the tertiary haemostasis which degrades the fibrin clot to restore homeostasis. Pathological thrombus formation (thrombosis), or pathological bleeding can occur whenever this process is dysregulated. Modified from https://healthjade.net/hemostasis. Created with BioRender.com.

1.1.1. Platelet function and thrombus formation

Platelets are enucleated discoid-shaped cell fragments produced from bone marrow progenitors called megakaryocytes. They circulate in the bloodstream for 7-10 days and subsequently, they are removed and destroyed by the spleen and the liver. A normal human platelet count ranges from 150,000 to 450,000 platelets per microliter

of blood. Platelets contains three types of granules: α-granules, dense granules, and lysosomes. In addition, platelets also have peroxisomes and T granules. Alpha-granules (α-granules) are unique to platelets and amount to 50-80 per platelet (White, 1998). They are 200-500 nm in size and contain mainly proteins such as membrane-associated receptors (e.g., GPIIb/IIIa complex [CD41/CD61] also known as αIIbβ3, P-selectin [CD62P]) and soluble molecules (e.g., platelet factor 4 [PF4] and fibrinogen). Proteomic studies have identified more than 300 soluble proteins in the α-granules involved in a variety of functions such as haemostasis (e.g., vWF and Factor V), inflammation (e.g., chemokines) and others (e.g., vascular endothelial growth factor, fibroblast growth factor) (Maynard et al., 2010). Dense granules (also known as δ-granules) are the second most abundant in platelets (3-8/platelet). They contain mainly adenine nucleotides (e.g., adenosine triphosphate [ATP]), adenosine diphosphate [ADP]), bioactive amines (e.g., serotonin and histamine) and cations such as calcium (Ca^{2+}). They derive their name from their electron-dense appearance due to the high cation concentration (Gerrard et al., 1977). Platelets also contain 1-3 lysosomes, peroxisomes and the presence of an electron-dense granule defined by Toll-like receptor 9 (TLR-9) has been described, however its existence remains controversial (Thon et al., 2012). In the platelets, there are also glycogen granules to supply energy sources.

Platelets are activated by several agonists, and they have a broad surface receptor repertoire. An overview of the major platelet receptors mediating platelet activation is summarised in the next paragraph and a description of the receptors relevant for this

thesis given in section **1.1.3** and **1.1.4**. The most relevant platelet receptors involved in haemostasis and thrombosis are summarised in **Figure 1.3**.

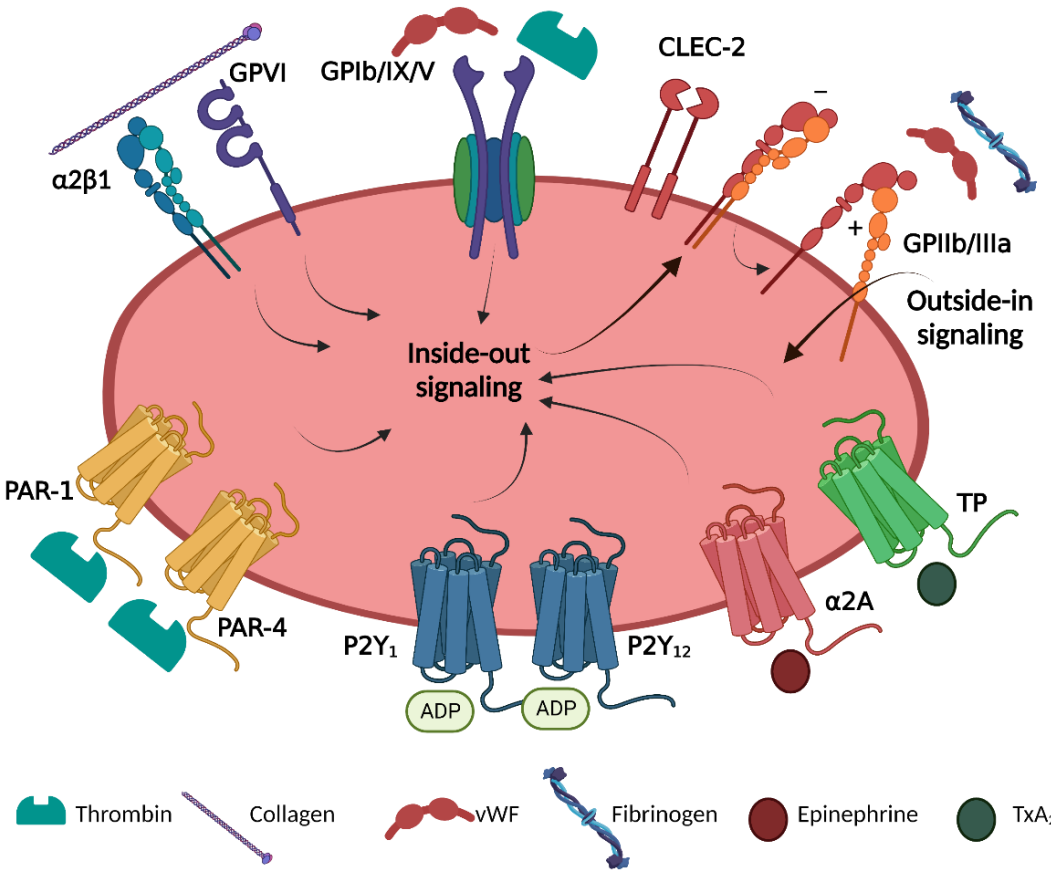


Figure 1.3 – Major receptor-ligand interactions on platelets. Diagram showing key platelet membrane receptors involved in platelet function during haemostasis and thrombosis. *Receptors mediating adhesion/recruitment* include GPIb/IX/V, α2β1 and glycoprotein VI (GPVI). *Receptors for secondary mediators* include the purinergic receptor P2Y G protein-coupled 1/12) (P2Y1/12), α_2A-adrenergic receptor (α2A) and Thromboxane (TP) receptors. Most of these receptors are involved in the activation of *integrins* such as GPIIb/IIIa which, in its open form, mediates aggregation between platelets. Protease-activated receptor 1 (PAR-1) and 4 (PAR-4) are activated by thrombin, subsequently to the activation of the coagulation cascade, which lead to shape change, secretion of granule content and further aggregation. Modified after Ref (Rivera et al., 2009). Created with BioRender.com.

1.1.2 Major Platelet membrane receptors

1.1.2.1. Integrins

Integrins are adhesion and signalling molecules made up of non-covalently bound heterodimers of α and β subunits. Platelets have three types of integrins: β1, β2 and β3. The β1 family has three main members on platelets, α2β1(or GPIa/IIa), an important collagen receptor; α5β1 and α6β1 mediating adhesion to fibronectin and laminin, respectively. β2 family has only αLβ2 (CD102) or intercellular adhesion molecule 2 (ICAM-2), important for platelet-neutrophil/leukocyte interactions. β3 family comprises the adhesion receptors αIIbβ3 (GPIIb/IIIa complex), the most abundant one, and αVβ3. Resting platelets express 80,000-100,000 molecules of GPIIb/IIIa on their surface which are also stored in the α-granules (Bennett et al., 2009). GPIIb/IIIa is translocated to the platelet plasma membrane following activation and is transformed from low affinity to high affinity state. This promotes binding to fibrinogen, fibrin, vWF, fibronectin, vitronectin and thrombospondin, promoting platelet adhesion and aggregation.

1.1.2.2. Selectins

Selectin receptors play a pivotal role in adhesion and include P-selectin, which is present in α-granules of resting platelets and is translocated to the membrane upon activation (Merten and Thiagarajan, 2000). P-selectin plays a crucial role in the binding of platelets and endothelial cells to neutrophils and monocytes. Furthermore, it recruits

microparticles that contain P-selectin glycoprotein ligand-1 (PSGL-1) and tissue factor helping thrombus formation in murine models (Falati et al., 2003).

1.1.2.3. G-protein coupled (seven-transmembrane) receptor (GPCR) family

G-protein coupled (seven-transmembrane) receptor (GPCR) family is the main agonist-stimulated receptor group on platelets. The main components of this group are the Thrombin Receptor, ADP receptors, prostaglandin receptors, lipid receptors and chemokine receptors. The first thrombin receptor identified on platelets was the protease activation receptor-1 (PAR1) (Willis Fox and Preston, 2020). Other PAR receptors are present on platelets such as PAR2 and PAR4 in humans. Unlike other members of this family, PAR receptor activation involves the cleavage of the N-terminal extracellular domain, which acts as a ligand to the receptor initiating signalling and playing an important role in platelet aggregation and activation. ADP is widely secreted by erythrocytes, endothelial cells and from dense granules of platelets to stimulate and potentiate platelet aggregation. In platelets, ADP induces mobilisation of Ca^{2+} from both intracellular stores and the extracellular environment (Puri and Colman, 1997). There are several distinct ADP receptors on platelets classified as P2X1, P2Y1 and P2Y12. ADP is involved in potentiation of platelet aggregation, Ca^{2+} mobilisation, shape change, production of thromboxane A2 (TxA2), GPIIb/IIIa activation and platelet aggregation (Puri and Colman, 1997). Relevant to this thesis are the P2Y12 antagonists, which are one of the most widely used anti-platelet drugs. This group is composed of the thienopyridines (ticlopidine, clopidogrel and prasugrel), whose active metabolites irreversibly block the P2Y12 receptor and of the newer cangrelor and

ticagrelor, which change the conformation of the receptor, resulting in reversible inhibition (Wallentin et al., 2009). These drugs are widely adopted for acute treatment and secondary prevention in ischemic cardiovascular disease and stroke (Chiarito et al., 2020, Gragnano et al., 2023, Johnston et al., 2018, Fernando et al., 2022, Winter et al., 2017). Thromboxane receptor (TP) is also a GPCR. TxA2 is the primary product of cyclo-oxygenase 1 and 2 (COX-1 and COX-2) from the metabolism of arachidonic acid. COX-1 is expressed constitutively in most tissues (Kirkby et al., 2012) whilst COX-2 is inducible and expressed at sites of inflammation (Nandakishore et al., 2014). However, platelets are anucleated cells and lack a robust transcription machinery; as a result, a small amount of COX2 has been shown to be constitutively expressed in platelets (Hu et al., 2017). COX1/COX2 mRNA and protein ratios in platelets are 3–7:1, and 17:1 respectively (Hu et al., 2017). TxA2 works in a paracrine manner through its receptor TP, activating adjacent platelets and amplifying the action of other platelet agonists. TP is present in two isoforms (α and β) but platelets express only the TPα (Habib et al., 1999). The TP coupling with protein G (G12/13) mediates stimulation of Rho signalling resulting in platelet shape change and platelet aggregation (Offermanns, 2006). Aspirin, another widely used anti-platelet drug, inhibits COX1 and COX2 via irreversible acetylation of active serine residues resulting in permanent suppression of TxA2 generation in platelets (Roth et al., 1975, DeWitt et al., 1990). Aspirin is a more potent inhibitor of COX-1 as compared to COX-2 (Gum et al., 2001, Vane et al., 1998). Indeed, aspirin irreversibly acetylates and inhibits the COX-1 isozyme resulting in complete platelet inhibition for the life of the platelet (Catella-Lawson et al., 2001). Briefly, aspirin prevents the access of the substrate (arachidonic acid) to the catalytic site of the enzyme and results in an irreversible inhibition of

platelet-dependent TxA2 formation (Schrör, 1997). Although, low aspirin doses have minimal anti-inflammatory effects as they leave vascular prostacyclin formation almost intact, high aspirin doses are anti-inflammatory due to the inhibition of COX-2 (De Gaetano et al., 2003, Altman et al., 2004). Non-steroidal anti-inflammatory drugs (NSAID), such as indomethacin, can block both COX-1 and COX-2 (Ghlichloo and Gerriets, 2023), whilst selective COX-2 inhibitors (such as celecoxib) only block COX-2 and are mainly used for their anti-inflammatory, anti-pyretic and analgesic properties (Zarghi and Arfaei, 2011).

1.1.2.4. Leucine-rich repeats (LRR) receptors

Leucine-rich repeats receptors (LRR) are involved in the formation of protein-protein interactions. They include the GPIb-IX-V complex, Toll-Like Receptors (TLRs) and matrix metalloproteinases (MMP). TLRs are transmembrane proteins and platelets express all TLRs mediating immune-driven platelet activation. TLR-4, the receptor for the gram-negative bacteria protein lipopolysaccharide (LPS), is among the most studied TLRs in platelets. LPS effect on platelets is still controversial as some studies showed no effect on platelet activation while other showed an increase in platelet activation. MMPs (MMP-1,2,3,9,14) are involved in platelet adhesion and aggregation (Choi et al., 2008).

1.1.3 GPIb-IX-V receptor complex

GPIb-IX-V complex is the major platelet adhesion receptor on platelets and is the second most abundant platelet receptor with around 50,000 copies per platelet. GPIb-IX-V complex is the main receptor for vWF on platelets initiating platelet recruitment and rolling on the exposed sub-endothelium at high shear rate. GPIb-IX-V complex consists of different subunits: GPIbα (CD42b), GPIX (CD42a), GPIbβ (CD42c) and GPV (CD42d) (Berndt et al., 2001). The four subunits of the GPIb-IX-V complex are encoded by different genes and assemble within the endoplasmic reticulum and are transported to the Golgi apparatus before their expression on the platelet surface (Dong et al., 1998). GPIbα (135kDa, 610 amino acids), GPIbβ (26kDa, 181 amino acids), GPIX (20 kDa, 160 amino acids) and GPV (82 kDa, 544 residues) are all members of the LRR family. GPIbα, GPIbβ and GPIX are necessary for the effective biosynthesis of the receptor and a lack of a single subunit significantly decreases the surface expression of the entire receptor complex as observed in patients with Bernard Soulier Syndrome (BSS) (Nurden, 2005, Lanza, 2006). Each subunit consists of an LRR ectodomain (extracellular domain), a single transmembrane helix and a short cytoplasmic tail. The GPIbα subunit is linked to the GPIbβ via disulfide bonds, while GPIX non-covalently associates to GPIbβ (**Figure 1.4**). The GPIX extracellular domain, has a LRR which shares more than 45% sequence identity with GPIbβ. The GPIX cytoplasmic tail is short consisting of 8 residues and is not known to associate with intracellular proteins (Li and Emsley, 2013). The extracellular domain of GPV has 13 LRR followed by a stalk region, the transmembrane sequence, and a short cytoplasmic tail. The GPV subunit is only weakly associated with the GPIb-IX complex through the

transmembrane domains and does not appear to be critical for signal transduction or for agonist binding (McEwan et al., 2011). GPV has minimal impact on the surface expression of GPIb-IX but GPIb-IX is required for efficient expression of GPV (Mo et al., 2012, Li and Emsley, 2013). The GPIbβ has an extracellular domain where both the N-caping and C-capping regions contain disulfide bonds. GPIb alpha is linked to beta through basic residues close to the plasma membrane.

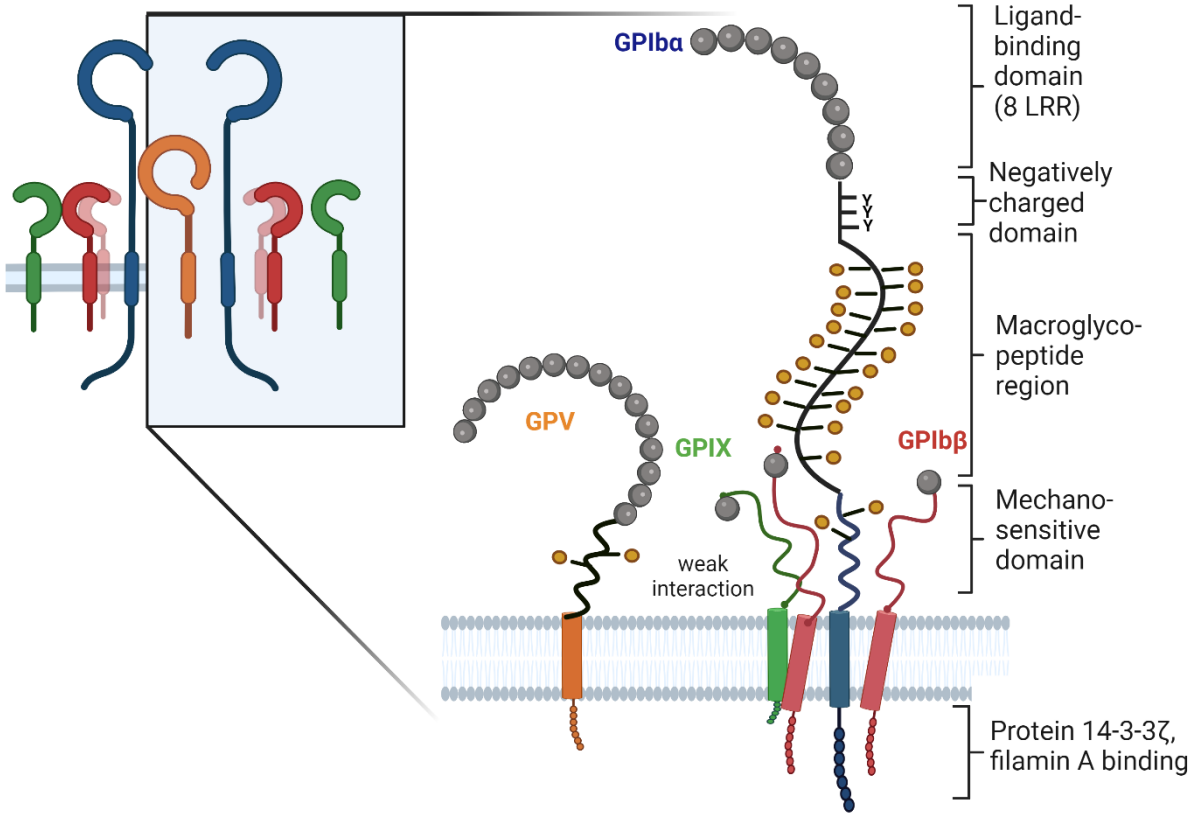


Figure 1.4 – Structure of GPIb-IX-V complex. The four polypeptide GPIbα, GPIbβ, GPIX and GPV are present on the platelet surface in a 2:4:2:1 stoichiometric distribution. The largest subunit of the complex is GPIbα, which consists of a N-terminal binding domain (made up of 8 leucine-rich repeats), a negatively charged domain, a sialomucin region (macroglycopeptide domain), the mechanosensitive domain (MSD), and intracellularly which interacts with the protein 14-3-3ζ and filamin A linking the complex to the cytoskeleton. The transmembrane domain provides a close interaction with GPIbβ and GPIX to form a stable bundle structure. This complex structure is further strengthened by the interplay of GPIbβ and

GPIX extracellular domains with the MSD in GPIbα. GPV is weakly associated with GPIb-IX by polar interactions. Modified after Ref (Bendas and Schlesinger, 2022). Created with BioRender.com.

1.1.3.1 GPIb-IX-V extracellular ligands

GPIbα interacts with multiple soluble proteins and membrane receptors regulating both thrombosis and inflammation. Along vWF, GPIbα is able to bind to multiple extracellular soluble proteins including α-thrombin, thrombospondin-1, clotting factors XI/XIIa, high-molecular-weight kininogen and membrane receptors including the Macrophage-1 antigen (Mac-1 or CD11b/Cd18), P-selectin, CD36 and FcγRIIA (Berndt et al., 2001, Jurk et al., 2003, Bonnefoy and Hoylaerts, 2008). vWF-GPIba and GPIbα-CD36/FcγRIIA interaction will be discussed in section 1.1.3.2 and 1.1.3.3. respectively. Many of the interactions of GPIbα with extracellular proteins occur through the N-terminal extracellular domain and the binding sites of different proteins are shown in **Figure 1.5**. Different monoclonal antibodies, including AK2 and SZ2, were shown to block selectively a binding site for vWF on GPIbα (Ward et al., 1996) (**Figure 1.5**). In addition, AK2 blocks thrombin binding to GPIbα without inhibition of thrombin-induced platelet aggregation. Whereas SZ2, which inhibits also type I collagen and Platelet Activating Factor (PAF)-induced aggregation, prevents thrombin-induced platelet aggregation, at low doses. Moreover, a peptide called OS-1 which changes the conformation of GPIbα was shown to inhibit its interaction with vWF (Benard et al., 2008, McEwan et al., 2009).

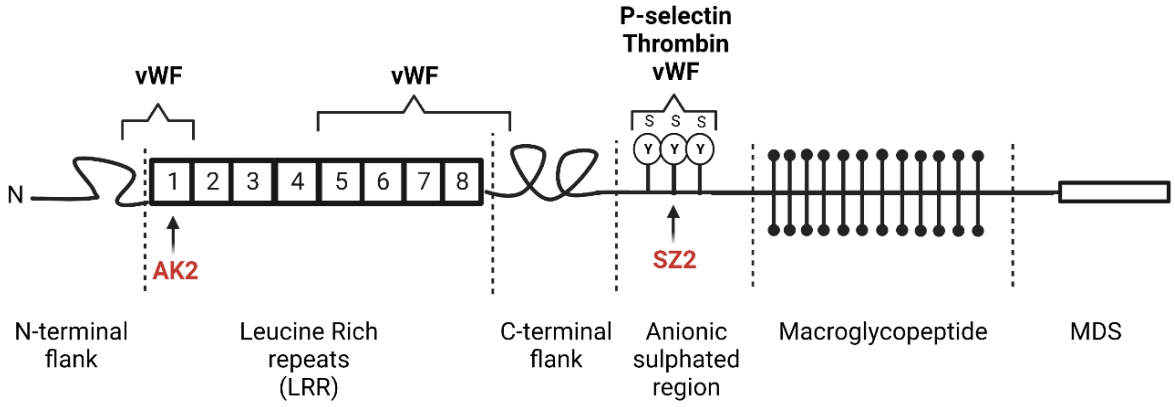


Figure 1.5 – Schematic illustration of GPIbα with its extracellular ligands. The extracellular domain of GPIbα consists of the N-terminal flank domain containing Cys4–Cys17 disulfide bond in the N-capping region; eight LRRs; a C-terminal flank composed of Cys209–Cys248 and Cys211–Cys264 disulfides; an anionic sequence containing three sulphated tyrosines and a highly glycosylated macroglycopeptide domain. vWF binds to GPIbα through two distinct sites: one site located at the LRR the second site at the anionic sulphated region. SZ2 and AK2 antibodies recognize the anionic tyrosine sulphated region (Tyr2-Glu282) and the first LRR (Leu36-Gln59, respectively and block vWF interaction with GPIbα. Mac-1:GPIbα binding is not shown in the figure but involves additional interactions consolidated by an elongated pocket flanking the GPIbα LRR capping α-helix at the N terminal domain (Morgan, 2019). Figure adapted from Ref (Huizinga et al., 2002, Shen et al., 2000). created with BioRender.com.

Before being exposed to the membrane surface, the receptor undergoes *post-translational modifications* which are very important for receptor-mediated adhesion and signalling. The sulfation of the three tyrosine residues (Tyr276, Tyr278, and Tyr279) regulates both vWF adhesion and thrombin binding (Dong et al., 2001, Tait et al., 2002, Marchese et al., 1995). The GPIb-IX-V complex is also highly glycosylated and the addition of O-linked oligosaccharides results in the stabilisation of the GPIbα structure (Moshfegh et al., 1998). Moreover, the macroglycopeptide region of GPIbα is required for platelet adhesion to immobilised vWF under flow (Li et al., 2002). Both GPIbα and GPIbβ can be phosphorylated on serine residues, the former at $Serine^{609}$ (Bodnar et al., 1999) and the latter at Ser^{166} (Fox et al., 1987, Wardell et al., 1989).

GPIbβ is also phosphorylated at Thr^{166} when platelets are stimulated with agents that increase intracellular cyclic adenosine monophosphate (cAMP) level (Wyler et al., 1986, Fox et al., 1987, Wardell et al., 1989). The phosphorylation at Ser^{609} is required for efficient binding of GPIbα to 14-3-3ζ, which is a crucial protein involved in the signal transduction pathway of GPIb (Bodnar et al., 1999). Phosphorylation of GPIbβ regulates the interaction between GPIbα and GPIbβ and controls the adhesive properties of the entire GPIb-V–IX complex (Perrault et al., 2003). Phosphorylation of GPIbβ correlates with inhibition of post-stimulatory cytoskeletal actin rearrangements and inhibition of shear-induced platelet aggregation (Berndt et al., 2001). Both GPIbβ and GPIX have been shown to be acylated in human platelets (Muszbeks and Laposatag, 1993), regulating their interaction and association. Palmitoylation has been found to be important for transmembrane signalling (Zhang et al., 1998) and protein anchoring to the lipid rafts (Webb et al., 2000, van't Hof and Resh, 2000). Desialylation of O-glycans on GPIbα, induces mechanosensitive domain unfolding, GPIb-IX signalling, and rapid platelet clearance (Wang et al., 2021).

The sulphated anionic region of GPIbα is a major binding site for thrombin and this receptor is able to bind two thrombin molecules at the same time (Tait et al., 2002, Dumas et al., 2003, Celikel et al., 2003). This binding enhances PAR-1 stimulation (De Candia et al., 2001). GPIb-IX signalling cooperates with PAR signalling to promote platelet response to low concentrations of thrombin (Estevez et al., 2015). Thrombin binding to GPIb induces GPIb-IX signalling, which greatly enhances platelet responses to PARs. There is evidence that thrombin binding to GPIb-IX can also transmit direct inducing platelet activation (Ramakrishnan et al., 2001, Adam et al., 2003, Zhang et

al., 2022). Interestingly Glycoprotein (GP) V is a major substrate cleaved by thrombin during thrombin-induced platelet activation. Platelets from GP V-null mice suggested a role for GP V as a negative modulator of platelet activation by thrombin (Ramakrishnan et al., 2001). Also, thrombin-mediated shedding of GPV limits thrombin-dependent generation of fibrin (Beck et al., 2023).

GPIbα has been shown to be a receptor for P-selectin and was shown to increase platelet rolling and adherence to activated endothelium, a binding inhibited by antibodies to both P-selectin and GPIbα (Romo et al., 1999). However, these data have to be taken cautiously has they have not been replicated. The domain of GPIbα involved in the binding of P-selectin is the sulphated anionic region. GpIbα binds also to the Mac-1, which is expressed on leukocytes and is involved in the adhesive interaction with endothelial cells during the inflammatory response, regulating cell migration, phagocytosis, oxidative burst, and signalling. Mice with Mac-1 deficiency (Mac-$1^{-/-}$) or mutation of the Mac-1-binding site for GPIbα show delayed arterial thrombosis after carotid artery and cremaster microvascular injury without affecting haemostasis (Wang et al., 2017b) . Factor XII and kininogen binding to platelets occur through GPIbα and they appear to have a modulatory role, controlling the extent of thrombin-induced platelet aggregation (Bradford et al., 1997, Bradford et al., 2000). Recently, it has been demonstrated that thrombospondin-1, a glycoprotein highly present in the atherosclerotic plaques, binds to the platelet GPIb-V–IX complex. Platelets were described to adhere to thrombospondin-1 via GPIbα in a shear dependent manner (Jurk et al., 2003). Furthermore, thrombospondin 1 requires the presence of vWF to modulate arterial thrombosis (Prakash et al., 2015). Soluble

GPIbα, as well as antibodies against GPIbα, blocked platelet adhesion to thrombospondin-1, confirming GPIb is a high shear adhesion-receptor for thrombospondin-1 under high shear stress conditions (Jurk et al., 2003). Therefore, GPIbα interacts with multiple proteins and regulates both inflammation and thrombosis.

1.1.3.2 vWF-GPIb interaction

vWF is a multimeric protein secreted from WPB and platelet α-granules (Andrews et al., 1997), and is present either in plasma or bound to collagen when the subendothelium is exposed. Soluble vWF circulates in a folded compact shape unable to bind to platelets, however immobilised vWF onto collagen or unfolding of vWF under high shear rate favour its interaction with platelets. vWF is composed of A, B, C and D domains and circulates in plasma as a carrier for factor VIII. The binding of vWF to the receptor, involves the A1 domain which recognises the LRR, the C-terminal flanking domain and the negatively charged region of GPIbα (Ward et al., 1996, Shen et al., 2002) (**Figure 1.6**).

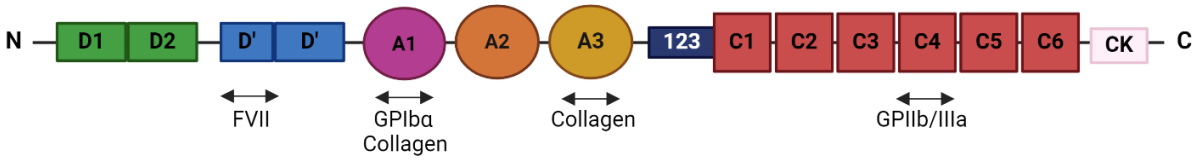


Figure 1.6 – Schematic representation of vWF domains and their major ligands, Modified after Refs (Crawley et al., 2011, Springer, 2014). Created with BioRender.com.

Soluble vWF requires a conformational change of the A1 domain to increase its affinity for the GPIb-IX-V complex, which takes place under hydrodynamic shear forces (Siedlecki et al., 1996). *In vitro*, soluble vWF can be activated by non-physiological modulators, the bacterial antibiotic ristocetin and the snake venom toxin botrocetin (Read et al., 1989, Scott et al., 1991). Shear stress causes an early wave of GPIb-dependent Ca^{2+} increase followed by a more pronounced and stable integrin-mediated Ca^{2+} elevation (Mazzucato et al., 2002). Therefore GPIb-IX acts as a shear force sensor, converting vWF-mediated mechanical force into intracellular signals (Chen et al., 2018, Zhang et al., 2022) (**Figure 1.7**).

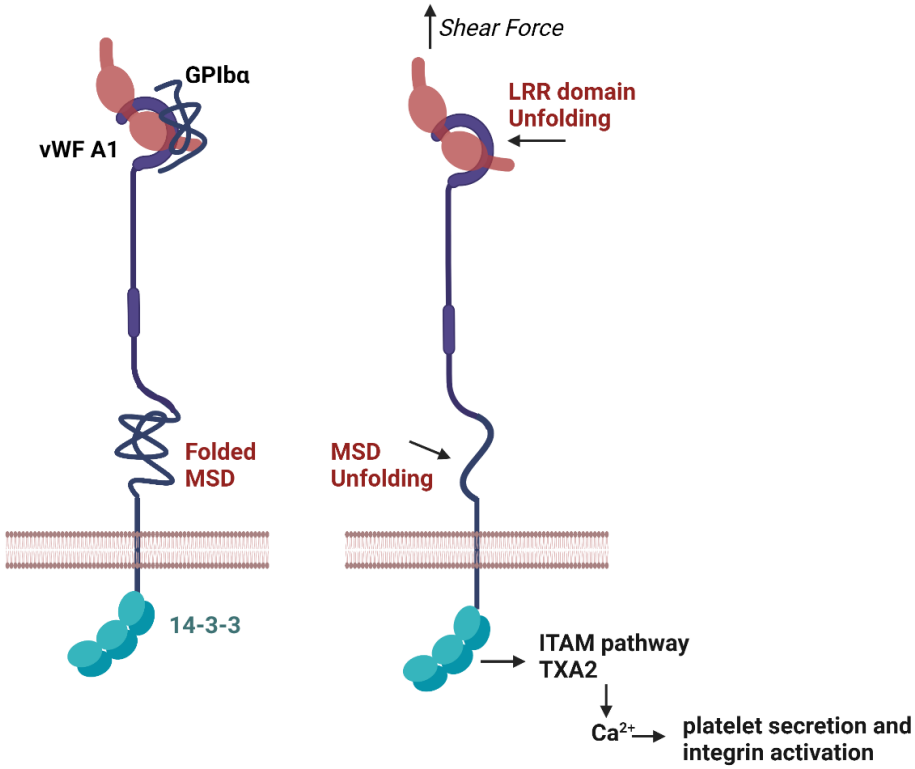


Figure 1.7 – vWF binding and GPIb-mediated mechanosensing. vWF binds to GPIb-IX in a conformation with folded MSD and LRRs. Pulling force generated by shear stress induces unfolding of

LRR and MSD and convert mechanical signal into GPIb membrane-proximal domain, which induces a 14-3-3–dependent signalling cascade leading to granule secretion and integrin activation. Modified after Ref (Chen et al., 2018). Created with BioRender.com.

The binding of vWF to the GPIbα N-terminal ligand binding domain under flow exerts a pulling force on GPIb-IX. vWF unfolds and extends the mechanosensitive domain (MSD) in the extracellular membrane-proximal region of GPIbα as well as the LRR domain (Ju et al., 2016, Zhang et al., 2014), leading to intracellular Ca^{2+} elevation (Ju et al., 2016). The GPIb conformational changes induces a 14-3-3–dependent signalling cascade leading to granule secretion and integrin activation (**Figure 1.7**).

1.1.3.3 GPIb-IX-V interaction with membrane receptors: CD36 and FcγRIIA

GPIb-IX-V interacts with different platelet membrane receptors such as CD36, GPVI, FcRγ, CLEC2 and FcγRIIA receptor (Arthur et al., 2005, Baker et al., 2004, Constantinescu-Bercu et al., 2022, Wu et al., 2001, Shao et al., 2022). The scavenger receptor CD36 which is constitutively expressed on the platelet surface (Kieffer et al., 1989, Burkhart et al., 2012) was shown to interact with GPIb and regulate its functions. CD36 has multiple ligands including collagen, thrombospondin-1, anionic phospholipids, long chain fatty acids and oxidized LDL (Endemannl et al., 1993, Podrez et al., 2002). It was also shown to bind multiple neutrophil-derived damage-associated molecular patterns (DAMPs) such as S100A8/A9 and S100A12. Binding of oxidised LDL to CD36 mediates activation of platelets (Korporaal et al., 2007, Assinger et al., 2010) and induces a hyperreactive platelet phenotype in the presence of ADP and collagen (Podrez et al., 2007, Silverstein, 2009). Thus, platelet CD36 increases

platelet reactivity to physiological ligands leading to a pro-thrombotic phenotype under conditions associated with metabolic and inflammatory stress. Blocking CD36 suppresses thrombin-induced platelet GPIIb/IIIa activation, granule exocytosis, binding of adhesion proteins and reduced FV, FVIII, FIX and FX binding to platelets in flow cytometry (Döhrmann et al., 2020). Specifically, soluble binding of fibrin to CD36 recruits distinct coagulation factors favouring the formation of procoagulant platelets, whose features are described in section 1.1.6 of this thesis. This CD36-dependent-thrombin generation has been demonstrated to be mediated by GPIbα via src family kinase pathways activation (Döhrmann et al., 2020). Another important receptor interacting with GPIbα is FcγRIIA. Those receptors have been shown to co-localise and physically interact on the platelet membrane and this interaction to be essential for tyrosine phosphorylation due to vWF binding (Sullam et al., 1998, Sun et al., 1999). Upon vWF binding, the association of GPIbα with FcRγ-chain has been shown to induce the formation of a signalosome composed of Src family kinase Fyn, and Lyn and tyrosine kinase Syk, which are all involved in platelet activation (Canobbio et al., 2004, Falati et al., 1999).

1.1.3.4 GpIb-IX-V intracellular interactions and signalling

The short cytoplasmic domains of the GPIb-IX-V subunits lack intrinsic enzymatic activity. However, by interacting with a number of intracellular proteins such as the actin-binding adaptor protein 14-3-3ζ (Du et al., 1996, Calverley et al., 1998), and calmodulin (Andrews et al., 2001), it regulates intracellular pathways involved in platelet rolling, adhesion (Schade et al., 2003, Williamson et al., 2002), cytoskeleton

rearrangement (Dyson et al., 2003) and signalling (Calverley et al., 1998, Gu et al., 1999, Englund et al., 2001). GPIbα cytoplasmic tail contains a binding site for actin-binding proteins, such as filamin A (Feng et al., 2003, Williamson et al., 2002), which anchor the entire receptor to the cellular cytoskeleton (Hartwig et al., 1999). This interaction is critical for vWF adhesion at high shear rate and to facilitate rolling on vWF (Cranmer et al., 1999, Schade et al., 2003). In addition, vWF-induced platelet activation and aggregation and shear-dependent tyrosine phosphorylation is reduced when this interaction is blocked (Feng et al., 2003, Christodoulides et al., 2001). GPIb-V–IX binds to 14-3-3ζ through both GPIb and GPV (**Figure 1.8**).

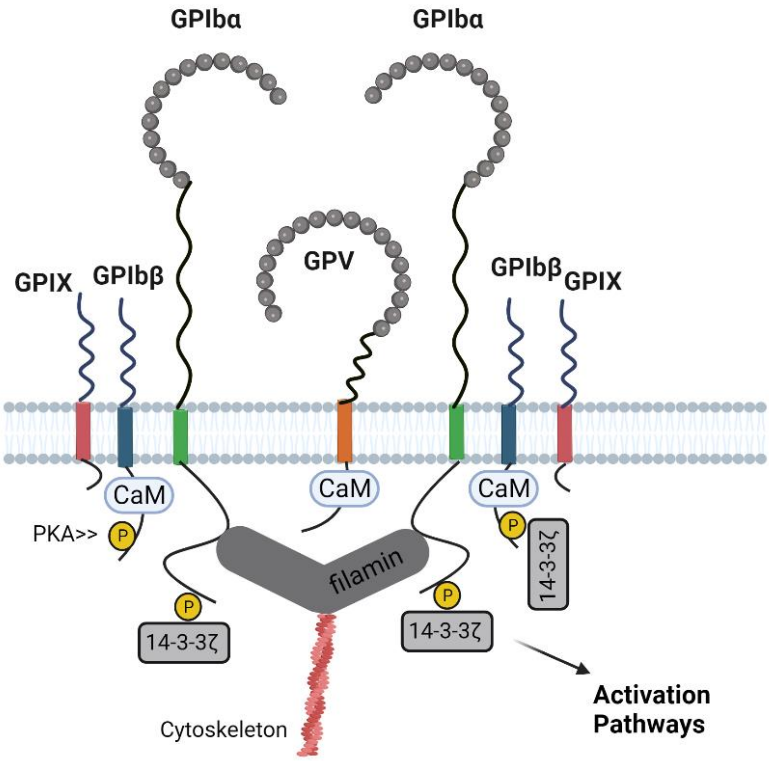


Figure 1.8 – Subunits of the GPIb-IX-V complex. Following vWF, phosphorylation of GPIbβ (by PKA) and GPIbα mediates association of the signalling protein, 14–3–3ζ. Interaction of 14–3–3ζ with GPIbα

is critical for the pathway leading from vWF binding at GPIb–IX to GPIIb/IIIa activation. Modified after Ref (Andrews et al., 2003). Created with BioRender.com.

Calmodulin has been described to be constitutively associated with GPIb-IX-V trough GPIbβ and GPV (Andrews et al., 2001). Upon thrombin stimulation, calmodulin translocate to the cytoskeleton leading to the activation cascade (Zhang et al., 2022).

The main intracellular pathways initiated by GPIb-IX-V complex are the activation of immunoreceptor tyrosine-based activation motif (ITAM)-containing receptors, phospholipase C and phospholipase A2, the cytoskeleton reorganisation pathway, and the crosstalk with integrin GPIIb/IIIa. Phosphorylation of two ITAM-containing receptors, FcγRIIA (Canobbio et al., 2001) and Fc receptor γ-chain (Wu et al., 2001, Falati et al., 1999) is involved in platelet activation by vWF as described in figure legend (**Figure 1.9**). FcγRIIA and FcR γ-chain are both phosphorylated downstream of GPIb-IX-V and FcR γ-chain associates with the GPIbα subunit. As described in the next section on platelet ITAM receptors, the phosphorylation of two tyrosine residues within the ITAM, allow recruitment of the tyrosine kinase Syk which initiates the intracellular cascade (Rayes et al., 2019a). Binding of vWF to GPIb-IX–V induces tyrosine phosphorylation of FcγRIIA in intact (not solubilised) platelets (Canobbio et al., 2001) and this mechanism is favoured by the physical proximity of these two receptors on the platelet surface (Sullam et al., 1998, Sun et al., 1999). The interaction between GPIb-V–IX and FcγRIIA occurs only within a specialised area of the membrane known as lipid rafts (Shrimpton et al., 2002).

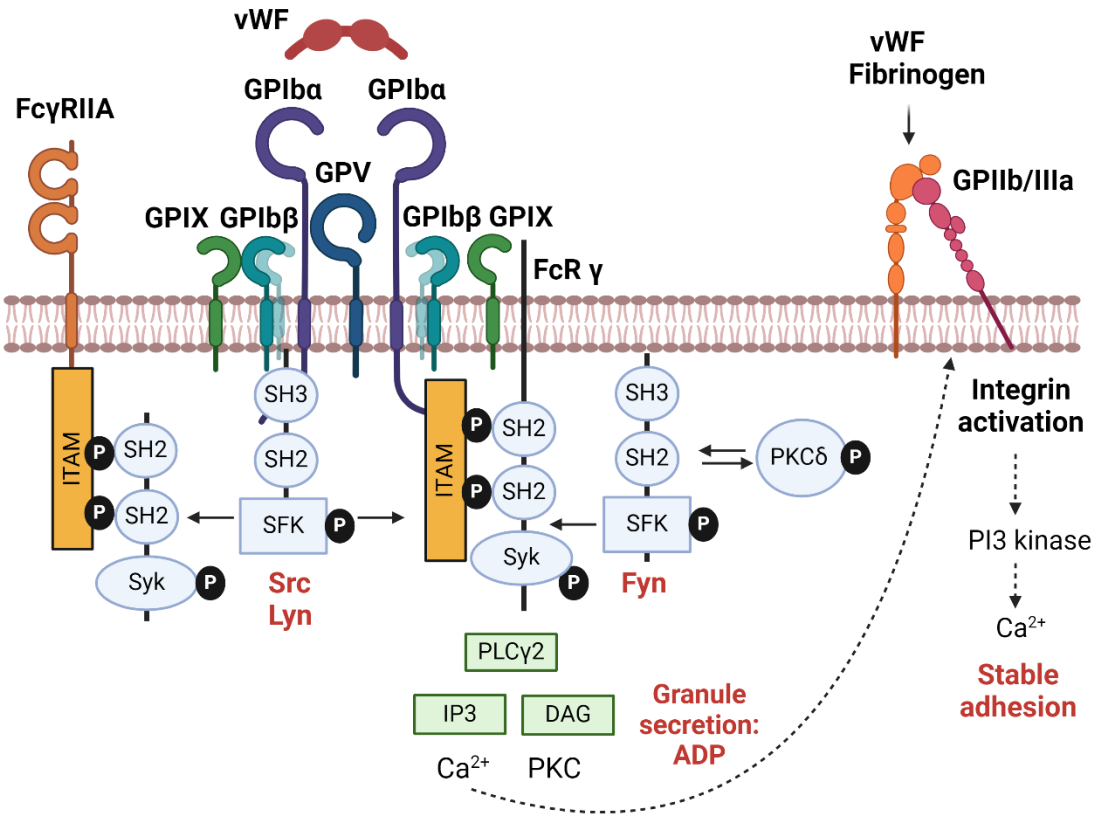


Figure 1.9 – Platelet signalling induced by GPIb-IX-V. Following vWF binding to GPIb-IX-V, Src and Lyn associate with the GPIbα subunit and initiate inside-out signalling. SFKs (Src and Lyn) are activated and phosphorylate downstream substrates (ITAM motifs of FcγRIIA or Fc receptor γ). ITAMs bind the SH2 domain of Syk with high affinity and activate second messengers, such as protein kinase C (PKC) δ. PKCδ has a role in cellular Ca^{2+} homeostasis. SFKs alone or in interaction with PI3-K activate PLCγ2 which produces DAG and IP3 that signal for platelet degranulation. The second messengers also facilitate the activation of integrin GPIIb/IIIa which is crucial for platelet stable adhesion. Modified after Refs (Senis et al., 2014, Bryckaert et al., 2015). Created with BioRender.com. Abbreviations: SFKs=Src family kinases, ITAM= immunoreceptor tyrosine−based activation motif containing receptors, IP3=inositol trisphosphate, DAG=diacylglycerol, PLC= phospholipase C (PLCγ2 subtype), PKC= protein kinase C, vWF=Von Willebrand Factor, PI3-K=PI3 kinase.

1.1.3.5 GPIb-V-IX in procoagulant platelet formation

Beyond inducing platelet aggregation, GPIb-V-IX was shown to regulate the formation of procoagulant platelets which express phosphatidylserine (PS) on their surface promoting the conversion of prothrombin to thrombin. Procoagulant platelets are described in detail in section 1.1.6 of this thesis. It has been demonstrated that 14-3-3ζ is a key regulator of PS exposure on the outer surface of the platelet membrane, linking GPIb-V-IX to procoagulant activity (Schoenwaelder et al., 2016). In GPIbβ$^{-/-}$ mice, thrombin generation was decreased in activated platelet-rich plasma and in washed platelets as well as PS exposure (Ravanat et al., 2010). In BSS patients, lacking GPIb-V-IX receptor, the addition of fibrin to PRP, did not increase thrombin generation (Béguin et al., 1999) supporting the evidence that the interaction of fibrin, vWF and GPIb triggers platelet procoagulant activity (Béguin et al., 1999, Béguin et al., 2004). The thrombin binding site on GPIb plays a key role in the exposure of negatively charged phospholipids on the platelet surface and thrombin generation (Dörmann et al., 2000). Although vWF binding to GPIb is not essential to initiate platelet procoagulant activity, this interaction reinforces procoagulant interactions between platelets and its procoagulant function (Béguin et al., 1999). Heparin and low molecular weight heparin interfere with the binding of vWF to GPIb and block thrombin-induced platelet procoagulant activity, an effect which may account, at least partly, for their clinical anti-coagulant functions.

1.1.3.6 Defect in vWF-GPIb interaction: Patients with BSS

Normal haemostasis is highly affected in severe bleeding disorders due to the defect of either the receptor (Bernard-Soulier-Syndrome and pseudo (platelet-type) Von Willebrand disease, Pseudo-VWD) or the ligand (VWD). VWD is a heterogeneous haemorrhagic disorder caused by a deficiency or dysfunction of vWF. In VWD, the defective interaction of vWF with platelets impairs primary haemostasis and leads to a bleeding disorder. Pseudo-VWD is a rare autosomal dominant bleeding disorder resulting from to the enhanced affinity of GPIbα, spontaneous binding and accelerated clearance from the circulation, resulting in thrombocytopenia and bleeding. Bernard−Soulier syndrome (BSS) is a genetic disorder, resulting from a defect in the biosynthesis or functional defect caused by bi-allelic nonsense/missense mutations in *GP1BA*, *GP1BB*, or *GP9* genes (which encodes GPIbα, GPIbβ or GPIX respectively) resulting in aberrant expression or function of GPIb-IX-V complex (Andrews and Berndt, 2013a, Savoia et al., 2014). This genetic defect results in a low platelet count, abnormally large platelets, and prolonged bleeding time (Andrews et al., 1997, López et al., 1998, Miller et al., 1992, Savoia et al., 2001, Noris et al., 2012). The main underlying cause for bleeding is the inability of platelets to adhere to vWF. Homozygous or heterozygous mutations in *GP1BA*, *GP1BB*, or *GP9* genes are responsible for the autosomal recessive forms and mutations in the *GP1BA* gene for the rare autosomal dominant trait. Loss of function of any 1 of 3 of the genes encoding these glycoproteins leads to autosomal recessive macrothrombocytopaenia complicated by bleeding, whilst a small number of variants in *GP1BA* have been reported to cause a milder and dominant form of macrothrombocytopaenia (Sivapalaratnam et al., 2017). A novel mutation in *GP1BB* has been described to cause mild macrothrombocytopaenia in heterozygous individuals (Barozzi et al., 2021). The

mutation frequency has been reported to be higher in GP9 than in the other two genes (Savoia et al., 2014), and several GP9 point mutations affect protein conformation and consequently attenuate the GPIb-IX-V complex expression (Savoia et al., 2014).

1.1.3.7 GPIb-IX-V as an immunological receptor

Beyond its role in thrombosis and haemostasis, GPIb-IX-V was shown to mediate innate immune cell activation. Under flow conditions, platelet priming through GPIb-vWF leads to the activation of GPIIb/IIIa and NETosis (Constantinescu-Bercu et al., 2020). Furthermore, reduced NET release was also observed in the absence of shear (Carestia et al., 2016), suggesting a critical role for GPIb in platelet-mediated NETosis. Blocking vWF markedly reduced platelet-mediated NET formation, suggesting vWF might act as a bridge between platelets and neutrophils (Carestia et al., 2016, Pendu et al., 2006) . Recently, it has been shown using intravital microscopy that, during deep vein thrombosis, murine platelet-neutrophil interaction is mediated by GPIbα (von Brühl et al., 2012). Indeed, it supports platelet recruitment during initial thrombus formation, leukocyte recruitment and triggers NET formation from neutrophils. Furthermore, GPIb-IX has also been described to mediate permanent surveillance of Kupffer cells in the liver and to modulate adapting immune responses shuttling opsonised bacteria to the spleen (Wong et al., 2013). Upon vascular injury, GPIbα is also involved in regulation of thrombus formation, influencing leukocyte recruitment and trafficking (Verschoor et al., 2011) and GPIbα from platelet-derived microparticles supports monocyte rolling on vWF, recruitment, and adhesion on endothelial cells (Bendas and Schlesinger, 2022, Chimen et al., 2020). Platelets can drive the polarization of

monocytes in presence of LPS towards an M1 inflammatory phenotype (Carestia et al., 2019). Interestingly, spreading platelets use lamellipodial protrusions to scan for fibrin/fibrinogen within the inflamed vessels, allowing individual platelets to optimize their position at the vascular wall and contribute to immune surveillance (Nicolai et al., 2020b).

1.1.4 Immunoglobulin superfamily receptors

Immunoglobulin superfamily receptors are a group of surface proteins involved in the binding and adhesion of cells. This family includes GPVI, a major receptor for collagen; FcRγ, which is a dimer that forms a high-affinity complex with two molecules of GPVI on the surface of the platelets and FcγRIIA (CD32), which exists in close proximity to GPIb-IX-V complex and mediates integrin GPIIb/IIIa outside-in signalling. FcγRIIA is also crucial for IgG dependent platelet activation as observed in heparin induced thrombocytopenia (HIT), vaccine induced prothrombotic immune thrombocytopenia (VITT) or in the case of IgG-opsonised bacteria. These receptors together with C-type lectin-like receptor 2 (CLEC-2) and the scavenger receptor CD36 (which is not an immunoglobulin receptor), signal in a tyrosine Kinase dependent manner.

Immunoreceptor-tyrosine-based-activation motifs (ITAMs) and hemi-ITAM (hemITAM) -receptors, are characterised by the presence of two or one YxxL/I motifs separated by 6–12 amino acids on the cytosolic chains of immunoglobulin receptors. These include Fc receptors, T- and B-cell antigen receptors (TCR and BCR), and on platelets, receptor C-type lectin 2 (CLEC-2), GPVI–FcRγ chain and FcγRIIA (Isakov, 1997). These receptors are now described.

1.1.4.1 Glycoprotein VI (GPVI)

Collagen is both a strong adhesive ligand that allows platelets to adhere to injured vessels and is also a potent platelet activator. These 2 responses are mediated by integrin α2β1 and GPVI respectively (Kuijpers et al., 2003, Watson et al., 2010). GPVI is an Ig domain-containing transmembrane protein exclusively expressed on platelets and megakaryocytes with 3000–4000 copies per human platelet (Nieswandt and Watson, 2003, Best et al., 2003). Its cytoplasmatic tail does not have intrinsic signalling activity but allows the association with the ITAM-containing FcRγ chain, which is responsible for the signalling via its ITAM. Upon GPVI-ligand interaction and dimerization, 2 tyrosine residues in the ITAM are phosphorylated, inducing binding and phosphorylation of spleen tyrosine kinase (Syk) through its tandem SH2 domains (**Figure 1.10**). The ensuing phosphorylation cascade leads to activation of phospholipase Cγ2 and phosphoinositide 3-kinases (PI3-K), intracellular Ca^{2+} increase and ultimately several platelet responses (Watson et al., 2005). GPVI has also been found to be a receptor for laminins (Ozaki et al., 2009), fibrin and fibrinogen (Xu et al., 2021), CD147 (Seizer et al., 2009) and galectin-9 (Zhi et al., 2022). GPVI was shown to be critical in arterial thrombosis at the site of atherosclerosis (Massberg et al., 2003), whereas it plays a minor role in haemostasis (Watson et al., 2010). It is important to note that GPVI deficiency was associated with increased thrombus instability (Perrella et al., 2021, Chen et al., 2021a, Ahmed et al., 2020) and reduced PS expression on platelets (Nagy et al., 2020).

1.1.4.2 FcγRIIA

Human but not mice platelets express FcγRIIA, however transgenic mice expressing the human receptor are available (McKenzie et al., 1999). Similar to GPVI, its crosslinking by IgG and immune complexes activation and downstream phosphorylation of target signalling molecules results in platelet activation. FcγRIIA allows platelets to play a role in innate immunity by binding to and becoming activated by pathogens opsonized with antibody favouring their clearance. Platelet activation through the FcγRIIA, plays a critical role in the pathogenesis of various immune-mediated thrombocytopenia and thrombotic syndromes, including heparin-induced thrombocytopenia, bacterial sepsis–associated thrombocytopenia, disseminated intravascular coagulation and the antiphospholipid syndrome (Arman and Krauel, 2015). In platelets, activation of FcγRIIA by anti-platelet autoantibodies triggers Ca^{2+} mobilisation (Ilkan et al., 2018), degranulation, upregulation of surface levels of P-selectin, generation of intraplatelet reactive oxygen species (ROS), and integrin-dependent platelet aggregation. Furthermore, FcγRIIA has been shown to be phosphorylated following ligand binding to platelet GPIIb/IIIa, contributing to the outside-in integrin signalling in human platelets (Boylan et al., 2008).

1.1.4.3 CLEC-2

CLEC-2 is a transmembrane protein containing an extracellular lectin-like domain and a short tail with a single YxxL sequence called hemITAM. CLEC-2 is expressed on platelets, megakaryocytes and at low levels on inflammatory dendritic cells. Human

and mouse platelets express between 2000-4000 and around 40,000 copies of CLEC-2, respectively.

CLEC-2 is expressed as a dimer and signals though a pathway detailed in figure 1.10 similarly to GPVI and FcγRIIA (**Figure 1.10**). CLEC-2 activation induces the hemITAM phosphorylation followed by Syk binding. Syk is therefore phosphorylated by SFK leading to recruitment of LAT and its effectors. The following cascade allows the phosphorylation and activation of PLCγ2 which induces the formation of the secondary messengers inositol 4,5,5 triphosphate (IP3) and 1,2 diacylglycerol (DAG). These lead to the mobilisation of intracellular Ca^{2+} and PKC activation followed by α- and dense granule secretion and inside out activation of GPIIb/IIIa. Mice with loss of CLEC-2 are characterised by blood-filled lymphatic vessels and near 100% perinatal mortality. This is mainly due to the role CLEC-2-podoplanin play in lung development. Targeted deletion of CLEC-2 in megakaryocytes/platelet (PF4-Cre.Clec-1bfl/fl) allows to obtain viable mice with around 30% thrombocytopenia (these mice have also other cell types affected by CLEC-2 deficiency). Postnatal deletion of platelet CLEC-2 does not induce blood-lymphatic mixing or thrombocytopenia, suggesting this receptor plays a crucial role during development (Haining et al., 2021). Treatment with the CLEC-2 monoclonal antibody INU1 results in CLEC-2 depletion in mouse platelets and severe but transient thrombocytopenia (Lorenz et al., 2015).

The major exogenous ligands of CLEC-2 are the snake venom toxin rhodocytin and type 1 human immunodeficiency virus (HIV-1). Rhodocytin induces platelet activation through receptor clustering (Chang et al., 2010). Other exogenous ligands include the sulphated polysaccharide fucoidan (Manne et al., 2013) and diesel exhaust particles

(Alshehri et al., 2015). The most important endogenous ligand for CLEC-2 is podoplanin, also known as GP38 or Aggrus. Podoplanin is not expressed in the vasculature but is expressed on lymphatic endothelial cells, kidney podocytes, lung epithelial cells and is upregulated during inflammation on macrophages and fibroblasts. Podoplanin is upregulated in cancer cells and its role was mostly studied in the context of cancer growth and metastasis but was also shown to participate in venous thrombosis (Quintanilla et al., 2019, Payne et al., 2017), atherosclerosis (Tang et al., 2021) and wound repair (Asai et al., 2016). Furthermore, CLEC-2/podoplanin axis facilitates blood/lymphatic vessel separation during embryonic development, maintains the lymph node vascular integrity, and participate in adaptive immune responses (Suzuki-Inoue et al., 2018, Astarita et al., 2015). Another molecule binding to CLEC-2 is the smooth muscle Ca^{2+} binding protein S100A13 which activates platelets and facilitates thrombus formation (Inoue et al., 2015) . We also demonstrated that hemin is novel endogenous ligand of CLEC-2 and is described and discussed in Chapter 4. Hemin is released following haemolysis and it is quenched by scavenging mechanisms. During severe haemolytic disease, heme induces platelet agglutination and aggregation by ferroptosis, in an ITAM-dependent manner. This will be further described in chapter 4. CLEC-2 plays a major role in thrombosis and a secondary role in physiological haemostatic processes. While there is compelling evidence that GPVI plays a significant role in arterial thrombosis following $FeCl_3$ injury or atherosclerotic plaque rupture in mice, there are fewer studies on CLEC-2. CLEC-2 antibody depletion or genetic depletion in platelets reduced thrombus formation following $FeCl_3$ injury, especially when CLEC-2 and GPVI are both depleted. Recently, it has been demonstrated that mice with genetic deletion of CLEC-2, have reduced thrombus size

post-$FeCl_3$-induced thrombosis, compared to littermates. Similarly, their blood, replenished with CLEC-2 deficient platelets *ex vivo* to match littermates, showed reduced aggregate formation at arterial flow rates (Bourne et al., 2022). This effect, mediated by the loss of CLEC-2 on mouse platelets, was not confirmed in human models of thrombosis (Bourne et al., 2022). Thrombosis is present not only in sterile injury but also as a consequence of systemic infection. In a mouse model of systemic Salmonella Typhimurium infection, thrombosis in the liver has been shown to be triggered by CLEC-2-podoplanin interaction (Beristain-Covarrubias et al., 2019a). Thrombosis was shown to peak at times when bacteraemia was absent whereas IFN-γ release enhanced the number of podoplanin-expressing monocytes and Kupffer cells in the hepatic parenchyma and perivascular sites. Thrombosis is inhibited in CLEC-2–deficient mice, suggesting that exposure of podoplanin induces CLEC-2–driven platelet activation (Payne et al., 2017). CLEC-2 has been shown to be involved in wound healing (Wichaiyo et al., 2019), sepsis (Rayes et al., 2017) , inflammation-driven venous thrombosis and macrophage migration as well as arterial mouse thrombosis (May et al., 2009, Suzuki-Inoue, 2011, Bender et al., 2013). This effect has been shown to take place in mice but not in humans, where CLEC-2 is unable to induce platelet aggregation at arterial shear (Bourne et al., 2022). Indeed, CLEC-2-agonist binding has been demonstrated to have important roles in immunomodulation and inflammation-driven thrombosis.

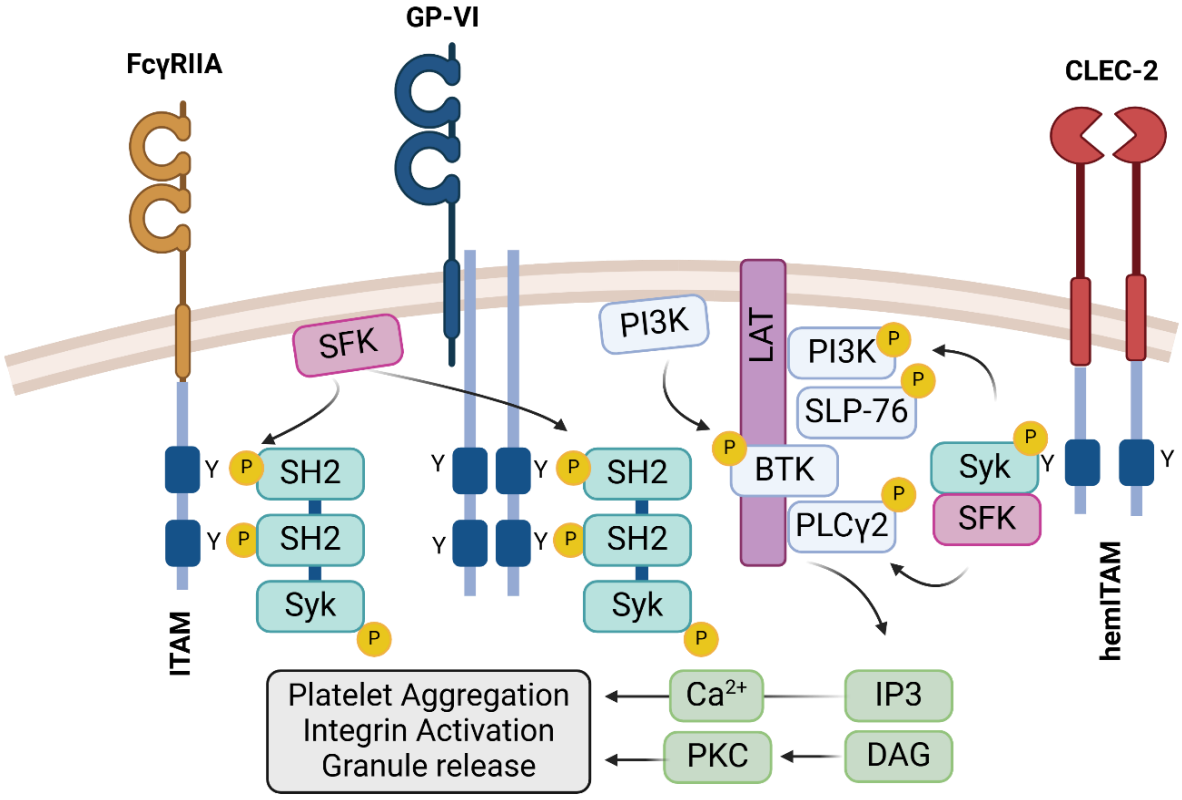


Figure 1.10 – ITAM and hemITAM signalling pathways in platelets. The signalling activity of (hem)ITAM receptors is activated at the cytosolic YXXL motifs which when phosphorylated serve as a docking site for SH2 domain containing signalling molecules. CLEC-2 exists as a homodimer on the platelet surface to allow the activation of the SH2 on the tyrosine kinase Syk. Both LAT and SLP-7645 contribute to the binding of phospholipase Cγ2 (PLCγ2), the enzyme involved in the generation of the second messengers, Ca^{2+} and diacylglycerol (DAG). Other adapters and effectors that associate with the ITAM signalosome, such as the tyrosine kinases Bruton's tyrosine kinase (Btk) and phosphatidyl inositol-3 (PI3) kinase also contribute to effective signalling. The signalling ensues via activated PLCγ2, mobilization of intracellular Ca2+, Ser/Thr kinase PKC isoforms and subsequently, platelets activation/aggregation (Boulaftali et al., 2014). Modified after Ref (Watson et al., 2005, Rayes et al., 2019b). Created with BioRender.com.

1.1.5 Multistep regulating classical haemostasis and thrombosis

Currently, two forms of haemostasis are recognised: classical and inflammatory haemostasis (Rayes et al., 2019b). The classical haemostasis takes places at sites of vascular injury, it is aimed at inducing platelet aggregation and vessel sealing and it is the focus of this chapter. Inflammatory haemostasis takes place in the context of inflammation without overt injury and covers an important role to secure vascular integrity at the site of inflammation. In the context of classical haemostasis, the dynamics of platelet plug formation requires a series of coordinated events: platelet arrest into the exposed endothelium (initiation phase), platelet recruitment and activation (extension phase) and finally stabilisation of the platelet plug (stabilisation phase) (**Figure 1.11**). The subendothelial components responsible to trigger platelet activation include different types of collagen, vWF, fibronectin, and other adhesive proteins such as vitronectin and thrombospondin. The adhesion of platelets to vessel wall is largely influenced by rheological conditions. Indeed, in veins and large arteries, at low shear rate, it mainly involves collagen, laminin and fibronectin while under conditions of elevated shear stress (or in stenotic arteries), vWF unfolding and interaction with platelets through GPIb-IX-V is essential in platelet recruitment. The ability of GPIb-IX-V complex to interact with vWF is critical to allow platelet rolling and subsequently firm adhesion to damaged vasculature (Berndt et al., 2001). This event initiates platelet activation, shape-change and granule secretion. Following vWF binding to GPIb-IX-V complex, the integrin GPIIb/IIIa inside-out signalling is activated allowing its binding to vWF or fibrinogen through the RGD sequence (López et al., 1998, Bodnar et al., 1999, Gu et al., 1999). vWF is not only involved in arterial

thrombosis but was also shown to be mediate venous thrombosis. Indeed, mice lacking vWF, do not develop deep vein thrombosis (DVT) (Brill et al., 2011), However it is important to note that mice lacking vWF don't contain WPB, therefore vWF deficiency is also associated with the lack of P-selectin and other proteins contained in WPB which influence immune cell recruitment and DVT. Arterial thrombosis is triggered by the transient interactions between GPIbα with vWF leading to platelet rolling.

Platelet rolling on vWF allows the interaction of GPVI and GPIa/IIa, with collagen establishing stable adhesion and inducing platelet activation. Upon activation, the cytoskeleton is quickly dismantled and reorganised resulting in platelet shape change. Initially the platelet become spherical as a consequence of actin filaments fragmentation and release of spectrin networks. Secondly, actin filaments assemble and cause membrane protrusion formation, called lamellipodia and filopodia (Sandmann and Köster, 2016). In the case of adherent platelets, the actin filaments polymerise, filopodial protrusion are formed, and cell flattening takes place. This mechanism is called platelet spreading, and allows the formation of a large adhesive surface supporting platelet and innate immune cell recruitment (Hartwig et al., 1999) and the release of granules content. Following platelet recruitment and activation, secretion of secondary mediators ADP and TxA2 supports the recruitment of circulating platelets, platelet aggregation and the formation of platelet-rich clot. This is supported by the activation of PARs on platelets by thrombin, which potentiates GPIIb/IIIa activation, that enables the formation of stable bridges between platelets and further increases cytosolic Ca^{2+} and reorganization of the platelet cytoskeleton

and platelet secretion. Ligand binding and integrin clustering induced outside-in signalling which are crucial for platelet signal amplification and clot stabilisation. Details of inside-out and outside-in GPIIb/IIIa are described in **Figure 1.11**.

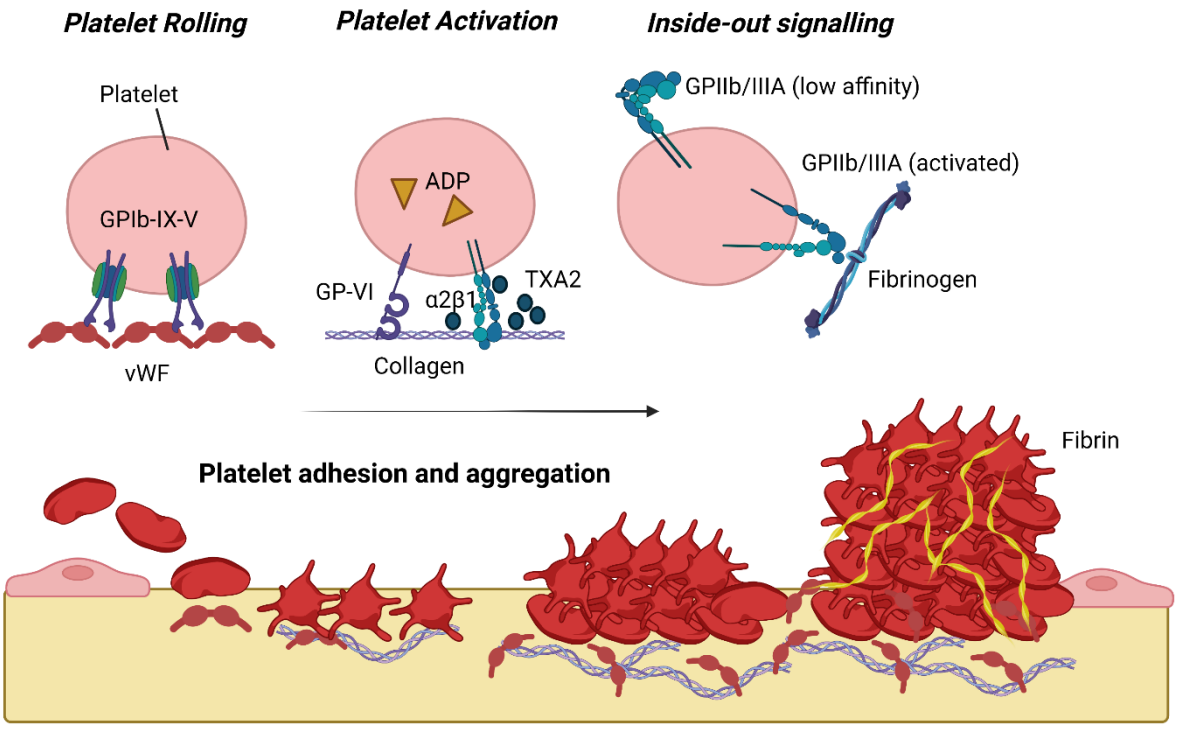


Figure 1.11 – Platelets activation and aggregation in thrombus formation. Platelets flowing into the blood vessels, transiently adhere to exposed collagen fibres, covered with von Willebrand factor (vWF), via the GPIb-IX-V complex initiating rolling. Stable platelet adhesion occurs by integrin α2β1 and glycoprotein VI (GPVI) and platelet recruitment is enhanced by ADP and thromboxane A_2 which recruit other platelets. GPIIb/IIIa becomes activated, and adjacent platelets connect by fibrinogen binding, resulting in platelet aggregation. After this initial phase, thrombin is generated, the coagulation cascade is activated and fibrin stabilises the thrombus. Modified after Ref (McFadyen et al., 2018). Created with BioRender.com.

1.1.5.1 Negative regulators of thrombus formation

Beside NO and PGI_2, the adhesion molecule PECAM-1 (also known as CD31), an immunoreceptor tyrosine-based inhibitory motif (ITIM) which becomes phosphorylated upon stimulation, inhibits kinase-dependent signalling and platelet activation. PECAM-1 plays a role in attenuating thrombus formation involving GPVI, GPIbα, and thrombin-mediated platelet activation, as well as normal integrin GPIIb/IIIa-mediated platelet function (Falati et al., 2006). Ectodomain shedding of platelet receptors, GPIb and GPVI as well as cleavage of vWF by ADAMS13 are other mechanisms contributing to thrombus growth restriction (De Meyer, 2019).

1.1.5.2 Coagulation and Fibrinolysis: platelet contribution to coagulation

Secondary haemostasis is required for stabilisation of the thrombus. First of all, GPIIb/IIIa bound to fibrinogen triggers essential outside-in events for thrombus growth and stabilization, such as cytoskeletal reorganization, stabilization of large platelet aggregates, development of a procoagulant surface (phosphatidylserine exposure) and for clot retraction. Two distinct pathways trigger the initiation of the coagulation cascade: (i) intrinsic (also known as contact activation pathway) and (ii) extrinsic (TF pathway) (**Figure 1.12**). Tissue Factor (TF) is derived from activated endothelial cells or exposed from the exposed sub-endothelium. Interestingly, in mice, neutrophils, monocytes and monocyte-derived microparticles captured by the thrombus through the interaction between P-selectin and PSGL-1 also express TF and contributes to thrombus formation and stability (Falati et al., 2003, Furie and Furie, 2004, Kappelmayer and Nagy, 2017). Irrespective of the trigger, both pathways (detailed in Figure 1.12) converge into thrombin generation. Generation of thrombin

requires several surface-mediated reactions carried out by the tenase complex (FIXa in complex with FVIIIa, which activate FX) and by the prothrombinase complex (FXa in complex with FVa, which activate FII). Phospholipid membranes with negatively charged phospholipids, (i.e. PS on platelets), greatly promote the binding of coagulation factors and the generation of Factor Xa and Thrombin (Heemskerk et al., 2002, Monroe et al., 2002). Beyond cleavage of fibrinogen to form fibrin, thrombin generation is also essential for the formation of procoagulant platelets which are described in section 1.1.6.

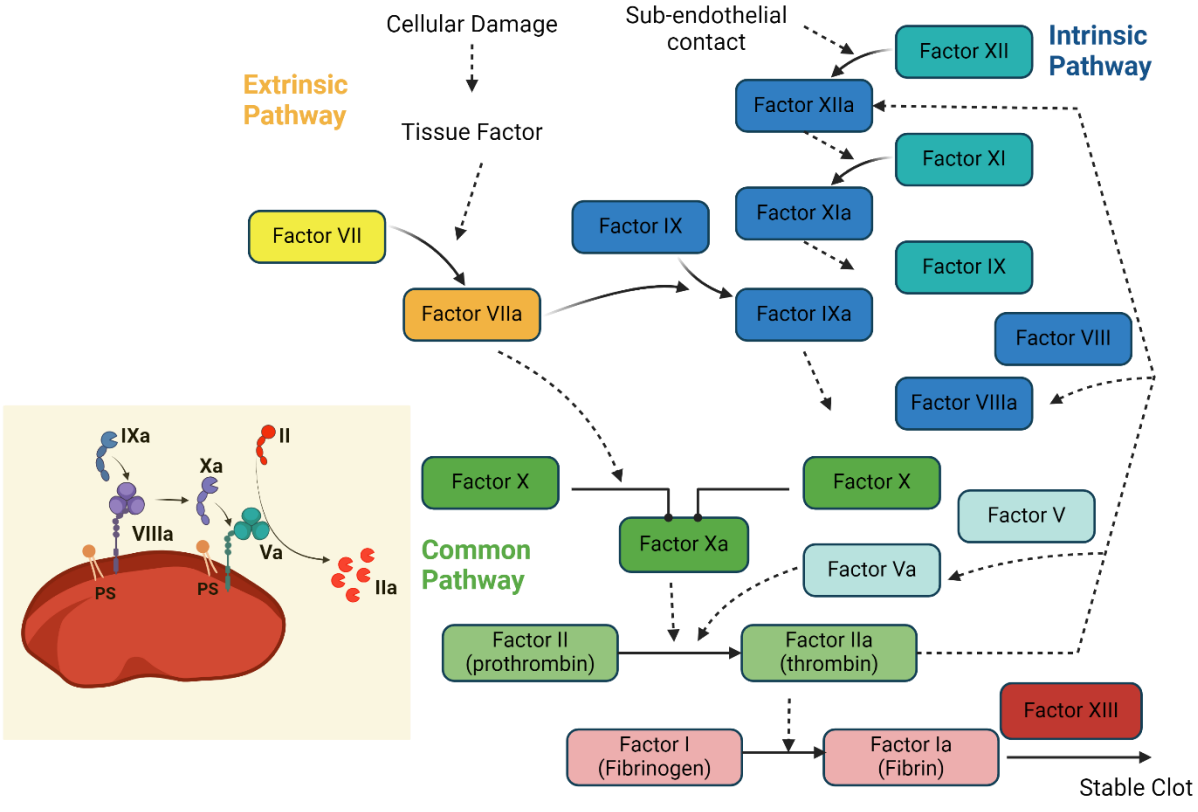


Figure 1.12 – Coagulation cascade. Steps of the coagulation cascade as described in the text. Procoagulant platelets favour blood coagulation. The serine protease factor IXa cleaves factor X into its active form, factor Xa. Factor X activation requires a PS-containing membrane surface for its assembly

with the factors VIIIa and IXa (the tenase complex). The prothrombinase complex consists of the non-enzymatic cofactor Va, the serine protease factor Xa and the substrate prothrombin. Procoagulant phospholipids (PS) stimulate prothrombinase assembly and thrombin formation Created with BioRender.com

Once released, thrombin exerts its function through the GPIbα subunit of the GP Ib/IX/V complex and PAR receptors leading to full platelet activation and aggregation. Finally, thrombin activates other coagulation factors such as Factor XIII, which contribute to fibrin stabilisation. The coagulation system is regulated by anticoagulant mechanisms which are naturally present in the body. The main molecules involved in this pathway are the following. Antithrombin (AT), a serine protease which mainly inhibits thrombin and inactivate factor IXa, Xa, XIa and XIIa. Tissue factor plasminogen inhibitor which affects the extrinsic pathway by inhibiting TF-VIIa complex. Finally, the Protein C pathway which consists of Activated Protein C (which inhibits activated factors V and VIII), Thrombomodulin (which binds to thrombin), Endothelial protein C receptor and Protein S (Palta et al., 2014).

The last stage of thrombus is clot contraction. The platelet GPIIb/IIIa creates a mechanical and structural bridge between extracellular fibrin and intracellular actin leading to mechanical contractile forces. This contraction induces a dramatic remodelling of the fibrin network, reducing clot volume and increasing its density (Litvinov and Weisel, 2022).

The final thrombus is very heterogeneous in composition of red blood cells, white cells and platelets. In particular arterial thrombus is rich in platelets while venous thrombus is mostly red cells and fibrin (Alkarithi et al., 2021). Besides being the core of the

thrombus, essential for aggregation and contraction of the clot, platelets are also crucial for accelerating the coagulation process. First of all, they are a source of coagulation factors, such as Factor V and XIII and prothrombin. Furthermore, a population of strongly activated platelets, called procoagulant platelets, described in the detail in section 1.6, provide a procoagulant surface and enhance fibrin generation. During thrombus contraction, these platelets are typically extruded and form a shell around the core of pro-aggregatory platelets inside the thrombus. This ring-like distribution allows them to control the spatial generation and deposit of thrombin and fibrin on the surface of the thrombus where they contribute to the regulation of the fibrinolytic process (Nechipurenko et al., 2019).

1.1.5.3 Fibrinolysis: tertiary haemostasis

The last stage of the haemostasis is the tertiary haemostasis, when the thrombus is lysed through the action of plasmin. Plasmin is generated from plasminogen on the surface of the clot or on cell surfaces by tissue plasminogen activator (tPA) or urokinase (uPA). Fibrinolysis is a highly regulated enzymatic process that prevents accumulation of fibrin and ensure removal of thrombi. Indeed, the activity of plasmin is negatively modulated at different levels to prevent hyperfibrinolysis. Plasminogen activator inhibitor type-1 (PAI-1) and type-2 (PAI-2) inhibit uPA and tPA, α2-antiplasmin and α2-macroglobulin regulate its activity in the plasma, while thrombin-activatable fibrinolysis inhibitor (TAFI) removes C-terminal lysine residues on partially degraded fibrin to decrease plasmin formation. In addition to procoagulant activity, platelets participate to fibrinolysis in different ways. Firstly, following clot retraction, the

availability of plasminogen and activators is attenuated due to their limited exposure on the surface. The cross-linking of α2-anti-plasmin-fibrin, decreases itself the binding of tPA (Kunitada et al., 1992, Aoki, 1993, White, 1998). Furthermore, platelets themselves secrete α2-antiplasmin, PAI-1, and TAFI, all of them highly concentrated in their α-granules (Suzuki et al., 2020). On the other hand, platelets have been also described to release plasminogen and its activators and to offer a binding site for plasminogen via GPIIb/IIIa. Interestingly, procoagulant platelets on the surface of the thrombus have been described to bind plasminogen to protruding “caps” via fibrinogen and display enhanced capacity to generate plasmin (Whyte et al., 2015). This evidence suggests an important role for platelets in modulating local fibrinolysis and regulating haemostasis. For further details on the role of platelets in tertiary haemostasis, I refer to an excellent review (Napolitano and Montuori, 2022). To conclude, it has become clear that platelets interact with the coagulation system during all the phases of the thrombus formation (Swieringa et al., 2018). Platelets are key actors in the maintenance of vascular integrity and distinct platelet pathways are coordinated in a spatial and temporal manner to guarantee all the stages of haemostasis are tightly regulated.

1.1.6 Procoagulant platelets: mechanisms, receptors, function

Procoagulant platelets are a subpopulation of activated platelets, expressing negatively charged phospholipids on their surface, which favour thrombin generation. They promote the deposition of fibrin at sites of vascular injury to stabilise and support primary plugs composed of aggregating platelets. An excess of procoagulant platelets

favours worse clinical outcome in conditions such as stroke, coronary artery disease and COVID-19-associated thrombosis (Wang et al., 2016, Kirkpatrick et al., 2020, Khattab et al., 2021) while a defect in procoagulant platelets can worsen conditions such as haemorrhagic stroke (Prodan et al., 2015). Current anti-platelet drugs, which target P2Y12 and COX, aim at reducing platelet activation and aggregation, while their effect on procoagulant platelet formation has only been partially investigated. For example, Clopidogrel has been shown to reduce procoagulant platelet activity in patients with diabetes mellitus but only at high dosing (Angiolillo et al., 2009).

Platelets reported in the literature to be procoagulant show several overlapping features and are known with different names such as ballooned platelets (BPs), sustained Ca^{2+}-induced platelets (SCIP), fibrinogen capped platelets (FIB-CAP), ballooned and procoagulant spread (BAPS) platelets, ballooned non-spread platelets (BNS), superactivated platelets (Mazepa et al., 2013), zombie platelets (Storrie, 2016), GSAO platelets, mPTP platelets and COATED (induced by collagen and thrombin) platelets. The earliest report of a ballooning platelet was in 1978 by Wester *et al*., who reported changes in platelet morphology depicted by electron microscopy. They described a subset of balloon-shaped platelets with fibrin deposition (Wester et al., 1978, Wester et al., 1979). After years, this phenotype was generated *in vitro* on collagen and platelets were described to externalise PS in presence of sustained intracellular Ca^{2+} increase. The ballooned area provides an extended surface area for the assembly of the tenase and prothrombinase complexes promoting coagulation processes at the wound site. Different pathways lead to the exposure of PS on platelets' outer membrane including apoptosis, necrosis, and necroptosis. Apoptosis is

a form of programmed cell death by which aged platelets are removed from the circulation. In platelets, apoptosis is mediated by Bak/Bax- signalling pathway, independently of platelets activation (Josefsson et al., 2014). Apoptotic platelets display cytochrome c, apoptotic protease-activating factor-1 (Apaf1) and caspases-9 and -3, which are all considered markers of apoptosis in nucleated cells. Necrosis is a form of programmed cell death resulting in inflammatory and reparative responses. In platelets, necrosis is triggered by different strong agonists stimulation leading to high sustained intracellular Ca^{2+} levels, mitochondrial dysfunction, and Cyclophilin-D activation. Cyclophilin-D is a mitochondrial isomerase that regulates mitochondrial function. Necroptosis is a form of regulated necrosis. The process of necroptosis leads to phosphorylation of MLKL (mixed lineage kinase domain-like pseudokinase) which forms octamer channels that translocate to the surface membrane of dying cells causing release of cellular contents. Details about procoagulant platelets and cell death pathways are discussed in the paragraph 1.1.6.3 In 2015, Hua *et al*., showed that co-labeling platelets with GSAO [4-(N-(S-glutathionylacetyl)amino) phenylarsonous acid] and an α-granule marker such as P-selectin, identified a subpopulation of activated platelets undergoing cyclophilin-D-dependent necrosis (Hua et al., 2015). GSAO is a cell death marker that pass through the procoagulant platelet membrane with elevated permeability, covalently bind to proteins with closely spaced dithiols, and is retained. Using a GSAO-fluorescein marker, flow cytometry can analyse procoagulant platelets (Tan et al., 2019, Pasalic et al., 2018). Cyclophilin-D is a modulatory component of the mitochondrial permeability transition pore (mPTP) formation and opening depolarises the inner mitochondrial membrane to decrease ATP and increase oxidative stress. Cyclophilin-D sensitises mPTP to Ca^{2+}, inorganic

phosphate and probably ROS, and mPTP opening is crucial for the formation of procoagulant platelets. Indeed, cyclophilin-D inhibition reduces the opening of the mPTP and the formation GSAO platelets (Jobe et al., 2008, Liu et al., 2013, Remenyi et al., 2005). From a clinical perspective, inhibiting CyPD to inhibit PTP opening protects against ischemia–reperfusion injury, making modulation of CyPD activity a potentially important therapeutic goal. GSAO platelets, were initially described by Dale *et al* to be formed following maximal co-stimulation with thrombin and collagen (Dale et al., 2002) and they are called “coated” because they express Factor Va (FVa) on their surface, transglutaminase substrates, fibrinogen, von Willebrand factor, thrombospondin, fibronectin and α2-antiplasmin. COATED platelet formation is associated with rapid loss of mitochondrial transmembrane potential (Remenyi et al., 2005, Dale et al., 2002, Alberio et al., 2000). The different definitions and features of the procoagulant platelet have differed depending on whether they were investigated in suspension (COATED, MPTP, BNS, FIB-CAP, or GSAO) or adherent to solid agonist-coated matrices (SCIP, MPTP, BNS, BAPS, FIB-CAP). Unlike in suspension, a solid matrix provides procoagulant platelets with an adhesion platform which allow features such as procoagulant-spreading, observed in SCIP and BAPS platelets (Agbani et al., 2015, Kulkarni and Jackson, 2004). Overall, several characteristics have been shared by procoagulant platelets. The main ones being externalisation of negatively charged phospholipids, Factor Xa/Va binding, surface exposure of Factor XIII (that stabilises the fibrin mesh), thrombin generation, high levels of intracellular Ca^{2+}, loss of mitochondrial membrane potential, cyclophilin D-dependence, microvesiculation, ballooning/cell membrane remodelling, GPIIb/IIIa inactivation (low capacity to aggregate) and procoagulant spreading (adherent only).

1.1.6.1 Agonists involved in procoagulant platelet formation

The procoagulant response can be activated by different agonists such as collagen (and related molecules such as collagen-related peptide (CRP), or convulxin and thrombin (Södergren and Ramström, 2018, Polgár et al., 1997, Munnix et al., 2007, Aliotta et al., 2021, Harper et al., 2013, Cosemans et al., 2008). Dual agonists and synergistic pathway activation allow sufficient and sustained Ca^{2+} mobilisation which is indispensable for the formation of procoagulant platelets (Sveshnikova et al., 2016). Other agonists such as adenosine diphosphate (ADP), arachidonic acid, ionophore A23187, or thrombin receptor activating peptide 6 (TRAP6) have been described to induce the formation of procoagulant platelets too. Immunoglobulin binding to FcγRIIA have been described to induce the formation of procoagulant platelets or potentiate the procoagulant effect of collagen (Batar and Dale, 2001). Furthermore, GPIb has also been shown to have a key role in thrombin generation and in the exposure of negatively charged phospholipids on platelet membrane (Dörmann et al., 2000).

1.1.6.2 Receptors involved in procoagulant platelet formation

Different receptors are involved in procoagulant platelet formation. GPVI is a key receptor involved in the formation of procoagulant platelet formation. Collagen induces more PS exposure than thrombin alone, but platelets become highly procoagulant when stimulated with dual agonists or when also exposed to shear stress (Delaney et al., 2014). Other receptors involved in procoagulant platelet formation are PAR1/PAR4. PAR1 agonists induce a weaker Ca^{2+} release and PS exposure than thrombin (Aliotta et al., 2020a), suggesting the additional interaction of thrombin with the GPIb receptor

plays a key role in this process. Importantly, in later activation, the GPIb-IX-V complex becomes a site where procoagulant factors adhere (Andrews and Berndt, 2013b). Furthermore, the activation of the ADP receptor P2Y12 amplifies the response initiated by collagen and thrombin, leading ultimately to reduced Ca^{2+} mobilisation (Keularts et al., 2000). Features of procoagulant platelets and receptors mediating those effects are summarised in **Figure 1.13**. The main agonists described in the literature to allow the formation of procoagulant platelets are summarised in table 1.1.

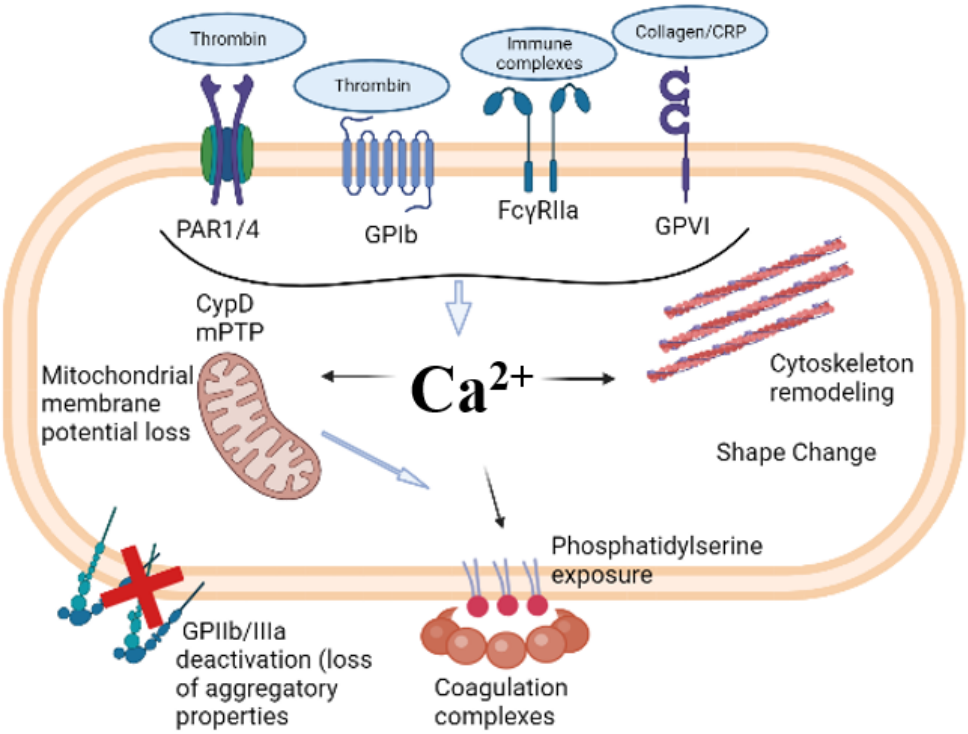


Figure 1.13 – Agonists triggering procoagulant Platelet formation. Procoagulant platelet formation is initiated by strong agonist stimulation of different platelet receptors, resulting in sustained increase in intracellular Ca^{2+}. Thrombin, collagen (or other GPVI agonists) and immunocomplexes can all lead to procoagulant activity. In the presence of Ca^{2+}, phosphatidylserine is flipped from the inner to the outer membrane of the platelet surface facilitating the assembly of coagulation complexes. Furthermore, the increase in Ca^{2+} drives the cytoskeletal remodelling and the mitochondrial potential loss. Created with BioRender.com. Adapted from Ref (Tohidi-Esfahani et al., 2020).

Sample Type	Agonist	Condition	% Procoagulant
Washed Platelets (5×10^4/µL) (Agbani et al., 2015)	Thrombin (0.1 U/ml) Collagen (5µg/ml) Thrombin (0.1 U/ml) + Collagen (5µg/ml)	37°C, 10 min37°C, 10 min 37°C, 10 min	59.2 % 45.76% 20.78%
Whole blood (Pasalic et al., 2018)	Collagen (10 µg/ml) Thrombin (2 U/ml) Thrombin (2 U/ml) + collagen (10 µg/ml)	RT, 10 min RT, 10 min RT, 10 min	1.2 % 13.15% 17.3%
Whole blood(Pasalic et al., 2017)	Thrombin (2 U/ml) + collagen (10 µg/ml)	37°C, 10 min	60%
Whole Blood (Tan et al., 2019)	Thrombin + collagen (final concentrations variable)	RT, 10 min	29.7%

Table 1.1 Main agonists which allow the formation of procoagulant platelets. RT=room temperature (RT=25-27°C), min=minutes. Adapted from Ref (Chu et al., 2021).

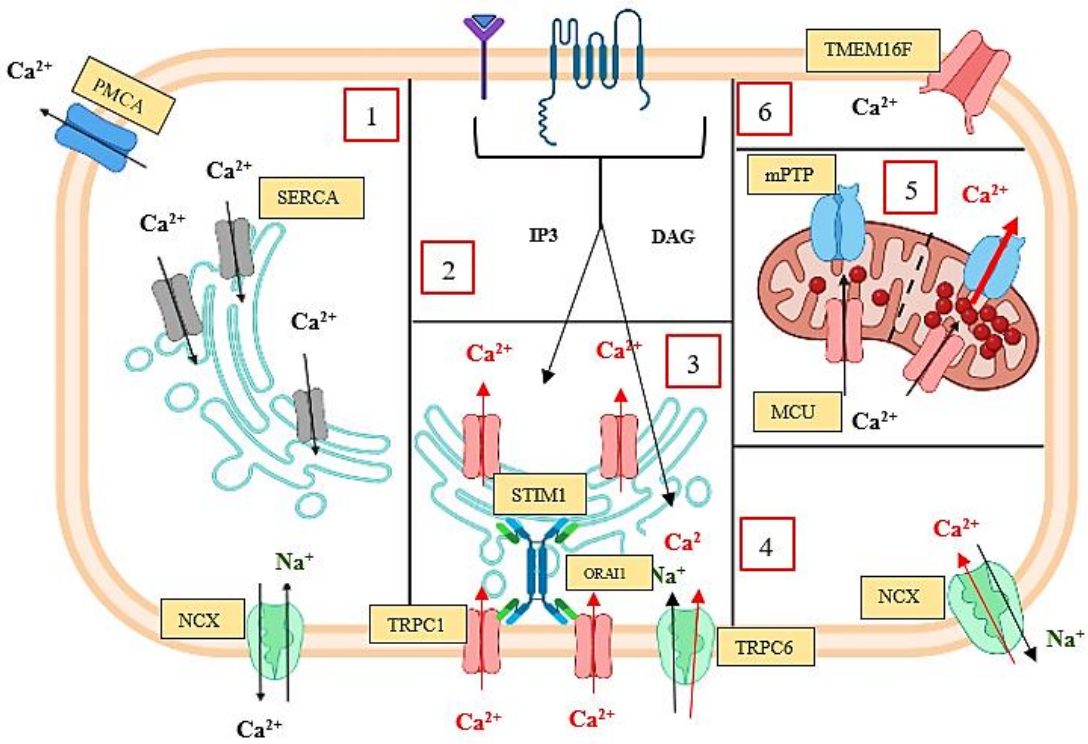
1.1.6.3 Mechanisms and signalling pathways involved in the formation of procoagulant platelets

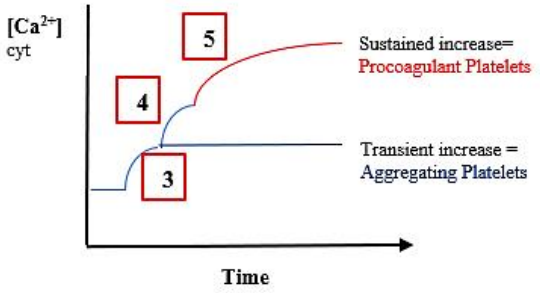
The main common features of procoagulant platelets are the 1) increase in intracellular Ca^{2+}, 2) loss of aggregating activity, 3) PS exposure 4) ballooned shape. The mechanisms leading to those features have been partially elucidated.

1.1.6.3.1 Role of intracellular Ca^{2+}in the formation of procoagulant platelets

When platelets are activated, acquiring either aggregating or procoagulant phenotypes, the cytosolic Ca^{2+} concentration increases. The key element leading to the procoagulant platelet features described above, is reaching a “supramaximal” cytosolic Ca^{2+} concentration (>100µM). This process involves three stages summarised in figure 1.14. Initially, Ca^{2+} is released from intracellular storages (mainly the ER) and this is further potentiated by extracellular Ca^{2+} entry. This process is mediated by receptor-operated (ROCE) and store-operated (SOCE) pathways detailed in the figure 1.14. Intracellular pathways converge to activate PLC (either PLCγ2 for GPVI and PLCβ for PAR1 and 4) following platelet activation. This leads to the formation of IP3 and DAG. IP3 triggers the release of Ca^{2+} from internal stores into the cytosol. The SOCE mechanism involves STIM1, a type 1A transmembrane Ca^{2+} sensor in the ER of platelets which can detect the release of Ca^{2+} from intracellular stores and regulate Orai1. Orai1is positively regulated by calpain, a cytosolic Ca^{2+} -activated protease and allows Ca^{2+} to enter from the extracellular space. DAG induces on its own the opening of the transient receptor potential cation channel subfamily C member 6 (TRPC6) allowing the entrance of extracellular Ca^{2+}. Once the Ca^{2+} is depleted from internal storages, TRPC1 is also activated (Zitt et al., 1996). Additional Ca^{2+} entry depends on high cytosolic Na^{+} levels. These are responsible for the switching of the NCX functional mode to its reverse mode. This mechanism allows the pumping of Ca^{2+} in the cytosol while expelling exceeding Na^{+} out of the cell. Ca^{2+} rise is also dependent on potassium (K^{+})-activated NCX (NCKX) (Pulcinelli et al., 2013). All these mechanisms allow the oscillatory intracellular Ca^{2+} to increase and the NCX

reverse mode allow the maintenance of high Ca^{2+} levels necessary to induce mitochondria depolarisation leading to the opening of the mPTP. The second stage involves the entry of Ca^{2+} inside the mitochondria. Mitochondria accumulate Ca^{2+} via MCU complex while they expel it through the mitochondrial H/Ca and Na^{+}/Ca^{2+} exchangers. Procoagulant platelet formation in MCU null platelets was significantly reduced. When this high non-physiological Ca^{2+} concentration is reached, the threshold for the mPTP opening is achieved and the third stage starts leading to the formation of procoagulant platelets. Indeed, high and sustained cytosolic Ca^{2+} is only achieved after the mPTP opening. Inhibitors of the mPTP, such as Cyclosporin A, reduce PS exposure and macrovesicle formation (Obydennyy et al., 2016) while activators, such as H_2O_2 increase their formation (Jobe et al., 2008, Remenyi et al., 2005). Cyclosporin A-inhibits cyclophilin D which allows mPTP opening. Finally, the Ca^{2+} -dependent scramblase activity of the TMEM16F (knows as Anoctamin 6) is responsible for PS exposure in platelets. This scramblase (as well as calpain) has low affinity for Ca^{2+}, and it exerts its role only when high Ca^{2+}, present only in procoagulant platelets, is reached (Kunzelmann et al., 2014).


Ca2+
PMCA
1
SERCA
Ca2+
Ca2+
Ca2+
IP3
DAG
2
3
Ca2+
Ca2+
STIM1
ORAI1
Ca2
Na+
TRPC1
TRPC6
Ca2+
Ca2+
Na+
NCX
Ca2+
TMEM16F
6
Ca2+
mPTP
5
Ca2+
MCU
Ca2+
4
Ca2+
NCX
Na+


[Ca2+]
cyt
5
4
3
Sustained increase=
Procoagulant Platelets
Transient increase =
Aggregating Platelets
Time

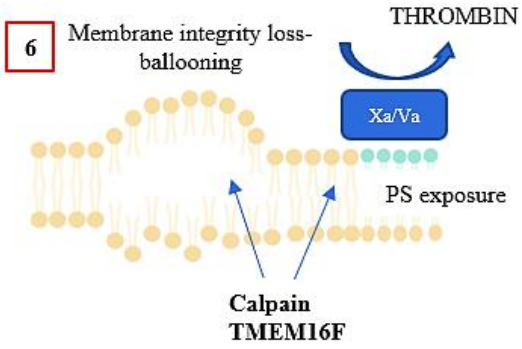

6
Membrane integrity loss-
ballooning
THROMBIN
Xa/Va
PS exposure
Calpain
TMEM16F

Figure 1.14 – Ca^{2+} regulation and mobilisation leading to the formation of procoagulant platelets. ***1.*** *Intracellular Ca^{2+} levels in resting platelets are regulated by the plasma membrane Ca2+ ATPase (PMCA) receptor and the forward mode of Na+/Ca2+ exchanger (NCX) which extrude Ca^{2+} towards the extracellular space, this is part of the receptor-operated (ROCE) pathway while Sarco-Endoplasmic Reticulum Calcium ATPase (SERCA) and the mitochondrial calcium uniporter (MCU) compartmentalise Ca^{2+} inside the intracellular organelles and mitochondria, respectively.* ***2.*** *Following activation of GPVI and PAR1/4, Inositol trisphosphate (IP3) and diacylglycerol (DAG) are produced.* ***3****. IP3 triggers the release of Ca^{2+} from internal stores while stromal interaction molecule 1(STIM1) sense the decrease in Ca^{2+} and activates ORAI1 to refill the stores. DAG activated TRCP6 and Ca^{2+} is increased intracellularly.* ***4****. The increase in cytosolic Na^{+} and activation of other mediators such as PKCα, induce the reverse mode of NCX allowing further Ca^{2+} to enter intracellularly.* ***5****. This increased Ca^{2+} is transferred by MCU inside mitochondria which become depolarised, when a specific threshold is reached, mPTP opens and a consistent amount of Ca^{2+} is released into the cytosol.* ***6****. When a sustained micromolar levels of intracellular Ca^{2+} are reached, calpain and TMEM16F are activated inducing the PS exposure and initiating all the other features described in procoagulant platelets. In the lower panel stepwise mobilisation of Ca^{2+} and details of PS exposure and membrane ballooning.* This figure is inspired and adapted from Refs (Chu et al., 2021, Veuthey et al., 2022). Created with BioRender.com.

1.1.6.3.2 Loss of aggregation activity

When platelets are exposed to stimulating agonists, they all rapidly activate their fibrinogen receptor and become pro-aggregatory via the inside-out activation of GPIIb/IIIa. The binding of fibrinogen to activated GPIIb/IIIa allows platelet bridging and induces an outside-in signalling leading to irreversible aggregation. Following this initial phase, a fraction of those activated platelets becomes procoagulant. These platelets downregulate GPIIb/IIIa and they start expressing PS (Alberio et al., 2017). GPIIb-IIIa inactivation prevents irreversible aggregation, release of granule content and

spreading of platelets. The mechanism leading to the downregulation of GPIIb/IIIa is not entirely clear. Different hypothesis has been investigated. One way is mediated by a calpain-2/TMEM16F-dependent mechanism following the increase in cytosolic Ca^{2+} (Mattheij et al., 2013) and mPTP opening (Liu et al., 2013, Kholmukhamedov et al., 2018, Alberio et al., 2017). It has also been described that GPIIb-IIIa outside-in signalling could be a negative regulator for procoagulant platelet formation (Aliotta et al., 2020b) blocking Ca^{2+} mobilisation and cytoskeleton changes required for the procoagulant response (Rosado et al., 2001).

1.1.6.3.3 PS exposure

The main phospholipids that have been described on the surface of procoagulant platelets are PS and phosphatidylethanolamine (PE) (Seigneuret and Devaux, 1984, Zwaal and Schroit, 1997). These phospholipids are crucial for the assembly of the intrinsic tenase complex (factors VIIIa-IXa) and the prothrombinase complex (FVa-Xa)(Stoilova-McPhie, 2021). In resting conditions, the presence of PS is restricted to the internal side of the platelet membrane due to adenosine triphosphate (ATP)-dependent flippase activity. However, when the cytosolic calcium concentration is increased, the flippase activity is inhibited and the transmembrane 16F (TMEM16F) scrambles PS/PE across the membrane (Millington-Burgess and Harper, 2020, Baig et al., 2016). In addition to PS/PE exposure, procoagulant platelets (in particular COAT platelets) retain on their membrane, α-granule-secreted procoagulant proteins (factor V/Va) and adhesive factors such as fibrinogen, fibronectin, vWF and thrombospondin. These proteins are retained on the surface by a serotonin and transglutaminase-

dependent mechanism (Dale et al., 2002). Transglutaminases catalyse the formation of iso-peptide bonds between specific glutamine residues and either lysine or simple amines and the α-granule proteins are all potential acyl-donors for transglutaminases. Furthermore, on COAT platelets, coagulation factor XIII is also externalised cross-linking α2-antiplasmin to fibrin, promoting fibrin fibres formation (Mattheij et al., 2016, Mitchell et al., 2014). Whereas PS exposure can be induced by different agonists, distinct characteristics are observed dependent on the stimulation. For instance, calcium ionophore induces PS exposure which does not require all the signalling pathways to be activated. Moreover, those platelets do not express coagulation factors on their membrane nor downregulate GPIIb/IIIa, suggesting they do not acquire the full and complete procoagulant phenotype (Alberio et al., 2000), despite PS expression.

1.1.6.3.4. Shape change

Another feature of procoagulant platelets is a distinct change in their shape. Procoagulant platelets undergo ballooning and blebbing formation which are led by actin remodelling through a calpain-mediated mechanism requiring high calcium concentrations (Cong et al., 1989). Ballooning takes place when blebs undergo expansion caused by the intracellular entry of water through TMEM16F and aquaporin1. When ballooning takes place, the process is irreversible (Agbani et al., 2015). The ballooning process allows the surface to increase on procoagulant platelets. Ballooning is mechanistically and structurally distinct from membrane blebbing and involves disruption to the platelet microtubule cytoskeleton and inflation through fluid entry. Membrane ballooning correlated with microparticle generation.

Inhibition of Na^+, Cl^-, or water entry impaired ballooning, procoagulant spreading, and microparticle generation, and it also diminished local thrombin generation. The high calcium concentration and the calpain pathway leads also to microvesicles shedding by procoagulant platelets (Nomura and Shimizu, 2015, Wei et al., 2020, Morel et al., 2011). Those PS-positive microvesicles, also positive for coagulation factors, support and propagate the coagulation cascade.

1.1.6.4 Distribution and function of procoagulant platelets in thrombosis

The data on the localisation of aggregating and procoagulant platelets within the thrombus have been controversial. Munnix *et al* , described procoagulant platelets to be distributed as patches around the thrombus either *in vitro* (flowing human or murine blood over collagen) and *in vivo* (ligating the carotid artery or via ferric chloride–induced injury) (Munnix et al., 2007). Similarly, Jørgensen et al. identified ballooned platelets and fibrin at the periphery of the thrombus (Jørgensen and Borchgrevink, 1963) using either carotid artery ferric chloride–induced injury or abdominal aorta mechanical injury in mice. In this work, the core of the thrombus is described to be composed of aggregating tight platelets, with reduced wash-out of agonists while at the periphery of the thrombus less activated and loosely adherent platelets are present. In addition, mice pulmonary neutrophils interact with procoagulant platelets present on the surface of thrombi (Yuan et al., 2017) and a similar pulmonary neutrophil-rich thrombotic response was described in humans with acute respiratory distress syndrome. Differently, laser-induced thrombi induced in mice mesenteric venules led to platelet surface PS expression to be initiated at the site of injury and then to penetrate into the

thrombus. PS-positive platelets were predominantly localized in the centre of the thrombus with fibrin (Hayashi et al., 2008). The surface localisation of platelets might seem a paradox because biochemically platelets require a very strong stimulus to become procoagulant. The strong activation is expected to be at the periphery rather than at the core of the thrombus. However, using transmission electron microscopy and fluorescence microscopy of arterial thrombi formed *in vivo* in mice, it was demonstrated that procoagulant platelets are located at the periphery of the formed thrombi (Nechipurenko et al., 2019). Therefore, it has been proposed that procoagulant platelets form within the thrombus and then are translocated to the outer shell by a mechanism of mechanical contraction (**Figure 15)**.

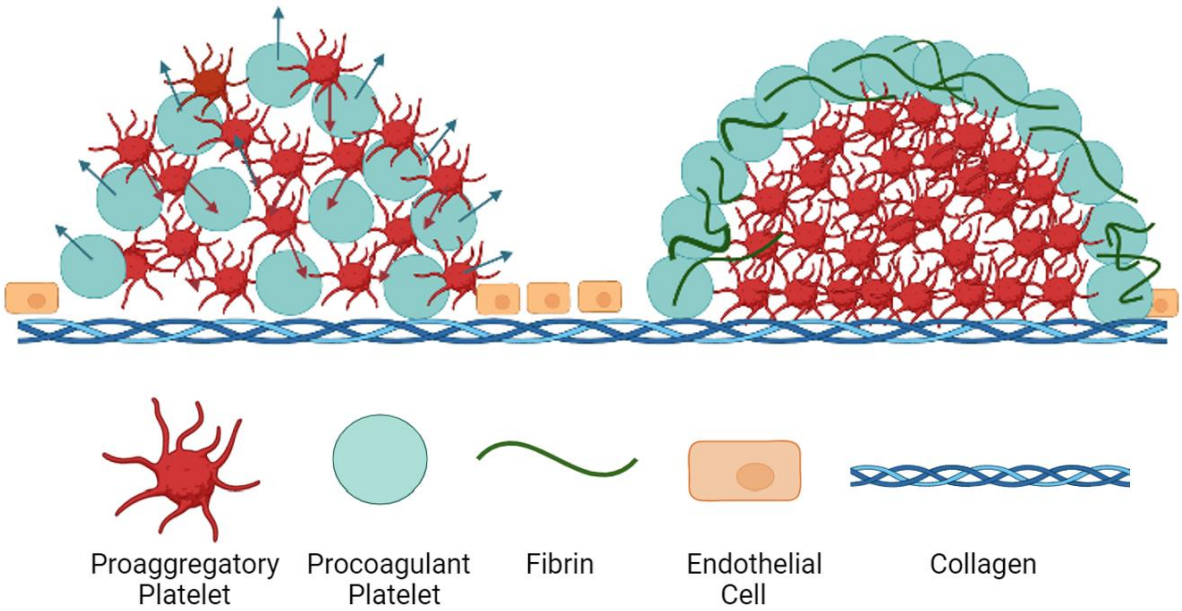


Figure 1.15 – The distribution of procoagulant platelet in thrombi. Following activation, platelets become pro-aggregatory and start forming the thrombus. Platelets aggregate via fibrinogen bridging activated GPIIb-IIIa receptors. Initial thrombin generation in combination with collagen further activates platelets in the thrombus core. The more the thrombus is growing, the more the platelets become activated, the increasing cytosolic level of calcium eventually creates procoagulant platelets. Procoagulant platelets downregulate their GPIIb-IIIa receptor and express negatively charged phospholipids binding

coagulation complexes, which localize and enhance thrombin generation. Thrombin converts fibrinogen into fibrin, which consolidates platelet clot. In parallel, the outside-in signalling of aggregating platelets inducing clot retraction squeezes procoagulant platelets out of core of the thrombus. Procoagulant platelets present at the external site of the thrombus, stop the bleeding and any further thrombus expansion. Adapted from Ref (Kholmukhamedov and Jobe, 2019) and illustrated using BioRender.com.

In this location, procoagulant platelets consolidate the thrombus by deposition of fibrin (Nechipurenko et al., 2019). Furthermore, they might prevent the recruitment of other platelets because of the inactivation of GPIIb/IIIa. Overall, most *in vivo* models report localisation of procoagulant platelets on the thrombus surface as represented in Figure 1.15.

1.1.6.5 Different mechanism for procoagulant platelet formation

A key aspect of platelet function is the relationship between a regulated activation response such as the procoagulant function, and cell death pathways which regulate platelet survival. Interestingly, procoagulant platelets are highly activated cells undergoing cell death. Indeed, they lose organelles, the cytoskeleton is disrupted, and they are unable to exert their primary function which is adhering and aggregating with other platelets. Nonetheless, procoagulant platelets retain functions as they support the assembly of coagulation complexes, thrombin formation and fibrin generation. Furthermore, they have increased reactivity towards neutrophils (Kulkarni et al., 2007), promoting inflammatory responses. One of the hallmarks of procoagulant platelets is PS exposure which is present in both apoptotic and necrotic platelets. It has been demonstrated that platelets undergo apoptosis to be removed from circulation. Many other features of apoptosis (cytoskeleton disruption, microvesiculation, caspase

activation) are also observed during prolonged platelet storage *ex vivo* or when platelets become procoagulant raising the possibility that apoptosis could also regulate platelet function and not only death (Kile, 2009, De Botton et al., 2002, Perrotta et al., 2003). However, suppressing the caspase, which is a modulator of apoptosis, does not affect the procoagulant function of platelets (Kulkarni and Jackson, 2004, Schoenwaelder et al., 2009), raising the possibility that alternative cell death pathways contribute to platelet procoagulant phenotype. There is increasing evidence that many biochemical processes regulating procoagulant function are typical of necrosis including the sustained high levels of calcium leading to the mPTP opening and the cyclophilin-D (CypD) dependent mitochondrial dysfunction. Indeed, platelet CypD deficiency reduces agonist-induced loss of mitochondrial membrane potential and platelet procoagulant activity (Jobe et al., 2008). Consistent with a necrotic process, calpains (not caspases) disassemble the cytoskeleton and promote the release of procoagulant microvesicles from the surface of platelets (Kulkarni and Jackson, 2004). Thus, necrotic cell death in platelets might have evolved as an early system to induce inflammatory and repair processes at sites of vascular injury via the formation of procoagulant platelets. It must be specified that in the absence of CypD, procoagulant activity was markedly impaired but not fully abrogated (Jobe et al., 2008) suggesting other pathways might be also involved. For example, other cell death pathways have been described in platelets such as pyroptosis in sepsis (Su et al., 2022), necroptosis during COVID-19 (Koupenova et al., 2021) and ferroptosis in several haemolytic conditions but their role on the formation of procoagulant platelets remains to be elucidated. Recently, a peculiar subpopulation of procoagulant platelet, which differs from the “classical” one, was identified. Those have been described to be as a low-

calcium PS-positive platelet with normal size, retained mitochondrial membrane potential and cytoplasmic membrane integrity, and combined retention of fibrin(ogen) with active αIIbβ3 (GPIIb/IIIa) and high pro-aggregatory function. Formation of low-Ca^{2+} PS-positive platelets was promoted by platelet concentration increase or shaking and was decreased by integrin GPIIb/IIIa antagonists, platelet dilution, or in platelets from kindlin-3-deficient and Glanzmann thrombasthenia patients (Topalov et al., 2012b). These platelets do not have necrotic nor apoptotic features, but they are PS positive. The physiological role of this new subpopulation remains to be established but might raise questions regarding the inefficacy of blocking necrotic-pathways to target the entire PS positive population. Finally, platelets can express PS also following stimulation with Ca^{2+} ionophores. In this case, the high intracellular Ca^{2+} concentration bypasses the requirement for mPTP opening. However, these platelets do not express coating (Aliotta and Alberio, 2022) nor downregulate GPIIb-IIIa (Alberio et al., 2000). These various features for procoagulant platelets suggest that the procoagulant phenotype is a spectrum of characteristics which depends on different stimulation and experimental conditions, and possibly different pathways are simultaneously activated or following a timely regulation.

1.1.6.6 Intrinsic and extrinsic factors driving procoagulant responses

The generation of procoagulant platelets is not due to a single mechanism but several intrinsic and extrinsic factors can lead to a procoagulant response. Considerations regarding the *in vivo* physiological and pathological production of procoagulant platelets are now addressed. Podoplelova *et al* (Podoplelova et al., 2021) and Veuthey

et al (Veuthey et al., 2022). have proposed platelet size, age and number of mitochondria present in platelets as determinants for the procoagulant response. A low number of mitochondria is also associated with high procoagulant activity. Wiskott-Aldricht syndrome, caused by a mutation in a protein regulating actin activity, is characterised by increased clearance of platelets. Those platelets have high PS on their membrane and low mitochondrial count (Obydennyi et al., 2020). It has been shown that platelets with a less than five mitochondria are more likely to express PS than platelets with a higher number possibly for a lower capacity to buffer cytosolic Ca^{2+}. Therefore, the PS exposure due to mPTP opening occurs more rapidly (Obydennyi et al., 2020). Another factor that has been proposed to affect the likelihood of platelet to become procoagulant, is the receptor/channel density (Sveshnikova et al., 2015, Borin and Siffert, 1990, Samson et al., 2001, Wright et al., 2016). The effect of platelet aging on PS exposure is not determined. Aged platelets expose PS in order to be cleared by autophagy. However, the age-induced PS exposure phenomenon is mediated by an apoptotic-like mechanism while platelets become functionally procoagulant only after a necrotic-induced mechanism. It has been demonstrated that only necrotic platelets were functionally procoagulant as measured by direct prothrombinase activity (Kholmukhamedov and Jobe, 2018), therefore, it is unlikely that old platelets can on their own support procoagulant function (Tohidi-Esfahani et al., 2021, Kholmukhamedov and Jobe, 2018). On the other hand, platelet aging induces ROS generation which has been proposed as driver of the procoagulant response (Masselli et al., 2020) but its real role in this context remains to be determined. GPIbα and GPVI function declines as platelets age and this might lead to less PS exposure (Bergmeier et al., 2004, Alberio et al., 2002). Another factor involved

in pro-coagulant platelet formation is shear-stress. Interestingly, elevated shear stress induces different pattern of platelet activation when compared to biochemical activation with high procoagulant features (Roka-Moiia et al., 2020). It has been demonstrated that physiological levels of shear present in blood enhance agonist-induced platelet PS exposure and microvesicles release (Miyazaki et al., 1996, Reininger et al., 2006) via vWF–GPIb-IX interaction. The shear alone is unable to induce a procoagulant response without the presence of collagen (Delaney et al., 2014). Finally, shear affects also vWF conformation, increasing the affinity of GPIb (Löf et al., 2018) but the extent of this effect on procoagulant platelet formation is not known (Roka-Moiia et al., 2020).

1.1.6.7. Decreased or increased procoagulant platelets in disease

The generation of procoagulant platelets in patients has also been described and underline the importance of developing drugs aimed at targeting procoagulant platelets. Diseases where procoagulant platelet formation is impaired are rare. Scott's syndrome is caused by a deficiency of TMEM16F (anoctamin 6) inducing a defect in PS exposure (Zwaal et al., 2004). A decreased generation of procoagulant platelets was also reported in a cohort of patients with bleeding disorders (Daskalakis et al., 2014). Patients with Stormoken's syndrome have platelets with increased basal Ca^{2+} levels due to a mutation in STIM1 gene. Those patients have platelets with high PS which are unable to respond normally to classical agonists due to a preactivated state (Misceo et al., 2014, Böhm and Laporte, 2018). Wiskott-Aldrich syndrome is characterised by platelets that expose PS via necrotic mechanism caused by their smaller size (Obydennyi et al., 2020). It has also been described in another acquired

platelet disorder with immune thrombocytopenia, the presence of high PS on platelets (Winkler et al., 2012, Ignatova et al., 2019, Martyanov et al., 2020). Patients with colon and gastric cancer have been identified to have significantly high levels of PS (Zhao et al., 2016, Yang et al., 2016) which might justify the high occurrence of venous thrombosis. Procoagulant platelets have been proposed to be a biomarker for the clinical assessment of different thrombotic conditions. For example, they correlate with stroke recurrence and transient ischemic attacks (Prodan and Dale, 2008, Prodan et al., 2011, Prodan et al., 2013, Kirkpatrick et al., 2014). Procoagulant platelets rise after aneurysmal haemorrhage and are associated with adverse outcome (Ray et al., 2019). Contrary, a low level of procoagulant platelets has been shown to be associated with more severe haemorrhage and increased mortality after spontaneous intracerebral haemorrhage (Prodan et al., 2009, Prodan et al., 2010, Prodan et al., 2015). In haemophilia patients with frequent bleeding episodes, the level of procoagulant platelets was described to be low (Saxena et al., 2010). Procoagulant platelets have been described to be predictive of worse prognosis and death in COVID-19 (Khattab et al., 2021). However, platelet isolated from COVID-19 patients showed a reduced ability to become procoagulant compared to those from matched healthy donors (Denorme et al., 2020). High procoagulant platelets have been described also in patients with acute myocardial infarction and ischemic heart disease (Gawaz et al., 1996, Morel et al., 2005). Interestingly also therapy can affect the levels of procoagulant platelets. Patients on dasatinib treatment have been shown to have reduced procoagulant platelets associated with gastrointestinal bleeding (Kostos et al., 2015, Podoplelova et al., 2021, Quintás-Cardama et al., 2009). Desmopressin, has been described to upregulate procoagulant platelets and thrombin generation, with a

mechanism that remains undetermined but open the possibility to use this drug in bleeding conditions (Horstman et al., 1995, Colucci et al., 2014). Procoagulant platelets are also known to release platelet derived microparticles which are also elevated in inflammatory disease such as rheumatoid arthritis. This suggest that procoagulant platelets could be a target for both anti-thrombosis and inflammatory conditions (frequently associated with thrombotic complications). It is interesting to note that Annexin V, a physiological anticoagulant protein present in the body, binds to PS, blocking the release of platelet microparticles (de Laat et al., 2007). This suggests that the body has already an inner mechanism to reduce the effect of procoagulant imbalances but possibly this protein is not enough to buffer the presence of procoagulant platelets.

1.1.6.8 Laboratory assay for detection of procoagulant platelets

The “gold-standard” assay for the detection of procoagulant platelets is measuring the formation of the prothrombinase complex (FXa/Va/II) and prothrombinase activity through thrombin generation. In addition, other surrogate measures have been used and rely predominantly on the detection of bound labelled Annexin V or lactadherin to PS exposed on procoagulant platelets. The downside of this method is that PS alone is not specific to detect functional procoagulant platelets. In this respect, GSAO detection in flow cytometry, appears to be a good surrogate marker for functionally procoagulant platelets. GSAO is a tripeptide trivalent arsenical that is unable to cross the plasma membrane of viable cells. However, when plasma membrane permeability changes in necrosis or late apoptosis, GSAO enters the cell and is retained in the

cytoplasm by covalent bonding to proteins containing closely spaced cysteine thiols. Co-expression of GSAO with P-selectin identifies procoagulant platelets while GSAO platelets alone are apoptotic (Hua et al., 2015). A communication from the scientific and standardization committee (SSC) of the ISTH recently published the recommendation of using P-selectin and PS to detect procoagulant platelets (Josefsson et al., 2023). The loss of mitochondrial membrane potential in procoagulant platelets can be measured by tetramethylrhodamine ethyl/methyl ester (TMRE/TMRM) retention by mitochondria. When mitochondria are no longer intact or metabolically active the signal is reduced or absent. TMRE/TMRM can be measured using fluorescent tags in microscopy and flow cytometry. Intracellular calcium can be studied at the single cell level using fluorescent indicators. For the detection of intracellular calcium, multiple dyes including BAPTA-AM, FURA-2 and Fluo-4 and 5 can be used.

In this thesis, in order to assess platelet adhesion, activation and aggregation, platelet adhesion was assessed performing spreading over surfaces coated with the molecules of interest. Platelet activation was assessed measuring surface expression of P-selectin and GPIIb/IIIa by flow cytometry and platelet aggregation via light transmission aggregometry. In order to assess and measure the presence of procoagulant platelets, P-selectin, Annexin V staining and calcium spiking within platelets were used. More details are provided in the materials and methods chapter. Procoagulant platelet are considered key mediators of thromboinflammation and they play a crucial role in pathological thrombosis (Denorme and Campbell, 2022).

1.2 Thromboinflammation

Thrombosis and inflammation are separate and different physiological processes which have been demonstrated to be tightly interdependent. Over the past decade, a sophisticated connection between platelet activation, innate immunity and coagulation, has been uncovered and this phenomenon is defined as thromboinflammation. When it is excessively activated, thromboinflammation can lead to immunothrombosis, which is immune driven thrombosis, this can be either protective or harmful. Indeed, immunothrombosis is part of the host response to limit systemic spreading of pathogens. However, in the absence of infection, it can cause vessel occlusion and organ damage. Immunothrombosis favours a systemic prothrombotic phenotype leading to several complications including cardiovascular diseases such as myocardial infarction, stroke and venous thromboembolism (Meier et al., 1998, Smeeth et al., 2004). This phenomenon is supported by the evidence that patients with inflammatory disease or during infections, are at increased risk of cardiovascular events. In the last three years, thromboinflammation has received even more attention due to the coronavirus disease 2019 (COVID-19) pandemic, which led patients to experience cardiovascular complication and pulmonary thrombosis as a consequence of a dysregulated host immune response to infection. Interestingly, this aberrant activation leads to immunothrombosis, not only driving the occurrence of thrombotic complications during acute infection but also triggering and promoting progression of non-infectious cardiovascular disease. This mechanism is valid for several chronic inflammatory conditions such as venous thromboembolism and atherosclerosis (Jackson, 2011, Mackman, 2008, Mackman, 2012). It is important to underline that

platelets and the coagulation system are targeted by several antithrombotic approaches. However, immunothrombosis can only be partially prevented or reversed by conventional antithrombotic strategies (Creel-Bulos et al., 2020, Cui et al., 2020, Klok et al., 2020a, Klok et al., 2020b, Poissy et al., 2020, Oxley et al., 2020, Bangalore et al., 2020, Stefanini et al., 2020), implicating the existence of a therapeutic gap, which is very likely to be inflammation itself. Indeed, clinical trials in the last few years, have demonstrated the anti-ischemic effects of therapeutical agents targeting inflammation, reinforcing the evidence of targeting inflammation to prevent cardiovascular complications in both acute infections and chronic inflammatory settings. In this section a description of immunothrombosis pathophysiology is described with a focus on its clinical relevance and current therapeutical evidence.

1.2.1 Mechanisms leading to Immunothrombosis

The spreading of bacteria in the bloodstream triggers immunothrombosis via several mechanisms. Platelets can recognise bacteria via TLRs and they have been shown to be able to migrate within the vasculature, collect bacteria and present them to neutrophils. This process is mediated by GPIIb/IIIa interaction with fibrinogen (Gaertner et al., 2017, Nicolai et al., 2020b) making them act as cellular scavengers. The contact between bacteria-loaded platelets and neutrophils triggers the formation of NETs, which are structures composed of DNA, histones and neutrophil-derived DAMPs which further help in containing bacterial infection. It has been demonstrated that activated platelets induce NETosis whereas resting platelets are unable to exert the same effect (Zucoloto and Jenne, 2019, Caudrillier et al., 2012). This mechanism

involve the binding of P-selectin and high mobility group protein B1 (HMGB1) to receptors present on neutrophils such as TLR4 and RAGE, and PSGL-1 (Etulain et al., 2015, Maugeri et al., 2014, Stark et al., 2016, Dong et al., 2021, Guglietta et al., 2016). These receptor activation leads to the release of decondensed chromatin and the formation of NETs. Under flow conditions, primed human platelets trigger GPIb and GPIIb/IIIa-dependent NETosis (Constantinescu-Bercu et al., 2020). Not only activated platelets trigger NETosis, but NETs formation leads to the release of prothrombotic molecules and acts itself as a scaffold to support platelet aggregation. NET-derived DAMPs including histones, S100A8/A9, myeloperoxidase, cathepsin G and neutrophil elastase induce direct and indirect platelet activation, and the formation of procoagulant platelets leading to thrombosis. For a detailed description of the role of NET-derived DAMPs inducing platelet activation, I refer to a recently published review we published in RPTH (Colicchia et al., 2023).

The dialogue between neutrophils and platelets extends over the formation of NETs. Indeed, the binding of P-selectin to PSGL1 has been demonstrated to increase neutrophils migratory capacity (Sreeramkumar et al., 2014). The communication of platelets with neutrophils depends not only on direct cell-interaction but also on soluble signals. For example, neutrophil-derived extracellular vesicles shuttle the cyclooxygenase 1 substrate arachidonic acid from neutrophils into platelets, promoting thromboxane A2 formation (Rossaint et al., 2016). Not only inflammation causes thrombosis but also thrombosis can trigger itself inflammation. Thrombin cleaves fibrinogen and activates the cytokine IL-1α, linking coagulation to inflammation. IL-1α drives innate immune responses (Burzynski et al., 2019). In a feedback loop, IL-1α

boosts thrombopoiesis by inducing the fragmentation of megakaryocytes in the bone marrow within hours. Similar mechanisms can also be found in viral infections. For example, platelets can be activated by the binding of influenza virus-IgG immune complexes to FcγRIIA receptor, triggering thrombin generation (Boilard et al., 2014, Koupenova et al., 2019). It is well established that immunothrombosis is a relevant mechanism to contain infection, however its exaggerated activation leads to detrimental effect, occlusive thrombosis and organ damage. The fact that increased risk of thrombosis in infection is independent of invading pathogen (Beristain-Covarrubias et al., 2019b), suggests that the host immune reaction to invading pathogens, is the main trigger of vascular events (Corrales-Medina et al., 2012, Dalager-Pedersen et al., 2014). The reader can refer to an extended review on thromboinflammation for in-depth details (Rayes et al., 2019a). Thromboinflammation is of particular interest as a consequence of the COVID-19 pandemic, which is one of the most relevant examples leading to aberrant immunothrombosis.

1.2.2 Immunothrombosis and coagulopathy in COVID-19

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection has been the cause of COVID-19 pandemic. One of the most feared complications of this disease is indeed cardiovascular events. Immunothrombosis has been demonstrated to be not just a bystander in COVID-19. First of all, coagulopathy has been described in COVID-19 patients who had increased plasma levels of fibrinogen and d-dimer correlating with disease severity and outcome. The picture initially described in China was similar to disseminated intravascular thrombosis (Zhou et al., 2020, Tang et al.,

2020, Chen et al., 2020a). SARS-CoV-2 infection of endothelial cells has been associated with immune cells and platelets recruitment (Varga et al., 2020). Furthermore COVID-19 patients have elevated blood levels of vWF (Mancini et al., 2021) and decreased concentrations of the metalloproteinase ADAMTS13, which regulates the activity of vWF (Mancini et al., 2021). Platelets from patients with COVID-19 have a hyperreactive phenotype and SARS-CoV-2 mRNA has been detected in platelets (Barrett et al., 2021a). In patients with COVID-19, the circulating levels of CXCL4 and CCL5, which are mainly platelet-derived and these chemokines trigger for NETosis (Cui et al., 2020).

Patients with ST-segment elevation MI (STEMI) and COVID-19 compared with patients with STEMI without COVID-19 (Blasco et al., 2020) have been demonstrated to have a higher level of NETs in their coronary thrombi. NETs are also found in pulmonary microthrombi in COVID-19 patients (Nicolai et al., 2020a). Markers of NET formation correlate with respiratory failure severity in patients with COVID-19 (Middleton et al., 2020). In severe COVID-19, platelets form aggregates with monocytes via GPIIb/IIIa- and P-selectin, inducing TF expression (Hottz et al., 2020). More information on COVID-19 and platelet hyperactivation are provided in Chapter 3.

1.2.3 Thromboinflammation as a therapeutic target

Increasing evidence shows that current therapies targeting thrombosis can affect inflammation (Bonaca et al., 2020, Borissoff et al., 2011, Eikelboom et al., 2017, Gadi et al., 2021, Hara et al., 2015, Vu et al., 1991) and anti-inflammatory strategies can

prevent thrombotic events (Nidorf et al., 2020, Tardif et al., 2019, Ridker et al., 2017, Ridker et al., 2019) as summarised in **Table 1.2**. The COMPASS and VOYAGER PAD clinical trials (Bonaca et al., 2020, Eikelboom et al., 2017), have demonstrated that anticoagulants can significantly affect plaque progression. Furthermore Rivaroxaban, a Factor Xa inhibitor, has been shown to reduce atherosclerosis progression in mice (Hara et al., 2015). In patients with atrial fibrillation, oral anticoagulation decreased the plasma levels of IL-6 and C-reactive protein (Kirchhof et al., 2020). Heparin, has also been described to reduce the effects of histones independently of its effects on coagulation (Wildhagen et al., 2014). However, these effects have been shown to be not selective and specific and more targeted approaches are needed. Ticagrelor, an anti-platelet drug, reduces the plasma levels of IL-6 and TNF in patients with diabetes mellitus and non-STEMI (Jeong et al., 2017). Aspirin is known to have anti-inflammatory effects mainly at high dose while the low dose, currently used for cardiovascular prevention, has been always considered to have anti-platelet function. Recently, low dose aspirin has also been demonstrated to attenuate leukocyte accumulation during inflammation via the production of lipoxins which reduce the interaction between immune cells and endothelium (Morris et al., 2009, Paul-Clark et al., 2004). Several studies have now demonstrated that targeting inflammation can prevent ischemic events (**Table 1.3**). Indeed, the CANTOS trial, showed that in patients with previous AMI and elevated plasma levels of CRP, the anti-IL-1β antibody, canakinumab, in addition to standard secondary prophylaxis, reduced the recurrence of subsequent re-stenosis or AMI (Ridker et al., 2017). There was no impact on all-cause mortality due to the high risk of fatal infections. On the other hand, the anti-inflammatory drug methotrexate, did not affect ischemic event rate in patients with

previous AMI in the CIRT trial. Colchicine, another anti-inflammatory drug, reduced the risk of stroke and AMI following within 30 days of an initial cardiovascular event in the COLCOT trial. Also in this clinical trial, colchicine had a strong immunosuppressive effect, which renders it difficult to implement in the clinical practice. Interestingly, in the LoDoCo2 trial, the administration of colchicine to patients with chronic cardiovascular disease, resulted in a marked reduction in inflammatory mediators such as NLRP3 inflammasome-associated molecules (IL-18, and MPO) (Opstal et al., 2020). Also, the levels of GPVI were reduced with colchicine treatment. It is evident that a close interaction between thrombosis and inflammation exists on the basis of these results but a better understanding of the systemic immune response to AMI as well as the development of therapeutic approaches targeting more specifically thromboinflammation are necessary. These new therapeutical strategies should be aimed at targeting thromboinflammation, whilst maintaining protective immunity.

Drug	**Antithrombotic target function**	**Anti-inflammatory properties**
Low-dose Aspirin (Morris et al., 2009, Paul-Clark et al., 2004)	Platelet activation	Increased synthesis of pro-resolution lipoxins
$P2Y_{12}$ Inhibitors (Liverani et al., 2016, Sexton et al., 2018)	Platelet activation	Decrease of pro-inflammatory mediators release
Heparin (Wildhagen et al., 2014, Fuchs et al., 2010, von Brühl et al., 2012)	Coagulation	Neutralisation of Histones and NETs
Direct Oral anticoagulants (DOACs)	Coagulation	Inhibition of PAR receptors, which mediate chemokines/cytokines expression

(Busch et al., 2005, Ichikawa et al., 2019)		

Table 1.2- Principal anti-thrombotic medications with proven anti-inflammatory effects.

Medication (Study name)	Clinical setting	Ischemic Events	Infections
Canakinumab (CANTOS)	Secondary myocardial infarction prevention	Reduction in non-fatal AMI /stroke or cardiovascular death	Increase in sepsis and infection events
Methotrexate (CIRT) (Ridker et al., 2019)	Secondary myocardial infarction prevention	No effect on myocardial infarction/stroke/cardiovascular death or hospitalisation for unstable angina	No effects on infectious events
Colchicine (COLCOT) (Tardif et al., 2019)	Within 30 days of myocardial infarction	Reduction of myocardial infarction	Increased infectious events
Colchicine (LoDoCo2) (Nidorf et al., 2020)	Chronic coronary artery disease	Reduced myocardial infarction, ischemic stroke, cardiovascular death	No effects on hospitalisation for infection

Table 1.3- Anti-inflammatory approaches affecting thrombosis (summary of recent clinical trials assessing the effects of anti-inflammatory medications on ischemic events).

Considering the relevance of finding new therapeutic pathways involved in immunothrombosis, understanding how different DAMPs selectively affect thrombosis would be essential. Indeed, the tailored inhibition of DAMPs could lead to the development of novel therapeutical molecules to be implemented into clinical practice.

1.3 Hypothesis and Aims of the thesis

Hypothesis: An increase in the levels of DAMPs is observed in pathogenic conditions such as infection, cardiovascular and haemolytic diseases. In particular, the levels of S100A8/A9 and free heme are associated with thrombotic complications. We hypothesise that these DAMPs have a direct effect on platelets and regulate platelet activation and thrombosis.

Aims: The aim of the work is to assess the direct effect of recombinant S100A8/A9 and free heme on platelet activation and thrombosis. The following queries will be addressed through this thesis:

1) Chapter 3 presents the characterisation of the mechanism by which S100A8/A9 is able to recognise and activate platelets.

2) In chapter 4, we assessed the effect of hemin (the oxidized form of heme) on platelet activation and identified the receptor involved in his process.

Overall, the work in this thesis identified for the first time two distinct mechanisms by which S100A8/A9 and heme contributes to thrombosis by altering platelet function.

CHAPTER 2

Materials and Methods

2.1 Ethical Approvals

Ethical approval for blood collection from healthy human volunteers was obtained by Birmingham University Internal Ethical Review (ERN_11-0175). Mouse experiments were conducted in accordance with UK laws (Animal Scientific Procedures Act 1986) with local ethical committee and UK Home Office approval (PPL P0e98D513 and PPL Pp9677279). Blood from Patients with COVID-19 disease, was collected under the ethical approval of Medical University of Vienna (EK1315/2020) as part of the Austrian Coronavirus Adaptive Clinical Trial (ACOVACT; ClinicalTrials. gov NCT04351724). Blood from a consenting patient with BSS was collected using ethical approval from the North-West – Haydock Research Ethics Committee (15/NW/0079, amendment 3). Ethical approval for collection of post-mortem tissues was granted by the North-East-Newcastle & North Tyneside 1 Research Ethics Committee (19/NE/0336). Collection of postmortem formalin-fixed and paraffin- embedded tissue was granted (IRAS: 197937) for samples obtained via prospective consent post-mortem and retrospective acquisition of tissue. For the latter, consent for use in research had already been obtained. Ethics for patient tissue were granted by the Health Research Authority with a National Health Service Research Ethics Committee. All patients and healthy volunteers written consent has been obtained and relevant institutional forms, archived. This research adheres to the Declaration of Helsinki.

2.2 Materials and antibodies

Table 2.1 contains the detailed list of antibodies used for flow cytometry, immunofluorescence staining, platelet spreading and western blot. All human and mouse platelet agonists and inhibitors are listed in **Tables 2.2** and **2.3** respectively.

Method	Antibody	Company	Reference
Flow cytometry	Anti-human P-selectin-PE	**BioLegend**	304906
	Anti-human GPIIb/IIIa-FITC (PAC-1)	**Invitrogen**	MA5-28564
	Anti-mouse P-selectin-PE	**BioLegend**	148306
	Anti-GPIIb/IIIa-PE (Jon/A)	**Emfret**	M023-2
	Annexin V PE/Cyanine7	**BioLegend**	640950
	Anti-CD66b-PEcy5.5	**BioLegend**	305107
	Anti-human P-selectin-PE/Cy5	**BioLegend**	304908
	Anti-human GPIIb/IIIa-Alexa Fluor 647	**BioLegend**	362806
Immunofluorescence	Rabbit anti-human S100A8/A9	**R&D Systems**	MAB45701
	Mouse anti-human Fibrin clone 59D8	**MERCK**	MABS2155
	Rabbit anti-human CD42b	**Abcam**	EPR6995
	Sheep anti-human S100A9	**R&D Systems**	AF5578
	Rabbit anti human MPO	**Abcam**	EPR20257
	Mouse anti-human CD42b	**BioLegend**	303902
Platelet spreading	Annexin-V-Alexa Fluor 647	**BioLegend**	640912
	P-selectin-Alexa Fluor 488	**BioLegend**	304916
Western Blot	Sheep anti-human S100A9	**R&D Systems**	AF5578
	Sheep anti-human S100A8	**R&D Systems**	AF4570
	Phospho-S100A9 (Thr113) polyclonal antibody	**ThermoFischer Scientific**	PA5-64853

	Phospho-Syk (Tyr525/526) Rabbit mAb	**Cell signalling**	2710S
	Phospho-PLCγ2 (Tyr759) Ab	**Cell signalling**	50535
	Syk Ab	**Cell signalling**	2712S
	PLCγ2 Ab	**Cell signalling**	3872S
Secondary Antibodies	Goat Anti rabbit 647	**ThermoFisher Scientific**	A-21245
	Goat Anti mouse 488	**ThermoFisher Scientific**	A-11001
	Goat Anti rabbit 488	**ThermoFisher Scientific**	A-11008
	Donkey Anti sheep 647	**ThermoFisher Scientific**	A-21448
	Anti rabbit Igg HRP	**Biocompare**	GENA931
	Anti sheep Igg HRP	**Sigma-Aldrich**	12-348

Table 2.1 – Antibodies used for flow cytometry, immunofluorescence staining, platelet spreading and western blot.

Agonist	Target	Source
Collagen Horm	GPVI/Integrins	Nycomed Pharma
Collagen-related peptide (CRP)	GPVI/Integrins	CRP-XL, Cambcol Laboratories
Thrombin Receptor Activating Peptide (TRAP-6)	PAR-1	Alta Biosciences
Recombinant heterodimeric S100A8/A9	CD36, TLR-4, GPIbα, RAGE	Biolegend
Hemin	TLR-4, CLEC-2	Frontier Scientific
vWF	GPIb	LFB Biopharmaceuticals limited

Table 2.2 – Platelet agonists

Inhibitor	Target	Source
TAK-242	TLR-4	Tocris
Anti-human S100A8/A9 heterodimer antibody	TLR-4, RAGE	Biolegend
CD36 blocking antibody	CD36 (oxLDL binding site)	Stemcell
Sulfosuccinimidyl Oleate (SSO)	CD36 downstream signalling	Cayman Chemicals
Paquinimod	TLR-4	BioVision
Azeliragon	RAGE	MCE
Cangrelor	P2Y12 inhibitor	Medicines Company
Indomethacin	COX-1/2 inhibitor	Sigma-Aldrich
PRT-060318	Syk kinase inhibitor	SYNkinase
PP2	Src kinase inhibitor	Selleckchem/Stratech scientific
Ibrutinib	Btk inhibitor	Selleckchem/Stratech scientific
Prostacyclin	PKA	Cayman Chemicals
Recombinant soluble GPIbα	S100A8/A9	R&D Bio-Techne

Table 2.3 – Platelet inhibitors

2.2.1 Recombinant S100A8/A9 purification and characterisation

For experiments testing the effect of S100A8/A9 on platelet function, both commercial recombinant S100A8/A9 (BioLegend) and in-house produced proteins were used. Recombinant human heterodimeric S100A8/A9 was produced as previously described by other authors (Futami et al., 2016) with minor changes as detailed here and described in supplementary material of our publication (Colicchia et al., 2022). Briefly,

E. Coli BL-21 were transfected with plasmid pET21-S100A8-S100A9 provided by Dr Futami, Japan. Protein production in bacteria were induced at Optical Density (OD) of 0.6 by the addition of 0.5 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubated for 3 h at 37 °C. The pelleted bacteria were washed once with NaCl (150mM), centrifuged and resuspended in BugBuster® protein extraction reagent (Merck Millipore) (5 mL/g wet weight) containing 1 mM PMSF protease inhibitor (ThermoFischer Scientific) and 0.33 units Benzonase® nuclease (Purity > 90%) per mL of BugBuster® reagent and incubated for 20 min at RT on a rotary mixer. Insoluble cell debris was removed by centrifugation at 16,000 × g for 20 min at 4 °C. The soluble fraction was diluted in 50 mM Tris-HCl buffer, 50mM NaCl, 5mM MgSO4, pH 7.5, containing 30 mM dithiothreitol (DTT) and incubated for 1h at 37 °C with 180 rpm shaking to reduce disulfide bonds. The protein was diluted 10 times in 50 mM sodium phosphate buffer, pH 6.0 at 4 °C. Recombinant protein was purified using a cation-exchange column chromatography (HiTrap SP Sepharose, Cytiva), with a linear gradient of NaCl (0–0.5 M) in 50 mM sodium phosphate buffer containing 2mM DTT, pH 6.0 followed by gel filtration chromatography using a Superdex 75 26/60. Polymyxin B beads were used to remove endotoxins from the purified proteins and protein is dialysed in PBS containing 2mM DTT. All proteins were snap frozen and stored at -80°C. In order to assess the protein purity of the in-house S100A8/A9, SDS-PAGE, native SDS-PAGE and Western Blotting was performed (Colicchia et al., 2022). Commercial (BioLegend) S100A8/A9 (40µg) and in-house produced S100A8/A9 were separated on the SDS-polyacrylamide gel electrophoresis (4-12%). This process was followed by staining with Coomasie blue for 15 minutes at room temperature under shaking conditions followed by washing in distilled water. For the native gel, proteins

were prepared with 1x running buffer and 5% G-250 sample additive. The gel was then fixed with 40% methanol and 10% acetic acid for 45 seconds with microwave irradiation for 15 min at RT. The gel was de-stained with 8% acetic acid for 45 seconds with microwave irradiation and left at room temperature overnight (Colicchia et al., 2022). For western blotting, *In-house* produced and commercial (BioLegend) S100A8/A9 (40 µg) were mixed with SDS reducing sample buffer and separated by SDS-polyacrylamide gel electrophoresis (4-12%). Polyvinylidene fluoride (PVDF) membranes were immunoblotted overnight at 4 °C with sheep anti-human S100A8 (1:200), sheep anti-human S100A9 (1:2500) or anti-human phospho-S100A9 (1:1000) (details in table 2). HRP-labelled secondary antibodies were used for detection using ECL kit (Thermofischer 32106). Characterisation of commercial and in-house produced S100A8/A9 is provided in **Figure 2.1**. The production and purity assessment of in-house S100A8/A9 was performed by Dr Gina Perrella and the support of Mrs Beata Grygielska (lab technician).

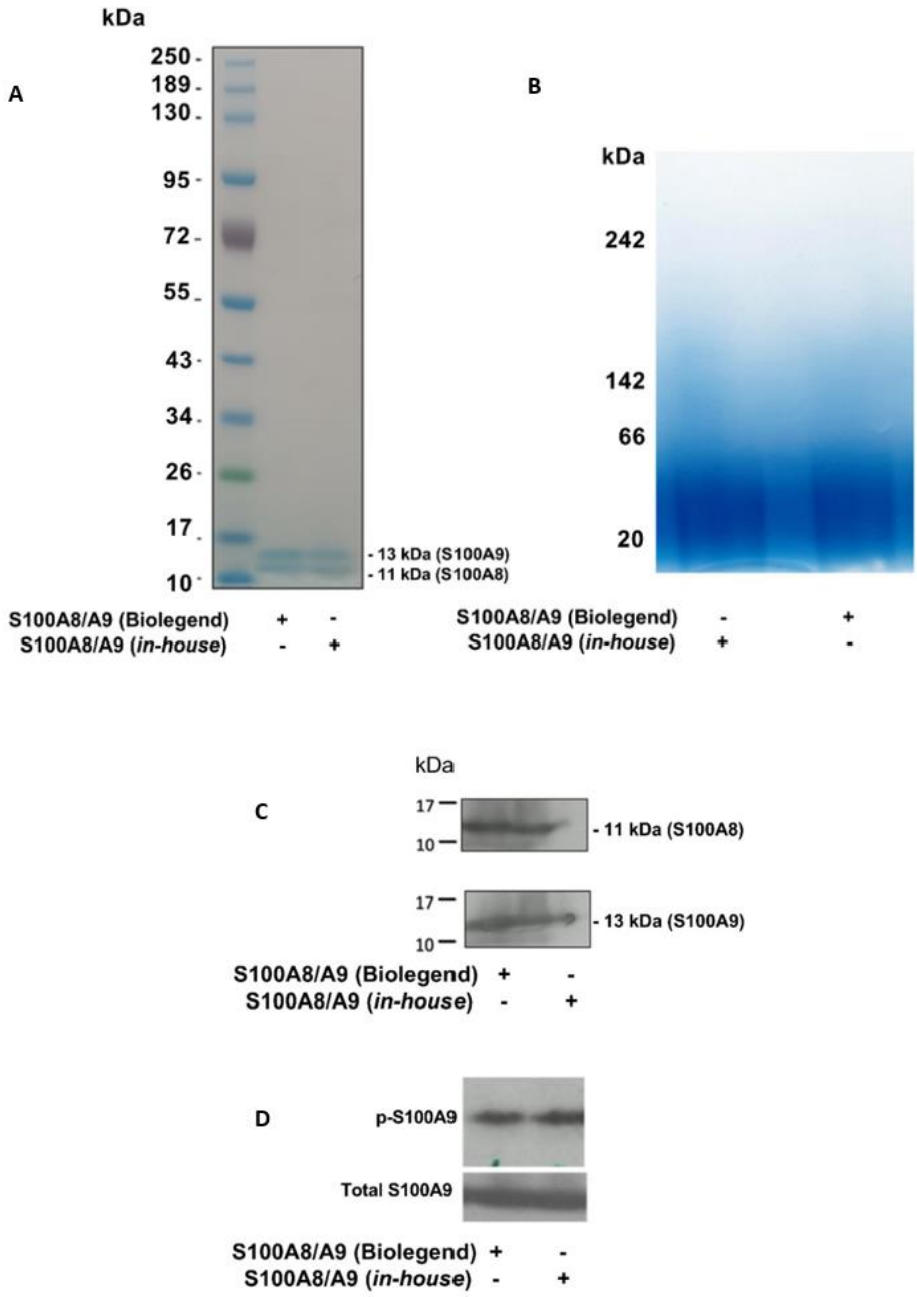


Figure 2.1 – Characterisation of in-house produced S100A8/A9 compared to commercial S100A8/A0. (A) SDS-PAGE gel and (B) Native-page gel for BioLegend and in-house produced recombinant S100A8/A9; (C) Western blot for total S100A8 and S100A9; (D) p-S100A9 and total S100A9 (loading control). Experiments performed and Figure generated by Dr Gina Perrella.

2.2.2. Human and Mouse recombinant CLEC-2 and GPVI

Human and mouse recombinant CLEC-2 and GPVI were produced by the Laboratory of Professor Steve Watson in human embryonic kidney HEK293 cells as Fc fusion proteins (hFC-CLEC-2, hFc-GPVI, Fc-mCLEC-2). Proteins were purified using chromatography affinity. Protein production, isolation and validation was performed in house and carried out my Mrs Ying Di and Dr Alex Slater (University of Birmingham).

2.2.3 Mice genetic alterations

Wild type (WT) mice C57BL/6 (8-14 weeks; male and females) were purchased from Harlan Laboratories (Oxford, UK). Mice with a platelet-specific CLEC-2 deficiency (Clec-1bfl/flGPIbcre+, CLEC-$2^{-/-}$) were generated as described by Haining *et al* (Nagy et al., 2020, Haining et al., 2021). GPVI-deficient ($GPVI^{-/-}$) had a global GPVI knockout as described by Lockyer *et al* (Lockyer et al., 2006). GPVI/CLEC-2 double-deficient ($GPVI^{-/-}$/CLEC-2fl/flGPIbcre+) mice and mice deficient for the extracellular domain of GPIbα (IL4Rα/GPIbα-tg) were generated as described by Haining *et al*. and Chimen *et al.* respectively (Haining et al., 2021, Chimen et al., 2020, Boulaftali et al., 2014, Kanaji et al., 2002). Clec-2 deficient mice (Clec-1bfl/fl PF4cre) and littermate controls adopted in Chapter 4, were previously described by other authors (Finney et al., 2012).

2.3 Platelet isolation

2.3.1 Human platelet isolation and preparation

Blood was drawn by venepuncture into the anticoagulant sodium citrate (4%). Subjects had not received anti-platelet medications or drugs interfering with platelet function for at least two weeks prior to blood collection. Washed platelets were isolated as previously described (Smith et al., 2022). Briefly, drawn blood was added with acid citrate dextrose (ACD, 1:10 v/v) and then centrifuged at 200xg for 20 minutes at room temperature (RT). Platelet rich plasma (PRP) was therefore obtained and centrifuged in the presence of 0.2 µg/ml prostacyclin (1000xg for 10 minutes at RT) and the collected platelet pellet suspended in modified Tyrode's buffer (129 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES, 5 mM Glucose, 1 mM $MgCl_2$; pH 7.3) supplemented with ACD. The platelets were washed by further centrifugation in the presence of 0.2 µg/ml prostacyclin (1000xg for 10 minutes at RT) and then the pellet resuspended in modified Tyrode's HEPES buffer and let to rest for 30 minutes prior to use in experiments (Suzuki-Inoue et al., 2006). The platelet count was measured using the Coulter Z_2 Particle Count and Size Analyser (Beckman Coulter Ltd.). The platelet concentration was adjusted in accordance with the experimental procedure.

2.3.2 Murine blood collection and platelet preparation

Blood was drawn using 1 ml syringe containing 10% ACD and then added to a 1.5 ml Eppendorf containing 200 µl Modified Tyrode's buffer. Mice were terminally bled and their death confirmed by cervical dislocation. Blood was collected from the inferior vena cava.

Collected blood was centrifuged in a Microcentaur (Sanyo) machine at 2,000 rpm for 5 minutes at RT. The PRP was collected together with and upper third of the erythrocytes present in the centrifuged tubes and transferred to new Eppendorf tubes. Following another centrifugation at 200xg for 6 minutes at RT, PRP was collected and added to new tubes. 1 ml of Modified Tyrode's buffer was added to the PRP and treated with PGI_2 (1 µg/ml) prior to centrifugation at 1000xg for 6 minutes at RT. Supernatant was discarded, the remaining platelet pellet resuspended in 400 µl Modified Tyrode's buffer and cells were normalised to the desired concentration following quantification using a Coulter Z2 Particle Count and Size Analyser. Platelets were rested for 30 minutes before their use as previously recommended (Suzuki-Inoue et al., 2006).

2.4 Platelet function assays

2.4.1 Light Transmission Aggregometry

Washed platelets ($2x10^8$ platelet/ml) were added to a glass aggregometer cuvette with a stirrer bar and pre-warmed at 37˚C for 2 minutes in a Chrono-log 700 Lumi-Aggregometer. An additional glass vial with the same volume of modified Tyrode's-HEPES buffer was used as blank during the experiment. Platelets were then stirred at 1200 rpm for the time indicated by every experimental design. Washed platelets were recalcified with Ca^{2+} (2 mM) prior to the agonist treatment unless specified otherwise in the figure legend. The function of inhibitors was assessed by pre-incubation of platelets with the different compounds for the time stated in each figure legends. Percentage of aggregation was quantified by measurement of final aggregation after 6

min (unless otherwise stated) compared to starting point, relative to the baseline. To induce platelet aggregation as positive control, Horm collagen at 10 (µg/ml) or collagen-related peptide (CRP) (CRP-XL, Cambcol Laboratories) (10 µg/ml) was added to washed platelet and the response measured for 6 minutes under constant stirring conditions. Adenosine triphosphate (ATP) secretion from dense granules was measured in washed platelets ($2x10^8$ platelet/ml) using luciferin-luciferase Chrono-lume regent (1:30 [v:v], Chrono-Log, Havertown, PA, USA). Briefly, platelets were stimulated with either vehicle or agonist of choice and left for 5 minutes before addition of ATP (2 nM). Calibration was performed with 2 nM ATP standard (Chrono-Log, Havertown, PA, USA) for each channel prior to the start of the experiment. This procedure allowed AGGRO/LINK8 software to calculate ATP release and AUC. For experiments in Chapter 3, recombinant human or mouse S100A8/A9 (Biolegend) was used, while for experiments in Chapter 4, fresh synthetic hemin solution (Frontiers Scientific, USA) was freshly prepared prior to each experiment. Inhibitors were freshly prepared and diluted for each experiment.

2.4.2 Flow Cytometry

For experiments described in Chapter 3, murine and human platelet activation was performed on washed platelets (10^6 cells/condition) resuspended in a fixed volume of Tyrode's buffer containing 2 mM Ca^{2+}. Human platelets were pre-stained for P-selectin and GPIIb/IIIa using anti-P selectin and anti-GPIIb/IIIa (PAC-1) antibodies respectively (**Table 2.1**). Mice platelets were prepared as for humans but using anti-mouse P selectin and GPIIb/IIIa (Jon/A) antibody. Phosphatidylserine exposure was assessed

by Annexin V binding to platelets. Platelets were then stimulated with different concentrations of recombinant human or mouse S100A8/A9. Platelets were incubated for 30 minutes at 37º C prior to fixation with formalin 2% (CellPath Ltd) for 15 minutes which was used to stop the reactions prior to data acquisition. CRP at 10 µg/ml and Thrombin Receptor Activating Peptide (TRAP-6) (PAR-1 peptide Alta Bioscience) at 100 µM were used as positive control for platelet activation. For the inhibitory experiments, platelets were preincubated for 10 minutes with respective inhibitors of choice prior to S100A8/A9 addition, unless stated otherwise in the figure legends. For competitive experiments using recombinant soluble GPIbα (rGPIbα) (His17-Leu505) (Recombinant Human CD42b/GPIb alpha Protein, R&D Systems), rGPIbα was preincubated with S100A8/9 for 10 minutes 37º C to addition of platelets and further incubated at 37º C for 30 minutes before fixation.

For experiments described in Chapter 4, human and mouse washed platelets (10^6 cells/condition) were stimulated by different concentrations of hemin for 20 minutes at 37ºC. For competition experiments, human and mouse recombinant CLEC-2 were preincubated with hemin for 15 minutes at 37ºC prior to addition to platelets. Samples were analysed using Accuri C6 flow cytometer (BD Bioscience, Oxford UK). Median fluorescence intensity (MFI) or percentage of positive expression compared to control was quantified using Accuri C6 and FlowJo v8 softwares. Fluorescence was calculated using cells with a known positive protein marker for each colour.

Gating Strategy for platelet microvesicles detection used for the experiments discussed in Chapter 3. Forward scatter (FSC) and side scatter (SSC) voltage parameters were set to log scale and the lowest thresholds allowed by the cytometer

were selected. To follow, in this gating window, the particles positive for CD41 were considered as platelet microvesicles and quantified. All the additional parameters (Annexin V, P-selectin) were measured as a percentage of the CD41 positive particles (**Figure 2.2**).

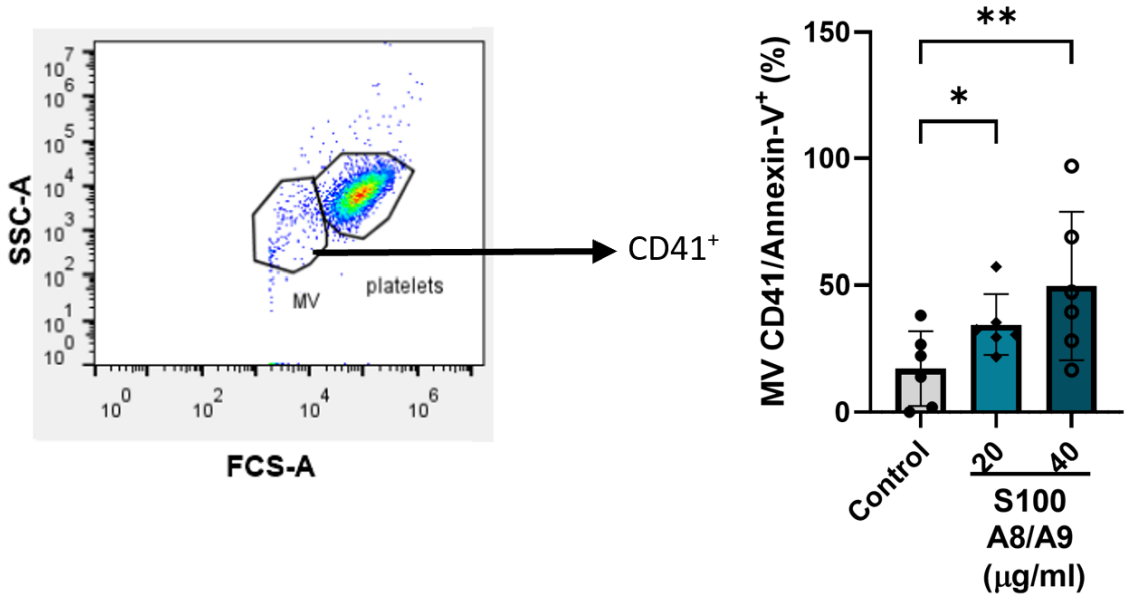


Figure 2.2 – Gating strategy to detect CD41⁺ microvesicles. Example of gating strategy to detect CD41⁺**/**Annexin V+ microvesicles (detailed in the text).

2.4.3 Platelet-neutrophil complexes in whole blood

Citrated whole blood was diluted in Tyrode's buffer (1:5) prior to incubation with human recombinant S100A8/A9 for 30 minutes at 37º C. Whole blood was stained with anti-CD42b (platelet marker) and CD66b (neutrophil marker). Following red cell lysis (Agilent), platelet-neutrophil complexes (CD42b⁺/CD66b⁺) were detected by flow cytometry.

2.4.4 Platelet spreading

Glass coverslips were coated with S100A8/A9 (20 or 40 µg/ml) or collagen (10 µg/ml) (overnight, 4°C) then blocked with 5 mg/ml fatty acid free BSA (1 h, room temperature) before the addition of platelets. BSA coating alone was used as negative control. Platelets were spread for 30 minutes (37°C) in Tyrode's buffer without Ca^{2+} before adding anti-P-selectin-AlexaFluor 488 (Invitrogen) and Annexin V-AlexaFluor 647 (BioLegend) in buffer containing Ca^{2+} (2mM) for an additional 15min. Platelets were fixed, washed and mounted using ProLong Gold Antifade Mountant (Life Technologies). Images were acquired using Zeiss Axio Observer 7 epifluorescence microscope with 63x, 1.4 Numerical Aperture (N.A.) objective lens.

2.4.5 Whole blood microfluidic flow experiments

Microfluidics experiments were performed using the Maastricht chamber. Glass coverslips were coated overnight with collagen Horn (50 µg/ml, Nycomed Pharma GmbH), with S100A8/A9 (40 µg/ml) at RT or combination of collagen and S100A8/A9. The spots used for the imaging on the coverslips were 3 mm apart and 1.5 mm in diameter. Coverslips were blocked with 1% bovine serum albumin (BSA) in HEPES buffer (pH 7.4) for 30 minutes to avoid unspecific platelet activation. Citrated blood was co-perfused with recalcification medium (32 mM MgCl2 and 63 mM CaCl2 in HEPES buffer pH 7.45). Two pulse-free micro-pumps (Harvard 70-4504 P308120 and World Precision Instruments AL-1000) were used together with a y-shaped mixed tubing. Volume ratio of 10 (whole blood) to 1 (recalcification medium) was the final ratio for

complete mixing. The final perfusion rate was of venous shear rate (100 s-1). Whole blood was added with AlexaFluor 647-Annexin V (BioLegend) and polyclonal rabbit anti-human fibrinogen FITC (Dako-Agilent) to assess PS exposure and fibrin generation respectively. Images were recorded at each of the three spots every 15 minutes using an EVOS AMF4300 microscope (Life Technologies) with a 60x, 1.42NA oil objective. Both brightfield and fluorescence microscopy images were analysed and quantified using pre-defined scripts (Van Geffen et al., 2019) as reported by other authors and formatted in Fiji package (Schindelin et al., 2012). The microfluidic experiments were performed with the support of Dr Gina Perrella.

2.4.6 Intracellular Ca^{2+} increase and dynamics in platelets

In order to assess intracellular Ca^{2+} release from platelets single cell, Ca^{2+} measurements was used as previously described (Clark et al., 2019). Micro-ibidi plates were coated with either S100A8/A9 (20 or 40 µg/ml), fibrinogen (10 µg/ml) or BSA and blocked with BSA (5 mg/ml) for 1 h at RT followed by 3 washes in PBS. Platelets were stained with Oregon green-488 BAPTA-1-AM calcium sensitive dye (Invitrogen) at 1µM. Dye-loaded platelets ($2x10^7$/ml) were transferred to the coated plates. Real-time platelet Ca^{2+} flux was monitored using a Zeiss Observer 7 epifluorescent microscope using a 63x 1.4NA oil immersion lens (Colibri 7 LED light source, Zeiss Filter set 38 for GFP/FITC and Hammamatsu ORCA Flash 4 LT sCMOS camera). The excitation wavelength 488 was used to measure calcium spiking, the acquisition was set at 50ms exposure time with 50% LED intensity as previously described (Clark et al., 2019). Images were analysed with MatLab (MathWorks) for quantification, using EZcalcium

script (Khan et al., 2022b). The presence of Ca^{2+} sparks was quantified as the peak fluorescence at time point zero (F0/Fmax). Imaging and imaging analysis were performed with the support of Dr Abdullah O Khan and Dr Jasmeet S. Reyat, respectively (University of Birmingham).

2.4.7 Thrombin Generation Test

Thrombin generation was assessed via Thrombin Generation Test (TGT) using calibrated automated thrombography (CAT) as previously described (Price et al., 2021, Hemker et al., 2003, Semeraro et al., 2011). 96-well round-bottomed plates were prepared using 80 µl of sample (S100A8/A9 or CRP) and 20 µl of either PRP reagent (5 pM tissue factor) or thrombin calibrator. In total for each experiment, 4 wells were used (2 test well with PRP reagent and 2 wells with thrombin calibrator). Double spun plasma (DSP), platelet-enhanced plasma (PEP), platelet poor plasms (PPP) and ionomycin were used as control. Plates were incubated for 10 minutes at 37º C followed by the addition of FLUCA reagent (20µl) (fluorescent thrombin substrate Z-Gly-Gly-Arg-aminomethylcoumarine, ZGGR-AMC) and $CaCl_2$ (CAT# 86197, Stago). FLUCA reagent was prepared according to manufactures instructions and kept in the dark until required. Following activation, a fluorescent plate reader was used (Fluoroskan Ascent, ThermoFischer Scientific™, UK). The parameter values for thrombin generation (lag time, peak time, time to peak, ETP area under the curve, start tail**, Figure 2.3**) were calculated by the Hemker software (thrombinoscope Software Version V5.0.0.742, Stago). Statistical analysis and figures were generated using R

and RStudio programmes. The calculation of the parameters was performed with the support of Dr Joshua Price (University of Birmingham).

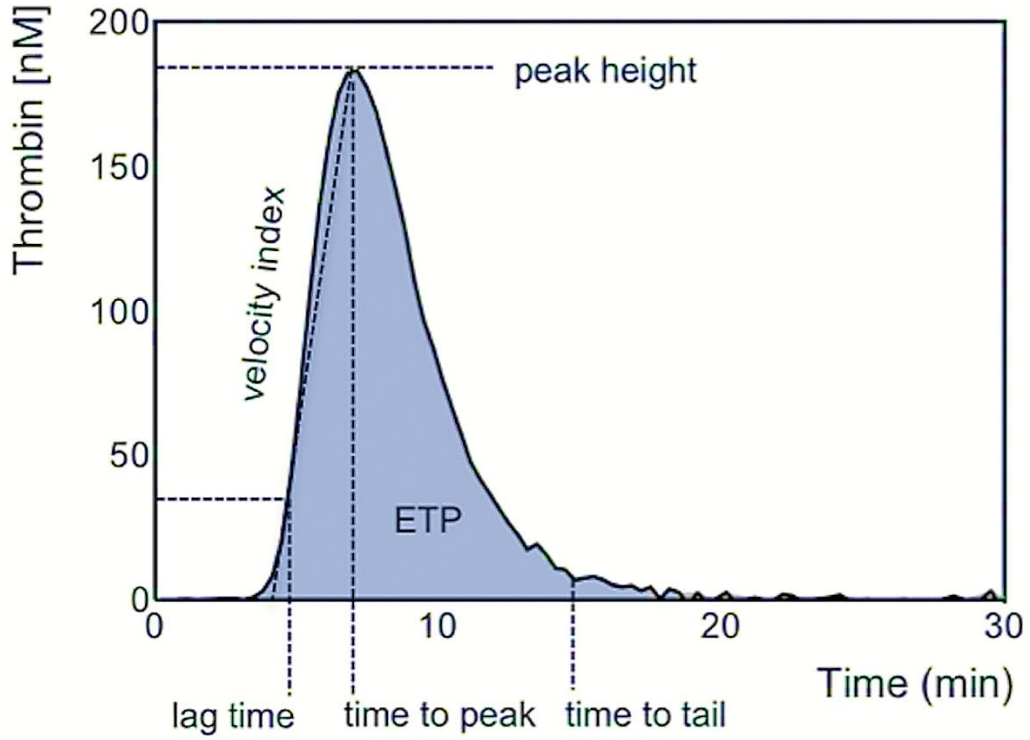


Figure 2.3 – Thrombin generation curve and parameters. Lag time (min) is the lag phase prior to thrombin generation. Peak (nmol/L) is the value of maximal thrombin generation. Time to peak (min) is the time taken to reach peak thrombin generation. The total area under the curve is the endogenous thrombin potential (ETP) (nmol/L x min). Start tail defines the time taken to reach the peak tail (min) or the time until the end point of thrombin generation. Figure adapted from Ref (Loeffen et al., 2014)

2.4.8 LDH quantification

In Chapter 4, LDH released by platelets was measured following stimulation with Hemin. Following Hemin-driven aggregation experiments (described in 2.3.1), platelets were centrifuged at 1,000xg for 10 minutes and LDH was measured in the remaining

supernatant using CyQUANT™ LDH Cytotoxicity Assay (ThermoFisher Scientific) according to manufacturer instructions.

2.5 Solid-phase binding assay

In order to assess the binding of S100A8/A9 to GPIbα, plates were coated with 0.5 μM S100A8/A9 for 1h and blocked with 3% BSA. Recombinant GPIbα ectodomain (R&D Systems) was added at a concentration ranging from 5000 nM to 2 nM for 1h at 37º C. BSA instead of GPIbα was used as negative control. Following incubation, primary antibody against GPIbα was added (mouse anti-CD42b/GPIbα antibody [Clone 486805], R&D BioTechne) for 1h followed by anti-mouse IgG horseradish peroxidase (HRP). Detection was performed with ABTS (azino-Bis(3-ethylbenzothiazoline-6-sulfonic acid)) (Sigma-Aldrich). Absorbance was measured with VersaMax microplate reader (Molecular Devices) at 405 nm. The BSA absorbance values were subtracted from the optical density (OD) of S100A8/A9-GPIb and normalised before plotting.

2.6 Surface plasma resonance and UV-spectroscopy

In order to assess the binding of human and mouse dimeric CLEC-2 to hemin, plasma resonance and UV spectroscopy were adopted. Recombinant CLEC-2-Fc was immobilised on a CM5 sensor chip (Cytiva) and real-time interaction profiles were obtained following injection of increasing concentrations of hemin (19.5-625 nM) over the immobilised CLEC-2. This allowed calculation of plasma surface resonance. The

association and dissociation were observed for 5 minutes each and data was quantified using a Langmuir global analysis model. Therefore, UV-vis absorbance spectroscopy was performed using spectra generated of CLEC-2-Fc (2 μM) together with different concentrations of hemin (0.5-16 μM). The spectra were obtained subtracting the spectra of hemin from the spectra of hemin in the presence of protein both at the same concentration. The measurements were performed at 25ºC in optical cell with 10 mm light path. These measurements were performed in Paris by Dr Jordan Dimitrov (University of Paris Sorbonne).

2.7 Monocyte preparation and TNF-α assay

Peripheral blood mononuclear cells (PBMCs) were separated from blood anti-coagulated with EDTA. Histopaque 1077 (Sigma-Aldrich) density gradient was used and $CD14^+$ monocytes were isolated by positive selection using CD14 magnetic bead isolation (Miltenyi Biotec) according to manufacturer instructions. Purified monocytes ($3.5x10^5$ cells) were placed in 24 well tissue culture treated plates (Falcon) with complete media (RPMI 1640 media [Gibco] supplemented with 10% foetal bovine serum (FBS) (Sigma-Aldrich) and 50 ng/ml human macrophage colony-stimulating factor (MCSF) (Peprotech) for 24 hours at 37ºC with 5% CO2. Supernatant was replaced by fresh media after 24h followed by cell stimulation. Cells were stimulated for 24 hours with S100A8/A9 (10μM) or with the TLR-4 inhibitor (TAK-242) for 10 minutes prior to S100A8/A9 (10μM). Supernatants were collected and centrifuged prior to TNF-α measurements by ELISA (R&D Systems) according to manufacturer

instructions. Monocyte experiments were performed with the support of Miss Jenefa Begum (University of Birmingham).

2.8 Plasma S100A8/A9 ELISA

Blood was collected from COVID-19 patients and aged matched controls and plasma obtained by blood centrifugation for 10 minutes at 1000g (4ºC) followed by 10 minutes centrifugation at 10.000g at room temperature. Plasma samples were collected and stored at -80º C until use for Enzyme-linked immunosorbent essay (ELISA). Plasma levels of S100A8/A9 were measured using LEGEND MAX Human MRP8/14 (Calprotectin) ELISA Kit (BioLegend) according to manufacturer instructions. These experiments were performed in collaboration with Waltraud C. Schrottmaier and Dr Alice Assinger (University of Vienna).

2.9 Western Blotting

In Chapter 3 and 4, Western Blot was performed. Reagents are listed in **Table 2.1.** For Chapter 3, in house produced and commercial S100A8/A9 (Biolegend) (40µg/ml) were mixed with SDS reducing sample buffer (300 mM NaCl, 20 mM Tris, 2 mM EGTA, 2 mM EDTA, 2 % NP40) and separated by SDS-polyacrilamide gel electrophoresis (4-12%), alongside a molecular weight ladder (ThermoFisher). Protein was transferred to Polyvinylidene fluoride (PVDF) membranes blocked with 5 % BSA-Tris-buffered saline with 0.1 % Tween 20 (TBS-T) for 1 h at RT and immunoblotted overnight at 4ºC with

different antibodies: anti-human S100A8 (1:200), anti-human S100A9 (1:2500) and anti-human phosphorylated S100A9 (1:1000). The membrane was thrice washed, before incubation in secondary antibody 1 h at RT, before thrice washing and revealing by ECL Western Blotting Substrate (ThermoFisher 32106) and imaging by chemiluminescent films. For Chapter 3, protein phosphorylation was assessed after 6 minutes of aggregation as previously described (Nicolson et al., 2018). Phospho-Syk Rabbit mAb, Phospho-PLCγ2 antibody, Syk antibody and PLCγ2 antibody were used (Cell signalling). For those samples, following protein electrophoresis, proteins were transferred to a PVDF membrane. Following blocking with 5% BSA-Tris-buffered saline with 0.1% Tween 20 (TBS-T) for 1 h at RT, membranes were incubated with primary antibodies overnight at 4ºC followed by HRP-labelled secondary antibodies. The detection was performed using ECL Western Blotting Substrate (TermoFisher Scientific) and imaged by an Odyssey Imaging System (LI-COR Bioscences). Western Blot experiments were performed by Dr Joshua Bourne (Chapter 4) and Dr Gina Perrella (Chapter 3) (University of Birmingham).

2.10 Immunofluorescence staining and microscopy

Human paraffin-embedded lung sections (6 µm) were prepared and stained as previously described (Khan et al., 2022a). The sections were incubated with primary antibodies against platelet CD42b, myeloperoxidase (MPO), fibrin, S100A8/A9 and S100A9 overnight at 4ºC. Therefore, fluorescently conjugated secondary antibodies were used. Materials are listed in **Table 2.1.** A commercial kit (Vector Laboratories) was used to quenched lung autofluorescence and the slides were mounted for imaging using ProLong Gold Antifade Mountant (LifeTechnology). The imaging of the sections

was performed using an Epifulorescent microscope or Zeiss Axioscan Z1 microscope and analysed using ZEN at 20x magnification and analysed using ImageJ software. Staining and imaging was performed by Dr Julie Rayes (University of Birmingham).

2.11 Data Analysis

All data in this thesis are presented as mean ± standard deviation (SD) unless stated otherwise. The statistical significance between 2 groups was analysed using unpaired t-test while the statistical difference between groups was analysed using either one-way or two-way Analysis of Variance (ANOVA) with multiple comparisons or Kruskal-Wallis test with multiple comparisons unless stated otherwise. The appropriated parametric on non-parametric test was decided on the basis of sample size, distribution, number of groups and the nature of the measured variable. The statistical analysis was performed using GraphPad Prism 7 or 8 (GraphPad Software Inc). Statistical significance was represented as follows: *p<0.05, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

CHAPTER 3

S100A8/A9 induces the formation of procoagulant platelets through GPIbα

Data from this chapter have been published:

Martina Colicchia, Waltraud C Schrottmaier, Gina Perrella, Jasmeet S Reyat, Jenefa Begum, Alexandre Slater, Joshua Price, Joanne C Clark, Zhaogong Zhi, Megan J Simpson, Joshua H Bourne, Natalie S Poulter, Abdullah O Khan, Phillip L R Nicolson, Matthew Pugh, Paul Harrison, Asif J Iqbal, George E Rainger, Steve P Watson, Mark R Thomas, Nicola J Mutch, Alice Assinger, Julie Rayes. **S100A8/A9 drives the formation of procoagulant platelets through GPIbα** (2022) *Blood*;140(24):2626-2643. doi: 10.1182/blood.2021014966.

Publication available in **Appendix 1**

3.1 Aims

The aim of this chapter is to explore whether S100A8/A9 induces platelet activation and to assess its direct effect on thrombosis. Using recombinant commercial and *in-house* produced S100A8/A9, we investigated whether S100A8/A9 influences human and mouse platelet function, its relevance to thrombosis and explored the levels of S100A8/A9 in patients with COVID-19, both in the plasma and in lung autopsies.

3.2 Introduction

3.2.1 S100 family and S100A8/A9

S100A8 and S100A9 (also known as calprotectin or Myeloid-related protein-8 and 14 [MRP-8 and MRP-14] or calgranulin A and B, respectively) are Ca^{2+} binding proteins belonging to the S100 family. The S100 protein family is the largest of Ca^{2+} binding helix E-loop-helix F (EF) proteins. These small acidic proteins were firstly identified in 1965 by B.W. Moore and have been named after their solubility in a 100%-saturated solution with ammonium sulphate at neutral pH (Moore, 1965). S100 proteins have a molecular weight of 10-12 kDa and contain two distinct EF-hands, 4 α-helical segments, a central region of variable length and the N-/C- terminal variable domains. At present, 25 proteins have been identified and most of them cluster at chromosome locus 1q21 (Moore, 1965, Moore and McGregor, 1965, Moore et al., 1968, Potts et al., 1995). They do not have intrinsic catalytic activity but there are two critical steps for their activation which are Ca^{2+} binding and homo/-hetero dimerization or

tetramerization. These events induce conformational changes and modulate their biological activity (Ikura, 1996). Indeed, the activity of the S100 proteins is regulated by metal ions (such as Ca^{2+}, zinc, and copper), which modulates the folding and oligomerization of the protein (Heizmann et al., 2002, Chazin, 2011). Ca^{2+} also contributes to the formation of S100 protein oligomers, especially calprotectin (S100A8/A9 tetramer and oligomers)(Brophy et al., 2012, Fritz et al., 2010, Korndörfer et al., 2007, Hayden et al., 2013). They have a broad variety of intracellular and extracellular functions encompassing inflammation, migration, protein phosphorylation, calcium balance, oxidative cell damage, differentiation, and proliferation (Santamaria-Kisiel et al., 2006). Based on their functional roles, the S100 proteins can be categorised into proteins that have only intracellular, only extracellular or both activities (Donato et al., 2013). The cell origin and functions of the main S100 proteins are summarised in **Table 3.1**.

S100 protein	Expression	Interactions	Involvement in disease
S100A1 (S100 alpha)	Skeleton muscle, Cardiomyocytes, Neuronal populations	*Extracellular:* RAGE. *Intracellular:* SERCA, RyR1 & 2, Fructose-bisphosphate aldolase	Deterioration of cardiac performance and heart failure (Ritterhoff and Most, 2012) Cancer metastasis (Stein et al., 2011)
S100A2 (S100L, CAN19)	Urothelium, respiratory, gastrointestinal and squamous epithelium	RAGE	Downregulated/upregulated in many cancers (Sugino and Sawada, 2022)

S100A3 (S100E)	Hair root cells and astrocytoma	RARα	Involved in HCC and tumour aggressiveness (Bai et al., 2023)
S100A4 (Metastasin1, Calvasculin)	Tumour and stromal cells, myeloid cells, adipocytes, fibroblasts, immunocytes, vascular cells	*Extracellular*: RAGE, EGFR, Gαq-coupled receptor. *Intracellular*: NMIIA, tropomyosin, actin, p53, S100A1, Annexin2	Upregulated in different cancers (Fei et al., 2017)
S100A5 (S100D)		RAGE	Upregulated in bladder cancer and recurrent meningiomas (Yao et al., 2007, Hancq et al., 2004)
S100A6 (Calcyclin)	Epithelial cells, fibroblasts and different kinds of cancer cells.	RAGE	Upregulated in anaplastic thyroid cancer (Chen et al., 2023)
S100A7 (Psoriasin1)	Keratinocytes	RAGE	Upregulated in psoriasis or other inflammatory skin conditions (D'Amico et al., 2016)
S100A8 (Calgranulin A)	Macrophages, dendritic cells, microvascular activated endothelial cells, epithelial cells, fibroblasts	*Extracellular*: GPCR, TLR4, Scavenger receptor CD36. *Intracellular*: telomerase	Overexpressed in inflammatory and autoimmune conditions (Holzinger et al., 2018)
S100A9 (Calgranulin B)	Monocytes, neutrophils, DC, Fibroblasts, mature macrophages, vascular endothelial cells and keratinocytes	*Extracellular:* RAGE, TLR4, Scavenger Receptor CD36 *Intracellular*: actin	Inflammatory conditions and autoimmune disease, Anti-inflammatory in healthy states (Chen et al., 2015, Cesaro et al., 2012)

S100A8/A9 (Calprotectin)	Monocytes, neutrophils, DC, Fibroblasts, mature macrophages, VEC and keratinocytes	RAGE, CD36, TLR4	Inflammation, atherosclerosis and CV evens, malignant progression, progression to psoriasis (Matsunaga et al., 2021, Wang et al., 2018, Gebhardt et al., 2006)
S100A10 (Calpactin-1)	Macrophages	Annexin2, serotonin 1B receptor	Downregulated in depressive-like states, implicated in antidepressant drugs action and seizures (Chen et al., 2022, Warner-Schmidt et al., 2010)
S100A11 (S100C) Calgizzarin	Chondrocytes, luteal cells, oviductal epithelial cells	*Extracellular*: RAGE. *Intracellular:* Nucleolin, Rad54B	Accelerate chondrocyte hypertrophy and matrix catabolism to promote osteoarthritis progression (Andrés Cerezo et al., 2017)
S100A12 (Calgranulin C)	Neutrophils, monocytes, induced in endothelial and epithelial cells and proinflammatory macrophages under inflammatory stimuli	*Extracellular*: RAGE, GPCR, Scavenger receptor. *Intracellular*: Aldolase, Nox-1.	Aortic aneurysms, VMSC dysfunction, leukocyte influx (Jiang et al., 2014)
S100A13	Fibroblasts, osteoblasts and melanoma cells	*Extracellular*: RAGE. *Intracellular*: FGF-1, p40 Syt1	High intratumoral angiogenesis and poor prognosis in stage I NSCLC (Miao et al., 2018)
S100A14	Lymphocytes, epithelial cells	*Extracellular*: RAGE. *Intracellular:* p53	Ectopic overexpression promotes motility and invasiveness of ESCC cells (Chen et al., 2013a)
S100A15 (S100A7A)	Keratinocytes in inflamed skin	GPCR	Potential therapeutic target for various human disorders

			including arthritis and cancer (Chen et al., 2017)
S100A16 (S100F)	Astrocytes, preadipocytes	*Intracellular:* p53	Upregulated in several tumours (Tanaka et al., 2015)
S100B	Astrocytes, Schwann cells, melanocytes, chondrocytes, adipocytes, skeletal myofibers, certain dendritic cell and lymphocyte populations	*Extracellular*: RAGE, FGFR. Intracell: Tubulin, actin-binding proteins, annexin 6, Rac1, SRC kinase, NDR kinase, p53, intermediates upstream of IKKβ/NF-κB.	Involved in brain, cartilage and muscle repair, activation of astrocytes in brain damage and neurodegenerative processes, melanomagenesis, gliomagenesis and cardiomyocyte remodelling after infarction (Michetti et al., 2012)
S100G (Calbindin-D9K)	Epithelial cells	Unknown	Unknown
S100P (S100E)	Lymphocytes, epithelial cells	*Extracellular:* RAGE. *Intracellular*: ezrin/radixin/moesin, IQGAP1	Overexpressed in clinically isolated tumours, associated with metastasis, drug resistance, and poor clinical outcome (Cong et al., 2020, Tóthová and Gibadulinová, 2013)
S100Z	Lymphocytes	Unknown	Downregulated in several tumours (Gribenko et al., 2001)

Table 3.1 List of S100 proteins: cell expression, receptors, and associated pathologies are represented in the table. HCC=hepatocellular carcinoma; CV=cardiovascular, VMSC=vascular smooth muscle cells, NSCLC= Non-small-cell lung carcinoma; ESCC= Oesophageal squamous cell carcinoma;

RAGE= Receptor for advanced glycation end products; SERCA=Sarco-Endoplasmic Reticulum Calcium ATPase); RyR1&2=RFGF Ryanodine receptor 1&2; EGFR= Epidermal growth factor receptor, NMIIA= Non-muscle myosin 1A; GPCR= G-protein coupled receptor; Toll-like receptor 4=TLR4; Nox-1=NADPH oxidase1; FGF-1=fibroblast growth factor 1; Syt1=synaptogamin1; IQGAP1=Ras GTPase-activating-like protein 1; Rac1= Rac Family Small GTPase 1, SRC kinase, NDR kinase= nuclear dbf2-related) *kinases*, p53, intermediates upstream of IKKβ/NF-κB= Inhibitor of nuclear factor kappa-B kinase subunit beta/ nuclear factor kappa-light-chain-enhancer of activated B cells. Table adapted from Refs (Gonzalez et al., 2020, Donato et al., 2013)

The main intracellular functions of S100 proteins involves membrane protein transportation and recruitment, transcriptional regulation (Komatsu et al., 2000, Melle et al., 2008), interaction with intracellular receptors or regulating enzymes and DNA repair (Donato, 2001, Berge and Mælandsmo, 2011, Mishra et al., 2012). Several S100 proteins, including S100B, S100A4, S100A8, S100A9, S100A12 and S100A13 are secreted and exert cytokine-like functions and this is summarised in **Table 3.1**. Considering the pleiotropic effect of S100 proteins, their expression is strictly regulated to maintain immune homeostasis. Once released in the extracellular space, S100 proteins have crucial roles in regulating immune homeostasis, tissue repair and inflammation. Extracellular S100 proteins act as cytokines to activate surface receptors on immune and non-immune cells, as damage-associated molecular patterns (DAMPs), favouring inflammatory responses. Of all the S100 proteins, S100A8 and S100A9, as alarmins, are significantly upregulated in almost all types of inflammation and represent one of the most attractive targets to monitor (as biomarkers) and treat several inflammatory conditions. S100A8/A9 has also been described to play a role in thrombotic disease. Below, a description of S100A8/A9 structure and function is detailed.

3.2.2 S100A8/A9

3.2.2.1 History of discovery of S100A8/A9 and structure

S100A8/A9 was discovered and initially characterised by *Fagerhol and Dale* in 1983 whom defined it as a "highly immunogenic human leukocyte protein" called L1 (Dale et al., 1983). This protein has been then studied and characterised in the last 30 years for possible clinical implications. The most known and largely adopted application has been as a biomarker of intestinal inflammation (Bjarnason, 2017). Indeed, in the late 80s, S100A8/A9 was found to be present and persistently stable in the stool and it has been used to monitor disease progression in patients with Inflammatory Bowel Disease (IBD). S100A8 and S100A9 are the S100 proteins with a major role in inflammation. The human S100A8 gene encodes a small molecule composed of 93 amino acids with a molecular mass of 10.8 kDa, while the human S100A9 gene encodes a protein of 113 amino acids with a molecular mass of 13.2 kDa (Zou et al., 2013, Wang et al., 2018). Structurally S100A8 and S100A9 are characterised by two Ca^{2+} binding sites of the EF-hand type and the dimer is known to bind four Ca^{2+} ions. In addition to Ca^{2+}, S100A8/A9 can also bind Zn^{2+}, Mn^{2+}, Cu^{2+}, and Fe^{2} and, by sequestrating these divalent cations in the extracellular environment, it acts as a potent antimicrobial agent (Vogl et al., 2006, Vogl et al., 2012). S100A8 and S100A9 can exist in several forms as homodimers, heterodimers and tetramers allowing for their biological function to change accordingly (Schiopu and Cotoi, 2013, Leukert et al., 2005, Leukert et al., 2006). Under physiological conditions, these proteins preferentially form heterodimers because of the poor stability as homodimers (Donato et al., 2013), despite S100A8 and S100A9 homodimers being able to maintain proinflammatory functions. The

formation of S100A8/A9 heterodimer does not require Ca^{2+} binding, while tetramerization and oligomerisation depends on Ca^{2+} or Zn^{2+}. The tetramerization has been shown to be an essential prerequisite for some biological functions such as microtubule formation in phagocytes (Leukert et al., 2006). Targeted deletion of S100A8 gene in mice is lethal while mice deficient in S100A9 have a healthy phenotype but they do not express S100A8 protein, showing that S100A9 is essential for maintaining the stability and function of S100A8 (Passey et al., 1999). The structure of S100A8/A9 heterodimer is shown in **Figure 3.1**.

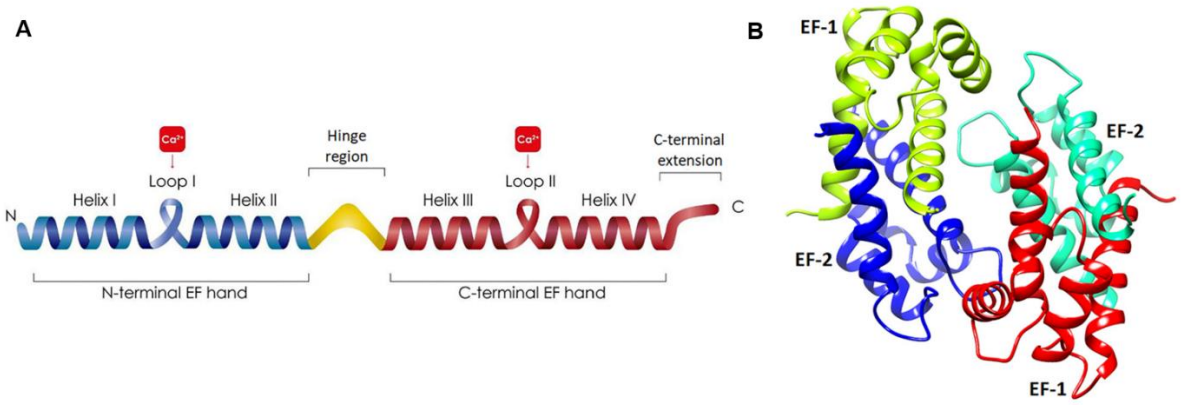


Figure 3.1 - (A) The structure of calprotectin (S100A8/S100A9); each calprotectin monomer consists of two Ca^{2+} binding EF-hand motifs in the N-terminal and C-terminal of sequence which are connected by a linker region. (B) The tertiary and quaternary structure of S100A8/S100A9 complex protein. Crystal structure adapted from Ref Shabani *et al*, 2020 (Shabani et al., 2020). Created with BioRender.com

3.2.2.2 S100A8/A9 cell origin

Acting as alarmins, S100A8 and S100A9 are passively released by necrotic cells or actively secreted by activated immune cells (Donato et al., 2013). S100A8 and S100A9 are constitutively expressed by myeloid cell and constitute approximately 40% of

neutrophils cytosolic proteins and 1-5% of monocytes cytosolic proteins (Hessian et al., 1993). Their expression is downregulated when monocytes become tissue macrophages but also macrophages can express it in inflamed tissues (Odink et al., 1987, Lagasse and Clerc, 1988, Zwadlo et al., 1988). S100A9 gene expression has been shown to be tightly regulated in a differentiation- and lineage-specific manner in the hematopoietic system (Kerkhoff et al., 2002). Indeed, primitive uncommitted and early myeloid progenitor cells lack the expression of S100A9 which starts to be expressed with CD11b and CD14 in monocytes and with CD15 in neutrophils. $CD19^+$ B-cells, $CD3^+$ T cells and erythroid cells have been classically described to be S100A9 negative (Kerkhoff et al., 2002) but recently S100A8 and S100A9 have been found to be expressed in a highly purified B-cell population and in platelets (Husson et al., 2002, Healy et al., 2006). S100A8/A9 is expressed in dendritic cells (DCs) which constitutively release S100A8/A9. Under inflammatory conditions, their expression is also induced in fibroblasts (Rahimi et al., 2005), mature macrophages (Ingersoll et al., 2010), vascular endothelial cells (McCormick et al., 2005, Yao and Brownlee, 2010, Landers-Ramos et al., 2017), vascular smooth muscle cells (Inaba et al., 2009), keratinocytes (Grimbaldeston et al., 2003), epithelial cells, and osteoclasts (Ehrchen et al., 2009). Exposure of endothelial cells to lipopolysaccharide (LPS) (Averill et al., 2011, Yen et al., 1997), IL-1, βTNF-α but also high glucose induced S100A8 and S100A9 expression *in vitro* (Yao and Brownlee, 2010), however, the protein was mainly cell associated and not released by cultured EC. In vascular smooth muscle cells (VMSCs) S100A9 is induced by gram-negative bacteria (Inaba et al., 2009) and LPS also stimulates S100A8/A9 expression in cardiomyocytes (Yao and Brownlee, 2010). Although immune cells are the main source of S100A8/A9, platelets and

megakaryocytes have been demonstrated to be enriched in S100A8/9 transcripts both in mice models and human samples (Healy et al., 2006, Wang et al., 2014). The quantity of S100A8/A9 released by non-myeloid cells is however significantly lower than the ones expressed by myeloid cells unless cell membrane is compromised, suggesting a possible local effect of S100A8/A9 expression in non-myeloid cells.

3.2.2.3 S100A8/A9 receptors and pathways

S100A8 and S100A9 can bind to different receptors on immune and stromal cells triggering multiple signalling pathways downstream of these receptors. S100A8 binds to toll-like receptor 4 (TLR-4)(Vogl et al., 2004, Fassl et al., 2015), receptor for advanced glycation end products (RAGE) (Ehlermann et al., 2006), heparan and N-glycans. S100A9 binds to TLR-4, RAGE, heparan sulphate, N-glycans and the extracellular matrix metalloproteinase inducer (EMMPRIN), also known as basigin or CD147. The complex S100A8/A9 binds to TLR-4, RAGE, glycosylated glycans and the scavenger receptor CD36. Considering the heterodimer is the main functional protein, those receptors are the ones that have mainly been targeted to prevent its function. TLR-4 and RAGE are the main receptors for S100A8/A9 on immune cells. S100A8/A9 has been demonstrated to promote migration and adhesion in neutrophils through TLR4 and RAGE in p38-MAPKs, JNK and ERK1/2-dependent manner (Ghavami et al., 2008). TLR-4 is the dominant receptor on monocytes and binding of S100A8/A9 results in NF-κB- and interferon regulatory factor (IRF)-mediated inflammatory gene transcription (Fassl et al., 2015). Interestingly, blockage of RAGE but not TLR-4 inhibits S100A9-mediated recruitment of macrophages and leukocytes *in vitro* (Chen et al.,

2015). On endothelial cells, S100A8/A9 predominantly binds to heparan, heparan sulfate glycosaminoglycans (Cecil et al., 2009) and carboxylated glycans (Srikrishna et al., 2001) leading to neutrophils binding to endothelial cells (Srikrishna et al., 2001). Blocking these N-glycans with specific antibodies inhibits leukocyte extravasation in a murine model of inflammation (Srikrishna et al., 2001). Binding of S100A8/A9 to RAGE leads to (PI3K)/Akt/mTOR pathway and mTOR complex 2 (mTORC2) activation increasing human umbilical vein endothelial cells (HUVECs) proliferation, viability, and migration (Zhong et al., 2020). On the other hand, interaction of S100A8/A9 with the scavenger receptor CD36 on endothelial cells facilitates their fatty acid uptake such as uptake of arachidonic acid (AA) (Kerkhoff et al., 2001). In cancer, S100A8/A9 at low concentration promotes tumour cell growth through RAGE signalling and activation of NF-κB (Ghavami et al., 2008). EMMPIRIN, a transmembrane glycoprotein that belongs to immunoglobulin superfamily, present on cancer cells (Hibino et al., 2013, Saha et al., 2010, Vogl et al., 2007), is also able to bind S100A9 but not S100A8 promoting inflammation and cancer metastasis (Muramatsu, 2016, Hibino et al., 2013).

3.2.2.4 Intracellular functions of S100A8/A9

S100A8/A9 exerts intracellular and extracellular activities which differentially alter cell behaviour. The main intracellular function exerted by S100A8/A9 in immune cells is cytoskeleton modulation and transfer of polyunsaturated fatty acids leading to respiratory burst. When Ca^{2+} is present, S100A8/A9 binds to cytosolic polyunsaturated fatty acid and transports them activating NADPH oxidase, a key step to generate ROS in phagocytes (Lim et al., 2009, Kerkhoff et al., 1999). S100A8/A9 has also been

described to act as a scaffold between arachidonic acid and NADPH oxidase facilitating their interaction (Benedyk et al., 2007, Kerkhoff et al., 2005) and subsequent respiratory burst enhancement. It has also been described that S100A8/A9-arachdonic acid complex is internalised by inflammatory cells favouring the synthesis of inflammatory mediators such as leukotriene B4 which is involved in inflammatory responses such as leukocyte degranulation and vascular endothelium damage (Han et al., 2007). In resting monocytes and neutrophils, S100A8/A9 stabilises microtubule polymerisation. Ca^{2+} -induced S100A8/A9 tetramerization seems to play a key role in stabilising the microtubule network (Roth et al., 1993, van den Bos et al., 1996, Vogl et al., 2004, Goebeler et al., 1995, Leukert et al., 2006). Once those immune cells are activated, S100A9 is rapidly phosphorylated through Ca^{2+} and p38 Mitogen-activated protein kinase (MAPK) signalling pathway, leading to microtubule rearrangement and effective cell migration (Vogl et al., 2004, Lominadze et al., 2005). Indeed S100A9-knockout mice show impaired leukocyte migration and $S100A9^{-/-}$ deficient neutrophils show a reduction in CD11b and polymerised tubulin suggesting that S100A8/A9 plays a crucial role in cytoskeleton modulation (Manitz et al., 2003). Monocytes from $S100A9^{-/-}$ mice have a reduced ability to migrate toward chemokines, while peritoneal macrophages isolated 48 hours after thioglycolate injection, show an impaired ability to release cytokines following LPS stimulation. In macrophages, S100A9 released from phagocytosed apoptotic neutrophils inhibits macrophage activation through regulation of Ca^{2+}-binding activity (De Lorenzo et al., 2010).

3.2.2.5 Extracellular functions of S100A8/A9

Once released extracellularly, S100A8/A9 exerts several anti-microbial, pro-inflammatory, and pro-thrombotic functions. Extracellular S100A8, S100A9 and S100A8/A9 are mainly released by immune cells throughout different mechanisms and activate different receptors expressed mainly on immune and endothelial cells as well as platelets (Chan et al., 2012, Newton and Dixit, 2012, de Haan et al., 2013).

3.2.2.5.1 Extracellular S100A8/A9 secretion from phagocytes

S100A8/A9 is mainly present in a soluble form, however, it can also be associated with large macrovesicles in stimulated and unstimulated neutrophils (Tardif et al., 2015). It was demonstrated that monensin and brefeldin A (known to block the vesicular traffic at the endoplasmic reticulum and Golgi) were unable to inhibit release of S100A8 and S100A9 suggesting these proteins are released by alternative pathways (Rammes et al., 1997, El Gazzar, 2015). It has been described that S100A8 and S100A9 lack the *signal peptide* (mostly hydrophobic residues at the N-terminal end) which act as a protein secretion marker (Bertheloot et al., 2016, Gross et al., 2013, Owji et al., 2018, Lang et al., 2022, Liaci and Förster, 2021). These secretion mechanisms are different according to cells and type of signal, leading to active or passive release of S100A8/A9. **Figure 3.2** summarises different mechanisms of S100A8/A9 secretion.

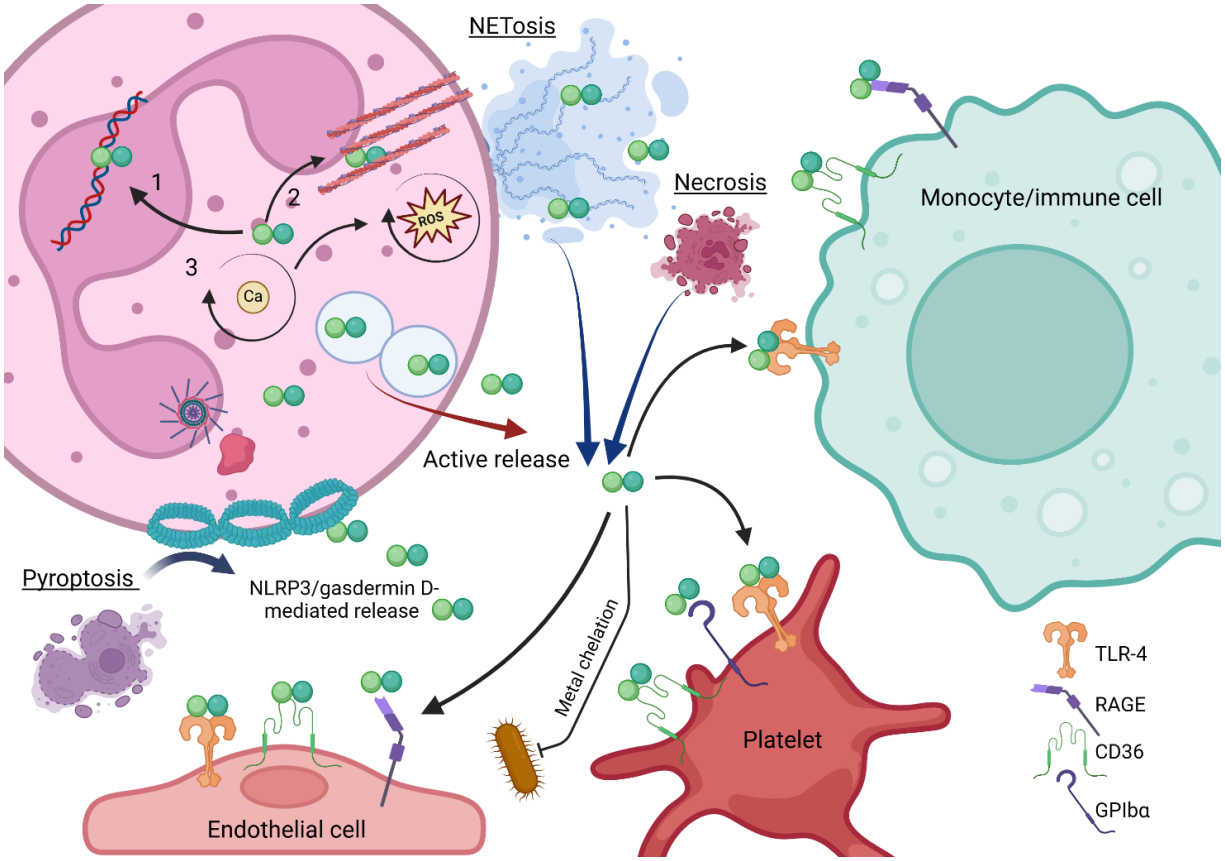


Figure 3.2 – Mechanisms of S100A8/A9 release in the extracellular space. Within the cell, S100A8/A9 is involved in diverse cell functions including transcriptional regulation and DNA repair (1)(Sun et al., 2024, Argyris et al., 2023, Song and Struhl, 2021), cytoskeleton organisation (2)(Wolf et al., 2023, Roth et al., 1993), calcium homeostasis (3)(Yang et al., 2020) and energy metabolism (Wang et al., 2023, van Dalen et al., 2018, Kerkhoff et al., 1999). The secretion of S100A8/A9 occurs either through passively mechanisms such as pyroptosis (Jorch et al., 2023), necrosis (Vogl et al., 2012, van Zoelen et al., 2009) and NETosis (Urban et al., 2009)(blue arrows) or actively following cell activation (red arrow). The active secretion is either vesicle-dependent (Chakraborty et al., 2015) or microtubule-dependent (Rammes et al., 1997). Extracellularly S100A8/A9 interacts with several receptors on the surface of different cells such as TLR-4, RAGE, CD36 and GPIbα. Furthermore, S100A8/A9 displays antimicrobial activity through the chelation of zinc and manganese ions. Abbreviations: NET, neutrophil extracellular trap; TLR4, toll-like receptor 4; RAGE, receptor for advanced end products; ROS, reactive oxygen species; NLRP3, nucleotide-binding domain, leucine-rich–containing family, pyrin domain–containing-3Adapted from Refs (Bertheloot and Latz, 2017, Wang et al., 2023). Created with Biorender.com.

The passive release of S100A8/A9 is mostly associated with neutrophil extracellular trap (NET) formation or necrosis (Pruenster et al., 2016). Conversely, the secretion of S100A8/A9 from activated monocytes is an energy-dependent process (Rammes et al., 1997), regulated by tubulin polymerisation and protein kinase C activation (Pruenster et al., 2015). Indeed, secretion of S100A8/A9 can be prevented by inhibition of microtubule polymerisation and uncoupling oxidative phosphorylation. At the site of inflammation, S100A8/A9 is released from activated monocytes following interaction with TNF-α-stimulated endothelial cells. In this setting, the secretion is triggered by increased intracellular calcium levels in monocytes while contact with resting endothelium inhibits protein kinase C-induced secretion. Therefore, the presence of inflammatory signals such as TNF-α at the site of inflammation favours the release of S100A8/A9 promoting monocytes migration (Viemann et al., 2005). Another interesting aspect is that in human monocytes, S100A8/A9 has been found to be effectively released only after two signals, a pro-inflammatory and one anti-inflammatory. Indeed, IL-10 leads to p38-MAPK activation and the phosphorylation of S100A8/A9 allowing its inclusion in plasma membranes while TNF-α promotes its release (Diercks et al., 2013). In neutrophils, the release of S100A8/A9 has been shown to be triggered by different signals including Monosodium urate (MSU) crystals in a Src kinase, Syk- and tubulin-dependent manner. Other molecules which can induce the release of S100A8/A9 from neutrophils include phorbol myristate acetate (PMA), H_2O_2, nanoparticles like silica, TiO_2, fullerene and fungi-derived protein Zymosan whereas chemotactic factors like FMLP, C5a, and IL-8 failed to induce S100A8/A9 secretion (Tardif et al., 2015). The release process in neutrophils has been demonstrated to be dependent on the production of ROS and requires exchange of K^+ ions through the

ATP-sensitive K^+ channels (Tardif et al., 2015). Despite S100A8/A9 being secreted in the phosphorylated and non-phosphorylated forms, it was shown that only S100A8/PhosphoA9 can induce cytokine secretion in differentiated neutrophil-like HL-60 cells (dHL-60) cells and purified human neutrophils via TLR-4 signalling (Schenten et al., 2018).

3.2.2.5.2 Anti-microbial functions of extracellular S100A8/A9

S100A8/A9 exhibits antimicrobial activity by chelation of zinc which is necessary for bacteria growth. Neither S100A8 or S100A9 alone showed antimicrobial properties when tested alone, suggesting the heterodimer is necessary to form a zinc-binding site capable of inhibiting microbial growth (Sohnle et al., 2000). Purified S100A8/A9 has been described to reduce or inhibit the growth of different species *in vitro* including Escherichia Coli, Candida Albicans, Staphylococcus Aureus., Klebsiella pneumonia, Salmonella typhimurium and Listeria monocytogenes (Zaia et al., 2009, Sohnle et al., 1991, Murthy et al., 1993). It has also been reported that S100A8/A9 in mucosal fluid or airways secretions limits the growth of commensal pathogen and the invasion of pathogens (Corbin et al., 2008). Accumulation of S100A8 and S100A9 has also been observed in mice infected with plasmodium (Mizobuchi et al., 2014). However, using animal models, deficiency in S100A9 did not alter bacterial growth during salmonella typhimurium infection (De Jong et al., 2015), suggesting that S100A8/A9 anti-microbial functions are dispensable in this model. It is likely that other compensatory mechanisms are involved and contribute to pathogen clearance.

3.2.2.5.3 Proinflammatory functions of extracellular S100A8/A9: chemotaxis and cytokine production

S100A8/A9 favours neutrophil chemotaxis, apoptosis and autophagy in various cell types including macrophages, lymphocytes and endothelial cells (Ryckman et al., 2003, Ghavami et al., 2010). Indeed, S100A8/A9 enhances the expression of β2 integrin CD11b affecting phagocyte's ability to adhere (Ryckman et al., 2003, Newton and Hogg, 1998). Exposing neutrophils with S100A9 enhances trans-endothelial migration while an anti-S100A9 antibody blocks leukocyte infiltration in a murine arthritis model (Chen et al., 2015). In response to chemokines, mouse S100A9$^{-/-}$ neutrophils show reduced migration due to cytoskeletal dynamics impairment into pneumococcal infected lungs (Chen et al., 2015, Lackmann et al., 1993, De Filippo et al., 2014). S100A8/A9 is also involved in immune cells extravasation as S100A8/A9 upregulates Macrophage-1 antigen Mac-1 (CD11b/CD18) expression and favours the binding of neutrophils to fibrinogen. On the other side, S100A9$^{-/-}$ mice have reduced Mac-1 expression (and activation) and reduced capacity to migrate through endothelial cells (Croce et al., 2009). Interestingly, neutrophils rolling triggers S100A8/A9 secretion which induces vascular cell adhesion molecule-1 (VCAM-1) and (Intercellular adhesion molecule 1) ICAM-1 expression on endothelial cells (Pruenster et al., 2015, Viemann et al., 2005). This mechanism increases the capacity of leukocytes to perform trans-endothelial migration (Viemann et al., 2005, Pruenster et al., 2015). Pre-treating mice with anti-mS100A8 and m-S1009 antibodies reduces macrophages migration to alveoli during streptococcal lung infection without affecting leukocyte count in the vasculature (Raquil et al., 2008). Beyond immune cell recruitment, the release of S100A8/A9

induces the secretion of multiple cytokines in immune cells including monocytes and neutrophils such as IL-1β and TNF-α (Holzinger et al., 2014, Vogl et al., 2007). S100A8/A9 binding to RAGE on endothelial cells cell membrane, promotes the secretion of pro-inflammatory cytokines such as TNF-α and IL-6, leading to acute inflammation and microglial cells dysfunction (Ma et al., 2017). Indeed, atherosclerosis was attenuated in *ApoE*$^{-/-}$/*RAGE*$^{-/-}$ mice compared with *ApoE*$^{-/-}$ mice. Compared to *ApoE*$^{-/-}$ mice, plasma collected from *ApoE*$^{-/-}$*RAGE*$^{-/-}$ mice showed significantly lower levels of soluble vascular cell adhesion molecule-1 (VCAM-1), one of the molecular markers of vascular inflammation in atherogenesis. (Harja et al., 2008). S100A8/A9 was also found to activate the scavenger receptor CD36 which mediated inflammatory responses in endothelial cells (Kerkhoff et al., 2001). Furthermore, it has been demonstrated a reduction in macrophages and neutrophils infiltration of S100A9-/- mice arteries together with a reduction in endothelial cell proliferation and atherosclerotic plaque infiltration (Croce et al., 2009). In neutrophils, S100A9 has been shown to potentiate IL-8 secretion induced by other neutrophils agonists (Simard et al., 2014). S100A8/A9 heterodimer increases proinflammatory cytokine production by macrophages in rheumatoid arthritis (Sunahori et al., 2006). Therefore, S100A8/A9 favours an inflammatory environment which can be further regulated by the organ and disease microenvironment affected and involved (Wang et al., 2018, Tammaro et al., 2018, Ma et al., 2024, Kleissner et al., 2021, Liang et al., 2024).

3.2.2.5.4 Anti-inflammatory functions of S100A8/A9

Although S100A8/A9 is mostly pro-inflammatory in pathological conditions, it has also been reported to have anti-inflammatory properties. S100A8 *in vitro* reduces mast cell degranulation and eosinophil infiltration in acutely asthmatic mice (Zhao et al., 2011). Pre-stimulating murine and human monocytes with S100A8 downregulates p38 phosphorylation with attenuation of IL-6 and TNF-α production in response to LPS and bacteria (Coveney et al., 2015). Interestingly, it has been demonstrated that S100A8/A9 traps and sequestrates IL1β, IL-6 and TNF-α (Hiroshima et al., 2014, Otsuka et al., 2009). Furthermore S100A8/A9 inhibits PMNs oxidative metabolism *in vitro* and scavenges ROS reducing oxidative damage in livers and lungs of LPS-treated mice. S100A8 promotes IL-10 expression in airway epithelial cells affecting neutrophils infiltration and reducing pro-inflammatory mediators (Sun et al., 2013, Başsorgun et al., 2014). S100A9 also reduces dendritic cells antigen presentation to T cells by affecting B7 expression (Shimizu et al., 2011). Additionally, S100A8/A9 regulates inflammatory responses also inducing apoptosis and phagocytosis of apoptotic cells. S100A8/A9 has been demonstrated to be implicated in inducing mitochondrial apoptotic pathways and also extracellular Zinc uptake promotes apoptosis being zinc an apoptotic inhibitor (Nakatani et al., 2005). The balance between anti-inflammatory and pro-inflammatory S100 activity seems to be dictated by the environment and subsequent biochemical changes occurring in those proteins.

3.2.2.5.5 Prothrombotic functions of extracellular S100A8/A9: endothelial cells and platelets

Beyond its inflammatory functions, S100A8/A9 was shown to contribute to arterial and venous thrombosis. The prothrombotic effect of S100A8/A9 is multifactorial and involved multiple cells and receptors. Using endothelial cells, S100A8/A9 was shown to increase endothelial cell inflammatory responses and impairs the integrity of endothelial monolayer loosening the tight junctions (Viemann et al., 2005), thereby increasing the exposure on the prothrombotic extracellular matrix. S100A8/A9 also enhances the secretion of IL-6 and MCP-1 and upregulates ICAM-1 and VCAM-1 in AGE-albumin pre-treated HUVEC in a dose-dependent manner and platelet adhesion on endothelial cells (Ehlermann et al., 2006). In periodontitis, S100A8/A9 has been demonstrated to activate platelets and increase the formation of platelet-leukocyte aggregates in whole blood, supporting a role for S100A8/A9 in thrombosis (Zhan et al., 2016). Indeed, S100A9-deficient mice showed a reduction in arterial thrombus formation, a prothrombotic function which was shown to be at least partially mediated by CD36 (Wang et al., 2014). Deficiency in S100A9 did not alter the haemostatic parameters suggesting that S100A9 is dispensable for haemostasis. Performing microcatheter sampling within the core of brain ischemia in human patients before mechanical thrombectomy, S100A8/A9 was found to be elevated and associated with the numbers of leukocytes that infiltrated the occluded compartment (Schuhmann et al., 2021). Indeed, high plasma S100A8/A9 concentrations are independently associated with increased risks of adverse clinical outcomes after 3 months following ischemic stroke (Guo et al., 2020) . Additionally, Tomohiro *et al* demonstrated that

immunization with an S100A9 vaccine resulted in long-term inhibition of thrombus formation in ischemic stroke in mice through inhibition of S100A9/CD36 signalling without increasing bleeding or autoimmune responses. These data show that S100A9 represent an anti-thrombotic target in arterial thrombosis. Moreover, S100A9-deficient mice were also protected from deep vein thrombosis as shown by a significant reduction in thrombus size compared to WT mice (Wang et al., 2017a) . Furthermore, the adoptive transfer of WT neutrophils or platelets, or the infusion of S100A8/A9 in $S100A9^{-/-}$ mice rescued thrombus formation defect suggesting that both neutrophil and platelet S100A8/A9 contributes to thrombosis. Interestingly, using a proteomic approach, it was recently shown that platelets isolated from healthy donors don't contain S100A8/A9 while the expression is significantly increased in patients with ST-elevation myocardial infarction (STEMI), systemic lupus erythematosus (SLE) and cardiovascular disease (CVD) (Lood et al., 2016). The authors showed using neutrophil-platelet co-culture experiments that neutrophils transfer S100A8/A9 to platelets, which is subsequently released during activation (Joshi et al., 2022). It is unlikely that transfer from neutrophil is the only mechanism leading to S100A8/A9 expression in platelets, and further research is needed to understand the expression and role of S100A8/A9 in platelets.

3.2.2.6 S100A8/A9 in metabolic, autoimmune and chronic inflammation

In metabolic diseases including gout, diabetes, and obesity, S100A8/A9 is found to be elevated in both sera and tissue. In gout patients, neutrophils migrate to gout-affected joints where they secrete S100A8/A9 accelerating inflammation (Goldberg et al., 2017,

Holzinger et al., 2014, Wang et al., 2015a, Oliva et al., 2013). Adipose tissue-derived S100A8/A9 stimulates TLR-4 cascade leading to inflammation and increased myelopoiesis (Murray, 2014). High epicardial S100A9 expression is present in patients with cardiovascular disease where small adipocytes produce a high volume of oxidative stress. S100A9 is upregulated in glial cells promoting amyloidosis and S100A8 is decreased in CD11c cells surrounding the amyloid plaques suggesting a possible beneficial role in Alzheimer's disease. S100A8/A9 is elevated in diabetic patients and associated with hyperglycaemia-induced inflammation. However, in diabetic foot ulcer patients, high local levels of S100A8/A9 are important to prevent bacterial infection.

Upregulation of S100A8/A9 occurs also in multiple autoimmune diseases. In patients with psoriatic arthritis S100A8/A9 is upregulated in plaques, synovial fluid and serum. In rheumatoid arthritis, S100A8 is released from activated macrophages correlating with inflammatory parameters such as C-reactive protein (CRP), erythrocyte sedimentation rate and rheumatoid factors. S100A8 being abundant in rheumatoid arthritis synovial fluid, promotes IL-6 expression in fibroblast-like synoviocytes via TLR4 and MAPK signalling (Lee et al., 2013, Kang et al., 2023). In systemic lupus erythematosus (SLE) S100A8/A9 is released from polymorphonuclear cells and high levels correlate with dsDNA antibodies and glomerulonephritis. Indeed, serum S100A8/A9 has been proposed as a biomarker to monitor disease activity in those patients (Wang et al., 2014, Tydén et al., 2017). SLE platelets expressed high levels of S100A8/A9 in close proximity to intracellular membrane and on the membrane surface upon activation with physiologic stimuli. Furthermore S100A8/A9 was enriched

at sites of membrane-membrane interaction indicating its crucial role in cell communication (Lood et al., 2016). In cardiac ischemia, the concentrations of S100A8, S100A9 are significantly increased in acute coronary syndrome (ACS), coronary artery calcification and cardiovascular intima hyperplasia (Buyukterzi et al., 2017, Wang et al., 2015b, Hirata et al., 2012). Clinical studies in patients with ACS have demonstrated that the concentration of S100A8/A9 in coronary blood was associated with thrombus formation, co-localized with leukocytes, and was associated with leukocyte activation (Sakuma et al., 2017). High serum levels of S100A8/A9 are associated with the development of late stent thrombosis, which is a thrombotic complication following percutaneous coronary interventions (Wang et al., 2020). When hyperglycaemia is present, myelopoiesis is induced by S100A8/A9 and neutrophils produce more S100A8/A9 favouring inflammatory processes (Nagareddy et al., 2013). Indeed, the levels of S100A8/A9 are elevated in patients with diabetes and blood concentration of S100A8/A9 in the blood correlates with neutrophil number, ACS events and cardiovascular death (Ortega et al., 2012, Cotoi et al., 2014). Using a mouse model of permanent ligation of the left anterior descending artery inducing AMI, it was demonstrated that neutrophils were rapidly recruited to the infarcted area releasing S100A8 and S100A9. These alarmins binding to TLR-4 promoted IL1β secretion which acting on the bone marrow induced granulopoiesis and inflammation. Marinkovic *et al.,* have demonstrated that in mouse models of re-perfused and non-re-perfused myocardial infarction, blocking S100A9 interaction with TLR4 and RAGE, attenuated neutrophils and monocytes recruitment promoting cardiac infiltration with reparative macrophages (Marinković et al., 2020). This led to attenuated systolic dysfunction and heart failure development. In addition to S100A8/A9 effect on immune cells also its

effect on endothelial cells, fibroblasts and cardiomyocytes resulted to be essential to determine inflammation or repair in the infarcted heart.

As a response to hyperglycaemia, in mice, neutrophil-derived S100A8/A9 have been found to interact with hepatic Kupffer cells in the liver resulting in increased IL-6 production. Reducing glucose levels or blocking the interaction with S100A8/A9 with RAGE receptor diminished this effect (Kraakman et al., 2017). Therefore, S100A8/A9 may represent an important target to reduce both inflammation and thrombosis in chronic inflammatory and metabolic disorders.

3.2.2.7 S100A8/A9 in infection: Sepsis and COVID-19

High plasma levels of S100A8/A9 have been demonstrated to indicate higher risk of death in septic shock patients (Dubois et al., 2019). In septic shock, the expression of S100A9 is continuously increased until the patient's death (Raju et al., 2016). Using mouse model of sepsis, S100A8/A9 has been demonstrated to contribute to neuroinflammation in sepsis survivor mice (Denstaedt et al., 2018). More recently, in a mouse model of caecal ligation and puncture-induced sepsis in mice, NETosis-driven S100A8/A9 release was shown to induce gasdermin-D-dependent platelet pyroptosis and the release of oxidised mitochondrial DNA which fuels NETosis. By creating a vicious circle of inflammation, S100A8/A9-induced platelets pyroptosis worsened inflammation leading to cytokine storm, thrombosis and death in a mechanism dependent on TLR4 (Su et al., 2022). S100A8/A9 has been demonstrated to be elevated in COVID-19 and govern hyper-inflammation. S100A8 and S100A9 transcripts are enriched in platelets isolated from patients with COVID-19 and SARS-

Cov-2 infection of megakaryocytes was shown to induce S100A8/A9 expression in these cells (Barrett et al., 2021a). As a consequence, increased S100A8/A9 was found to be released in a greater extent by patient with COVID-19 when compared to control contributing to hypercoagulable phenotypes and endotheliophathy (Barrett et al., 2021a, Barrett et al., 2021b). Beside platelets, a subpopulation of neutrophils that contributes to the uncontrolled pathological damage and onset of COVID-19 was shown to dramatically increase S100A8/A9 production and these aberrant neutrophils contributes to the pathogenesis of COVID-19 (Guo et al., 2021). Therefore, S100A8/A9 is a direct contributor to the inflammatory response during infection including sepsis and COVID-19, however, its direct effect on thrombosis in these models is not known.

3.2.2.8 S100A8/A9 as a biomarker of inflammation

The possible use of S100A8/A9 as a biomarker or therapeutic target are increasing. S100A8/A9 has been proposed to be used as a biomarker for many inflammatory diseases and thrombotic conditions such as inflammatory arthritis (Kane et al., 2003, Kang et al., 2014), juvenile Idiopathic arthritis (Walscheid et al., 2015) and juvenile rheumatoid arthritis (RA) (Frosch et al., 2000), skin conditions (Zhong et al., 2016, Chimenti et al., 2016, Marionnet et al., 2003), transplantation (Nakata et al., 2023, Ikemoto et al., 2003), inflammatory bowel disease (Okada et al., 2019, Pruenster et al., 2016), glomerulonephritis (Pepper et al., 2013), diabetes (Nakatsuji et al., 2012, Bouma et al., 2004), cystic fibrosis (Gray et al., 2010, Cohen et al., 2005), periodontitis (Kim et al., 2021), autoimmune synovitis (Blom et al., 2020, De Rycke et al., 2005), peritonitis (Schwarz et al., 2007), uterine cervix inflammation (Xin et al., 2024, Kunimi

et al., 2006), diabetic nephropathy (Burkhardt et al., 2009), CVD (Cai et al., 2020, Kleissner et al., 2021, Healy et al., 2006, Altwegg et al., 2007, Saito et al., 2012), infections (van Zoelen et al., 2009) and juvenile dermatomyositis (Nistala et al., 2013). In sepsis, S100A8/A9 can be an independent predictor of 28 days mortality and a promising marker for risk stratification (Gao et al., 2015). In pregnant women, S100A8 present in the amniotic fluid was found to be a predictor for early onset sepsis incidence in neonates (Buhimschi and Buhimschi, 2010). In diabetes, not only S100A8/A9 is a biomarker for the disease itself but it has been associated with atherosclerosis development in diabetic patients (Nakajima et al., 2015, Bouma et al., 2004, Hirata et al., 2012). Faecal S100A8/A9 has been used to differentiate between IBD from functional gut disorders and monitor disease progression (Boyapati et al., 2016). S100A8/A9 is considered a marker to differentiate between RA and osteoarthritis (Kang et al., 2014) and to evaluate patients' responsiveness to biologic disease-modifying anti-rheumatic treatments (Kang et al., 2014, Nordal et al., 2017). The level of faecal S100A8/A9 is also found high in infants with food allergy and could monitor their disease progression (Zhu et al., 2017). In patients with psoriatic arthritis, S100A8/A9 became a better predictor of ongoing disease than CRP (Hansson et al., 2014). S100A8/A9 is considered a sensitive biomarker for inflammatory progression in RA, SLE and juvenile idiopathic arthritis (Gupta et al., 2016, Kopeć-Mędrek et al., 2016, Soyfoo et al., 2009, Ometto et al., 2017). Indeed, circulating S100A8/A9 has been proposed as a biomarker to assess and monitor patients with COVID-19 (Norman et al., 2022). Overall, it has been demonstrated that S100A8/A9 can be largely used as a biomarker in conditions where inflammation is involved for both prediction and treatment monitoring. Considering the poor specificity of the biomarker, more defined

cut-offs and the association with other disease-specific molecules will be required for accurate diagnosis.

3.2.2.9 S100A8/A9 as a therapeutic target

Currently, few data are available to demonstrate *in vivo* the effective blocking of S100A8/A9 expression. The use of specific hairpin RNAs to reduce S100A8/A9 expression in human myeloid cells in patients with myelodysplastic syndrome had reduced the release of pro-inflammatory cytokines but its applicability is questionable considering the physiological roles S100A8/A9 has in those cells (Wei et al., 2011). Another approach has been targeting the alternative transport pathways which are shared by S100 proteins. Blocking those pathways would not affect the release of the majority of classically secreted proteins. This approach has been investigated in patients affected by familial Mediterranean Fever, who have an extraordinary level of S100A8 an S100A9 driven by caspase-1 and IL-1 (Dzhndoian, 2012). Colchicine, which blocks microtubule polymerisation has been shown to reduce S100A8 and S100A9 release from monocytes (Rammes et al., 1997). Blocking this alternative pathway targeting caspase-1 is another approach currently under development (Poreba et al., 2013, Keller et al., 2008). Another approach would be targeting intracellular signalling pathways induced by S100A8/A9. Anti-TLR4 antibodies have been used and inhibited the production of pro-inflammatory cytokines in a mouse model of arthritis (Monnet et al., 2015). However, blocking TLR-4 is associated with a high risk of adverse events considering its important role in infection responses (Gasparoto et al., 2010). Small molecules inhibitors such as F-04494700 have been

developed to target RAGE binding but they are not specific for S100A8/A9 (Sabbagh et al., 2011). S100A8/A9 interact with TLR4 using peptide sequenced located within one of the calcium-binding sites. Upon release, the formation of tetramers hides this TLR-4 binding site, restricting the activity of S100A8/A9 locally. The loss of this autoinhibitory mechanism results in fatal inflammation in mice (Vogl et al., 2018). Therefore, adopting molecules that force S100A8/A9 to acquire tetramer form could be another therapeutic strategy. Another group of drugs that have been tested is known as Q compounds which can block the recruitment of neutrophils and macrophages activation in a murine model of SLE (Pruenster et al., 2015). These compounds block the interaction between S100A9 and TLR4 or RAGE (Björk et al., 2009). One of these compounds called laquinimod, has been used in phase III randomised clinical trial and demonstrated that was effective to treat relapsing-remitting multiple sclerosis. Laquinimod was also proved to be safe, well tolerated and with positive effects in patients affected by Crohn's disease patients (Comi et al., 2012, D'Haens et al., 2015) as well as Paquinimod in systemic sclerosis (Hesselstrand et al., 2021) and lupus (Bengtsson et al., 2012). Azeliragon is a small molecule inhibitor of the receptor of AGE (RAGE) and it is in phase II clinical trial for Alzheimer disease (AD). Animal models of AD treated with Azeliragon showed reduced Aβ plaque deposition, decreased inflammatory cytokines, reduced cognitive decline, and improved cerebral blood flow (Burstein et al., 2014). Other approaches could involve targeting CD36-S100A8/A9 interaction or signalling pathways but it has not been tested yet in disease settings. A vaccine against S100A9/CD36 signalling in platelets was tested in mice and was demonstrated to be inhibit thrombosis without increasing the risk of bleeding in ischemic stroke (Kawano et al., 2018). Overall, more research is required to target

specifically S100 proteins specifically without compromising physiological cell/immune responses.

In this chapter we investigate the possible direct effect of S100A8/A9 on platelets and try to decipher its intracellular pathways.

3.3 Results

3.3.1 S100A8/A9 levels are sustained in patients with COVID-19 and correlate with adverse outcome

Several studies demonstrated that patients with COVID-19 suffer from thromboinflammation. S100A8/A9 levels have been described to be elevated in COVID-19 patients and their levels to correlate with thrombosis (Guo et al., 2021, Mahler et al., 2021, Wang et al., 2014, Barrett et al., 2021a, Hogg et al., 1989). A cohort of 87 patients with demonstrated COVID-19 disease were recruited and their demographical characteristics are summarised in **Table 3.2**. Study inclusion occurred up to 72 hours after hospital admission, other inclusion/exclusion criteria are summarised in **Table 3.3**. Patients were stratified according to clinical severity into uncomplicated (not requiring treatment in intensive care) and complicated (requiring intensive care treatment). Complicated patients were further divided into intensive care unit survivors and non-survivors. Plasma was collected during their hospitalisation and frozen until use. Plasma levels of S100A8/A9 were measured by ELISA on the plasma collected over the first week of enrolment into the study. It is important to specify that

these patients were recruited in Austria, where hospitalisation for COVID-19 patients, occurred on test positiveness, independently of symptoms presence and severity. The results show that on day of study recruitment, the levels of S100A8/A9 were elevated in all COVID-19 patients when compared to healthy controls (**Figure 3.3 A**). However, no differences could be detected between uncomplicated or complicated patients. Interestingly, over the further 7 days of monitoring, the levels of S100A8/A9 remained high only in patients with complicated outcome but decreased in uncomplicated patients. Within the complicated group, S100A8/A9 levels reached significantly higher levels, in the non-survivor group (10-40 µg/ml) (**Figure 3.3 B, C**). These data suggest that S100A8/A9 levels correlates with disease severity and worse outcome. Our data confirmed data reported from other authors (Chen et al., 2020b, Bagheri-Hosseinabadi et al., 2022).

	Males (n=60)	**Females** (n=27)
Age (years)	62.8±16	70.8±12
Covid classification on admission*:		
Mild	4 (7%)	6 (22%)
Moderate	33 (56%)	12 (44%)
Severe	12 (20%)	7 (26%)
Critical	10 (17%)	2 (7%)
Outcome:		
Uncomplicated	33 (55%)	15 (55%)
ICU survivors	20 (33%)	6 (22%)

ICU non-survivors	7 (12%)	6 (22%)
Obesity (BMI>25)	26 (47%)	9 (37%)
DM II	19 (32%)	9 (33%)
CKI	4 (7%)	3 (11%)
CVD*	20 (33%)	9 (33%)
COPD	7 (12%)	2 (7%)
Hypertension	45 (75%)	18 (68%)
Chronic Liver Disease	4 (7%)	2 (7%)
Thyroid Dysfunction (ipo/iper)	6 (10%)	7 (26%)
Atrial Fibrillation	7 (12%)	4 (15%)
PAD	3 (5%)	2 (7%)
Malignancies	7 (12%)	4 (15%)
Rheumatic Disease	2 (3%)	1 (4%)
Platelet Count (x10^9/L):**		
Admission	199.4±86.6	208.55±83.7
Day 1	227.76±83.3	210.5±68.6
Day 3	284±106.5	303.2±126
Day 5	333.1±122.8	334.2±127
Day 7	334.9±117.3	394±197.3
D-Dimer (mg/L)** admission		
Uncomplicated	2.7 (0.2-33)	0.9 (0.3-3.7)
ICU survivors	3.3 (0.2-33)	0.6 (0.3-0.8)
ICU non-survivors	2.6 (0.8-5)	0.8 (0.3-1.8)
D-Dimer (mg/L)** all Day 3	2.2 (0.2-24)	1.1 (0.2-4.2)
D-Dimer (mg/L)** all Day 7	1.69 (0.3-8)	4.6 (0.4-33)

Table 3.2 Patient demographic and blood finding for COVID-19 patients. *More detailed demographic for the cohort at Ref (Schrottmaier et al., 2021).** Missing data, average performed on available data. Percentages are calculated on respectively total males and total females. Diabetes Mellitus Type II (DM II), Chronic Kidney Insufficiency (CKI), cardiovascular disease (CVD) includes coronary artery disease, heart failure, atrial fibrillation, peripheral artery disease, Chronic Obstructive Pulmonary disease (COPD), peripheral artery disease (PAD).

Inclusion Criteria	Exclusion Criteria
• SARS-CoV-2 infection (confirmed by RT-PCR or oropharyngeal swab) • ≥ 18 years of age	• Life expectancy < 1 month (e.g due to comorbidities) • Pregnancy/breast feeding • Stage 4 kidney disease • Severe Liver Dysfunction

Table 3.3 Inclusion/exclusion criteria for COVID-19 patients. Patients recruited in this study were all part of the ACOVACT trial (see methods). They have been recruited to the Clinic Favoriten, Vienna, Austria between 17th April 2020 and 28th October 2020. The study inclusion was possible within 72 hours following hospital admission. Subjects with severe liver dysfunction were excluded because of the possible effect on haemostasis. Blood was drawn upon inclusion (day 0) up to a week. Additional blood drawing was omitted if patient was anaemic (haemoglobin<11 g/dl). More information on this clinical trial can be found at relative publications (Schrottmaier et al., 2022, Schrottmaier et al., 2021).

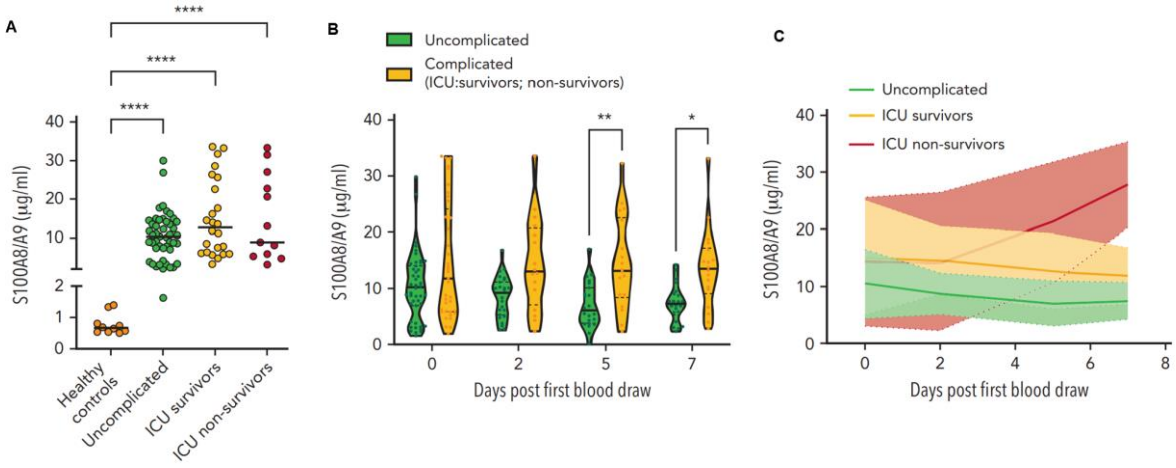


Figure 3.3 – Patients with COVID-19 have sustained high plasma levels of S100A8/A9. (A-C) Plasma levels of S100A8/A9 were detected and measured in patients with COVID-19 disease (uncomplicated [n=48], ICU survivors [26], ICU non-survivors [n=13]) and healthy controls [n=10]. Measures occurred over the first 7 days of hospitalisation. (A) S100A8/A9 levels on admission day. (B, C) S100A8/A9 levels in the following hospitalisations days. Results are shown as mean ± standard error of the mean (SEM). Statistical significance was analysed using two-way ANOVA with Tukey's multiple comparison test between groups. Data for COVID-19 patients collected by Schrottmaier *et al*. as stated in methods. Figure 3.2 C created by Schrottmaier *et al*. *P<0.5, **P<0.05, ***P<0.001, ****P<0.0001. Figures adapted from Ref (Colicchia et al., 2022).

3.3.2 S100A8/A9 is detected in neutrophils and in the lung vessel walls of COVID-19 patients

Other studies described that S100A8/A9 derived from immune cells, drives and support leukocyte recruitment through deposition on venule endothelial cells. This mechanism allows the interaction of myeloid cells to vascular endothelium and promotes immune cell transmigration (Hogg et al., 1989). We hypothesised that also in COVID-19 patients, S100A8/A9 could deposit on the vessel wall and contribute to cell transmigration and increased permeability. Lung autopsies from non-survivor COVID-19 patients (n=8) vs control sections from aged-matched patients suffering

from other lung disease (n=3) were assessed to detect the presence of deposited S100A8/A9. The heterodimer S100A8/A9 was found to be present in the cytoplasm of neutrophils, that we identified by the presence of the neutrophil-marker MPO, and lining the vessel wall (**Figure 3.4 A, B**).

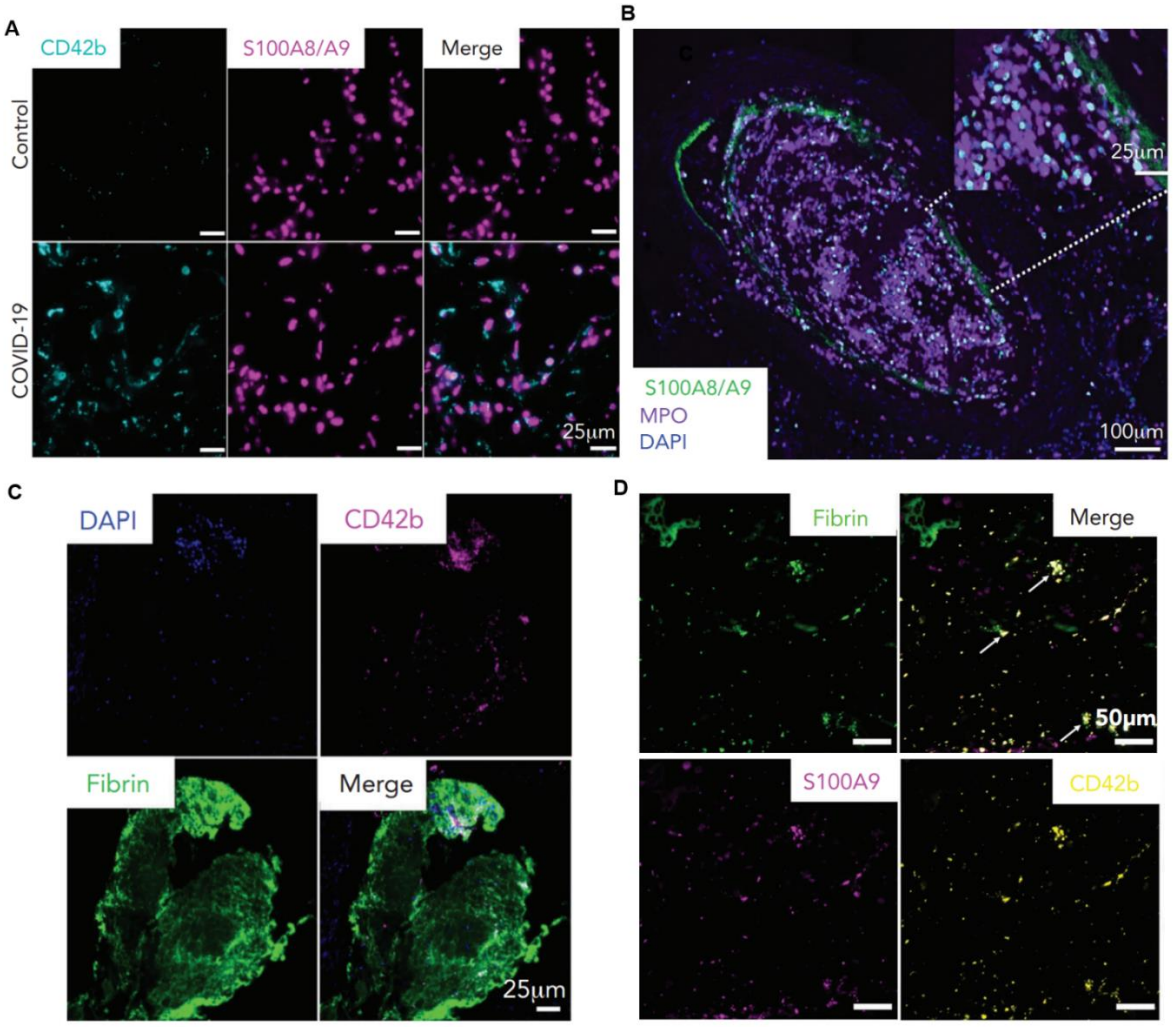


Figure 3.4 – S100A8/A9 is detected in the lungs of COVID-19 patients. Immunofluorescence staining representative images of lung autopsies obtained from COVID-19 patients and aged-matched controls suffering from other lung diseases. (A) S100A8/A9 and platelet marker CD42b staining in lung parenchyma and microcirculation of lung sections. (B) Staining of a large vessel in the lung of a patient with COVID-19 who suffered thrombotic complications. Section was stained for S100A8/A9, nuclei (DAPI) and MPO (neutrophil marker). (C) Staining for nuclei (DAPI), platelets (CD42b), fibrin in a lung

large vessel thrombus of a patient with COVID-19. (D) S100A9, CD42b and fibrin staining in lung parenchyma of COVID-19 patient. Arrows show platelet-fibrin microaggregates. Images were captured with Epi fluorescence microscope and slide Axioscanner Z1. Microscope images prepared and captured by Dr Julie Rayes. Figures adapted from Ref (Colicchia et al., 2022).

Looking into CD42b-positive cells (platelets), we found a fraction of those cells to be positive for S100A8/A9 but only in the lungs of COVID-19 patients and not in controls (**Figure 3.4 A**). Interestingly, using an antibody against S100A9 only, revealed a stronger co-localisation between CD42b and S100A9 in the lung tissue of COVID-19 patients (**Figure 3.4 D**). Platelets were found to be lining the endothelium as single platelets or as small aggregates positive for fibrin or integrated in large fibrin-rich thrombi in the lung of COVID-19 patients (**Figure 3.4 C, D**), suggesting they play multiple pathophysiological roles in this context (as component of thrombi or as mediators of fibrin production). These results in addition to the ELISA data described in 3.3.1, show that S100A8/A9 is not only increased as a soluble protein in the bloodstream of COVID-19 patients but also deposited in the vasculature and parenchyma of COVID-19-lung tissues. On the basis of the evidence that we gained, we hypothesised that S100A8/A9 may activate platelets. Platelet-derived S100A8/A9 was previously shown to induce thrombosis in mice through CD36 (Wang et al., 2014) but the direct effect of S100A8/A9 on platelets was never assessed before. Indeed, platelets from COVID-19 patients have been shown to be enriched in S100A8/A9 and platelet releasate to promote endothelial cell activation via CD36 (Barrett et al., 2021b). Here we assessed the effect of both soluble and immobilised recombinant S100A8/A9 on platelet activation and fibrin generation using blood from healthy donors.

3.3.3 Immobilised S100A8/A9 increases fibrin generation

Commercial and in-house produced recombinant S100A8/A9 were used to assess fibrin generation in whole blood from healthy donors. As described in the Material and Methods chapter, we characterised the in-house S100A8/A9 prior to its use in our experiments. We initially tried to assess the effect of soluble S100A8/A9 on thrombin generation using calibrated automated thrombography (CAT). We obtained PRP from healthy donor's blood and we observed no induction of thrombin generation when PRP exposed to S100A8/A9 was compared to PRP alone (**Figure 3.5**). No significant difference was found in CAT parameters (endogenous thrombin potential [ETP], Time to Peak, Lag Time, Peak Thrombin Generation) between PRP alone and PRP exposed to S100A8/A9.

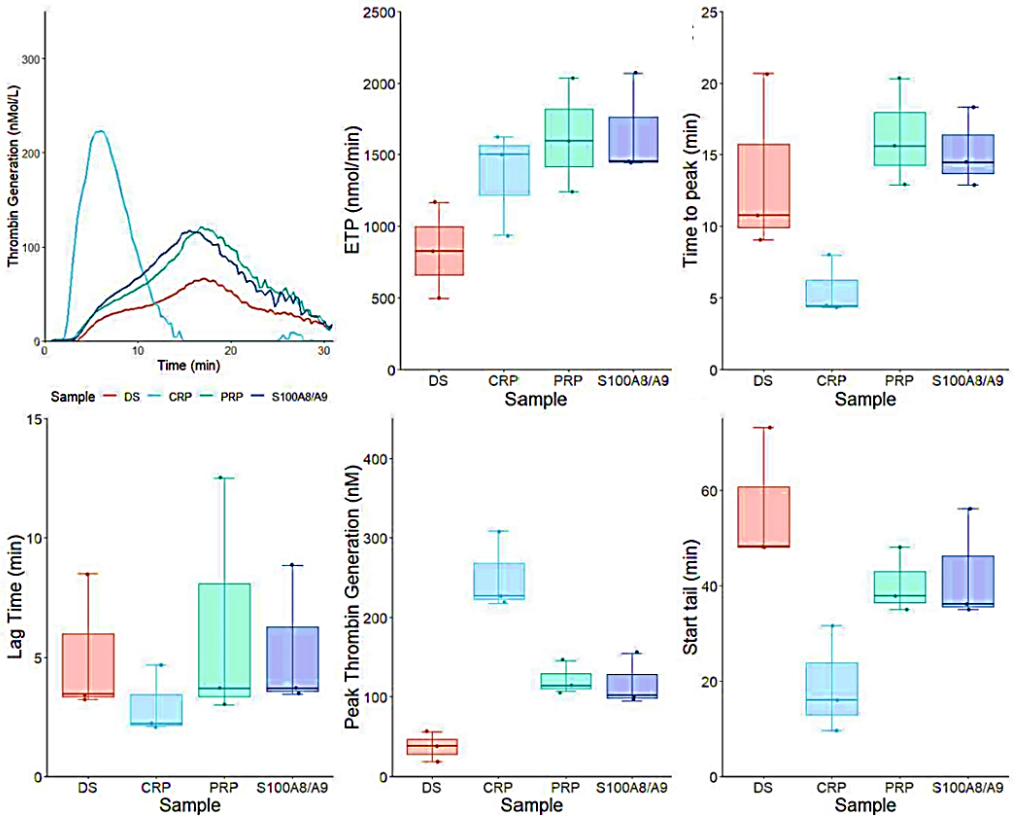


Figure 3.5 – Calibrated automated thrombography CAT thrombin generation performed on PRP reagent of double spun plasma (DS), unstimulated PRP, PRP stimulated with either CRP (10 µg/ml) or PRP exposed to S100A8/A9 (20 or 40µg/ml) (n=3, measured in duplicate). Top raw (left to right): Trace (nmol/L), area under the thrombin generation curve (the endogenous thrombin potential; ETP (nmol/min), time to peak (min). Bottom raw (left to right): lag time (min), (E) peak thrombin generation (nM), (F) start tail (min). Figures adapted from Ref (Colicchia et al., 2022). Thrombin generation analysis performed with the support of Dr Joshua Price.

However, perfusing recalcified whole blood obtained from healthy donors over S100A8/A9-coated surfaces at venous shear rate (100 s^{-1}), induced platelet adhesion and fibrin generation (**Figure 3.6**). Interestingly, we noticed early recruitment of Annexin-V positive platelets. Using anti-fibrin/fibrinogen antibody and assessing Annexin-V binding, we demonstrated that compared to S100A8/A9 or collagen alone, surfaces coated with S100A8/A9 in combination with collagen induced recruitment of Annexin-V platelets more rapidly and the formation of widespread fibrin (**Figure 3.6**). In particular, blood flow was rapidly limited by the formation of multiple fibrin-rich areas and fibrin network. These data indicate that immobilised S100A8/A9 accelerates the formation of fibrin-rich thrombi at venous shear. The same effect on thrombin generation did not seem to be induced by soluble S100A8/A9 (**Figure 3.6**).

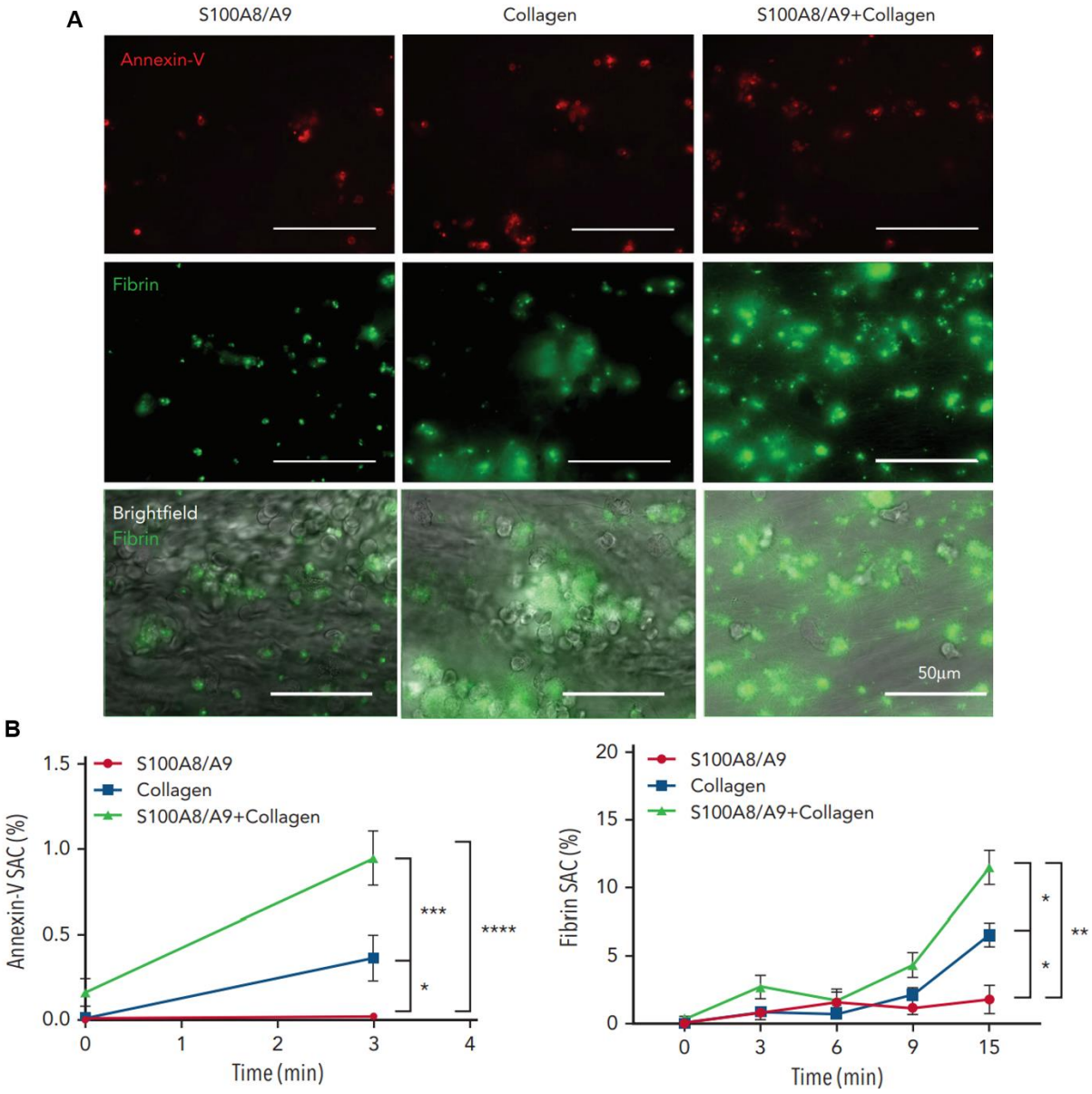


Figure 3.6 – S100A8/A9 induces Annexin-V-positive platelets and fibrin generation at venous shear rate. Whole blood under recalcified conditions was perfused over chambers coated with S100A8/A9 (40 µg/ml), collagen (100 µg/ml) or a combination of both at a venous shear rate (100 s^{-1}). Using Annexin-V-Alexa Fluor 647 and FITC-anti-fibrinogen/fibrin antibodies, the presence of PS-positive platelets and fibrin respectively, was assessed. Images were taken every 5 minutes for 15 minutes. (A) Representative images taken at 15 minutes. (B) Graphs showing quantification of Annexin-V signal (3 minutes) (n=5) and fibrin signal time course (n=5). Quantification was calculated using predefined semiautomated scripts in Fiji as described in Methods. Microscopy details and experimental procedures described in Methods. Results are shown as mean ± standard error of the mean (SEM). Statistical significance was analysed using two-way ANOVA with Tukey's multiple comparison test between groups. *P<0.5, **P<0.05, ***P<0.001, ****P<0.0001. Flow assays performed by Dr Gina Perrella.

Figures adapted from Ref (Colicchia et al., 2022). Figures in A and flow experiments performed by Dr Gina Perrella.

3.3.4 Recombinant S100A8/A9 induces human and mouse platelet activation *in vitro*

To assess whether S100A8/A9 directly activates platelets leading to thrombosis, we tested the effect of soluble recombinant S100A8/A9 on platelets *in vitro*. We incubated human washed platelets with S100A8/A9 and we demonstrated platelet activation by flow cytometry increase in P-selectin expression (**Figure 3.7 A, B**). S100A8/A9 was used at increasing concentrations in the range previously detected in COVID-19 patients and in diseases characterised by thromboinflammation. Interestingly, S100A8/A9 induced a slow increase of P-selectin as measured by anti-P-selectin antibody, indicating α-granules release (**Figure 3.7 E**). Healthy donors responded differently to S100A8/A9, and the P-selectin upregulation was not homogeneous. Donors divided into high and low responders, but there was no clear association between gender or known demographic (e.g. age) with either high or low response. We used CRP and TRAP-6 as positive controls in our experiments and high responders reached the P-selectin increase obtained with these strong agonists even when low dose of S100A8/A9 was used (10-20 µg/ml). Furthermore, S100A8/A9 induced GPIIb/IIIa activation as shown by the binding of PAC-1 antibody (anti-CD61/41) to platelets (**Figure 3.7 C**). PAC-1 recognises the activated form of the integrin. The increase of P-selectin was slower when compared to the GPIIb/IIIa one which occurred rapidly after the addition of S100A8/A9 (**Figure 3.7 F**). P-selectin is known to be the ligand for P-selectin glycoprotein ligand-1 (PSGL-1) or CD162 on

neutrophils. We found that stimulation of diluted whole blood (1:5) with S100A8/A9, induced the increase in platelet-neutrophil aggregates (**Figure 3.7 D**).

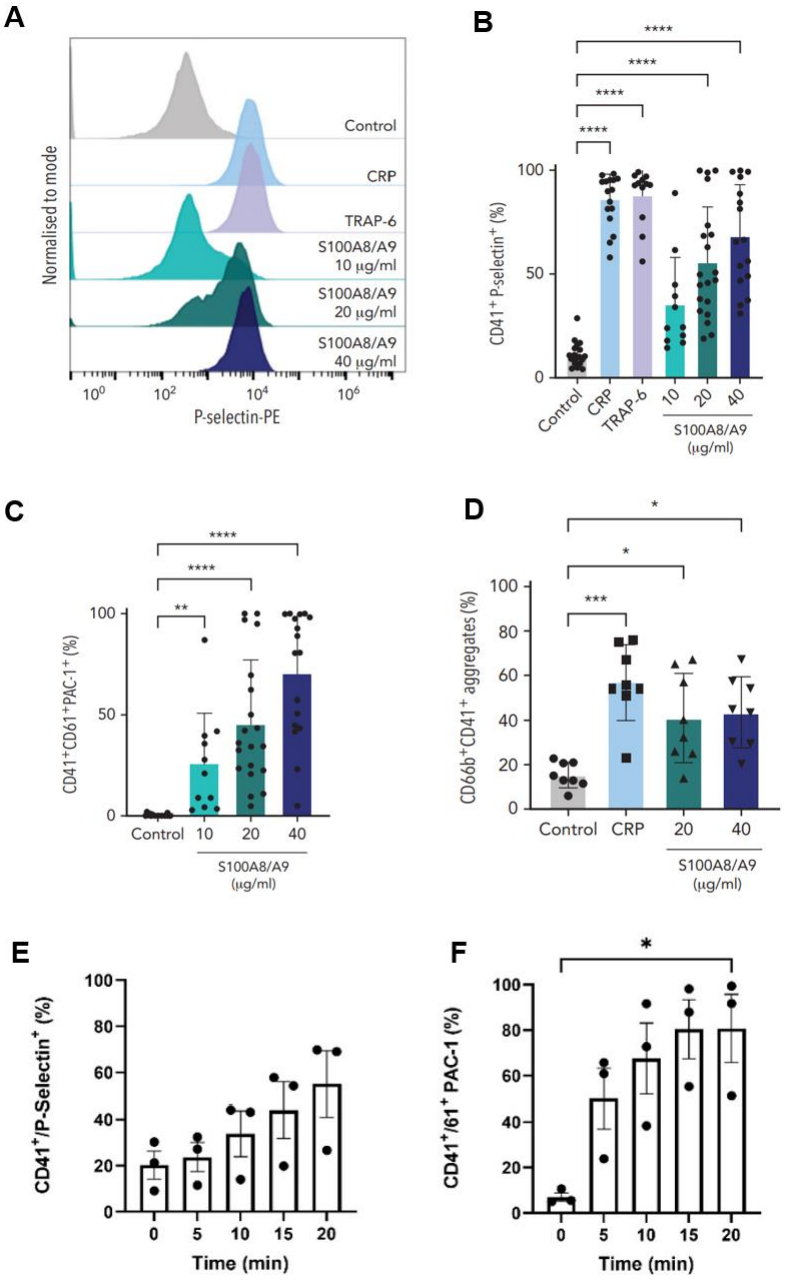
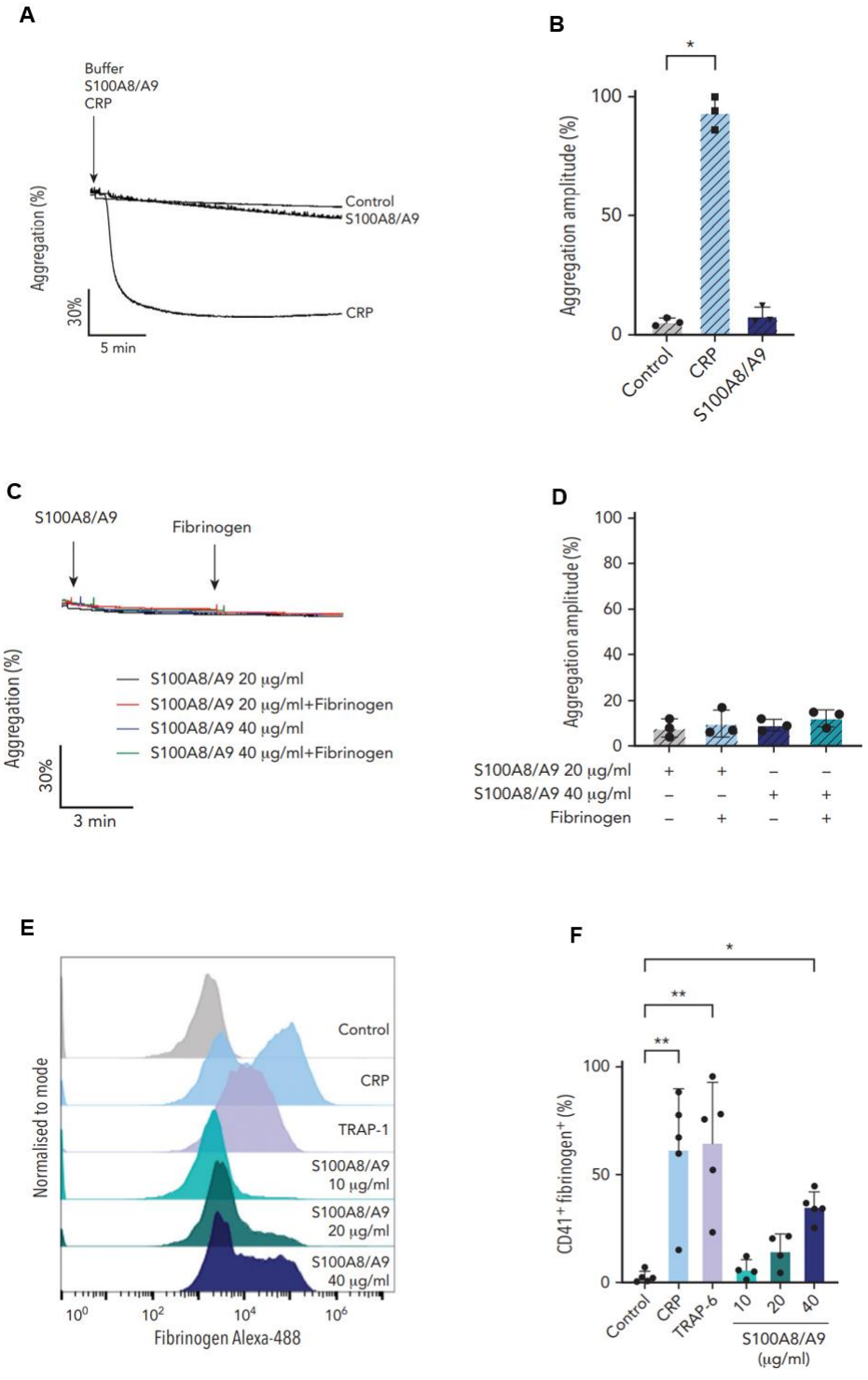


Figure 3.7 – S100A8/A9 induces human platelet activation *in vitro*. (A, B, C) Human washed platelets (10^6 platelets/condition) were stimulated with increasing concentrations of recombinant human

S100A8/A9 (10, 20 and 40 µg/ml) for 30 minutes at 37ºC. Platelet activation was assessed by flow cytometry using anti-P-selectin antibodies. (A) Representative plot for P-selectin in healthy donor, histogram normalised to mode for each condition. (B) Percentage of $CD41^{+}$P-$selectin^{+}$ platelets. (C) Percentage of $CD41^{+}$ platelets positive for PAC-1. (D) Diluted whole blood (1:5) was stimulated with S100A8/A9 (20 and 40 µg/ml) for 30 minutes at 37ºC (n=8). The percentage of platelet-neutrophil aggregates (CD66b+/CD41+) was calculated. Data are shown as mean ± SD. (E, F) Kinetic of platelet activation by S100A8/A9 (20 µg/ml) at 5min interval. Results are shown as mean ± (SEM). Statistical significance was analysed using ordinary one-way ANOVA (Kruskal-Wallis Test). *P<0.5, **P<0.05, ***P<0.001, ****P<0.0001. Figures adapted from Ref (Colicchia et al., 2022).

3.3.5 Recombinant human S100A8/A9 does not induces platelet aggregation *in vitro*

In order to assess whether the GPIIb/IIIa activation was associated with S100A8/A9-induced platelet aggregation, we performed light transmission aggregometry. S100A8/A9 was not able to induce platelet aggregation using our experimental conditions (**Figure 3.8 A, B**). This was also confirmed when we tried to supplement the media with exogenous fibrinogen to rule out a possible defect in fibrinogen secretion (**Figure 3.8 C, D**). A possible explanation for these results is that S100A8/A9-stimulated platelets develop an alteration in fibrinogen binding. Indeed, addition of exogenous labelled fibrinogen to S100A8/A9-stimulated platelets, showed low binding capacity when compared to CRP (**Figure 3.8 E, F)**. We also demonstrated that S100A8/A9 does not induce dense granule release by testing ATP increase (**Figure 3.8 G, H**). The platelet activation induced by S100A8/A9, suggest a novel mechanism might be taking place. As assessed by flow cytometry and light transmission aggregometry, S100A8/A9 does induce P-selectin upregulation and GPIIb/IIIa activation without platelet aggregation.


A
Buffer
S100A8/A9
CRP
Aggregation (%)
Control
S100A8/A9
CRP
30%
5 min
B
*
Aggregation amplitude (%)
100
50
0
Control
CRP
S100A8/A9
C
S100A8/A9
Fibrinogen
Aggregation (%)
S100A8/A9 20 μg/ml
S100A8/A9 20 μg/ml+Fibrinogen
S100A8/A9 40 μg/ml
S100A8/A9 40 μg/ml+Fibrinogen
30%
3 min
D
Aggregation amplitude (%)
100
80
60
40
20
0
S100A8/A9 20 μg/ml + + – –
S100A8/A9 40 μg/ml – – + +
Fibrinogen – + – +
E
Normalised to mode
Control
CRP
TRAP-1
S100A8/A9 10 μg/ml
S100A8/A9 20 μg/ml
S100A8/A9 40 μg/ml
10⁰ 10² 10⁴ 10⁶
Fibrinogen Alexa-488
F
*
**
**
CD41⁺ fibrinogen⁺ (%)
100
50
0
Control
CRP
TRAP-6
10
20
40
S100A8/A9 (μg/ml)

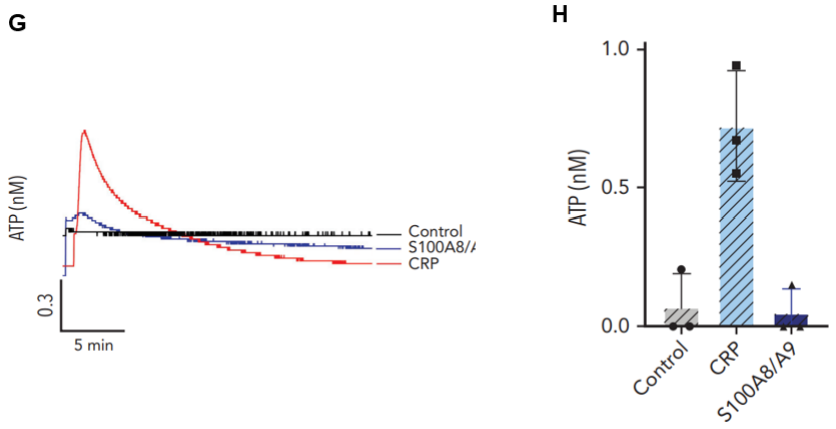
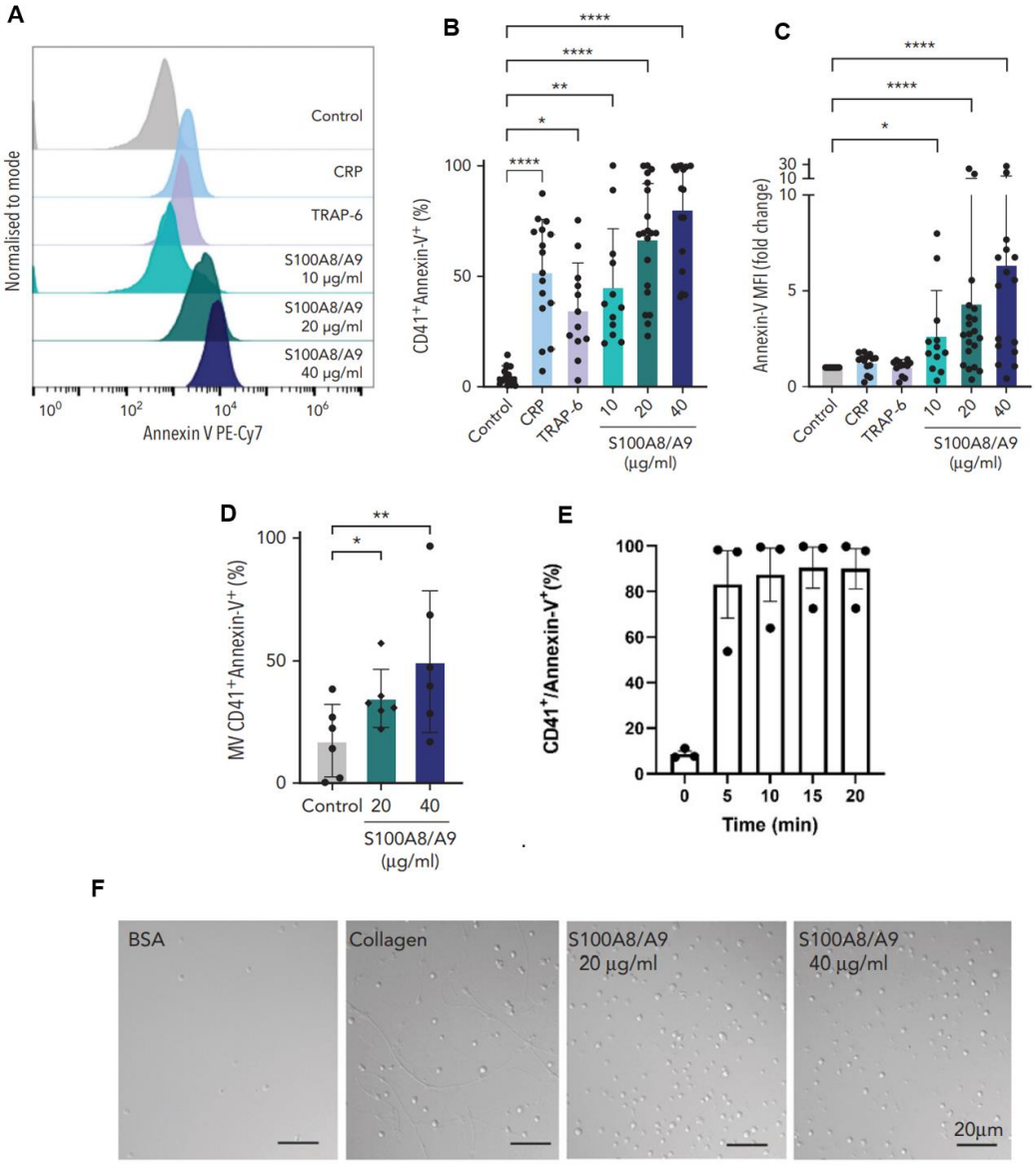


Figure 3.8 – S100A8/A9 does not induce human platelet aggregation. (A, B) Human washed platelets (2x10^8 platelets/condition) were incubated with S100A8/A9 (40 µg/ml) or CRP (10 µg/ml) and platelet aggregation was assessed for 20 minutes using light transmission aggregometry (n=3). (C, D) Human washed platelets (2x10^8 platelets/condition) were incubated with S100A8/A9 (20 or 40 µg/ml) for 6 minutes followed by the addition of exogenous fibrinogen (200 µg/ml). Platelet aggregation was assessed (n=3). (E, F) Human washed platelets (10^6 platelets/condition) were stimulated with either CRP (10 µg/ml), TRAP-6 (100 µM) or (S100A8/A9 (20 or 40 µg/ml) and fibrinogen binding was assessed by flow cytometry. Percentage of platelets positive for Alexa-Fluor 488 labelled fibrinogen is shown. (G, H) Human washed platelets (2x10^8 platelets/condition) were incubated with S100A8/A9 (40 µg/ml) or CRP (10 µg/ml) and ATP generation was assessed for 6 minutes with chrono-lume luciferin: luciferase assay kit from Chronolog (n=3). (A, C, G) representative traces. (G) Representative for fibrinogen binding. Data are shown as mean ± SD. Statistical significance was analysed using ordinary one-way ANOVA (Kruskal-Wallis Test). *P<0.5, **P<0.05, ***P<0.001, ****P<0.0001. Figures adapted from Ref (Colicchia et al., 2022).

3.3.6 Recombinant S100A8/A9 induces phosphatidylserine exposure on human and mouse platelets

Recently, a particular subtype of platelets has been described as detailed in the introduction. They expose high levels of phosphatidylserine (PS) on their membrane surface and are called procoagulant platelets. Different populations of procoagulant platelets have been described and most of them have very low capacity to aggregate

despite carrying GPIIb/IIIa activation (Topalov et al., 2012a, Topalov et al., 2012b). Therefore, we assessed the presence of PS on S100A8/A9-activated platelets by measuring Annexin-V binding in flow cytometry. As hypothesised, S100A8/A9 induced PS exposure on the platelet surface (**Figure 3.9 A, B, C)** and this event takes place very rapidly following stimulation (**Figure 3.9 E)**. Furthermore, we could also detect high levels of PS on CD41-positive microvesicles (**Figure 3.9 D**). We also tested the ability of washed platelets to spread on S100A8/A9 and indeed platelets were able to spread (**Figure 3.9 F**). Interestingly, the adherent platelets showed predominantly high expression of either PS or P-selectin, suggesting a commitment towards either classical activation or procoagulant platelet development (**Figure 3.9 G, H**). We could not notice any specific distribution in the studied slides of P-selectin vs Annexin-V positive platelets. Increasing the concentration of S100A8/A9 on the coated areas, stimulated the formation of PS-positive platelets and microvesicles and P-selectin^{+} platelets. This evidence confirmed that both soluble and immobilised S100A8/A9 are capable of inducing platelet activation and indeed, the formation of procoagulant platelets. To further confirm the procoagulant platelet nature of S100A8/A9-stimulated platelets, we tested Ca^{2+} increase in adherent and spread platelets. Platelets adherent to S100A8/A9 showed high Ca^{2+} signal confirming their procoagulant nature (**Figure 3.10**). We also tested whether the overall platelet activation that we have described, was induced by either S100A8 or S100A9 alone, but we demonstrated that only the heterodimer is able to induce those effects (**Figure 3.11 A, B, C**). To this point we can conclude that the heterodimer S100A8/A9 induces platelets activation in the form of P-selectin increase, GPIIb/IIIa activation without aggregation, PS exposure and release of PS-positive microvesicles.


A
Normalised to mode
Control
CRP
TRAP-6
S100A8/A9 10 µg/ml
S100A8/A9 20 µg/ml
S100A8/A9 40 µg/ml
Annexin V PE-Cy7
B
CD41+Annexin-V+ (%)
Control
CRP
TRAP-6
S100A8/A9 (µg/ml)
C
Annexin-V MFI (fold change)
D
MV CD41+Annexin-V+ (%)
E
CD41+/Annexin-V+(%)
Time (min)
F
BSA
Collagen
S100A8/A9 20 µg/ml
S100A8/A9 40 µg/ml
20µm

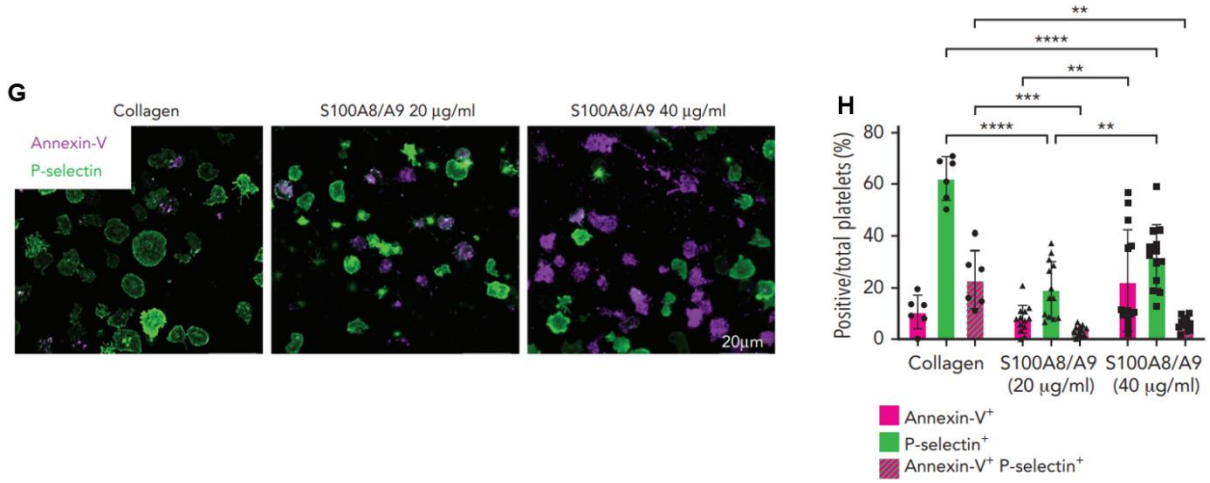


Figure 3.9 – S100A8/A9 induces the formation of procoagulant platelet. (A, B, C, D) Human washed platelets (10^6 platelets/condition) were incubated with different concentrations of S100A8/A9 (10, 20, 40 µg/ml) or CRP (10 µg/ml) or TRAP-6 (100 µM) for 30 minutes at 37ºC. PS exposure was assessed by flow cytometry using PE-Cy7-labeled annexin-V (n=17). (A) Representative histogram for Annexin-V staining normalised to mode (each peak normalised to each condition mode). (B) Percentage of $CD41^+$Annexin-V^+ platelets and (C) fold change in the mean fluorescence intensity (MFI) of agonist-activated platelets over control. (D) Percentage of $CD41^+$Annexin-V^+ MVs (n=6). (E) Human washed platelets (10^6 platelet/condition) were incubated with S100A8/A9 (20 µg/ml), S100A8 (20, 40 µg/ml) or S100A9 (20, 40 µg/ml). Kinetic of platelet activation by S100A8/A9 at 5min interval. Data shown as mean ± SEM. (F, G, H) Human washed platelets spreading on collagen or S100A8/A9. (F) Representative images of spread platelets differential interference contrast (DIC). (G) Representative images of annexin-V and P-selectin staining for adherent platelets. Annexin-V (magenta) and P-selectin (green). (H) Quantification of platelets adherent on collagen and S100A8/A9 and positive for annexin-V, P-selectin or both markers (positive/total platelets). Data are shown as mean ± SD. The statistical significance was analysed using two-way ANOVA with Tukey's multiple comparison test between all groups. *P<0.5, **P<0.05, ***P<0.001, ****P<0.0001. Figures adapted from Ref (Colicchia et al., 2022).

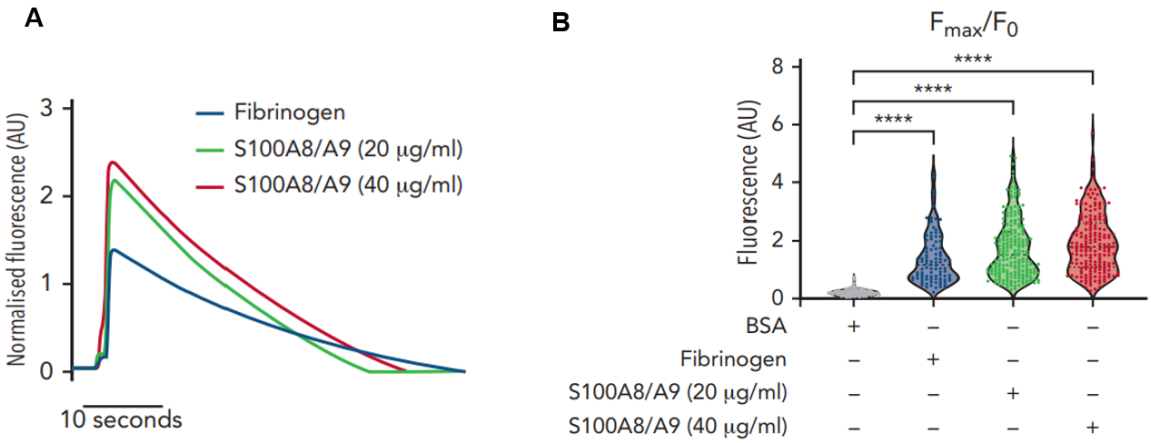


Figure 3.10 – S100A8/A9 induces intracellular Ca^{2+} release. (A, B) Assessment of intracellular Ca^{2+} release from platelets spread over surfaces coated with S100A8/A9 (20 and 40 µg/ml), fibrinogen or bovine serum albumin. The Ca^{2+} release was assessed using Oregon Green-488 BAPTA-1-AM Ca^{2+} sensitive dye. (A) Representative traces Data are shown as mean ± SD. The statistical significance was analysed using two-way ANOVA with Tukey's multiple comparison test between all groups. *P<0.5, **P<0.05, ***P<0.001, ****P<0.0001. Capture of Ca^{++} release at epi microscope performed by Dr Abdullah Khan. Figures adapted from Ref (Colicchia et al., 2022).

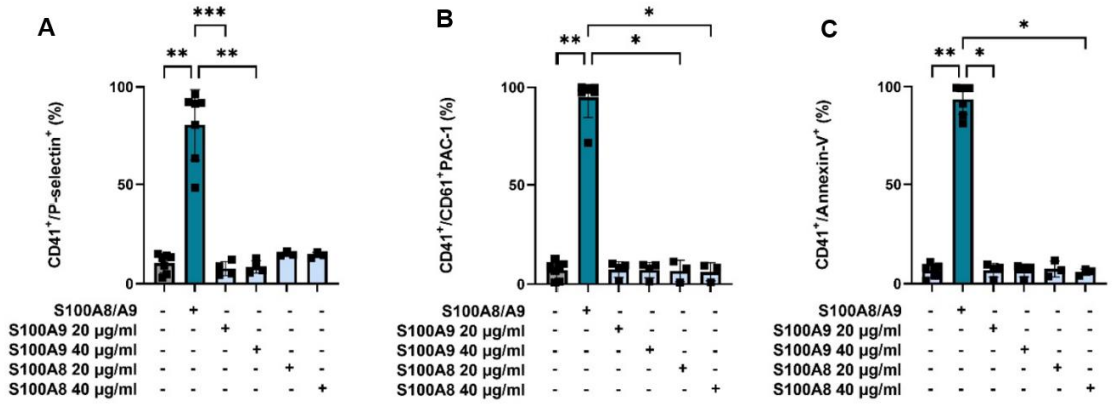


Figure 3.11 – S100A8 or S100A9 do not induce platelet activation (A, B, C) Measurement of platelet activation by S100A8/A9, S100A8 or S100A9 at 30min. (A) P-selectin expression, (B) CD41/CD61 PAC-1 and (C) Annexin V binding. Data is shown as the percentage of platelets (CD41+) positive for these markers. Data shown as mean ± SD. Statistical significance was analysed using one-way ANOVA (Kruskal-Wallis Test). ✱ p<0.05, ✱✱ p<0.005, ✱✱✱✱ p<0.0001. Figures adapted from Ref (Colicchia et al., 2022).

3.3.7 The effect of S100A8/A9 on platelets is differently reduced by CD36 blockade and immunoreceptor-based activation motif receptor inhibitors

The next step was to understand the receptors and mechanisms involved in S100A8/A9-platelet activation. S100A8/A9 has been described to have 3 known receptors on endothelial cells and immune cells: RAGE, TLR-4 and CD36 as described in 3.2.1. All those receptors are present on platelets therefore we initially assessed the effect of inhibitors targeting those receptors in S100A8/A9-stimulated washed platelets. Azeliragon, an inhibitor for RAGE receptor and Paquinimod, which alters the binding of S100A8/A9 to TLR-4, did not affect S100A8/A9-induced platelet activation (**Figure 3.12 A, B, C**). Blocking the downstream signalling of TLR-4 using the small molecule inhibitor TAK-242 or preventing the binding of S100A8/A9 to TLR-4 and RAGE by a specific antibody (Ab clone A1505B), did not alter platelet activation too (**Figure 3.12 D, E, F**). When we antagonised irreversibly the downstream signalling of CD36 using Sulfo-N-succinimidyl oleate (SSO), P-selectin expression was not affected but GPIIb/IIIa activation and PS exposure were partially reduced (**Figure 3.12 F, G, H**). Conversely, blocking CD36 by using an antibody that blocks the oxidised low-density lipoprotein binding site on this receptor, there was no reduction of S100A8/A9-induced platelet activation (**Figure 3.12 I, J,**). This specific data suggests that CD36 downstream signalling may play a role in S100A8/A-induced effect on platelets but the binding site is different from the ox-LDL one.

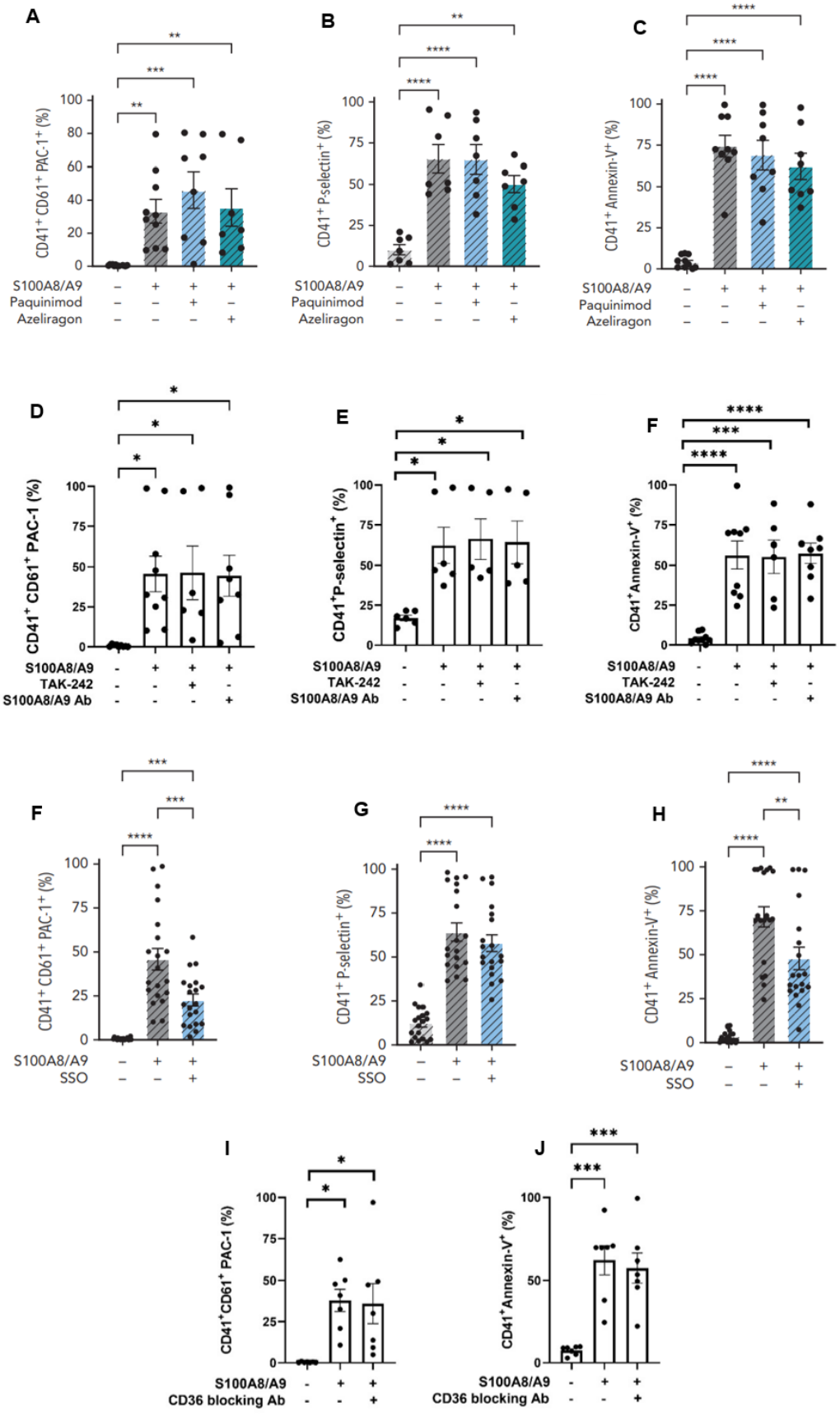

A
B
C
D
E
F
F
G
H
I
J
CD41+ CD61+ PAC-1+ (%)
CD41+ P-selectin+ (%)
CD41+ Annexin-V+ (%)
S100A8/A9
Paquinimod
Azeliragon
TAK-242
S100A8/A9 Ab
SSO
CD36 blocking Ab

Figure 3.12 – CD36 blockade partially decreases GPIIb/IIIa activation and PS exposure. Human washed platelets (10^6 platelets/condition) were incubated with S100A8/A9 (20 µg/mL) with or without different inhibitors for 30 minutes at 37◦C. Inhibitors were preincubated with platelets for 10 minutes before addition of S100A8/A9: Paquinimod (blocks S100A9 binding to TLR4) (10 µM), RAGE inhibitor Azeliragon (1 µM), CD36 inhibitor SSO (25 µM), TLR-4 inhibitor TAK-242 (10 µM), S100A8/A9 blocking antibody (20 µg/ml), CD36 blocking antibody (50 µg/ml). Platelet activation was determined by flow cytometry using anti-CD41/CD61 PAC-1 antibody, anti–P-selectin antibody, and annexin-V binding. Data are shown as the percentage of platelets positive (CD41+ for these markers. Data are shown as mean ± SD. For D, E, F, I ,J data shown as mean ± SEM. Statistical significance was analysed using ordinary one-way ANOVA. *P < .05, **P < .005, ***P < .001, ****P < .000. Figures adapted from Ref (Colicchia et al., 2022).

Considering Syk is part of CD36 downstream signalling pathway, we tested the effect on S100A8/A9-platelet activation of platelet immunoreceptor tyrosine-based activation motif receptors (ITAMs) inhibition using PP2, PRT-060318 and ibrutinib, blocking Src, Syk and Btk respectively. Interestingly, those inhibitors significantly reduced P-selectin increase but they did not affect GPIIb/IIIa activation and PS exposure (**Figure 3.13 A, B, C**). Syk inhibition showed the strongest P-selectin decrease which was associated with reduction in platelet-neutrophils aggregates in whole blood (**Figure 3.13 D**). Importantly, classical anti-platelets drugs that are currently used to prevent platelet aggregation in the clinical practice, did not affect S100A8/A9-induced platelet activation. Indomethacin and cangrelor, which inhibit respectively COX and P2Y12, did not alter platelet activation and PS exposure (**Figure 3.13 E, F, G**). These data suggest that the S100A8/A9-induced P-selectin increase on platelets is mediated by Syk/Src while PS exposure and GPIIb/IIIa activation are partially upregulated via CD36.

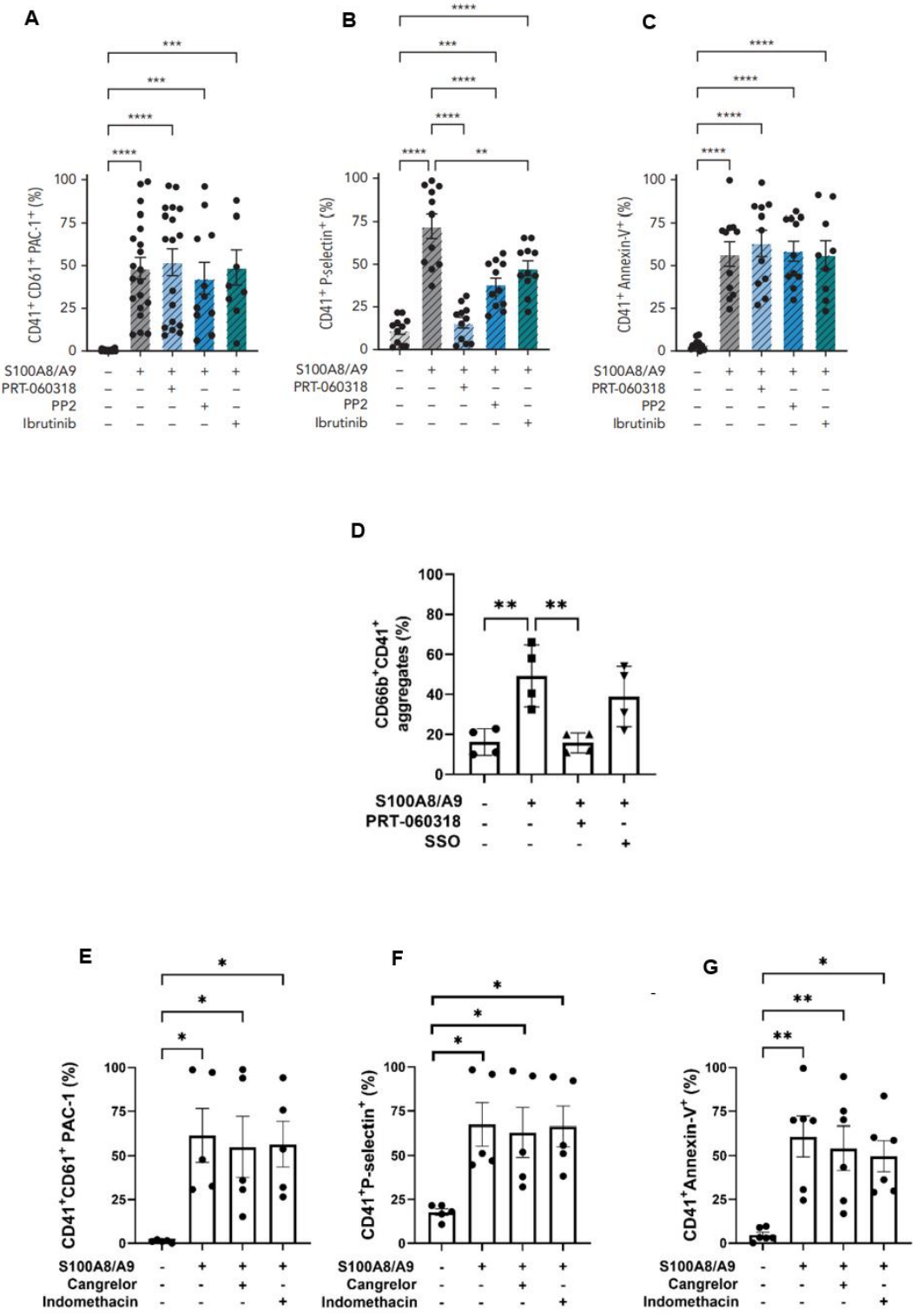


Figure 3.13 – Immunoreceptor tyrosine-based activation motif receptor inhibition reduces P-selectin. Human washed platelets (106 platelets/condition) were incubated with S100A8/A9 (20 µg/mL)

with or without different inhibitors for 30 minutes at 37◦C. Inhibitors were preincubated with platelets for 10 minutes before addition of S100A8/A9. Syk inhibitor PRT-060318 (10 μM), Src inhibitor PP2 (20 μM), and BTK inhibitor Ibrutinib (500 Nm), P2Y12 inhibitor Cangrelor (10 μM) or COX-1 inhibitor Indomethacin (10 μM) were used. Platelet activation was determined by flow cytometry using anti-CD41/CD61 PAC-1 antibody, anti–P-selectin antibody, and annexin-V binding. (D) Platelet (CD41+) and neutrophil (CD66b+) aggregates were measured in the presence of PRT-060318 and SSO. Data are shown as the percentage of platelets positive (CD41+) for these markers. Data are shown as mean ± SD. D, E, F, G data are shown as mean ± SEM. Statistical significance was analysed using ordinary one-way ANOVA. *P < .05, **P < .005, ***P < .001, ****P < .0001. Figures adapted from Ref (Colicchia et al., 2022).

3.3.8 S100A8/A9 activate human and mouse platelets through GPIbα

Considering platelet ITAM receptors (CLEC-2, GPVI and GPIb) and CD36 signal through Src/Syk, we tested the effect of S100A8/A9 on murine platelets deficient in CLEC-2, GPVI, CLEC-2 and GPVI or platelets deficient in the extracellular domain of GPIbα (IL4R/GPIbα-Tg mice). Initially we tested whether murine recombinant S100A8/A9 (mS100A8/A9) was able to activate platelets in WT mice. Indeed, we demonstrated that similarly to human data, S100A8/A9 was able to increase P-selectin and Annexin-V in a dose dependent manner (**Figure 3.14 A, B**). Surprisingly, P-selectin expression and Annexin-V binding were not significantly affected in mS100A8/A9-stimulated platelets isolated from mice deficient for GPVI, CLEC-2 or both receptors when compared to WT mice (**Figure 3.14 C, D)**. Of notice, GPVI and CLEC2/GPVI deficient platelets showed a bimodal P-selectin expression. Platelets from mice deficient in the extracellular domain of GPIbα (lLR4/GPIbα-Tg) showed a significant reduction in P-selectin expression, GPIIb/IIIa activation and Annexin-V binding (**Figure 3.14 E, F, G, H, I, J)**. Interestingly in mice, S100A8/A9 induced only modest GPIIb/IIIa activation. We hypothesised that the CD36 receptor could be

involved in the crosstalk with GPIb for S100A8/A9-induced platelet activation. Therefore, we used the CD36 inhibitor on S100A8/A9-activated platelets isolated from ILR4/GPIbα-Tg mice and we could demonstrate a significant complete reduction in platelet activation ((**Figure 3.13 K)**. These data indicate that GPIbα could be the main receptor for S100A8/A9 on mouse platelets with a supportive role for CD36.

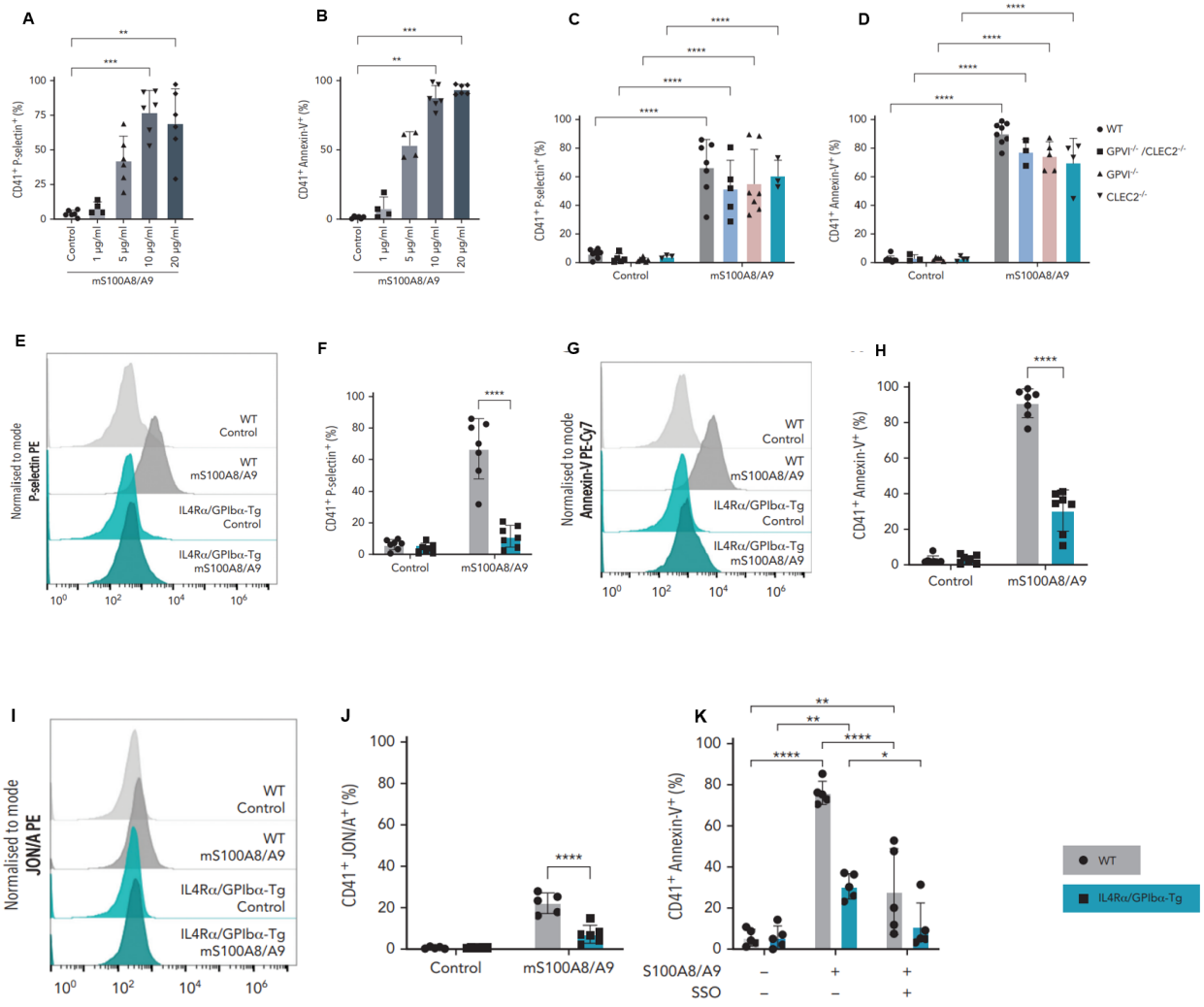


Figure 3.14 – Murine S100A8/A9 activates mouse platelets through GPIbα and CD36. Washed murine platelets (10^6 platelets/condition) were incubated with recombinant mouse S100A8/A9 at different doses for 30 minutes at 37◦C. Platelet activation was determined by flow cytometry using (A)

anti–P-selectin antibody and (B) annexin-V binding. (C-K) Washed platelets, previously isolated from WT, GPVI (GPVI$^{-/-}$), CLEC-2 (CLEC-2$^{-/-}$), GPVI/CLEC-2 knock-out (GPVI$^{-/-}$/CLEC-2$^{-/-}$), or GPIbα$^{-/-}$ mice (IL4R/GPIbα−Tg mice) were incubated with S100A8/A9 (10 μg/mL) for 30 minutes at 37◦C. The percentage of platelets positive for P-selectin, GPIIb/IIIa activation (JON/A), and annexin-V binding were measured by flow cytometry. Data are shown as percentage platelets (CD41^{+}) positive for specific markers. (K) Platelets isolated from WT or IL4R/GPIbα−Tg mice were pretreated with SSO (25 μM) prior to addition of S100A8/A9 (10 μg/mL) for 30 minutes at 37◦C, and annexin-V binding was assessed by flow cytometry. The statistical significance was assessed by nonparametric test (Kruskal-Wallis test) (A-B), ordinary two-way ANOVA with Sidak's multiple comparison test (C, D, F, H, J), and two-way ANOVA with Tukey's multiple comparison test (K). Data are shown as mean ± SD. *P < .05, **P < .005, ***P < .001. Adapted from Ref (Colicchia et al., 2022).

To assess whether GPIbα is the main receptor for S100A8/A9 or regulates S100A8/A9-platelet activation indirectly, we performed a receptor binding assay (**Figure 3.15)**. The binding between GPIbα and S100A8/A9 was tested by ELISA and we found that human S100A8/A9 binds to recombinant GPIbα with an EC50 of 130±75 nM. In order to confirm that GPIbα is the main receptor for S100A8/A9 on human platelets, we adopted a competitive strategy. Platelet activation by S100A8/A9 was assessed in the presence of recombinant soluble GPIbα (rGPIbα). Indeed, human washed platelets failed to respond to recombinant S100A8/A9 when equimolar concentrations of rGPIbα (1.7 μM) were used. This competitive effect was measured by failure in P-selectin upregulation, GPIIb/IIIa activation and PS exposure by flow cytometry (**Figure 3.16 A, B, C, D, E, F, G, H)**. In human platelets the effect of CD36 is possibly less relevant than in mice, as competition by GPIbα was sufficient to reduce completely the stimulatory effect of S100A8/A9 on platelets. rGPIbα was also able to inhibit the formation of PS-positive MV, reducing the platelet procoagulant effect further (**Figure 3.16 I)**. The key role of GPIbα in S100A8/A9-driven human platelet activation was

confirmed by the data obtained on platelets obtained from a patient with Bernard-Soulier syndrome. This syndrome, as described in chapter 1, is characterised by a deficiency in the GPIb-V-IX complex. The patient recruited for our experiment, has a homozygous *GP9* missense variant (Cys24Arg) resulting in the absence of GPIbα functional expression on platelets. The genetic mutation of the BSS patient was tested at Oxford Regional Genetics Laboratories, Churchill Hospital Old Road, Headington (www.ouh.nhs.uk/geneticslab). Indeed, platelets from this patient responded normally to CRP but failed to respond to S100A8/A9 (**Figure 3.16 J, K, L)**. These results were confirmed for all the activation markers. Overall, all these data confirmed that GPIbα is the main receptor for S100A8/A9 on mice and human platelets inducing the formation of procoagulant platelets. The role of CD36 on human platelets remains to be fully discovered and more research into this topic is needed.

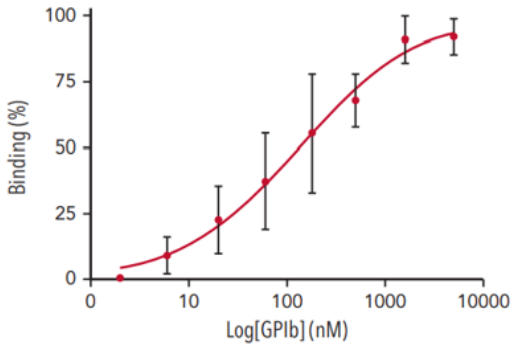


Figure 3.15 – Binding of GPIbα to S100A8/A9. Binding of rGPIbα to immobilized S100A8/A9. Plates were coated with 0.5 µM S100A8/A9 and recombinant GPIbα ectodomain (R&D systems) was added at concentrations ranging from 5000 nM to 2 nM. BSA is used as negative control. Data presented as mean ± SEM (n = 3). Adapted from Ref (Colicchia et al., 2022).

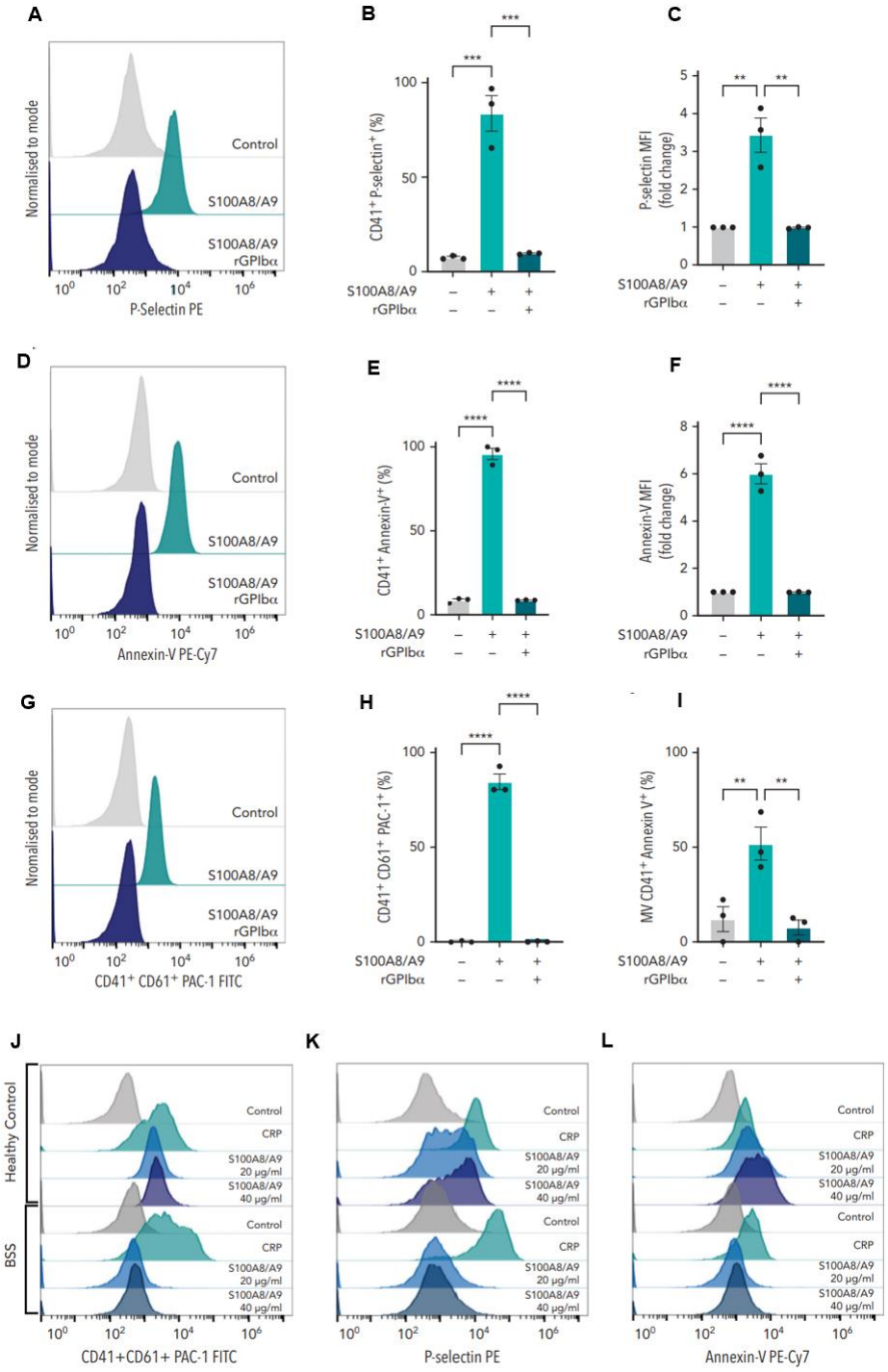


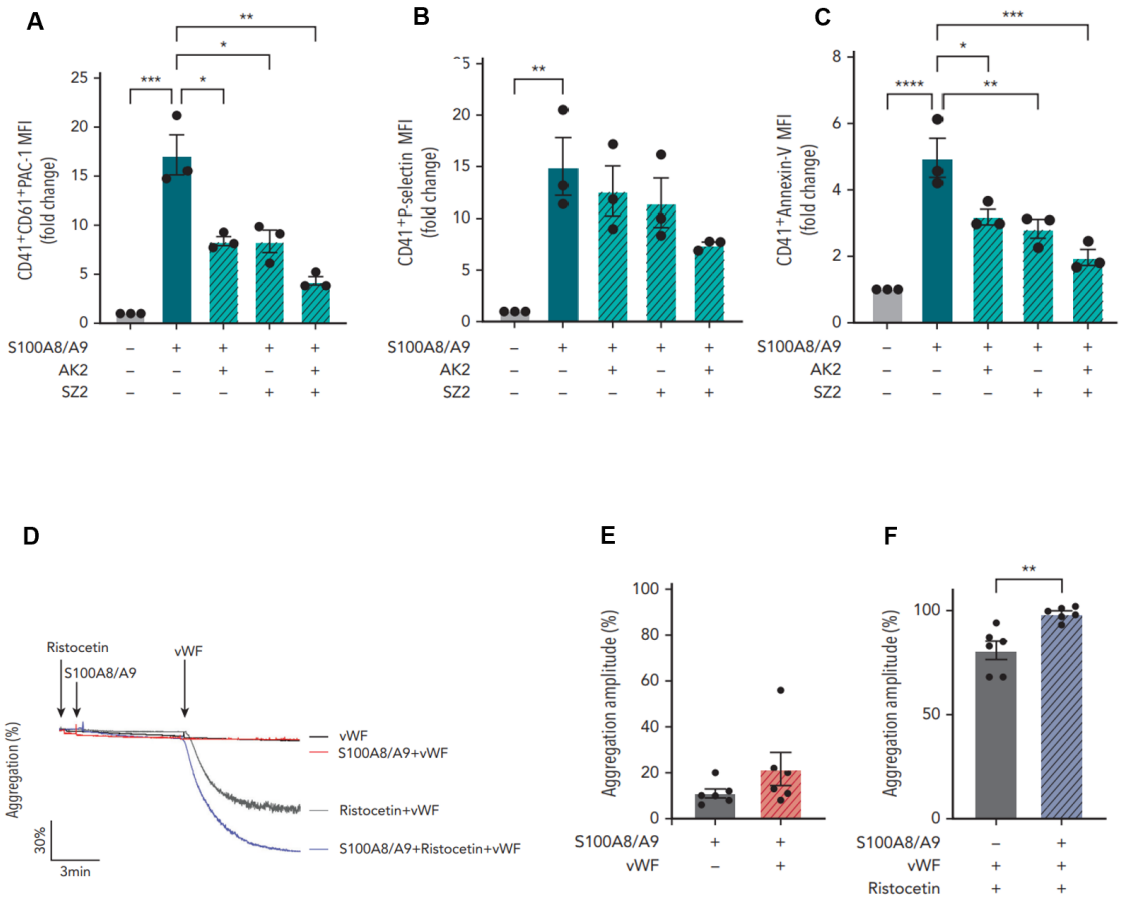
Figure 3.16 – Recombinant human GPIbα blocks platelet response to S100A8/A9. Washed human platelets (10^6 platelets/condition) were incubated with S100A8/A9 (40 µg/mL) in the absence or presence of recombinant human GPIbα (rGPIbα; 1.7 µM) for 30 minutes at 37◦C (n = 3). Platelet activation was assessed by flow cytometry using anti–P-selectin, PS exposure using annexin-V, and GPIIb/IIIa activation using PAC-1 antibody (A-H); (I) Annexin-V on platelet microvesicles (MV). (J, K, L)

Washed human platelets (10^6) from a healthy donor or a Bernard-Soulier syndrome patient were incubated with S100A8/A9 (20 and 40 µg/mL) for 30 minutes at 37◦C. CRP (10 µg/mL) was used as positive control. The statistical significance was analysed. using one-way ANOVA. Data presented as mean ± SEM. *P < .05, **P < .005, ***P < .001, ****P < .0001. adapted from Ref (Colicchia et al., 2022).

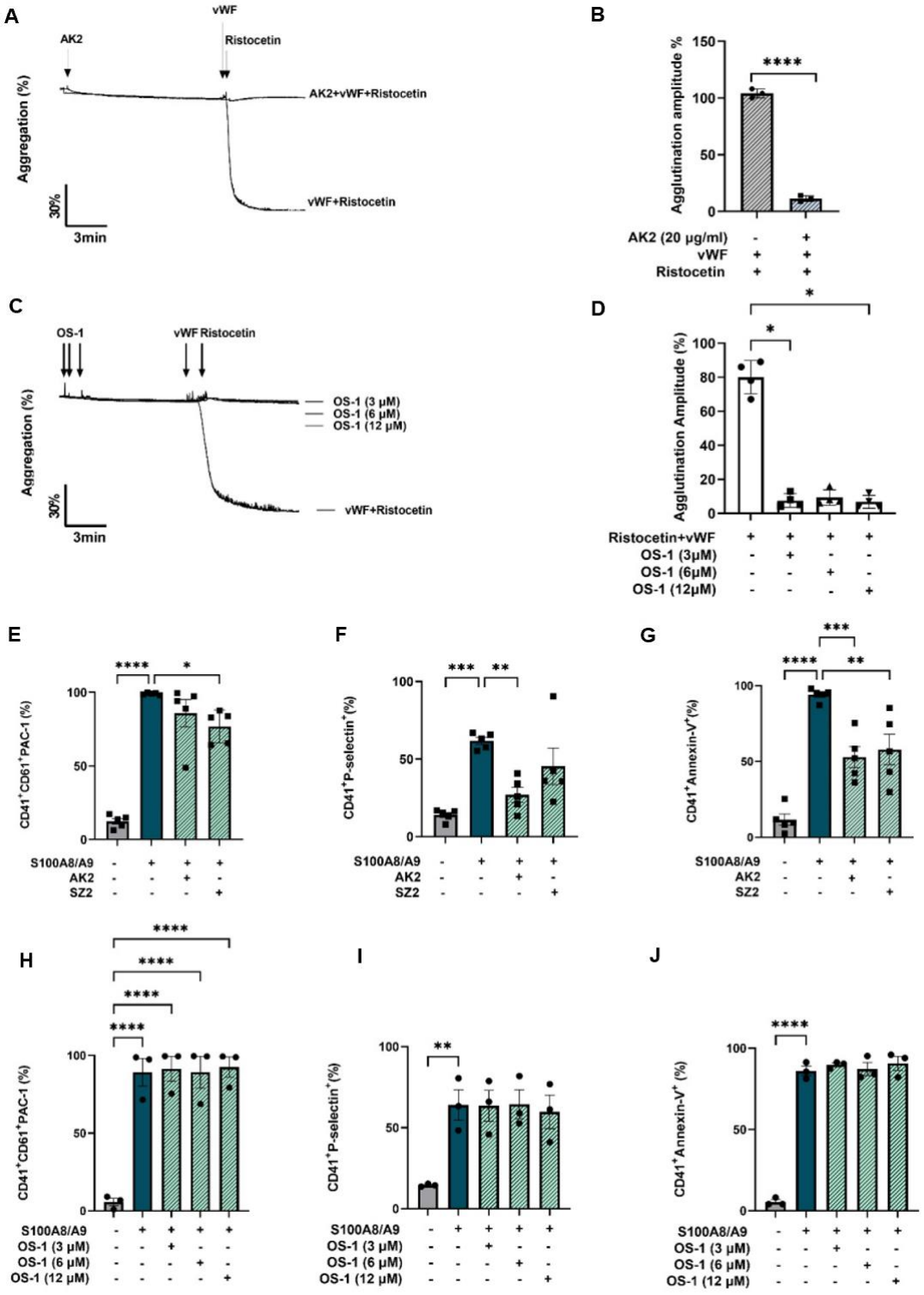
3.3.9 S100A8/A9 potentiate von Willebrand factor-mediated agglutination and its binding site overlaps with one of the vWF-bind sites on platelets

To assess whether the S100A8/A9 binding site on GPIbα overlaps with vWF, we tested the effect of specific antibodies that were previously described to block this interaction. This is important for therapeutical purposes, considering the crucial role that GPIbα plays in haemostasis. We used the antibodies AK2 and SZ2, which recognise different epitopes located on GPIbα. The anionic/sulphated tyrosine region (Tyr2-Glu282) and the first leucine-rich repeat (Leu36-Gln59) respectively. Furthermore, we used the peptide OS-1, which was previously described to prevent allosterically the interaction between GPIbα and vWF. All these molecules were tested at concentrations reported in the literature to inhibit vWF-led platelet agglutination (Shen et al., 2000, Ward et al., 1996, McEwan et al., 2009). In the presence of AK2 and SZ2, the effect of human S100A8/A9 was significantly decreased for PS exposure and GPIIb/IIIa activation (**Figure 3.17 A-C**). This effect was further augmented when a combination of both antibodies was used. OS-1 peptide, which inhibited vWF-induced platelet agglutination in the presence of ristocetin, was not able to prevent the effect of S100A8/A9 on human platelets (**Figure 3.18 H-J**). This effect did not change when we increased the dose of OS-1. These data show that S100A8/A9 binds very likely to overlapping vWF sites on

GPIbα. Furthermore, we showed that priming platelets with S100A8/A9 did not induce platelet agglutination in the presence of vWF without ristocetin. However, S100A8/A9 potentiated vWF-mediated platelet agglutination in the presence of ristocetin (**Figure 3.17 D-F**). The effect of SZ2, AK2 and OS-1 on platelet agglutination is represented in Figure 3.18. This figure shows that the minimum dose of AK2 (**Figure 3.18 A, B**) and OS-1(**Figure 3.18 C, D**) that we adopted in the experiments was able to inhibit platelet agglutination induced by ristocetin and vWF. AK2 and SZ2 were shown to be able to partially inhibit the effect of the effect of human S100A8/A9 already at lower concentrations (**Figure 3.18 E-G**).



3.17 – S100A8/A9 and increases vWF-dependent platelet agglutination. Washed human platelets (10^6/condition) were preincubated with or without AK2 Ab (40 µg/mL), SZ2 (40 µg/mL), or both for 10 minutes at 37◦C and then stimulated with S100A8/A9 (20 µg/mL) for 30 minutes at 37◦C. Platelet activation was determined by flow cytometry using anti-CD41/CD61 PAC-1 antibody, anti–P-selectin antibody, and annexin-V binding. (A-C) Data are shown as MFI of treated platelets over control (unstimulated) for different markers. Ristocetin-induced platelet aggregation was assessed by addition of vWF (1 µg/mL) and ristocetin (1.5 mg/mL) to washed platelets. Platelets were primed with S100A8/A9 and ristocetin for 6 minutes before addition of vWF. Platelet aggregation was monitored for 6 minutes after vWF addition (n = 6). (E) Representative trace. Data are shown as mean ± SEM. The statistical significance was analysed using ordinary one-way ANOVA (B-D) and nonparametric Mann–Whitney t test (Kruskal-Wallis test) (F-G). *P < .05, **P < .01, ***P<.001, ****P < .0001. Adapted from Ref (Colicchia et al., 2022).



3.18 – Effect of SZ2 and OS-1 on vWF-mediated agglutination and S100A8/A9 Washed human platelets (10^6/condition) were pre-incubated with (A, B) anti human GPIbα-clone AK2 (20 µg/ml) for 10 min and stimulated with plasma-derived vWF (2 µg/ml) and ristocetin (0.5mg/ml) (n=3). (C, D) OS-1 peptide at different (3, 6, 12 µM) was added to platelets for 10 min followed by vWF (10 µg/ml) and

ristocetin (1.5mg/ml) (n=4). Platelet aggregation was assessed for 6 min by light transmission aggregometry and statistical significance was analysed for (B) using Mann-Whitney test or (D) using one-way ANOVA (Kruskal-Wallis Test). Data is shown as mean ± SD. (A, C) Representative traces. Platelets were preincubated with (E, F, G) AK2 Ab (20 µg/ml) or SZ2 Ab (20 µg/ml) or (H, I, J) OS-1 (3, 6, 12 µM) for 10 min at 37° prior to S100A8/A9 (20 µg/ml). Platelet activation was determined by flow cytometry using anti-$CD41^{+}CD61^{+}$PAC-1 antibody, anti-P-selectin antibody and Annexin-V binding. (E, F, G) Data is shown as percentage of platelets ($CD41^{+}$) positive for respective markers ± SEM (n=5). (H, I, J) Data is shown as percentage of platelets ($CD41^{+}$) positive for respective markers ± SEM (n=3). Statistical significance was assessed using Kruskal-Wallis Test (B, D) and ordinary one-way ANOVA (E-J). ∗ $p<0.05$, ∗∗ $p<0.005$, ∗∗∗∗ $p<0.0001$. Adapted from Ref (Colicchia et al., 2022).

3.4 Discussion

High levels of S100A8/A9 and thrombotic complications, have been described in multiple thromboinflammatory disease including infections such as COVID-19 (Mellett and Khader, 2021), cardiovascular disease (Sakuma et al., 2017) and DVT (Han et al., 2022). In addition, the presence of procoagulant platelets have been detected in COVID-19, correlating with raised D-dimer levels, organ failure and thrombotic complications as well as the formation of procoagulant MVs (Khismatullin et al., 2022, Khattab et al., 2021, Mellett and Khader, 2021, Althaus et al., 2020). Mice deficient in S100A8/A9 have reduced arterial and venous thrombosis (Wang et al., 2017a, Wang et al., 2014). In this study we confirmed that S100A8/A9 circulating levels are increased in COVID-19 patients and persistent high levels correlate with worse prognosis. Furthermore, we demonstrated that S100A8/A9 deposits on lung vessel walls, in lung tissue neutrophils and CD42b-positive cells. Importantly, we show that S100A8/A9-coated surfaces accelerate fibrin generation at venous shear rate, and soluble S100A8/A9 induce α-granule secretion and PS exposure in both human and mouse

platelets. Despite GPIIb/IIIa activation, S100A8/A9 failed to induce platelet activation, possibly for reduced fibrinogen binding. We demonstrated that S100A8/A9-induced platelet activation and procoagulant platelet formation occurred via GPIbα with a supportive role for CD36. Furthermore S100A8/A9-mediated platelet activation does not involve RAGE and TLR-4 and is not affected by antiplatelet drugs used in clinical practice targeting COX-2 and P2Y12. Overall, we identified a novel mechanism triggering the formation of procoagulant platelets via the S100A8/A9-GPIbα axis. In COVID-19, other studies have previously reported that the formation of procoagulant platelets is antibody driven (Althaus et al., 2020). Here we describe an alternative mechanism which could be responsible of procoagulant platelet formation. Furthermore, platelets isolated from COVID-19 patients, have been shown to be enriched in S100A8 and A9 mRNA and endothelial cells to be activated by platelet release from COVID-19 patients via CD36 (Barrett et al., 2021b). In addition, is has been reported that neutrophils transfer S100A8 and S100A9 to platelets during necrosis or NETosis and the levels of platelet-derived S100A8/A9 correlate with neutrophil count in myocardial infarction (Joshi et al., 2022). In our cohort, S100A9 was detected in CD42b positive cells in a more pronounced way rather than the heterodimer S100A8/A9. It remains to be further investigated for the presence of S100A8 and S100A9 as monomers or dimers in platelets and whether they both have proinflammatory and/or pro-thrombotic effects.

We showed that immobilised S100A8/A9 support early PS^+ platelet formation and fibrin generation at venous shear rate. However, it was not possible to induce thrombin generation in S100A8/A9-stimulated PRP. These results suggest that deposited

S100A8/A9 could be a stronger thrombogenic agonists compared to soluble S100A8/A9. COVID-19 complications have been associated with the formation of fibrin-rich thrombi and the deposition of S100A8/A9 on the vasculature, which could contribute to this effect but it remains to be demonstrated.

Another important aspect to note, is the presence of high and low responders to S100A8/A9. We could not identify in our cohort, any epidemiological characteristics such as age and gender that could correlate with the degree of response. We were not aware of any additional diseases that these subjects could carry and might have affected the responses. Interestingly, we repeated the experiments multiple times on the same subjects at distance of months and the response was equal. This suggests that some chronic conditions might affect the platelet response to S100A8/A9. It is tempting to speculate that circulating binding proteins could buffer S100A8/A9 and prevent unwanted aggregation in the circulation and therefore some subjects might have a reduce buffering capacity or more pronounced S100A8/A9 levels circulating. We could not identify a higher level of CD36 or GPIbα expression on platelets of high responder subjects. However, their platelets might be more prone to respond to S100A8/A9. This aspect remains a very interesting matter to question and further experiments should be performed.

Our data on S100A8/A9-induced fibrin generation and the formation of PS positive platelets with increased intracellular Ca^{2+} indicate the possible formation of procoagulant platelets. The field of procoagulant platelets has been expanding recently and different populations of procoagulant platelets have been described (Chu et al., 2021, Heemskerk et al., 2013). Following stimulation with collagen and thrombin (GPVI

and PAR receptors activation) or with calcium ionophore A23187, 2 types of procoagulant platelets are described (Andersen et al., 1999, Dale et al., 2002, Veuthey et al., 2022). The first one, with low capacity to aggregate, inactive GPIIb/IIIa, depolarised mitochondrial membrane with subsequent sustained high levels of intracellular Ca^{2+}. The second one with active GPIIb/IIIa, preserved low aggregating capacity, retained mitochondrial membrane potential and only spikes of high intracellular Ca^{2+} returning to normal levels (Topalov et al., 2012b). Our data indicate that S100A8/A9-induced procoagulant platelets share intermediate features of these two types. Indeed, they have inability to aggregate with active GPIIb/IIIa with a reduced ability to bind fibrinogen. We also showed that those platelets retain mitochondrial membrane potential (data not published and discussed in this thesis) and they have high intracellular Ca^{2+}. Whether this is due to different S100A8/A9 pathways induced in platelets or to the presence of a time-led, agonist-dependent (quality and quantity) spectrum of procoagulant platelets phenotypes, remains to be described. We further identified GPIbα as the main receptor of S100A8/A9 on platelets both in mice and humans. GPIb has been already described to be mediating procoagulant platelet formation, but this is the first time, it is described as a receptor for S100A8/A9. Interestingly, GPIbα has multiple extracellular agonists including vWF, thrombin but also P-selectin and Mac-1, which are well known to be involved in thromboinflammation (Mezger et al., 2019).

Using antibodies recognising different epitopes on GPIbα involved in vWF binding, we show that the effect of S100A8/A9 on platelets is regulated by at least two binding sites. It is possible that the binding of S100A8/A9 to these sites, further increase the

effect of other agonists involved in either thrombosis or/and inflammation. Indeed, the binding of S100A8/A9 to GPIbα further increase the agglutination properties of vWF, suggesting synergy between S100 and vWF. The anionic/sulphated region on GPIbα, having negative charge and sulphate groups, share similarities with the anticoagulant heparin which has been demonstrated to bind S100A8/A9. Whether the binding of S100A8/A9 to this region, is specifically involved in the formation of procoagulant platelets preventing the therapeutical effect of heparin, remains to be demonstrated.

Another aspect that we identified in our experiments and that requires further investigation is the role of CD36. CD36 has been already described to partially reduce S100A8/A9-induced thrombosis in mouse models of DVT, carotid injury and laser-induced injury to cremasteric circulation. Our data support the role of CD36 to GPIIb/IIIa activation and PS exposure without effect on alpha granule secretion. Indeed, blocking CD36 with SSO, reduces those activation markers and in Tg-mice abolishes completely the effect of S100. It is likely that CD36 plays a supportive role in S100A8/A9-mediated platelet activation by a crosstalk with GPIbα but the exact mechanism remains to be elucidated. Being a scavenger receptor, it is tempting to speculate that the initial role of CD36 is to eliminate S100 from the circulation and by this mechanism S100 is incorporated into platelets and induces their activation.

There are some limitations related to this study. First of all, it remains to be elucidated which role GPIIb/IIIa activation has in S100A8/A9-activated platelets. Currently we only know that it is less capable to bind fibrinogen and its activation is not associated with aggregation. Whether its binding to vWF is augmented by S100A8/A9-driven activation remains to be demonstrated. Furthermore, currently, specific GPIIb/IIIa antagonists are

considered the most powerful anti-thrombotic agents in acute thrombosis (Madan et al., 1998, Phillips et al., 1997, Topol et al., 1997). However, they carry bleeding risks (Scarborough et al., 1999, Madan et al., 1999, Horwitz et al., 2003). Whether the effect of those drugs in S100A8/A9 driven GPIIb/IIIa-activation are reduced or could be used to reduce procoagulant effects, remains to be tested. In order to further confirm the procoagulant properties those platelets have a panel of additional tests could be performed (Josefsson et al., 2023) such as measuring the effect of S100A8/A9 on mitochondrial membrane potential and excluding caspase activation in those platelets. Lastly, it is important to highlight that during our experiments, we registered that recombinant S100A8/A9 strength in activating platelets was changing according to batches. It is possible that post translational modifications on S100A8/A9 occur affecting its effect on platelets and this could potentially be a regulatory mechanism requiring further investigation. Indeed, this phenomenon has already been described in different research models and cells (van Dieck et al., 2009, Nolan and Peet, 2023).

Our study carries several limitations. This work has been conducted *in vitro*, assessing the effects of S100A8/A9 and potential inhibitor compounds on healthy human and murine samples. It remains to be confirmed that the mechanism described, takes place *in vivo.* Therefore, further work on animal models is required to assess the relevance of this discovery in different disease states. Furthermore, it remains to be demonstrated that the interaction between S100A8/A9 and GPIbα, favours clotting pathways *in vivo* in healthy volunteers and in patients. For this purpose, plasma form human donors will be required. Whether the response to S100A8/A9 and/or the S100A8/A9-driven responses are different between healthy donors and patients affected by either acute

or chronic diseases or infective vs sterile inflammation, remains to be explored. Another aspect that limits our results, is the number of donors that we have assessed. Increasing the number of donors and their demographic characteristics (age, race, gender, ethnicity and comorbidities) could allow the identification of risk factors and mechanisms involved in S100A8/A9-induced variable responses that we have observed.

To conclude, our data identified for the first time the S100A8/A9-GPIbα axis as a novel immune-driven pathway for platelet activation and formation of procoagulant platelets. This mechanism appears to be independent from platelet aggregation. It is important to underline that classical anti-platelet drugs such as COX and P2Y12 inhibitors, are currently first choice in thrombotic diseases, does not affect S100A8/A9 effect on platelets. As well as drugs targeting RAGE and TLR-4. Existing emerging drugs such as Syk inhibitors can only affect S100A8/A9-driven P-selectin increase and platelet-neutrophil aggregates. However, targeting GPIbα alone or in conjunction with CD36 is essential to reduce PS exposure and GPIIb/IIIa activation supporting the clinical need to discover novel molecules or convert current therapies to limit the formation of S100A8/A9-induced procoagulant platelets. It is important to specify that targeting the entire GPIb would be not possible, considering its crucial role in haemostasis as well as sequestrating fully S100A8/A9 because of its important anti-bacterial properties. Therefore, specific binding sites of S100A8/A9 on GPIbα should be identified and new molecules targeting only those epitopes should be developed.

CHAPTER 4

Hemin induces platelet aggregation via CLEC-2

Data from this chapter have been published:

Heme induces human and mouse platelet activation through C-type-lectin-like receptor-2

Joshua H. Bourne*, Martina Colicchia*, Ying Di, Eleyna Martin, Alexander Slater, Lubka T. Roumenina, Jordan D. Dimitrov, Steve P. Watson and Julie Rayes. **Heme induces human and mouse platelet activation through C-type-lectin-like receptor-2** (2021) Haematologica; Vol. 106 No. 2.

*Joint first author

Publication available in **Appendix 2**

4.1 Aims

The aim of this chapter is to explore whether free hemin, the oxidised form of heme, activates human and mouse platelets and which mechanisms are involved in this effect. The experiments and data obtained from this chapter were performed by Dr Julie Rayes, Dr Joshua Bourne and myself.

4.2 Introduction

4.2.1 Heme

Haemoglobin (Hb), is an iron-containing protein present in the red blood cells (erythrocytes), responsible of delivering oxygen to the tissues and transporting carbon dioxide to the lungs (Gell, 2018). Haemoglobin is composed of two α-globin and two β-globin chains, attached to a non-protein compound (the prosthetic group) known as heme (Perutz et al., 1960). The oxidised form of haemoglobin is called methaemoglobin which has decreased affinity for heme, leading to the release of free heme in the circulation (Bunn and Jandl, 1968, Kassa et al., 2016). Heme exists as a prosthetic group in numerous enzymes and other hemoproteins, with which it is involved in oxygen transport and storage (Kumar and Bandyopadhyay, 2005, Rodgers, 1999). Heme is a coordination complex of ferrous iron and a tetrapyrrole ring protoporphyrin IX (Ogun et al., 2023), (**Figure 4.1**). Heme is maintained in its reduced ferrous (Fe^{2+}) state by energy-demanding metabolic processes. Upon release into the extracellular space, heme is rapidly oxidised to a ferric (Fe^{3+}) state, becoming hemin.

Indeed, heme is beneficial when it remains compartmentalized within erythrocytes allowing oxygen transport throughout the body. However, free extracellular heme is a source of redox-active iron that can produce toxic free radicals. For this reason, an extended scavenging system exists and upon its exhaustion, as observed in haemolytic disease, oxidative stress and lipid peroxidation takes place. Heme iron can easily change its oxidation state and interact with multiple haemoproteins and receptors. Accumulating evidence shows that besides proteins that use heme as a firmly bound cofactor, other intracellular proteins are able to interact with it in a transient manner such as transcription factors, proteins implicated in cell signalling and cycle as well ion channels. Furthermore, heme can bind and oxidise lipoproteins (LDL), albumin, complement proteins, coagulation factors and immunoglobulins. Heme can also interact with membrane-bound receptors such as TLR4, RAGE, CLEC-2. Heme-CLEC-2 interaction will be further detailed below.

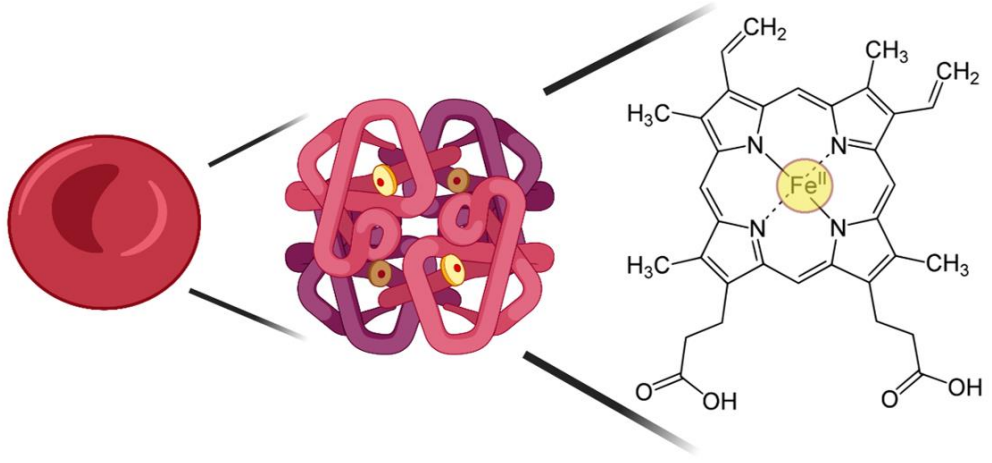


Figure 4.1 – Heme structure. Heme is a ring-shaped molecule holding an iron molecule. It is made of 4 pyrroles, small pentagon-shaped molecules made of 4 carbons and 1 nitrogen. Four pyrroles together form a tetrapyrrole. When the tetrapyrrole has substitutions allowing it to hold a metal ion, it is called a porphyrin. Thus, heme is an iron-holding porphyrin. The nitrogen molecules point toward the inside the ring they create. The double and single bonds which connect the pyrroles are balanced and the entire molecule remains stable making it an aromatic molecule. Heme combines with globin proteins to form haemoglobin in erythrocytes which carries oxygen from the lungs to the rest of the body. Haemoglobin comprises four subunits, each having one heme group. Each human erythrocyte contains *approximately 270 million haemoglobin molecules* (Pierigè et al., 2008, Nemkov et al., 2018, Yoshida et al., 2019). Created with BioRender.com

Within cells, heme levels are maintained by a balance of heme production, degradation, and distribution. Besides its role as a gas carrier, heme groups exert multiple other roles, such as being involved in the mitochondrial electron transport chain and signal transduction.

4.2.2 High levels of free heme in haemolytic and non-haemolytic diseases

Haemolysis is a pathological process characterised by the loss of erythrocyte membrane integrity. This causes the release of the cytosolic content of red cells into the extracellular space. Haemolysis can be triggered by several factors including genetic haemoglobin abnormalities, as observed in patients with sickle cell disease or β-thalassemia, or acquired factors (dysregulation in the complement system, pathogens, trauma, blood transfusions and other). Haemolysis is categorised into intravascular or extravascular: in the former, the destruction of red blood cells takes

place within the blood vessels, while in the latter, red blood cells lysis takes place mainly in the liver and spleen by reticuloendothelial macrophages. During intravascular haemolysis, intracellular erythrocytes content such as haemoglobin, are released into the circulation becoming extremely toxic. Beyond the decrease in the red blood cell count, haemolysis leads to the release of DAMPs such as haemoglobin and heme (Dimitrov et al., 2023) which once released extracellularly, exert dangerous functions associated with activation of the immune system, oxidative reactions, vascular damage, immune dysregulation, and thrombosis (May et al., 2021, May et al., 2018, Higdon et al., 2012, Soares and Bozza, 2016) (**Figure 4.2**). Patients with haemolytic diseases are at high risk of thrombotic complications such as venous thromboembolism and stroke as well as secondary infections.

4.2.3 Scavenging system for heme

Considering the dangerous effects of free haemoglobin and heme, a system for scavenging and disposal of these DAMPs exists. The main plasma proteins specialized in binding, neutralization and transport of haemoglobin and heme are haptoglobin and hemopexin, respectively. These proteins bind to their ligands and transport them for degradation in the spleen and liver. Haptoglobin binds to haemoglobin with high affinity and the haptoglobin-haemoglobin complex is cleared by liver Kupfer cells or hepatocytes which recognise the complex through the receptor CD163 (Schaer et al., 2006, Kristiansen et al., 2001). Heme is sequestered by hemopexin and cleared in the liver and spleen. Hemopexin is produced in the liver and

its synthesis is further upregulated during inflammation. It binds to heme with high affinity which can be considered as potentially an irreversible binding. The hemopexin-heme complex is recognized by the scavenge receptor CD91, also known as low-density lipoprotein receptor-related protein 1 (LRP1) by macrophages and hepatocytes. Upon cellular internalization, heme is catabolised by heme oxygenase-1 (HO-1) leading to the release of free ferrous iron, carbon monoxide and biliverdin. Free iron is then recycled and repurposed for the production of haemoproteins (**Figure 4.2**).

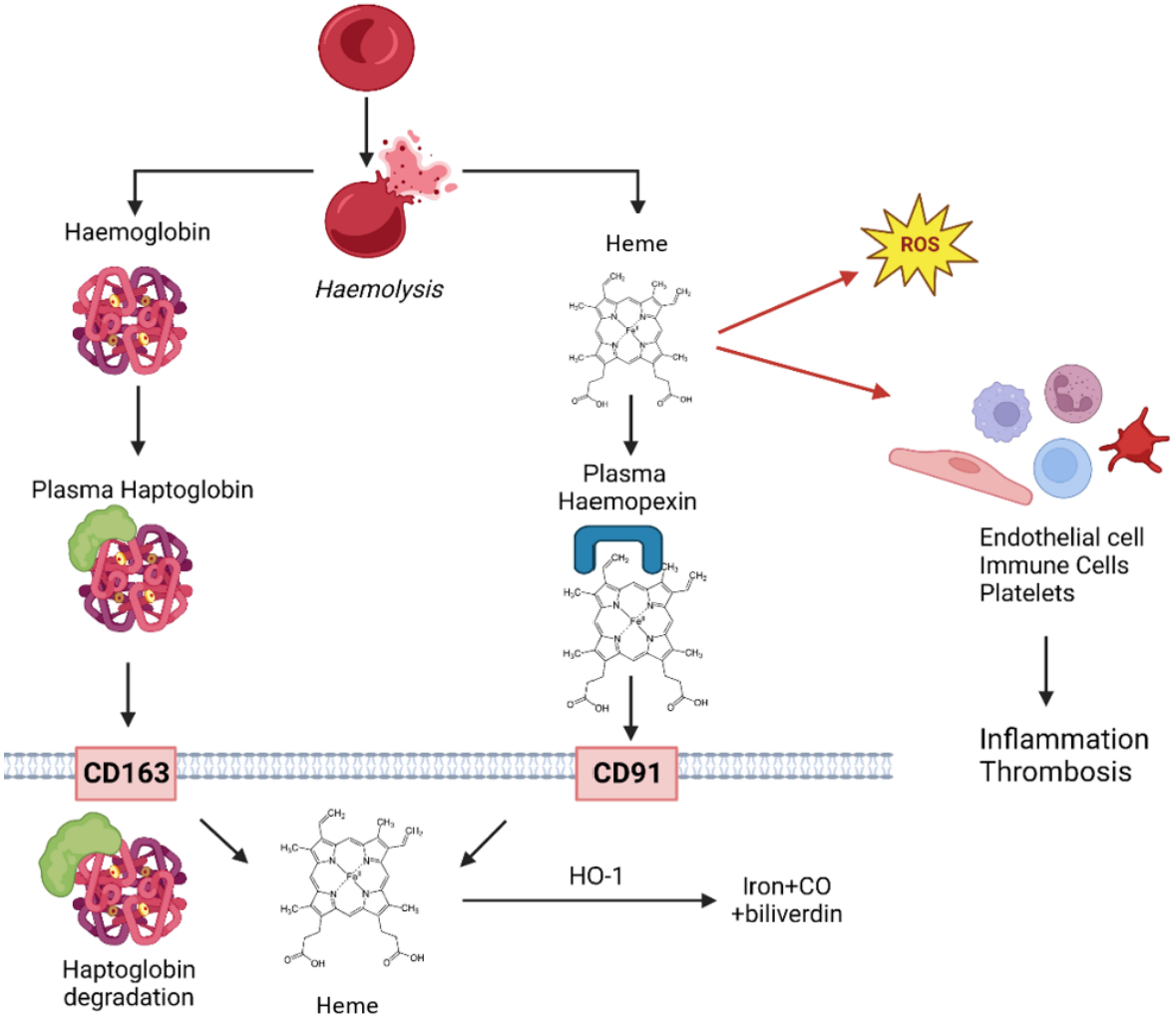


Figure 4.2 – Mechanisms of heme and haemoglobin scavenging during haemolysis. Following destruction of erythrocytes haemoglobin and heme are released to the extracellular space. Free haemoglobin is quickly bound by haptoglobin and the complex engulfed by macrophages through the scavenge receptor CD163, expressed on tissue macrophages such as liver Kupffer cells. Heme is bound by hemopexin and recognised by cell receptor CD91. Intracellularly heme is degraded by heme oxygenase-1 (HO-1) 1 into iron, carbon monoxide (CO) and biliverdin. During haemolytic diseases elevated levels of heme are released, overwhelming the haptoglobin/hemopexin system with subsequent saturation of their binding capacity. This leads to the presence of free heme able to trigger immune dysregulation, oxidative stress and thrombosis. Furthermore, heme can also generate ROS directly and indirectly through iron causing lipid peroxidation, DNA damage and protein damage. Adapted from Refs (Schmidt et al., 2019, Wu et al., 2019). Created with BioRender.com.

HO-1 expression is upregulated during haemolytic diseases such as sickle cell disease and pathological conditions associated with haemolysis such as sepsis. Although haemolysis itself increases the plasma levels of haptoglobin, hemopexin, HO-1 and ferritin, in severe disease, the scavenging system fails to metabolise haemoglobin and heme. In these circumstances, haemolysis-derived DAMPs exert inflammatory and pro-thrombotic effects and contribute to the pathogenesis of haemolytic diseases.

4.2.4 Heme oxidative effects

The pro-oxidant nature of free heme catalyses oxidative reactions leading to the formation of ROS which can oxidise other molecules and exert pro-inflammatory properties. ROS damage lipid membranes, proteins and nucleic acids, activate

proinflammatory transcription factors, alter protein expression, and affect membrane channels (Vercellotti et al., 1994, Jeney et al., 2002). Heme, due to its lipophilic nature, can intercalate within the hydrophobic phospholipid bilayer promoting the oxidation of cell membrane and causing the formation of cytotoxic lipid peroxide, which enhances membrane permeability (Balla et al., 1991, Ryter and Tyrrell, 2000, Kumar and Bandyopadhyay, 2005, Tolosano et al., 2010). Heme is also a strong pro-inflammatory molecule, inducing leukocytes, platelets and red blood cells recruitment to vascular endothelium, oxidizing LDL and consuming nitric oxide, an important regulator of vascular homeostasis.

4.2.5 Heme interaction with endothelial cells and the innate immune system

4.2.5.1 Heme and Endothelial Cells

As a consequence of its release into the circulation, heme interacts with multiple cells in the vasculature promoting inflammation and thrombosis. Endothelial cells are considered the main target of haemolysis-associated DAMPs. Interestingly, some endothelial cells such as brain microvascular and hepatic sinusoidal endothelial cells, can also engulf damaged red cells and clear them from the circulation (Fens et al., 2012). Upon haemolytic conditions, endothelial cell activation represents the main driver for ischemic events. Heme can directly activate endothelial cells through TLR-4 inducing the recruitment of platelet and immune cells, favouring vascular occlusion

(Belcher et al., 2014). Indeed, heme was shown to trigger vaso-occlusive crisis (VOC) in an experimental model of sickle cell disease (SCD) (Belcher et al., 2014). TLR-4 expression on endothelial was shown to initiate VOC in SCD mice. Heme triggers Weibel-Palade bodies release, the expression of tissue factor and the increase in adhesion molecules on endothelial cells. Interestingly, endothelial cells from patients with sickle cell disease express high levels of adhesion molecules promoting immune cell recruitment to the endothelial wall (Wagner et al., 2004). vWF release from Weibel-Palade bodies favours platelet recruitment and tissue factor upregulation triggers coagulation (Peyvandi et al., 2011, Ruggeri, 2007)**.** The adhesion of platelets, the activation of coagulation and of the immune system, all contribute to increased thrombosis (Roth, 1994, Wagener et al., 1997, Sparkenbaugh et al., 2015). To further confirm the central role of endothelial cells in haemolytic crisis, the anti-P-selectin antibody Crizanlizumab, has been shown to reduce the frequencies of VOC in SCD patients (Ataga et al., 2017). Heme has also been shown to activate the NLRP3 inflammasome through ROS in endothelial cells leading to IL-1β release, promoting inflammation. Furthermore, it has been shown to induce lipid peroxidation and cause mitochondrial dysfunction in endothelial cells (Higdon et al., 2012) while high concentrations induce apoptosis and necroptosis (Camus et al., 2015, Singla et al., 2017). Heme increases complement deposition on endothelial cells conferring selectivity to more vulnerable organs to innate immunity-driven thrombosis (Frimat et al., 2013, Kerr and Richards, 2012, Dutra and Bozza, 2014).

4.2.5.2 Heme and neutrophils, macrophages, complement

Heme, as a DAMP, is able to activate immune cells, particularly neutrophils, monocytes and macrophages. Exposure of human neutrophils to heme, induces their activation, the expression of the pro-inflammatory cytokine IL-8, the oxidative burst (with ROS synthesis) and increase their chemotactic activity (Graça-Souza et al., 2002, Miguel et al., 2021). Furthermore, in neutrophils, heme induces anti-apoptotic pathways, increasing their survival and pro-inflammatory role (Arruda et al., 2004). These effects are mediated not only by TLR-4 but also involve G protein-coupled receptors (Porto et al., 2007). Besides these roles, heme induces the formation of neutrophil extracellular traps (NETs) which contribute to VOC in SCD patients (Kono et al., 2014, Chen et al., 2014). Interestingly, heme induces NETs only in TNF-α primed neutrophils or in presence of neutrophils and heme-stimulated platelets, supporting the cooperative role of neutrophils and platelets in haemolytic disease (NaveenKumar et al., 2023). Moreover, platelet-neutrophil aggregates are present in patients affected by haemolytic disease, and this interaction is mediated by platelet P-selectin expression (Polanowska-Grabowska et al., 2010). Targeting P-selectin or $P2Y_{12}$ reduces platelet-neutrophil aggregates in SCD mice (Polanowska-Grabowska et al., 2010).

Heme also binds also to TLR-4 on macrophages at a distinct site to LPS, inducing their activation (Figueiredo et al., 2007). Furthermore, it has been shown to induce differentiation of macrophages specialised in phagocytosis and disposal of erythrocytes (Haldar et al., 2014, Pfefferlé et al., 2020). On the other hand, heme has

also be shown to impair macrophage function by altering actin polymerisation and cytoskeleton in those cells (Martins et al., 2016, Martins and Knapp, 2018).

Heme was also shown to interact with complement, which is an integral part of the innate immune system, amplifying erythrocyte destruction (Pawluczkowycz et al., 2007, Lindorfer et al., 2016). Indirectly, the heme-induced expression of P-selectin on endothelial cells, activate complement, worsening thrombotic effects in haemolytic disease (Frimat et al., 2019).

4.2.6 Heme interaction with platelets and thrombosis

During haemolysis, several mechanisms contribute to platelet activation and can favour thromboinflammation. The release of ADP from damaged red cells can activate P2Y1 and P2Y12 on platelets promoting aggregation and interaction with immune cells. ADP released during haemolysis was shown to induce the formation of platelet-rich thrombi and targeting its pathways could decrease thrombotic complications. However, Prasugrel, a P2Y12 inhibitor, showed no amelioration in VOC rate in SCD patients (Heeney et al., 2016), suggesting multiple mechanisms take place during haemolysis-induced ischemic events. It was shown that free heme, forming dimers and oligomers, causes damage to platelets and their death (NaveenKumar et al., 2019, NaveenKumar et al., 2018, Belcher et al., 2010). Interestingly, injection of heme in mice, show that high concentrations induce bleeding while low concentrations induce hypercoagulability, suggesting the delicate role this DAMP exerts in haemostatic balance (Hopp and Imhof, 2021). In terms of coagulation, heme, binding to different

coagulation factors (FVIII, FIX, FXII, protein C) affects their procoagulant or anti-coagulant functions (Hopp et al., 2021). Also, through its iron ion and the protoporphyrin ring, binds and crosslink fibrinogen and inhibits its conversion to fibrin. Through its oxidant function, heme was described to induce ROS generation (Porto et al., 2007), inflammasome activation (Salgar et al., 2023) and ferroptosis in platelets (NaveenKumar et al., 2018). Ferroptosis is an iron-dependent cell death characterised by high levels of ROS where mitochondrial damage takes place. Morphologically, cells which undergo ferroptosis, differ from necrotic or apoptotic cells. Heme has been described to induce platelet ferroptosis through the release of iron and free radical ions. In the context of non-severe haemolytic disease, heme still contribute to thrombosis. In patients with acute coronary syndrome, high levels of heme are associated with higher risk of thrombotic complications (Cubedo et al., 2017). Furthermore, heme accumulation under ischemic conditions in the heart, lead to cell toxicity and heart failure (Sansbury et al., 2014). In a mouse model of DVT, heme has been associated with thrombus formation as hemopexin deficient mice showed larger thrombi (Nath et al., 2020).

4.2.7 Haemolysis in diseases

Haemolysis arises for either congenital or acquired conditions. Genetic predisposition can lead to diseases such as SCD while acquired conditions can lead to haemolysis secondary to antibodies, trauma, infections, toxic agents or drugs. A classification of haemolytic diseases is shown in **Figure 4.3**.

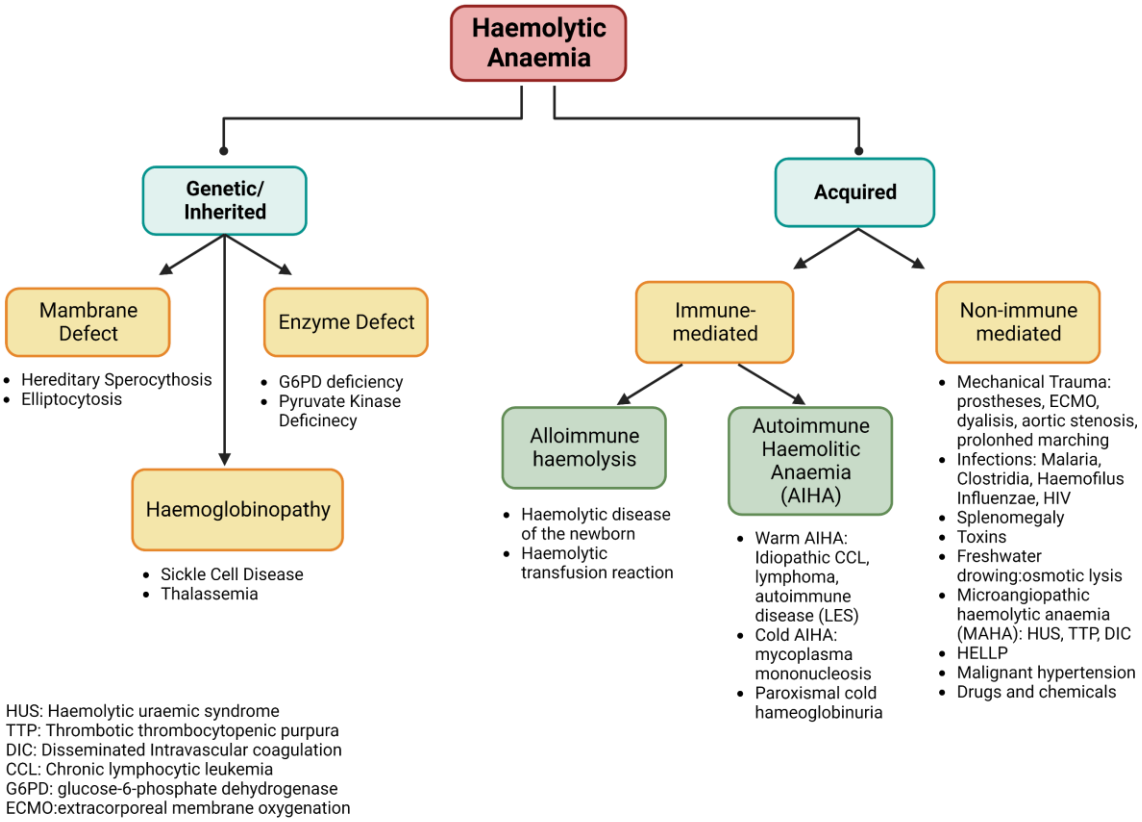


Figure 4.3 – Causes of haemolytic anaemia. Adapted from Kumar & Clark's Clinical Medicine Oxford Handbook of Clinical Medicine. Created with BioRender.com

Symptoms of haemolysis are caused by anaemia, as a result of erythrocyte lysis not being compensated by the production of cells and by the detrimental effects of haemolysis itself on driving inflammation and thrombosis. As discussed in this chapter, the effect of heme on different types of cells, induces tissue damage, acute renal injury, organ dysfunction and thrombosis. Beyond this role, heme favours bacterial infections dysregulating the innate immune response and allowing the proliferation of pathogenic bacteria (Nairz and Weiss, 2020).

In this chapter we investigate the mechanism by which heme activates human and mouse platelets and we identify its functional receptor on platelets.

4.3 Results

The experiments presented in the following paragraphs, have been all performed using hemin, the oxidised form of heme unless otherwise specified. Therefore, the words heme and hemin are used interchangeably.

4.3.1 Hemin activates human platelets in a bell-curved shape

Here we demonstrate that hemin induces human platelet aggregation *in vitro*. Blood plasma concentration of hemin in patients affected by haemolytic diseases, range from 2 to 50 µM (Gouveia et al., 2017). However, the level of free labile heme was determined in the range of 2-6 µM in patients with haemolytic diseases. We therefore used this range of heme concentration to assess platelet activation and aggregation *in vitro*. Interestingly, lower concentration of hemin (3.12-12.5 µM) elicited rapid human washed platelet aggregation while higher concentrations (>25 µM) induced lower magnitude of platelet aggregation (**Figure 4.4**). These results suggest distinct mechanisms might be involved in hemin-induced platelet aggregation and this response appears to be in a bell-curved shape.

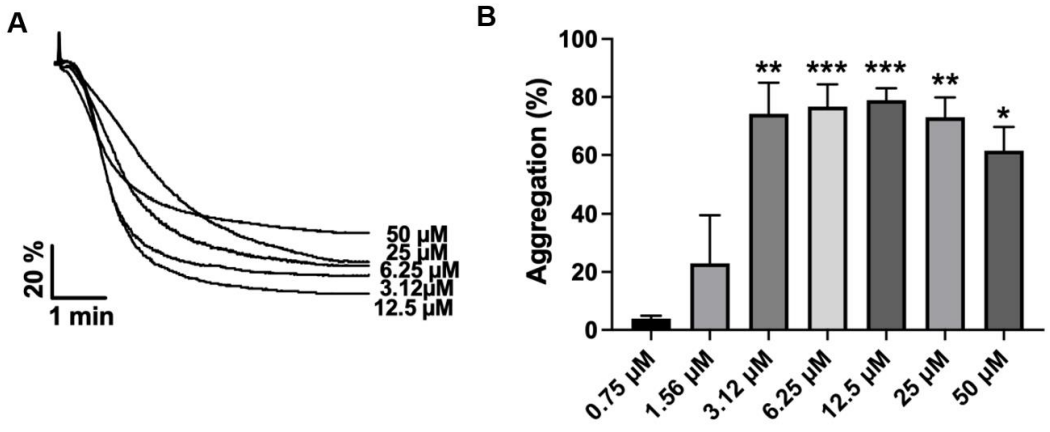


Figure 4.4 – Hemin induces human washed platelet aggregation in a dose-dependent manner. Human washed platelet ($2x10^8$/ml) were incubated with increasing doses of hemin and platelet aggregation was assessed for 6 minutes (min) using light transmission aggregometry (n=5). Experiments were performed at 37°C in the presence of Ca^{2+} (2mM). (**A**) Representative platelet aggregation traces. (**B**) Percentage (%) of maximal aggregation (n=6). The statistical significance was analysed using a one-way ANOVA with Tukey's multiple comparisons test, Significance is shown compared to control (absence of hemin), **p*<00.5 ** *p*<00.1 ****p*<000.1. Adapted from Ref (Bourne et al., 2021).

Using flow cytometry, we demonstrated that hemin induces human platelet activation *in vitro* as shown by α granule release (P-selectin expression) and GPIIb/IIIa activation (**Figure 4.5 A, B**). This effect on platelets was achieved with both low/mid (3.12-12.5 µM) and high doses of hemin (>25 µM). Interestingly, only high doses of hemin (>25 µM) significantly increased PS exposure on platelets as assessed by Annexin V staining (**Figure 4.5 C**). This result suggests a hemin-induced toxic platelet effect confirmed by high levels of lactate dehydrogenase (LDH) in the supernatant of high dose-hemin-activated platelets (**Figure 4.5 D**). To further confirm platelet activation, we showed that hemin induces the phosphorylation of Syk and PLCγ2 at all concentrations but to a lower extent at high concentration (50 µM) (**Figure 4.5 E**). The

western blot data suggest that platelet response due to intracellular pathways activation is more likely to be activated at low-mid doses of hemin, while higher doses induce platelet toxicity.

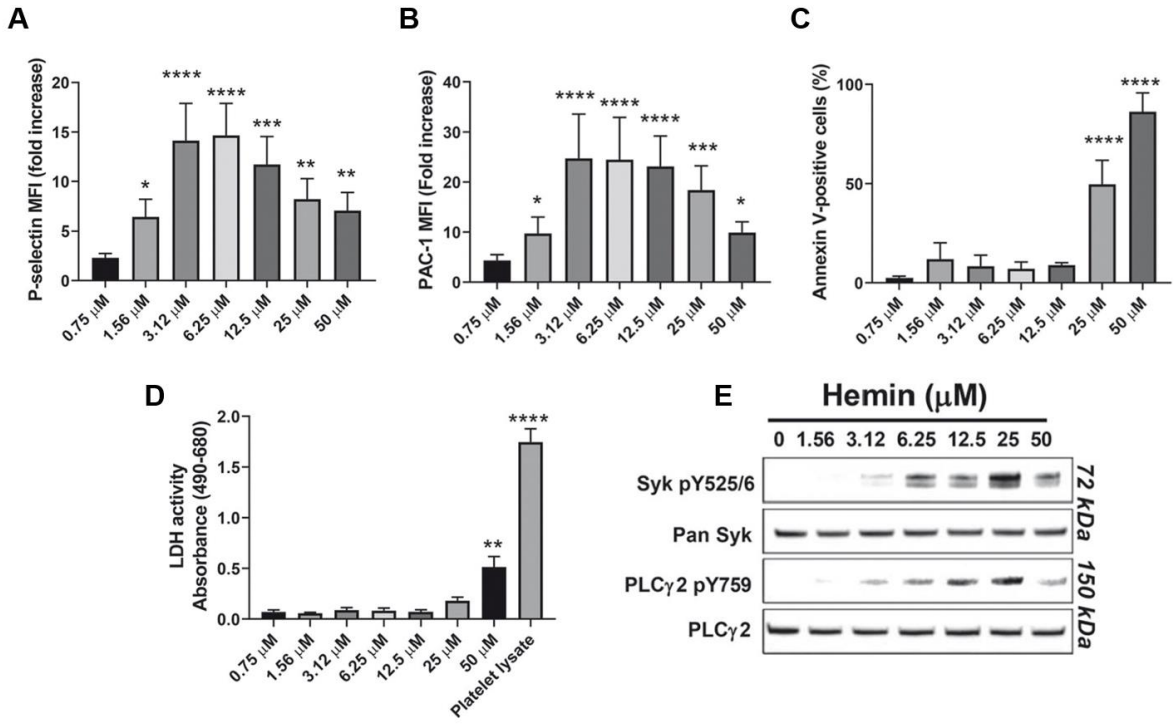


Figure 4.5 – Hemin induces human washed platelet activation and PS exposure. (**A**-**D**) Human washed platelets ($1x10^6$) were incubated with different concentrations of heminfor 20 min at 37°C. Platelet activation was determined by flow cytometry using (A) anti-P-selectin and (B) GPIIb/IIIa PAC-1 antibodies (n=4). Data is shown as fold increase of median of fluorescence (MFI) of treated platelets over control (absence of hemin). (C) Phosphatidylserine exposure was assessed using Annexin V staining. (D) Lactate dehydrogenase (LDH) levels were measured in the supernatant following hemin-stimulated platelet aggregation (6 min). Platelet lysate was used to detect the total level of LDH in platelets. Histogram data are shown as mean standard deviation. (E) Whole platelet lysate following hemin stimulation (6 min aggregation) was assessed for protein levels of Syk and PLCγ2 phosphorylation, alongside total Syk and PLCγ2 by western blotting. Western blots are representative of five independent experiments. The statistical significance was analysed using a one-way ANOVA with

Tukey's multiple comparisons test. Significance is shown compared to control (absence of hemin), *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Adapted from Ref (Bourne et al., 2021)

We also used scanning electron microscopy (EM) to image aggregated platelets in the presence of heme (5 µM). The EM images show a potent platelet aggregation and microvesicles release following hemin stimulation (**Figure 4.6**). At higher concentration (25 µM), significant damage to the platelet membrane was observed with the formation of platelet aggregates rich in microvesicles (**Figure 4.6**). EM images and data were was performed by the EM facility at the University of Birmingham and have not been included in the publication.

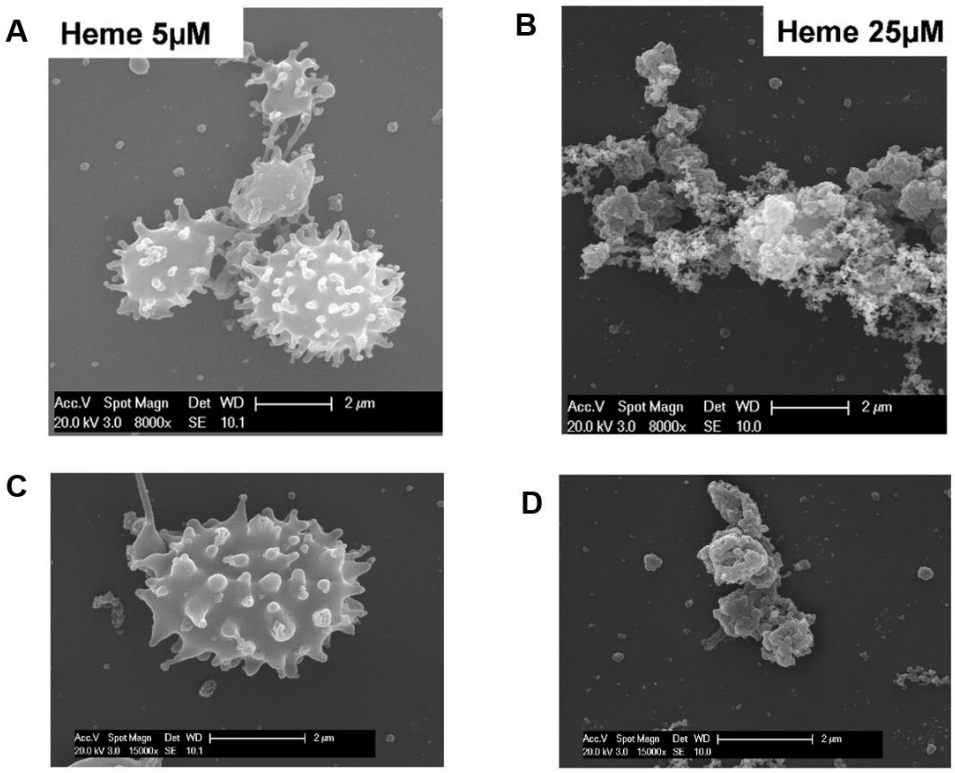


Figure 4.6 – Human washed platelets ($2x10^8$) were subjected to light transmission aggregometry for 6 minutes at 37°C in the presence of hemin 5 µM (A, C) or 25 µM (B, D) and Ca^{2+} (2mM). Platelets were

fixed and subjected to scanning electron microscopy. Platelet aggregation by heme was assessed in Tyrode's modified buffer containing 2mM Calcium for 6 minutes. At the end of the aggregation, platelets were fixed using Glutaraldehyde 0.3% and sample processed for electron scanning microscopy. Experiments performed by Dr Julie Rayes.

4.3.2 Hemin activates human platelets through CLEC-2

Hemin has already been reported to bind to murine endothelial cells via TLR4 resulting in endothelial cell activation (Belcher et al., 2014). In order to identify the receptor involved in hemin-induced platelet activation, we screened several inhibitors for platelet receptors using light transmission aggregometry (**Figure 4.7**). The concentration of hemin used in the experiments was 6.25 µM which is a mid-range value that we proved to induce maximal aggregation and platelet activation without platelet death. Using TAK-242, which blocks TRL4 signalling on platelets, we were unable to prevent hemin-induced platelet activation suggesting other receptors and pathways involvement. The same results were obtained using drugs targeting COX (indomethacin) and P2Y12 (Cangrelor) which are the most common anti-platelet compounds used in the clinical practice. Hemin-induced human platelet aggregation was significantly inhibited by Syk inhibitor (PRT-060318), Src family kinase inhibitor (PP2) and BTK inhibitor (Ibrutinib) and partially reduced by GPIIb/IIIa inhibitor epitifibatide (Integrilin). These results suggest involvement of immunoreceptor-tyrosine-based activation-motif (ITAM) receptor-pathway in heme-mediated platelet activation. In order to identify the specific receptor(s) involved in hemin-induced platelet activation, we adopted a blocking strategy using recombinant dimeric forms of C-type-lectin-like receptor-2 (CLEC-2) and collagen receptor glycoprotein VI (GPVI) (hFc-

CLEC-2 and hFc-GPVI, respectively). Pre-incubation of hemin with hFc-CLEC-2 blocked platelet aggregation while the same effect was not induced by hFc-GPVI (**Figure 4.7)**.

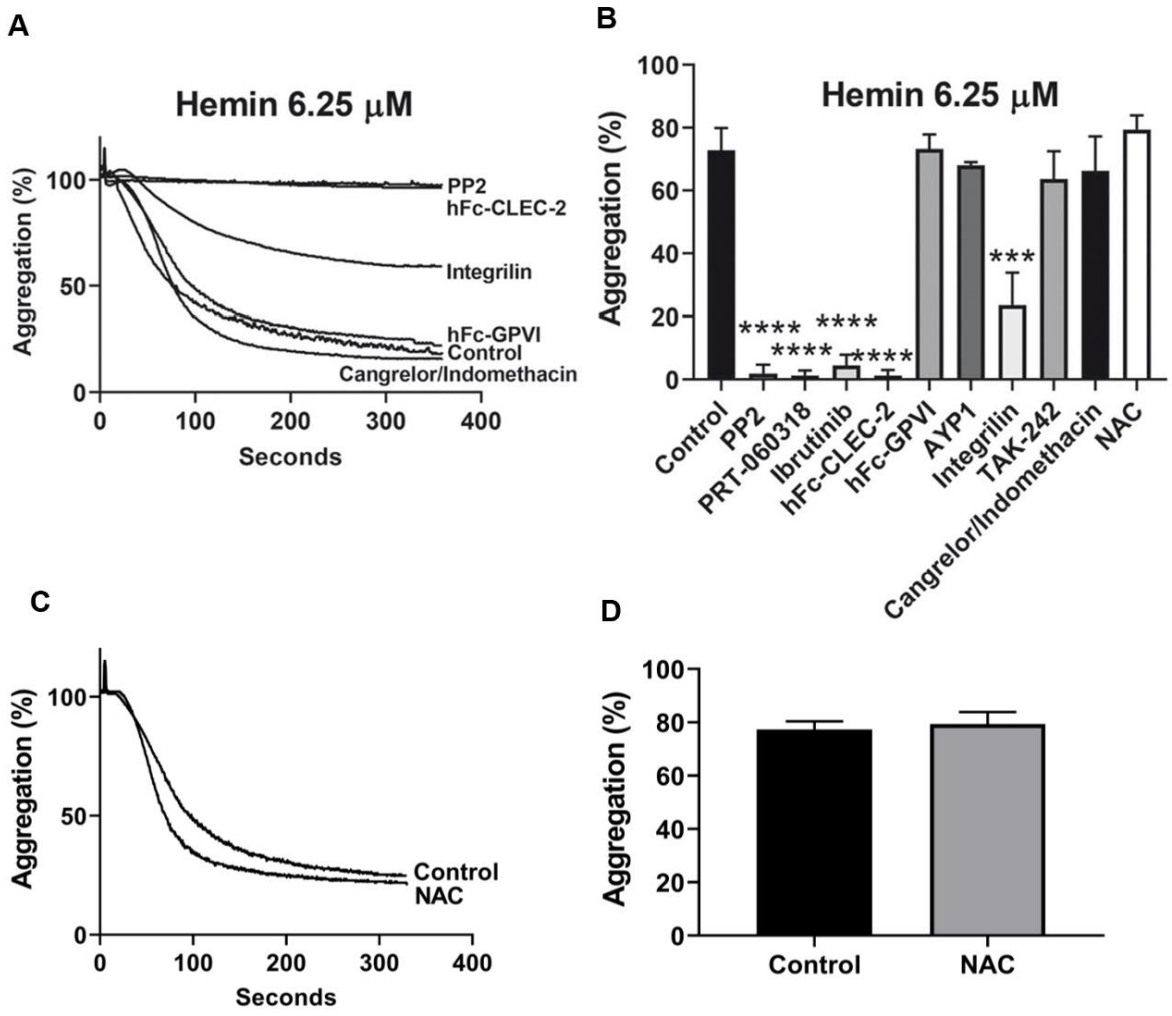


Figure 4.7 – Low-dose hemin mediates human platelet activation through C-type-lectin-like receptor-2. (A, B, C, D) Human washed platelets ($2x10^8$/mL) were incubated with increasing concentrations of hemin in the presence of Ca^{2+} (2 mM) and platelet aggregation was assessed for 6 minutes (min) using light transmission aggregometry at 37°C (n=5). Inhibitors used: Btk inhibitor Ibrutinib (500 nM), Src family kinase inhibitor PP2 (20 μM), Syk inhibitor PRT-060318 (20 μM), TLR4 inhibitor TAK-242 (10 μM), P2Y12 inhibitor Cangrelor (10 μM), cyclooxygenase inhibitor indomethacin (10 μM),

dimeric C-type-lectin-like receptor-2 (CLEC-2) (hFc-CLEC-2, 20 mg/mL), dimeric collagen receptor glycoprotein VI (GPVI) (hFc-GPVI, 20 mg/mL) or AYP1 (10 µg/ml) or N-acetyl cysteine (NAC, 100 µM). Platelets were pre-incubated with different inhibitors for 5 min at 37°C prior to the addition of hemin (6.25 µM). (A) Representative aggregation traces of washed platelets incubated with inhibitors and hemin 6.25 µM (only PP2, hFc-CLEC-2, Integrilin, hFc-GPVI, Cangrelor/Indomethacin are shown). (B) Percentage (%) of maximal aggregation (n=5). (C) Representative aggregation traces of washed platelets incubated with NAC (100 µM) and hemin 6.25 µM. (D) Percentage (%) of maximal aggregation (n=3). Mean ± SD. The statistical significance was analysed using a one-way ANOVA with Tukey's multiple comparisons test. Significance is shown compared to control (absence of hemin), *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Adapted from Ref (Bourne et al., 2021)

These experiments identify CLEC-2 as the hemin receptor mediating human platelet activation. Hemin-induced platelet aggregation was not affected by AYP1, an antibody that blocks the interaction between CLEC-2 and podoplanin, suggesting that hemin binding site on CLEC-2 is distinct to the podoplanin one. Furthermore, platelet aggregation was not affected by the antioxidant N-acetyl cysteine (NAC) suggesting it does not depend on oxidative stress (**Figure 4.7**). In all those experiments a low dose (6.25 µM) of hemin was used. Integrilin, Src, Syk or Btk inhibitors were unable to inhibit high concentration (>25 µM) of hemin-induced platelet aggregation (**Figure 4.8**). These data suggest that high concentration of hemin might induce agglutination through cell death rather than aggregation.

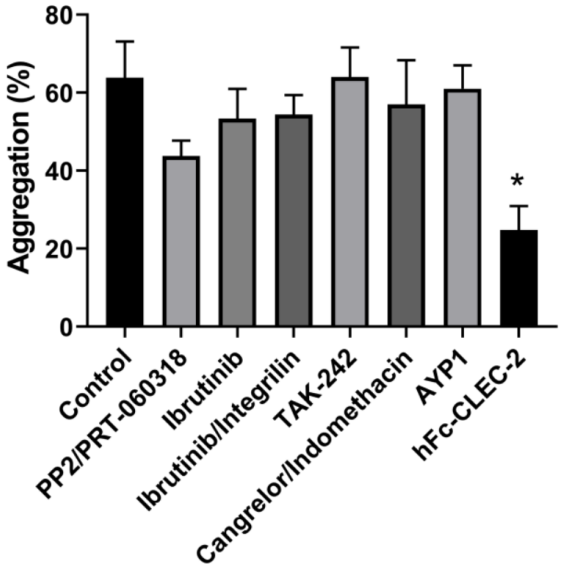


Figure 4.8 – Recombinant human CLEC-2 (hFc-CLEC-2) inhibits platelet agglutination by high concentrations of hemin. Human washed platelets ($2x10^8$/mL) were incubated with hemin (50μM) in the presence of $Ca2^+$ (2 mM) and platelet aggregation was assessed for 6 minutes (min) using light transmission aggregometry at 37°C (n=3). Human washed platelets ($2x10^8$/ml) were incubated with hemin (50μM). Platelet aggregation was assessed using light transmission aggregometry. Ibrutinib (500nM), PP2 (20μM), PRT-060318 (20μM), TAK242 (10μM), Cangrelor (10μM), Indomethacin (10μM) were preincubated for 5 min with platelets prior hemin addition. Recombinant hFc-CLEC-2 (50μg/ml) was preincubated with hemin for 15 minutes at 37°C prior addition to platelets. Percentage (%) of maximal aggregation (mean ± SD). The statistical significance was analysed using a one-way ANOVA with Tukey's multiple comparisons test. Significance is shown compared to control *$p < 0.05$. Adapted from Ref (Bourne et al., 2021)

4.3.3 Heme induces P-selectin expression in human platelets independently of oxidative stress

Hemin has been described to induce mitochondrial ROS (mtROS) generation in platelets through TLR-4 (Annarapu et al., 2021). Scavenging mtROS prevented the release of the α-granule thrombospondin-1 and of other granules proteins (IL-1β,

CXCL7, FGFb, PDGF-B, angiostatin, TGF-β, kininogen). To test whether platelet P-selectin release was independent of heme-induced oxidative stress, we tested the effect of different inhibitors targeting intracellular metabolic pathways. ROS can be either formed in the cytoplasm of platelets or in the mitochondria. In human platelets, both NADPH oxidase 1 and 2 (NOX1 and NOX2) significantly contribute to ROS production, and they are considered responsible for the regulation of thrombus formation (Walsh et al., 2014). NOX can be inhibited by diphenyleneiodonium chloride (DPI) or 2-acetylphenothiazine (2-APT). We used N-Acetyl Cysteine (NAC), which is an antioxidant compound, able to neutralise free radicals and scavenge oxidant species and MitoTEMPO, a mtROS scavenger. Interestingly, inhibition of ROS using NAC (1mM) at a higher dose in comparison to the aggregation experiment or mitochondrial ROS using MitoTEMPO did not alter CD62P expression (**Figure 4.9A**). Blocking NOX in human platelet using 2-APT or DPI did not alter hemin-induced human platelet activation. CD62P expression was reduced by chelating intracellular calcium using Bapta-AM and by chelating extracellular calcium using EDTA (**Figure 4.9A**), supporting the role of calcium in heme-induced platelet activation. In accordance with the aggregation data, addition of Btk inhibitor Ibrutinib, Src inhibitor PP2, Syk inhibitor PRT-030618 or recombinant CLEC-2-Fc inhibited heme-induced CD62P expression while P2Y12 or COX inhibition had no effect on CD62P expression (**Figure 4.9B**). These results, (unpublished), suggest that hemin-induced P-selectin release, is independent from oxidative stress and targeting Btk, Syk and Src in human platelets or using recombinant CLEC-2 represents a successful strategy to block heme-mediated platelet aggregation and P-selectin expression.

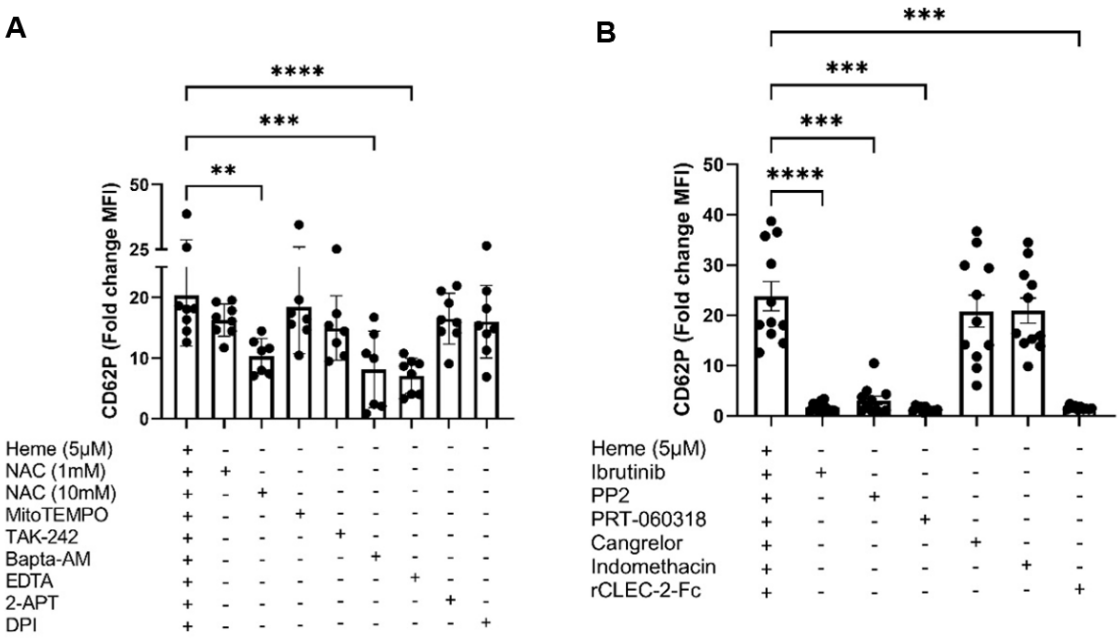


Figure 4.9 – Hemin-mediated P-selectin and phosphatidylserine exposure on human platelets does not depend on ROS generation and platelet oxidation. Human washed platelets (10^6/condition) were left untreated or incubated with hemin (5 µM) (heme on the figure) in the presence of 2mM Ca^{2+} for 30 minutes at 37°C. (A, B) CD62P was assessed by flow cytometry. Inhibitors were preincubated with platelets for 10 minutes at RT before addition of hemin: Ibrutinib (500nM), PP2 (20µM), PRT-060318 (10 µM), Cangrelor (10 µM), Indomethacin (10 µM), rCLEC-2-Fc (20 µg/ml), N-acetylcyteine (NAC) (1 or 10mM), MitoTEMPO (10 µM), TAK-242 (10 µM), Bapta-AM (10 µM), EDTA (10 mM), 2-APT (10 µM) and DPI (10 µM). The statistical significance was analysed using a Tukey's one-way ANOVA with multiple comparison. **p <0.01, *p <0.05.

4.3.4 Hemin induces mouse platelet aggregation

Similar to human platelets, hemin induced murine platelet aggregation at concentrations higher than 12.5 µM (**Figure 4.10**). This suggests that mouse platelets are less sensitive to hemin than human platelets. Similar to human platelets, hemin-induced platelet aggregation was inhibited by PP2, PRT-060318, ibrutinib,

recombinant mouse Fc-mCLEC-2 and Integrilin. TLR-4, and P2Y12 inhibitors had no effect on hemin-induced platelet aggregation.

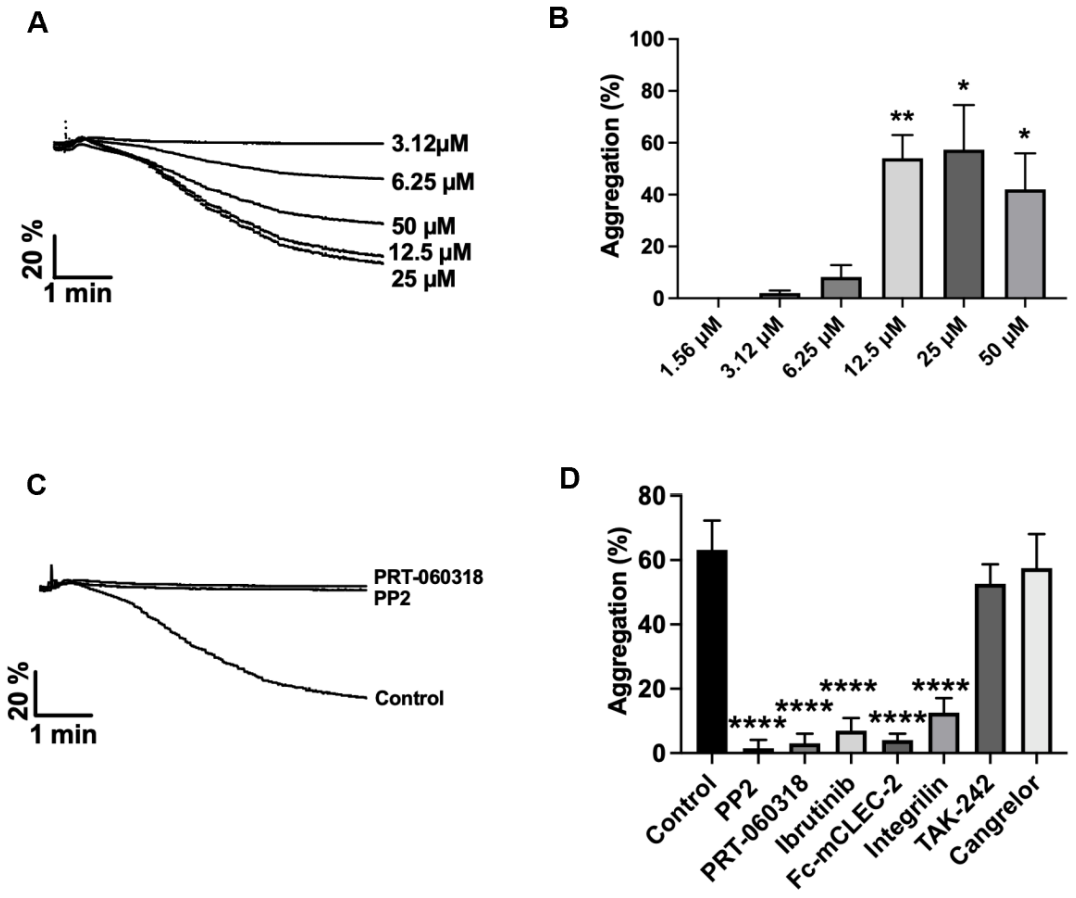


Figure 4.10 – Hemin induces mouse platelet aggregation. (A, B) Mouse washed platelets (2x10^8/mL) were incubated with increasing concentrations of hemin in the presence of Ca^{2+} (2 mM) at 37°C. (**A**) Representative traces of platelet aggregation. Platelet aggregation was assessed using light transmission aggregometry. (B) Percentage (%) of maximal aggregation. (C, D) Hemin-mediated platelet aggregation (12.5 µM hemin) was assessed in the presence of Ibrutinib (2 µM), PP2 (20 µM), PRT-060318 (20 µM), TAK-242 (10 µM), Cangrelor (10 µM), recombinant mouse CLEC-2 (Fc-mCLEC-2, 10 µg/mL) (n=4). (C) Representative traces of platelet aggregation. (D) Histogram data with percentage (%) of maximal aggregation are shown as mean standard deviation. The statistical

significance was analysed using a one-way ANOVA with Tukey's multiple comparisons test using Prism 8. Significance is shown compared to control ****P<0.0001. Adapted from Ref (Bourne et al., 2021)

We investigated the possibility of a redundant role for CLEC-2 and GPVI in mouse platelets using washed platelets from WT, platelet CLEC-2 deficient mice (CLEC-2$^{-/-}$) CLEC-1bfl/flGPIbcre), GPVI$^{-/-}$ and mice deficient in platelet CLEC-2 and GPVI (CLEC-2$^{-/-}$GPVI$^{-/-}$) (CLEC-1bfl/flGPIbcre/GP6$^{-/-}$) by performing flow cytometry. We observed that platelet aggregation by heme (10 µM) was significantly reduced in CLEC-2$^{-/-}$, GPVI$^{-/-}$ mice and abolished in CLEC-2$^{-/-}$/GPVI$^{-/-}$ mice (**Figure 4.11**). At higher doses, aggregation was inhibited only in CLEC-2$^{-/-}$/GPVI$^{-/-}$ mice. Deletion of CLEC-2 or GPVI from platelets did not significantly reduce P-selectin (CD62P) exposure at low doses, whereas platelets from CLEC-2$^{-/-}$/GPVI$^{-/-}$ had significantly lower expression of CD62P-positive platelets suggesting the contribution of both receptors in mice. At higher doses, CLEC-2$^{-/-}$/GPVI$^{-/-}$ had similar CD62P expression compared to WT suggesting a cell mechanism similar to human platelets. Contrary to human platelets, PS exposure, assessed by Annexin V binding, was marginally increased at lower dose of hemin but significantly increased at 25µM. Annexin V binding was not altered by the absence of CLEC-2 or GPVI suggesting that heme-induced membrane damage is independent of these receptors. Both concentrations of hemin decreased the mitochondrial membrane potential as measured by flow cytometry using TMRM, therefore the absence of PS exposure is not related to distinct effect on mitochondrial membrane potential and requires further investigation. Flow Cytometry was not included in the publication.

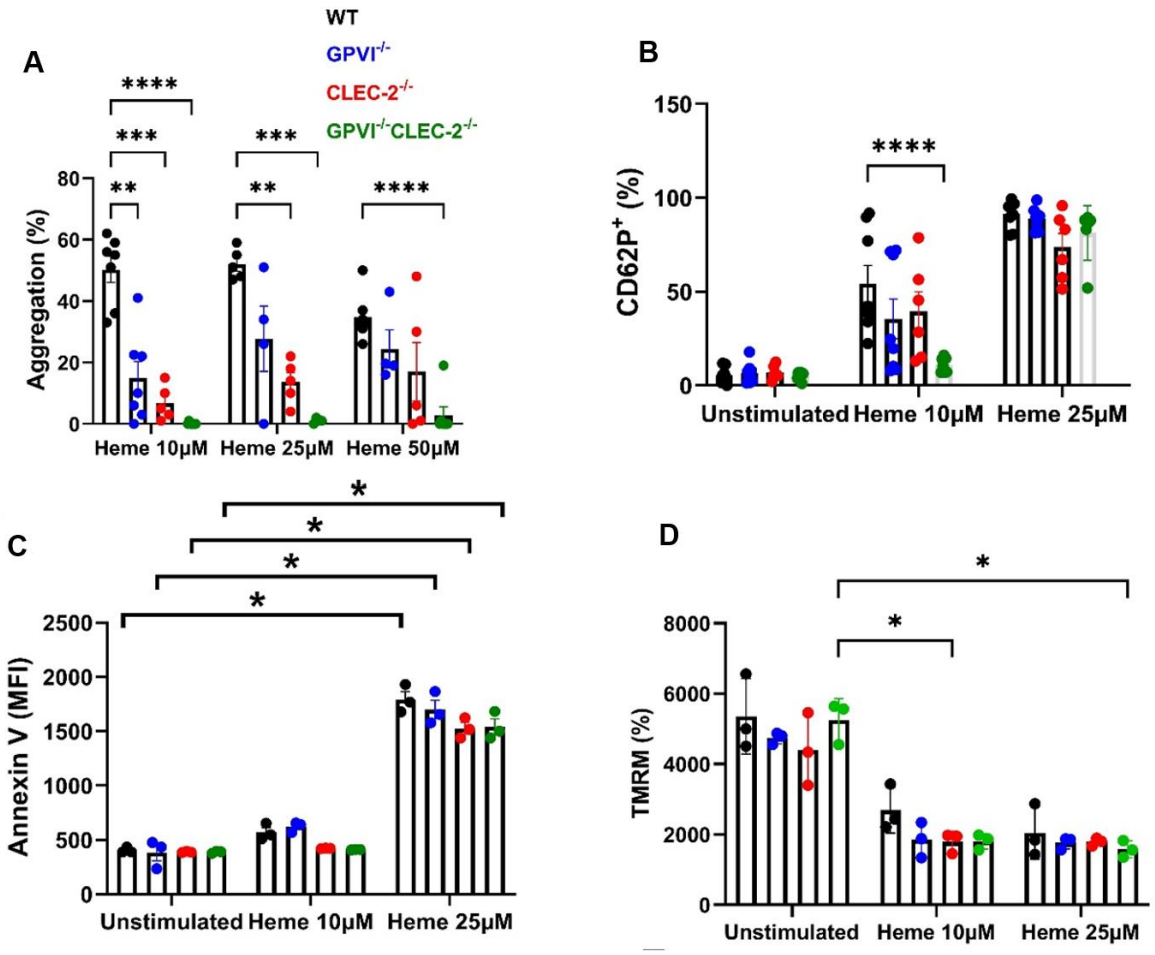


Figure 4.11 – Mouse platelet susceptibility to hemin is dependent on CLEC-2 and GPVI. (A) Mouse washed platelets ($2x10^8$/ml) isolated from WT, GPVI$^{-/-}$, CLEC-2$^{-/-}$ and GPVI$^{-/-}$CLEC-2$^{-/-}$ mice were incubated with different concentrations of hemin (10, 25, and 50 μM) in the presence of $CaCl_2$ (2mM) and platelet aggregation assessed for 6 min using light transmission aggregation. (B-D) Washed platelets (10^6 platelets/condition) were incubated with hemin for 30min at 37ºC in the presence of $CaCl_2$. (B) Platelet CD62P expression, (C) PS exposure and (D) mitochondrial membrane potential were assessed using anti CD62P antibody, Annexin V binding and TMRM uptake, respectively, by flow cytometry. The statistical significance was analysed using mixed effect multiple comparisons 2-way ANOVA test. Statistical difference is shown comparing KO platelets to WT platelets with the same treatment. ****p <0.0001, ****p <0.001, **p <0.01, *p <0.05. (Data unpublished)

P-selectin expression was significantly reduced by PP2 and PRT in mouse platelets but annexin V remained unchanged (**Figure 4.12 A, C**). Interestingly, contrary to human platelets, P-selectin expression was drastically inhibited by the antioxidant NAC (1mM) and NOX inhibitor 2-APT but was not altered by mitochondrial ROS inhibitor MitoTEMPO or BAPTA-AM. CD62P expression was completely abolished using EDTA, supporting the role of extracellular calcium in hemin-mediated mouse platelet activation (**Figure 4.12B**). PS exposure was inhibited by NAC but not altered by any other inhibitor (**Figure 4.12D**). These data support a key role for hemin redox activity in mouse platelet activation independent of ITAM receptors.

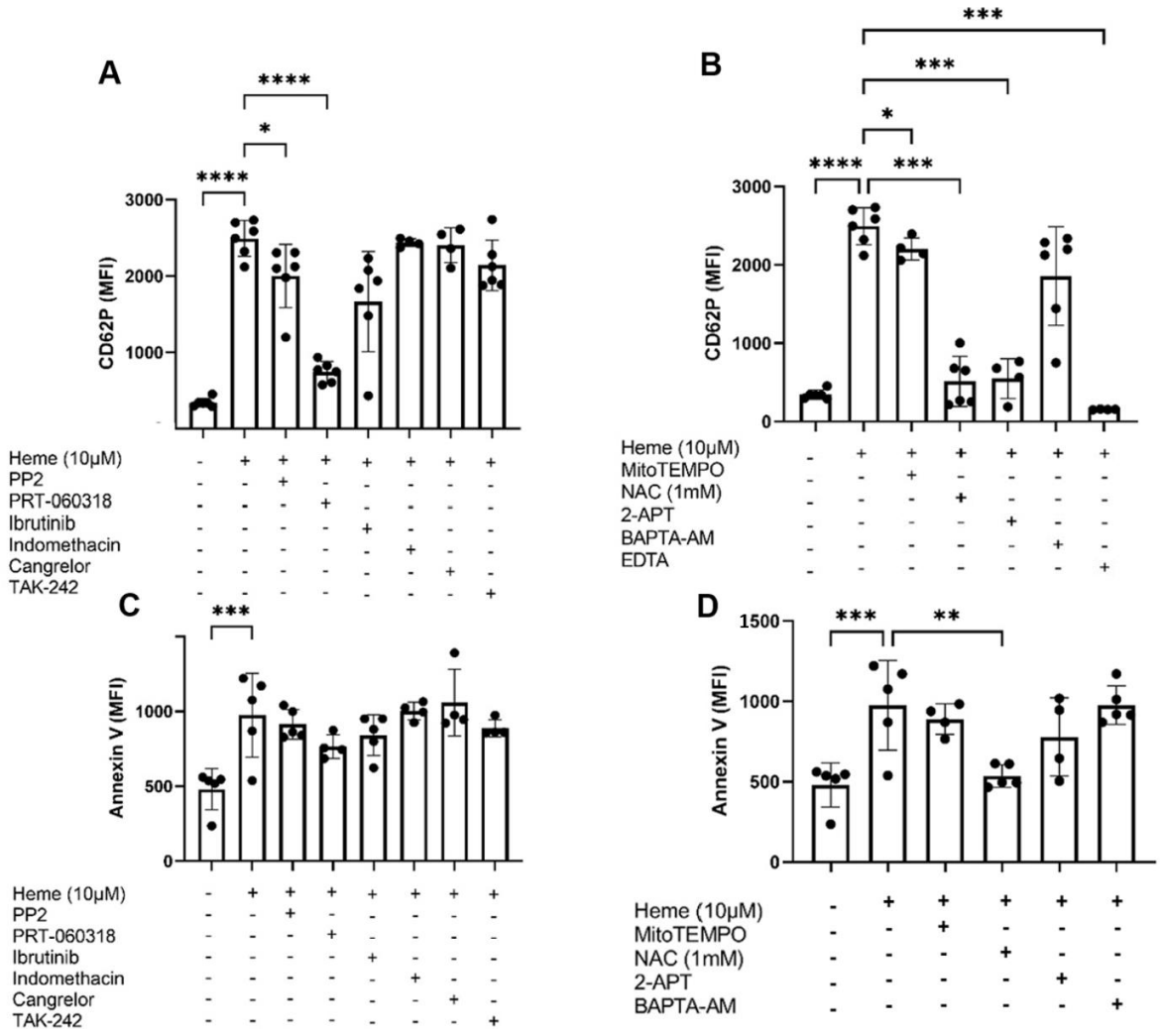


Figure 4.12 – Hemin-mediated mouse platelet activation depends on oxidative stress. Mouse washed platelets (10^6 platelets) were incubated with hemin (10μM) for 30min at 37ºC in the presence of $CaCl_2$ (2mM). Inhibitors were added 5min prior to heme. (A, B) Platelet CD62P expression and (C, D) PS exposure was assessed using anti CD62P antibody and Annexin V binding, respectively, by flow cytometry. Inhibitors were preincubated with platelets for 10 minutes at RT before addition of hemin. Inhibitors doses as per methods. Data represented as fold change in the mean fluorescence intensity (MFI) of agonist-activated platelets over control. The statistical significance was analysed using a Tukey's one-way ANOVA with multiple comparison and statistical difference showed compared to heme-stimulated platelets ****p <0.0001, ****p <0.001, **p <0.01, *p <0.05. Adapted from Ref (Bourne et al., 2021)

4.3.5 Hemin binds to human and mouse recombinant CLEC-2

The inhibition of hemin-mediated platelet aggregation by recombinant CLEC-2, suggests a direct interaction between hemin and CLEC-2. Here we demonstrated that hemin binds to both murine and human recombinant CLEC-2 with an affinity of ~200 nM, using surface plasmon resonance-based technique (**Figure 4.13**).

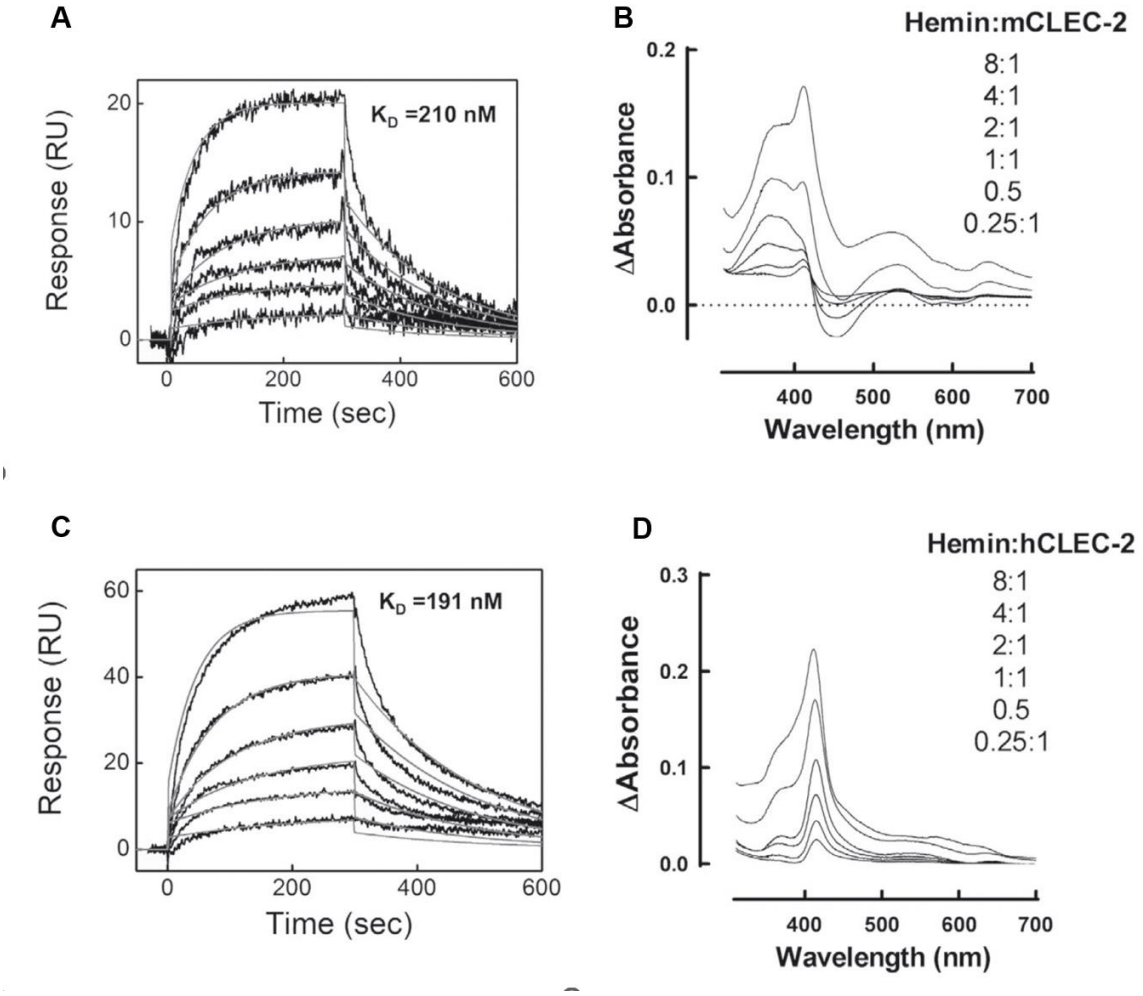


Figure 4.13 – Hemin binds to mouse and human recombinant CLEC-2. (A, C) Hemin (19.5–625 nM) at increasing doses was injected over recombinant mouse (A) and (C) human CLEC-2 immobilised on CM5 sensor chip for surface plasma resonance. Realtime interaction profiles were recorded. Association/dissociation was followed for 5 minutes. The analysis was performed with Langmuir global

analyses model. (B, D) Generation of differential spectra was obtained following titration of (B)mouse or (D) human dimeric CLEC-2 (2 μM) with increasing concentrations of hemin (0.5–16 μM) for UV-vis absorbance spectroscopy. The differential spectra were obtained after subtraction of the spectra of hemin at a given concentration from the spectra of the same concentration of hemin in the presence of the protein. Measures done at 25ºC in optical cell with 10 mm light path. Experiments performed and analysed by Dr Jordan Dimitrov. Adapted from Ref (Bourne et al., 2021)

Similar values of association and dissociation kinetic rate were found for hemin binding to either mouse CLEC-2 or human CLEC-2. The interaction of hemin with human and mouse CLEC-2 was further confirmed via absorbance spectroscopy by UV. The differential absorbance spectra profiles showed significant shifts of the spectra of hemin towards higher wavelength confirming direct binding.

4.3.6 Iron coordination in porphyrin ring mediates platelet aggregation

In order to try to identify heme components responsible for platelet activation, we investigated the possible interactions responsible for hemin-mediated platelet aggregation. It has been previously shown that platelet ferroptosis is inhibited by the iron chelator FS-1 (NaveenKumar et al., 2018), therefore we used different porphyrins and iron in the ferrous and ferric state (**Figure 4.14**). Hemin contains oxidized Fe^{3+} iron while protoporphyrin IX (PPIX), lacks the iron ion. PPIX failed to induce platelet activation (**Figure 4.14 B-D**) suggesting hemin-induced aggregation is dependent on the presence of the iron ion. This effect is independent of the concentrations used with only a non-significant platelet aggregation/agglutination induced at higher

concentrations (above 25 μM). Interestingly, the iron ion alone, at equivalent concentrations to hemin, is not sufficient to induce platelet aggregation as $FeCl_3$ (Fe^{3+}) or $FeSO_4$ (Fe^{2+}) failed to induce platelet aggregation (**Figure 4.14E**). These results suggest a key role for iron coordination within the tetrapyrrole structure for platelet aggregation. The importance of iron coordination in the tetrapyrrole structure is also seen when Fe (III) Deuteroporphyrin IX chloride (FeDP), Fe (III) Mesoporphyrin IX chloride (FeMP) or Fe (III) Protoporphyrin IX dimethyl ester chloride (FeDME) were used. These contain the iron ion with modifications in the tetrapyrrole structure. Protoporphyrin IX cobalt chloride (PP IX cobalt), a compound structurally similar to hemin aside from its cobalt core, induces platelet aggregation, although the amplitude of platelet aggregation is reduced compared to heme (**Figure 4.14 B-D**). A significant reduction in platelet aggregation is observed using different porphyrins suggesting that iron ion coordination and other interactions may also be involved in platelet aggregation. PPIX and cobalt hematoporphyrin were previously shown to inhibit podoplanin-CLEC-2 interaction without inducing platelet activation (Tsukiji et al., 2018). The type of interaction and the possible different binding sites of different porphyrins on CLEC-2, requires further investigation. These data are not included in the publication however offer preliminary data to develop molecules able to specific inhibit heme-CLEC-2 interaction.

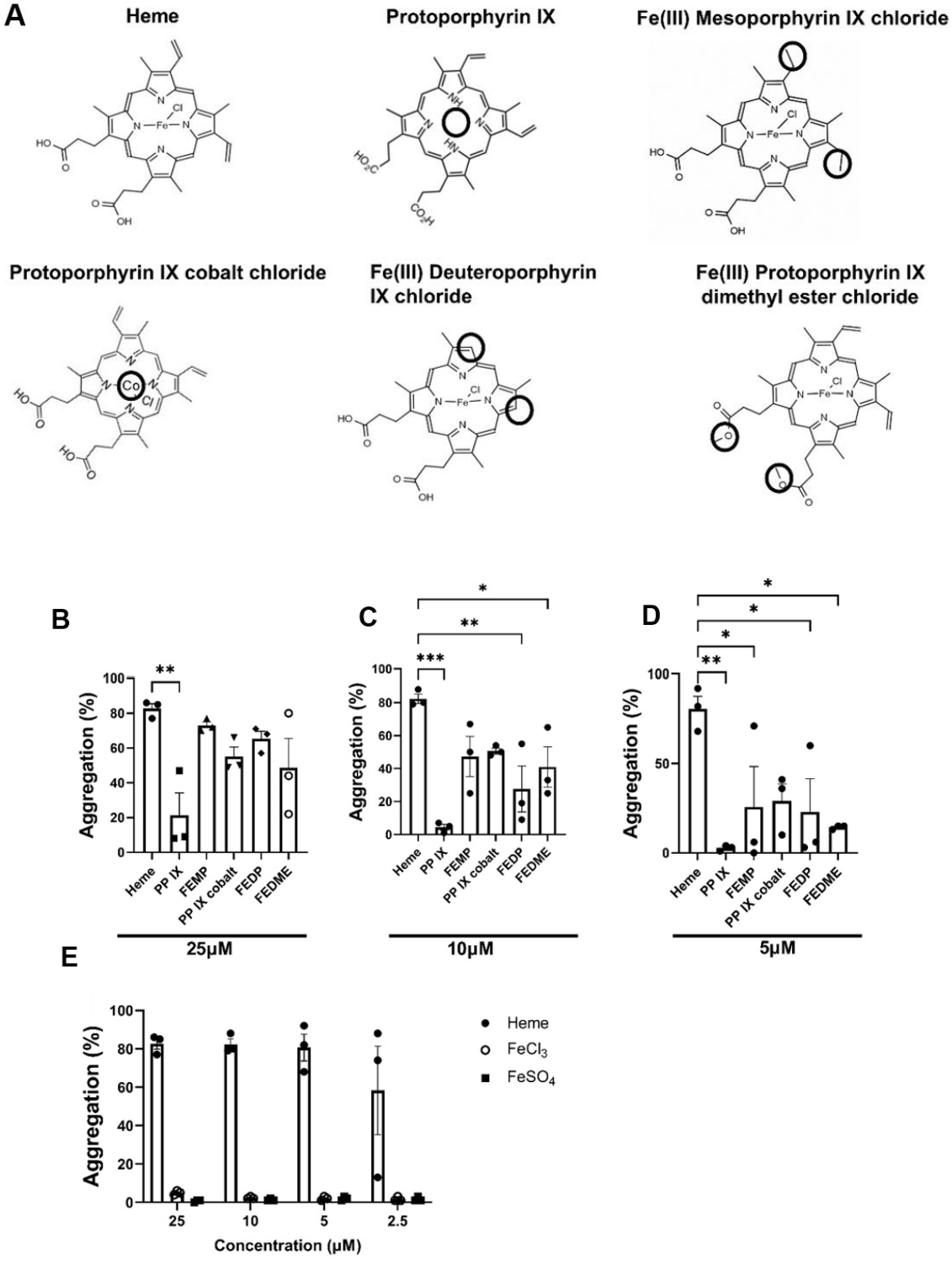

A
Heme
Protoporphyrin IX
Fe(III) Mesoporphyrin IX chloride
Protoporphyrin IX cobalt chloride
Fe(III) Deuteroporphyrin IX chloride
Fe(III) Protoporphyrin IX dimethyl ester chloride
B
C
D
Aggregation (%)
Heme
PP IX
FEMP
PP IX cobalt
FEDP
FEDME
25μM
10μM
5μM
E
Heme
FeCl3
FeSO4
Concentration (μM)

Figure 4.14 – Hemin-mediated platelet aggregation depends on the iron and its coordination in the porphyrin ring. Human washed platelets ($2x10^8$/ml) were incubated with different concentrations of hemin or other porphyrins in the presence of Ca^{2+} (2mM) and platelet aggregation assessed for 6 minutes using light transmission aggregometry. (A). Structure of porphyrins used in the experiment. (B, C, D). Platelet aggregation by porphyrins. (E). Platelet aggregation induced by $FeCl_3$ or $FeSO_4$ at 25 µM, 10µM and 5 µM. The statistical significance was analysed using a Tukey's one-way ANOVA with multiple comparison and statistical difference showed compared to untreated platelets **p <0.01, *p <0.05. Data not published.

4.3.7 Discussion

Thrombotic complications associated with haemolytic diseases are unpredictable and therapeutic intervention is quite challenging. During acute haemolytic events, the scavenging system becomes exhausted, leading to increased "free" heme in plasma which activates immune cells, endothelial cells and platelets. Platelet activation by hemin was previously reported in terms of cell death or ferroptosis (NaveenKumar et al., 2019) however identifying the precise mechanism leading to platelet activation in this context, is of extreme relevance.

This chapter shows that hemin is an endogenous agonist for CLEC-2 leading to platelet activation at low concentrations and possibly, agglutination of damaged platelets at high concentrations. The high concentration adopted in this work (50µM), corresponds to the one that can be detected in the plasma of patients with sickle cell disease during crisis, although it might represent the local accumulation of free heme on specific sites rather than systemic. Although the proportion of "free" heme and heme bound with low

affinity plasma proteins like albumin in patients cannot be easily discriminated using classical assays, the level of free heme during haemolytic conditions range between 2-8 µM, which correspond to the low doses used in this study. Indeed, at the site of acute haemolysis, the level of free heme could even exceed the one in the plasma. We observed high level of PS exposure on human platelets with high levels of heme, whether those cells exert procoagulant roles, requires further investigation. Importantly, platelet activation by heme was not altered by inhibiting $P2Y_{12}$ and COX-1, suggesting that heme-induced platelet activation is refractory to anti-platelet drugs.

Interestingly, the binding site of hemin on CLEC-2 seems to differ from the binding site for podoplanin (Tsukiji et al., 2018). Surface plasmon resonance demonstrates high affinity of hemin for its receptor, confirming direct binding. It is well known that heme binds also to other receptors (May et al., 2021, Oishi et al., 2021, Bourne et al., 2021) and TLR-4 has among the ones with highest affinities being within the micromolar range (Hopp et al., 2024). Although the research here described suggests that heme-induced platelet activation is independent of TLR4 and is mediated by CLEC-2, heme-mediated TLR-4 activation induces ROS generation in platelets (Annarapu et al., 2021). This suggests that complex mechanisms might affect heme preferential/competitive or parallel binding to different receptors and this requires further research. Mice platelets appear to be activated to hemin at a higher concentration than human platelets and importantly, a second platelet receptor together with CLEC-2, seems to be involved in mice. This receptor has been reported to be GPVI (Oishi et al., 2021). Indeed, CLEC-2 and GPVI deficiency has been described to improve rhabdomyolysis-induced acute kidney injury in mice (Oishi et al.,

2021). Our results show that this is specific for mice platelets and not human ones. Indeed, hemin-induced human platelet aggregation is fully prevented using hFc-CLEC-2 but not by hFc-GPVI, suggesting an exclusive role for CLEC-2 in human platelet activation. It has to be said that higher concentrations of hFc-GPVI than the ones described in this work, have been tested in our lab (data not shown), and this has been demonstrated to be able to block the effect of heme too. In consideration of the data from Oishi *et al.* who show that both CLEC-2 and GPVI have been identified as receptors for heme for human and mouse platelets, our data, might be related to the low blocking hFc-GPVI concentrations used.

Hemin was shown to induce ferroptosis in platelets, an iron-dependent form of cell death associated with lipid peroxidation associated with the depletion of the glutathione (GHS), which is responsible in reducing lipid peroxidation (NaveenKumar et al., 2019). Interestingly, it was demonstrated that ferroptosis induced by heme is mediated through ROS-driven proteasome activity and inflammasome activation in platelets (NaveenKumar et al., 2019). The electron microscopy images suggest the presence of lipid peroxidation leading to the formation of heme-dependent pores in dead platelets. Our data are in line with previous studies showing that platelet activation by hemin is dependent on the presence of the iron ion. Here we show that iron ion alone in the ferrous or ferric form is not sufficient to induce platelet activation, whereas the coordination of the iron ion in the tetrapyrrole is required. Moreover, addition of protoporphyrin IX lacking the iron, does not induce platelet aggregation. Protoporphyrin IX cobalt chloride was also able to induce platelet aggregation although it was less potent than hemin, suggesting that coordination of divalent ion in the tetrapyrrole

induces platelet aggregation. However, iron coordination seems more potent than cobalt. Cobalt Protoporphyrin IX was recently shown to inhibit CLEC-2-podoplanin interaction and to reduce thrombosis and cancer metastasis in mouse models (Tsukiji et al., 2018). It can be speculated that a competition with heme for CLEC-2 activation might exists in this context. Therefore, different porphyrins interacting with platelets can act as inhibitors or agonists for platelet activation. Whether different porphyrins can activate platelets differently and to a different extent, requires further investigation and could open the door for new treatments in haemolytic diseases.

Interestingly hemin-dependent aggregation and P-selectin expression is independent from oxidative stress in human platelets but are required for mouse platelets. These data support the data from Annarapu *et al*., which showed hemin-driven mtROS induced secretion of only specific proteins from platelet granules. It is possible that heme induces specifically P-selectin increase and platelet aggregation via CLEC-2 while ROS generation and the secretion of other proteins (such as thrombospondin) via other receptors such as TLR-4. In this respect, heme-induced ROS does not seem to be important for platelet aggregation. The independence of P-selectin expression from oxidative stress, suggest that also interaction with immune cells and endothelial cells might not be reduced by anti-oxidant compounds. Another explanation for the difference described between mouse and human, is the contribution of GPVI in mice and not in human heme repsonses. Indeed, GPVI signalling involves NOX activation and this could contribute to mouse platelet activation by heme (Walsh et al., 2014). This selective activation requires further studies and might be useful in the diseases.

In clinical practice, different molecules are either on the market or currently under clinical trial, to target heme and its toxic effects. In mammals, heme homeostasisis primarily controlled indirectly by the binding of haemoglobin through haptoglobin, and directly by both hemopexin, which scavenge heme in the circulation and HO-1, which degrades heme intracellularly. There are also other minor proteins, such as albumin (Little and Neilands, 1960), α1-microglobulin (Allhorn et al., 2003)and α1-antitrypsin (Karnaukhova et al., 2012) which can bind heme in the circulation. Furthermore, intracellularly, proteins such as glutathione-S-transferases, heme-binding protein/ liver fatty-acid binding protein, heme-binding protein 23/peroxiredoxin 1, p22 heme binding protein and glyceraldehyde-3-phosphate dehydrogenase, can bind heme and could prevent its potential toxic effect or its degradation (Liem et al., 1994, Yuan et al., 2016, Harvey and Beutler, 1982, Taketani et al., 1998, Chakravarti et al., 2010). Any intervention aimed at increasing the levels or the function of those proteins, could be beneficial in the clinical setting of heme-induced haemolysis. Indeed, sequestration of free heme by Hemopexin has been shown to block heme-driven tumour growth and metastases (Canesin et al., 2020). Administration of haptoglobin, has been proposed to be adopted to reduce the toxic effect of heme (Vallelian et al., 2022, Graw et al., 2016). These two molecules have the potential to be injected intravenously. Due to the known protective effects of Hemopexin and Haptoglobin in haemolysis, it is reasonable to assume that combined treatment with these two proteins may provide a synergistic protection (Graw et al., 2016, Deuel et al., 2015, Schaer et al., 2014). In contrast, potential interventions with HO-1 appear to be more complex because this molecule is upregulated directly by heme and haemoglobin themselves, therefore inducing its

upregulation might induce toxic effects itself. Potential therapeutic applications of HO-1 may involve cell type-specific modulation (for example immune cells), or gene therapy selectively targeted at increasing this molecule (Funes et al., 2020, Abraham et al., 2007). Further studies are required before HO-1 therapeutic strategies become applicable in clinical practice. The other approach to prevent the deleterious effects of heme would be interfering with its receptors and pathways on heme-target cells. Drugs targeting platelet-ITAM receptors could be beneficial and one of them, Ibrutinib, has already been implemented in the clinical practice. Indeed, Ibrutinib has been shown to be an effective treatment of autoimmune haemolytic anaemia in the contest of leukaemia (Manda et al., 2015) and multiple clinical trials are still ongoing or being published (Kander et al., 2019). More information regarding clinical trials assessing therapeutic benefit of heme scavengers in haemolytic diseases can be found at https://clinicaltrials.gov/. Its effect is the results of both immunomodulatory and anti-thrombotic functions. Further translational studies would need to be conducted.

This study shows some limitations. All work was performed in washed platelets *in vitro* and therefore the relevance of CLEC-2 in haemolytic diseases would need to be addressed in animal models (such as sickle cell disease).

In conclusion, we provide evidence that heme is a novel agonist to platelet receptor CLEC-2. Recombinant CLEC-2 is shown to be able to block both mechanisms suggesting this might be a therapeutic intervention in haemolytic disease. Adopting antioxidant treatments as NAC, does not seem to offer additional support in preventing human platelet aggregation and activation. Heme is not only elevated in haemolytic

disease but also in other conditions such as DVT, ischemic heart disease and heart failure and can cause secondary infections in patients with comorbidities. Therefore, further work on heme and thromboinflammation might bring an extended benefit.

CHAPTER 5

General Discussion

5.1 Summary of Results

The work presented in this thesis describes the mechanisms by which two different DAMPs, S100A8/A9 and hemin, affect platelet function and thromboinflammation. In particular we studied the effect of a non-disease specific DAMP (S100A8/A9) released mainly by neutrophils during NETosis, which is tightly linked to thrombosis and elevated non-specifically during inflammation and infection. Furthermore, we studied a disease specific DAMP, hemin, which is released by erythrocytes during haemolysis. We hypothesised that both those DAMPs are not only biomarkers of disease severity and thrombotic risk but they exert direct effects on platelets. We aimed to determine the mechanism by which these DAMPs affect platelet function and to identify possible therapeutical targets. We identified: (*i*) a role for S100A8/A9 in activating platelets inducing predominantly a pro-coagulant phenotype; (*ii*) a role for hemin, in activating platelets at lower concentration and causing agglutination by cell death at higher concentration; (*iii*) we demonstrated that platelet activation induced by S100A8/A9 or hemin is resistant to COX and P2Y12 inhibitors; (*iiii*); we identified CLEC-2 as the main receptor for hemin on platelets mediating platelet activation; and (*iiiii*) we showed a key role for GPIbα in mediating S100A8/A9-induced procoagulant platelet formation, highlighting the existence of multiple pathways by which DAMPs regulate platelet activation in disease state. Overall, we showed that S100A8/A9 and hemin contribute to platelet activation and therefore we highlighted the need to target those DAMPs in the context of thrombotic diseases especially when associated with inflammation. According to the type of disease, there would be a DAMP-specific therapy to be associated with currently used anti-platelet and anti-coagulant drugs. DAMPs can

either be aspecifically associated with inflammation or be very specific to the type of disease, as for S100A8/A9 and hemin respectively. Our data reinforce and implement the existing literature opening the field to further research.

5.2 NETs-associated DAMP: S100A8/A9

This interlink between danger molecules released through inflammatory mechanisms and thrombosis has been described in multiple disease conditions. Inflammation and coagulation have a close relationship and this has an evolutionary origin (Levi et al., 2004). Indeed, effective host defence upon pathogen invasion of the bloodstream requires both proinflammatory and prothrombotic responses. However, dysregulation in the balance between these components can result in uncontrolled inflammation and thrombosis (Foley and Conway, 2016). Under physiological conditions, platelets circulate in the bloodstream in a quiescent state. The endothelium is covered by a protective glycocalyx (Megens et al., 2007, Wang et al., 2005, Zeng and Tarbell, 2014) and releases molecules that prevent platelet activation such as CD39 (Kanthi et al., 2019), nitric oxide and prostacyclin (Su, 2015). Upon endothelial activation, platelets become activated and adhere to the exposed endothelium favouring thrombotic events. Inflammation induces platelets, leukocyte and endothelial cell activation leading to endothelial cell dysfunction and thrombosis (van der Poll and Parker, 2020). Once inflammation is triggered, various inflammatory mediators are secreted such as chemokines, cytokines and growth factors. Those can activate immune and endothelial cells supporting inflammation and fuelling thromboinflammation. Moreover, some

signals can directly activate platelets (Cognasse et al., 2015). The dialogue between inflammatory and thrombotic processes is bidirectional. For example, thrombin has been described to augment the expression of proinflammatory proteins such as IL-1, IL-6, and IL-8, MCP-1, ICAM-1 and VCAM-1) by endothelial cells whilst exerting prothrombotic effects (Rahman et al., 2002, Colotta et al., 1994, Marin et al., 2001, Minami and Aird, 2001, Okada et al., 2006). On the other side, chemokines and cytokines increase thrombotic pathways. In particular neutrophils, in addition to their ability to undergo phagocytosis, they also release NETs, which are a network of extracellular fibres composed of DNA-histone complexes and various proteins such as MPO, elastase, histones, S100A8/A9 and others. NETs constitute a crucial link between inflammation and thrombosis providing a scaffold for platelet adhesion and aggregation (Ma, 2022, Fuchs et al., 2010) but also carrying and secreting prothrombotic danger molecules (Thålin et al., 2019, Ma, 2022). Some of these DAMPs have been described to play a supporting role in platelet activation (Colicchia et al., 2023). (**Figure 5.1**).

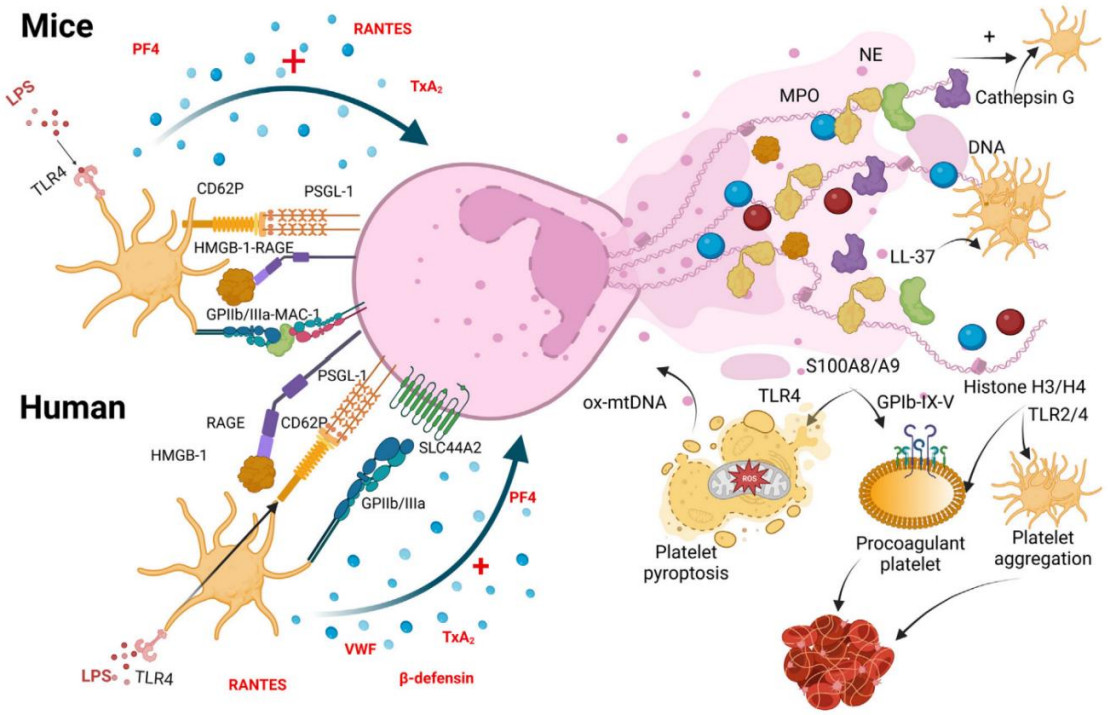


Figure 5.1 – Effect of NET-associated DAMPs on platelets. Platelet activation supports NETosis through different agonists and receptors. In LPS-stimulated platelets, CD62P contributes to platelet-dependent NETosis, HMGB1 supports NETosis through RAGE in humans and mice while GPIIb/IIIa mediates NETosis through Mac-1 in mice. vWF-GPIbα priming of human platelets triggers GPIIb/IIIa-dependent NETosis through SLC44A2. Multiple NET-associated DAMPs trigger platelet activation. Cathepsin G induces platelet aggregation, and neutrophil elastase amplifies this effect. Histones, induce platelet aggregation and the formation of procoagulant platelets mainly through TLR2/TLR4. S100A8/A9 supports the formation of procoagulant platelets through a mechanism involving GPIb-IX-V and induces platelet pyroptosis through TLR4 with the subsequent release of oxidized mitochondrial DNA (ox-mtDNA) supporting NETosis and amplifying thromboinflammation. Figure from Ref (Colicchia *et al.* 2023).

Several molecules have been described to be involved in thromboinflammation with S100A8/A9 emerging as a marker of inflammation and disease severity. Moreover, S100A8/A9 levels are associated with thrombotic complications. S100A8/A9 has been associated with diseases such as cardiovascular disease, diabetes, rheumatoid

arthritis and sepsis. S100A8/A9 can be detected in several bodily fluids and it has already been studied as a potential biomarker in cystic fibrosis, intestinal bowel disease and rheumatoid arthritis (Ometto et al., 2017, Wang et al., 2018). In those conditions the levels of S100A8/A9 predicted severity of disease and complications. Recently, high levels of S100A8/A9 have been described to be persistently detectable during COVID-19 by multiple studies. S100A8/A9 has been described to be a biomarker of disease severity in COVID-19 (Wang et al., 2018, Chen et al., 2020b, Silvin et al., 2020, Malik et al., 2021, Ponti et al., 2020, Henry et al., 2020). Indeed, patients with COVID-19 and high levels of S100A8/A9 on admission, were more likely to suffer from multi organ failure within 72h (Bauer et al., 2021). In our work we confirm that S100A8/A9 is persistently elevated in patients with COVID-19 and is associated with ITU admission and death. While serum levels in healthy individuals are typically below 1 ug/mL, these levels are reported to be nearly 100-fold higher during inflammation (Wang et al., 2018, Ometto et al., 2017). Although being a non-specific biomarker of inflammation, S100A8/A9 has some advantages when compared to other commonly used inflammatory biomarkers such as C-reactive protein (CRP) or calcitonin (CT). It is rapidly secreted during inflammation and furthermore, it has a half-life of 5 hours and it is stable at room temperature, making it a favourable biomarker for diseases as shown in COVID-19 (Silvin et al., 2020, Liao et al., 2020). Being a non-specific inflammatory molecule, plasma levels of S100A8/A9 levels have been demonstrated to be higher in patients with COVID-19 and other inflammatory comorbidities compared to those without them (Silvin et al., 2020). Nevertheless, the observed increase in S100A8/A9 in patients with severe COVID-19 exceeded

correlations associated with comorbidities such as obesity, diabetes mellitus, cancer, cardiovascular and respiratory disease (Silvin et al., 2020). It is important to perform further studies to determine potential confounding factors before using S100A8/A9 as a clinical biomarker.

Multiple studies have also described the predictive capacity of S100A8/A9 specifically for thrombotic complications in both COVID-19 and other diseases. It has been already described that S100A8/A9 pro-thrombotic effects include its role on primed endothelial cells (Viemann et al., 2005) and S100A8/A9 deficiency in mice protects from thrombosis (Wang et al., 2014). Indeed, S100A8/A9 has been shown to trigger endothelial activation in AGE-primed endothelial cells, characterised by increased production of cytokines and adhesion molecules (Bresnick, 2018, Chen et al., 2013b, Scott et al., 2020). Furthermore, it increases platelet aggregation on the endothelial surface. Additionally, endothelial cells exposed to S100A8/A9, were shown to downregulate genes responsible for the endothelial cell integrity (Bresnick, 2018, Scott et al., 2020) and extended S100A8/A9 exposure was shown to induce endothelial permeability (Scott et al., 2020). Interestingly, platelets from patients with COVID-19 were shown to upregulate S100A8/A9 and platelet releasate induces endothelial cell activation. In our work we showed that S100A8/A9 contribute to thrombosis by direct activation of platelets and the formation of procoagulant platelets *in vitro* involved in increased fibrin formation.

In our work we found a fraction of platelets containing S100A8/A9 and a recent publication showed S100A8/A9 is also contained in a fraction of megakaryocytes from

severe COVID-19 patients (Fortmann et al., 2023). Those megakaryocytes were detectable in the peripheral blood and were shown to have an inflammatory phenotype. It is possible to speculate that platelets or megakaryocytes are capable of producing S100A8/A9 as S100A8 mRNA was found in ST-myocardial infarction platelets. Whether platelets contribute to the source of S100A8A9 and whether S100A8A9 could reflect platelet activation has not been fully clarified yet. It is also possible that neutrophils, which are the main source of S100A8/A9, transfer S100A8/A9 to platelets which can subsequently be released and act locally at the site of inflammation to induce procoagulant platelet formation. Our data doesn't show the cellular origin of S100A8/A9 but, considering S100A8/A9 levels transcripts in platelets from patients with COVID-19 were found to be 1000 times less than in neutrophils (Fortmann et al., 2023), it is likely that neutrophil-derived S100A8/A9 represents the major source of plasma S100A8/A9. After being released, neutrophil-derived S100A8/A9 can bind to platelets and support procoagulant platelet formation. More recently, S100A8/A9 was shown to induce pyroptosis in platelets during sepsis, leading to the release of oxidised mitochondrial DNA which fuel NET formation and further S100A8/A9 release favouring thrombosis(Su et al., 2022). This mechanism is dependent on the classical receptor for S100A8/A9, TLR4. Interestingly, we didn't observe a role for TLR4 in the formation of procoagulant platelets by using a small molecule inhibitor (TAK-242) or by addition of antibodies blocking S100A8/A9 binding to TLR4. Preliminary work shows that S100A8/A9 seems to preferentially bind to distinct platelet populations which can result in differential activation mechanisms, these data were not part of the current research

discussed. Whether TLR4, CD36 and GPIb cooperate to modulate these different binding capacity and activation pathways requires further investigation.

5.3 Targeting S100A8/A9 in thromboinflammation

Due to the relevant pro-thrombotic and pro-inflammatory effects of S100A8/A9, it has already been proposed as a valuable therapeutic target. The main therapeutical strategies involve targeting S100A8/A9 receptors. Paquinimod, is a molecule specifically able to inhibit the binding of S100A9 to TLR4, suggesting its potential role as S100A8/A9 blocker (Schelbergen et al., 2015, Björk et al., 2009). Paquinimod treatment in SARS CoV-2-infected mice, lowered the accumulation of aberrant neutrophil subsets and inhibited viral replication (Guo et al., 2021). Differently, SARS-Cov-2-infected mice treated with azeliragon, a RAGE receptor inhibitor, did not affect mice prognosis, suggesting TLR-4 could be the main driver for inflammatory responses in mice. Pharmacological inhibition using Paquinimod through targeting S100A8/A9-TLR4 signalling axis, improved survival in mice with CLP-induced sepsis by suppressing platelet pyroptosis (Su et al., 2022). Another therapeutical molecule (ABR-238901), which targets both RAGE and TLR-4 receptor, showed reduced myocardial inflammation and post-ischemic myocardial injury in a mouse model of coronary artery ligation (Mareş et al., 2023, Marinković et al., 2019). Furthermore, it appeared to prevent myocardial compensatory hypertrophy, and post-ischemic stress (Boteanu et al., 2022, Marinković et al., 2019, Mares et al., 2024, Jakobsson et al., 2023). On the other side, due to the broad cellular effects of S100A8/A9 on many cell types,

therapeutic targeting of S100A8/A9 especially at its inflammatory side, may pose major challenges and further studies are required. Indeed, unselected long-term targeting of S100A8/A9 has been already proven to lead to gradual deterioration of cardiac function and accelerate left ventricular remodelling post myocardial infarction (Marinković et al., 2020). Indeed, blockade of S100A8/A9 during sepsis could represent a therapeutic strategy, as surviving patients have decreased S100A8/A9 levels compared with non-survivors (Dubois et al., 2019, Hofer et al., 2016, Miyashita et al., 2022). However, it remains to be proven that blocking S100A8/A9 during infections would not interfere with pathogens clearance.

Our data showed that S100A8/A9-induced platelet effects are mainly mediated by GPIb receptor. This data is unexpected and little information on the interaction of GPIb, solely, or in complex with other receptors are available. As GPIb is considered a receptor with weak signalling, along that the formation of procoagulant platelets requires strong and sustained intracellular calcium, it is likely that GPIb cooperates with other receptors, including CD36, to induce procoagulant platelet formation.

The main concern with blocking GPIb receptor, includes its central role in binding vWF, with the subsequent risk of inducing excessive bleeding when completely blocked. However, blocking GPIb is has been shown to reduce stroke size and improve functional outcome without increasing the risk of intracerebral haemorrhage in mice while it increased the clinical severity in preclinical models of sepsis (Schuhmann et al., 2017, Corken et al., 2014). Therefore, as antibodies targeting the binding site of GPIb to vWF decreased S100A8/A9 effect, it is likely that targeting GPIb might

increase the bleeding risk. A better understanding of the role of GPIb in this process and the identification of other partners and their cooperation is essential for development of new treatments.

A peptide called R9α557, which comprises a sequence of amino acids from the GPIb cytoplasmic region, reduced vWF-mediated adhesion in human platelets (David et al., 2006). Additionally, the monoclonal antibody, 6B4, that targeted the GPIb receptor in nonhuman primates, could reduce thrombus formation without significant increase in bleeding time (Cauwenberghs et al., 2000). In rat, a platelet GP1b receptor antagonist, called VCL, reduced platelet adhesion and the extent of intimal thickening following balloon injury to the femoral artery (Zahger et al., 1995). Another compound, called anfibatide, was shown to inhibit the GPIbα-vWF interaction both *in vitro* and *in vivo* (Li et al., 2021, Cheng et al., 2000). The safety and efficacy of anfibatide has been also tested in Phase I healthy human individuals, showing antithrombotic properties (Hou et al., 2013). Following this initial study, anfibatide has also been tested in Phase Ib/IIa study in patients with ischemic cardiovascular disease (Zheng et al., 2021). This study showed that intravenous administration of anfibatide was able to inhibit platelet aggregation without increasing the risk of thrombocytopenia and bleeding in non-ST elevation myocardial infarction (NSTEMI) patients undergoing percutaneous reperfusion (PCI). Further investigations are warranted to determine the safety profile for patients with a high intracoronary thrombus burden and optimal dosing.

5.4 Disease-specific DAMP: Heme

Differently from S100A8/A9, heme is a DAMP mostly released under haemolytic conditions. It also can be released from myoglobin during muscle injury and act extracellularly as a DAMP. Several pathological conditions are associated with haemolysis or myolisis causing the plasma levels of free heme to increase (sickle cell anaemia, thalassemia, malaria, paroxysmal nocturnal haemoglobinuria, rhabdomyolysis) (Gozzelino et al., 2010). The increase in free heme could be either secondary to an alteration in the metabolism of heme or accelerated erythrocytes and muscle breakdown. Heme is a potent proinflammatory and prooxidant molecule which can act on a large number of host cells leading to organ damage. Free heme is a source of redox-active iron involved in the production of radicals. ROS damage lipid membranes, nucleic acids and proteins, activating the transcription of proinflammatory factors and altering membrane channels (Vercellotti et al., 1994, Jeney et al., 2002). Its ability to intercalate into lipid membranes, heme alters membrane permeability, cell lysis and death (Balla et al., 1991, Ryter and Tyrrell, 2000, Kumar and Bandyopadhyay, 2005, Tolosano et al., 2010). Indeed, in our studies, we showed that high concentrations of heme induce platelet death, independent of platelet receptors. This data shows that heme acts in receptor-dependent and independent manner leading to a plethora of actions on host cells. Heme is also a strong pro-inflammatory molecule inducing the recruitment of leukocytes, platelets and red blood cells to the vascular endothelium, and consuming nitric oxide, thus impairing vascular function. Many of these inflammatory functions depends on the interaction of heme with its receptor

TLR4. Interestingly, we showed that TLR4 was not involved in heme-mediated platelet activation and we identified CLEC-2 as the major receptor for heme on platelets. GPVI was also shown to contribute to heme-mediated platelet activation in mice suggesting that both ITAM receptors are involved in heme-mediated platelet activation. However, Annarapu *et al* showed that extracellularly heme acts through TLR-4 on platelets by inducing mitochondrial ROS production. This shows that heme can bind multiple receptors on platelets inducing several pro-oxidant and prothrombotic functions as ROS stimulate platelet granule secretion and indirectly platelet activation (Annarapu et al., 2021). Therefore, heme by acting through multiple receptors, and in receptor-independent manner, it is likely that combination of therapies or heme-specific therapies are required to inhibit its effect on host cells.

5.5 Targeting heme in thromboinflammation

Currently, no clinical trials targeting CLEC-2 in thromboinflammation are available. This is perhaps due to the concern regarding the important role of CLEC-2 in haemostasis. Data from our lab show a negligible role of CLEC-2 in human thrombus-growth providing evidence that it could be a valid therapeutic target. There are specific molecules, such as Fostamatinib, a Syk inhibitor, which is clinically approved to treat thrombocytopaenia (Connell and Berliner, 2019), or BTK inhibitors, such as Ibrutinib (Davids and Brown, 2014, Byrd et al., 2013, Byrd et al., 2015, Wen et al., 2021), which are currently widely used for haematological diseases which inhibit platelet ITAM receptor and could be considered in thromboinflammation. In particular low-dose BTK

inhibitors have been shown to block specifically CLEC-2 without affecting GPVI (Nicolson et al., 2021), offering a potential way to target thrombosis without affecting bleeding. Our data show that heme concentration induces different effects on platelets. This evidence suggests that therapies aimed at preventing heme effects on platelets might vary according to the levels of heme. It is important to note that low concentrations of heme reflect the systemic levels whereas high concentrations of heme reflex the level of heme that could be accumulating at the site of haemolysis. Considering the effect of heme on ROS production, lipid peroxidation and the role of mitochondrial ROS on platelet granule release, anti-oxidant agents, alongside CLEC-2 inhibition could be studied in haemolytic conditions. This combination of treatment may be of benefit for both thrombosis and further haemolysis exacerbation. Currently, hemopexin, which selectively binds and inhibit heme was shown to limit vaso-occlusive crisis and to improve organ function in sickle cell patients (Gentinetta et al., 2022, Xue and Li, 2024). As hemopexin inhibits heme interaction with all its receptors, it is a promising strategy to limit its detrimental functions (Gentinetta et al., 2022).

5.6 Cardiovascular Implications

Although not specifically explored in this thesis, both S100A8/A9 and hemin play a crucial role in cardiovascular disease and offer a potential therapeutic target in this context. Cardiovascular disease is the main cause of death worldwide and cardiovascular inflammation is becoming an interesting target to prevent its

progression. There have been multiple lines of research on inflammation associated with cardiovascular disease.

Both S100A8/A9 and heme contribute to the development and worsening of atherosclerosis. The atherosclerotic plaques, especially the necrotic core, contain apoptotic macrophages, erythrocytes and its metabolites (including heme), and cytotoxic substances such as oxidized lipids, fibrin and calcium (Li et al., 2006). RBCs, and their products (haemoglobin, heme, iron) contribute to plaque inflammation and toxicity (Nagy et al., 2010). When exposed to oxidized atherosclerotic plaque materials, erythrocytes undergo lysis and liberate heme which can be captured by lipoproteins such as low-density lipoprotein (LDL) and high-density lipoprotein (HDL) when the plasma heme scavenger Hemopexin is saturated (Chistiakov et al., 2015). At the same time, the lipophilic properties of heme enable its combination with oxygen radicals (Van der Zee et al., 1996), stimulating the lipid peroxidation cascades and the production of oxidized LDLs (ox-LDLs) itself. Also, S100A8-S100A9 complexes of different sizes were found in human carotid plaques contributing to plaque inflammation (McCormick et al., 2005). Whether targeting S100A8/A9 and heme on plaques could prevent their progression and their interaction with platelets, would be relevant to investigate. Heme is highly involved not only in atherosclerosis but also other cardiovascular conditions.

S100A8/A9 plays a major role after AMI, as its highly expressed in neutrophils, which dominate the early immune response post-acute cardiovascular events (Sreejit et al., 2020b, Sreejit et al., 2020a). The released S100A8/A9 interact with TLR4 on naïve neutrophils, priming the Nlrp3 inflammasome and promoting IL-1β release which

increments inflammation itself (Sager et al., 2015). While S100A8/A9 acutely appears to be detrimental in ACS, it seems to me crucial for myocardial repair during the chronic phase following AMI (Chen et al., 2021b). Interestingly, heme, plays a similar dual effect. While being toxic to cardiomyocytes, it also activates heme oxygenase-1 (HO-1), an enzyme with anti-inflammatory and cytoprotective properties and it has been shown to be able to switch macrophages into an anti-inflammatory phenotype, improving infarct healing and repair (Ben-Mordechai et al., 2017). Plasma levels of S100A8/A9 are an early biomarker of ACS (Altwegg et al., 2007). Whether S100A8/A9 and heme contribute directly to platelet aggregation and worsening of thrombus burden during ACS, remains to be demonstrated.

Compounds like ABR-238901, tasquinimod (ABR-215050) and paquinimod (ABR-215757) have been shown to block the interaction of S100A8/A9 with TLR4 and RAGE (Björk et al., 2009, Källberg et al., 2012). These compounds have been reported to reduce inflammation and disease progression in a number of inflammatory models (Raymond et al., 2014, Fransén Pettersson et al., 2018, Tahvili et al., 2018, Wache et al., 2015, Kraakman et al., 2017). Those could be potentially repurposed to target cardiac inflammation post-AMI. Interventions to reduce S100A8/A9 secretion by inhibiting NETosis may also have suppressive effect on inflammasome activation and subsequent secretion of IL-1β (Nagareddy et al., 2020) Therapeutically, short-term pharmacological inhibition of S100A8/A9 with ABR-238901 mitigated both local and systemic inflammation (Marinković et al., 2019). Interestingly, long-term inhibition of S100A8/A9 with the same drug led to deterioration of cardiac function over time likely for defective clearance of apoptotic cardiomyocytes and larger myocardial scar

(Marinković et al., 2020). Therefore, targeting S100A8/A9 could require a close monitoring of its plasma levels and should be tailored on disease progression timing. Less data is available for heme, but its dual effect might need to be taken in consideration for potential therapies.

Both those DAMPs have been shown to contribute to the development of heart failure. Heme has been demonstrated to be toxic for cardiomyocytes. Heme-injured myocytes exhibit reduced beating rates, and increases enzymes losses (Jeney et al., 2002) by post-translational modification of contractile proteins and oxidation of myofilament proteins (Alvarado et al., 2015). In haemolytic mice, free heme accumulates in the heart, affect Ca^{2+} homeostasis, and impairs systolic function (Ingoglia et al., 2017). All of those can be detrimental for the development of myocardial ischemia-reperfusion injury and heart failure. Indeed, in a murine model for haemolytic diseases, hemopexin, restores myocardial function (Vinchi et al., 2013). Heme has been shown to be elevated in human failing hearts mainly for increase in the δ-aminolevulinic acid synthase 2 (ALAS2), the rate-limiting enzyme in heme production. Furthermore, heme-induced ferroptosis drives cardiomyopathy (Menon et al., 2021). S100A8/A9 released from infiltrating neutrophils causes damage to the mitochondrial electron transport chain leading to cardiomyocyte death. In elderly patients with severe heart failure, the high levels of S100A8/A9 proteins positively correlated to IL-6 predicting the mortality of patients for 6 and 12 months (Ma et al., 2012). Whether the effects of S100A8/A9 and heme in the failing heart are only due to pro-oxidant effects in cardiomyocytes or there is also a platelet contribution, remains to be demonstrated.

This evidence shows that in the foreseeable future, ‘cardio-inflammatory’ patient phenotypes could be identified by multiple biomarkers (S100A8/A9, NETs biomarkers, heme). Such individual might benefit for prevention or as an additional therapy of DAMPs-targeted therapies as enhanced treatment options. This stratification could be relevant for several sterile and infectious pro-thrombotic clinical conditions.

5.7 Limitations of the study and future work

The work we conducted on purified S100A8/A9 and heme has some important limitations and offer good potential for further investigations. This work highlighted some important results that require further studies.

First of all, we described variability in the response intensity to S100A8/A9 between subjects. The small number of healthy volunteers we have studied, did not allow to identify demographical or pathogenic features associated with high or low response to S100A8/A9. As GPIb might cooperate with other receptors, it is possible that the expression and distribution of other receptors determine the variation among donors. This suggests that, describing the risk factors associated with high response to this alarmin, could allow the identification of subjects that require specific additional treatments to prevent and treat thromboinflammation. In the era of personalised medicine, patients could be stratified in high/low risk of S100A8/A9-driven risk of thrombotic complications and be treated accordingly. However, this work requires a better understand of the mechanism by which S100A8/A9 affect platelet function and

identify the interplay between GPIb and other receptor in this process. In the context of heme, we described a different platelet response according to heme dose. Heme circulatory levels could predict either platelet aggregation or agglutination as well as mainly thrombotic vs pro-oxidant effects which could dictate specific therapies. In this context, further studies are required to identify cut-offs that could define prognosis and offer a specific therapeutical plan. It is also important to measure the level of free heme and not only total heme in the plasma as its prothrombotic effect is strictly related to the free form. However, most clinical assays can't distinguish between the two forms and further work is necessary to determine the local and system concentrations of free heme in patients.

It is important to note that both S100A8/A9 and heme have other receptors rather than only GPIbα and CLEC-2 respectively, including TLR-4 and RAGE. Those receptors are both present on endothelial cells and immune cells and they are upregulated during acute and chronic inflammation. It could be speculated that when S100A8/A9 or hemin increase their level rapidly, they might bound initially to those receptors and only after saturation, to platelet GPIbα and CLEC-2, exerting pro-thrombotic effects. Although not explored in this thesis, we can't rule out a cooperation between GPIb, CLEC-2 and TLR4/RAGE and that these DAMPs act through complex mechanisms in which GPIb and CLEC-2 play central roles. In a condition of chronic inflammation or chronic haemolysis, both S100A8/A9 and hemin could be elevated in the circulation and tissues, saturating their immune and endothelial cell receptors baseline and activating platelets upon any additional acute inflammatory or haemolytic stimulus. In chronic inflammatory and haemolytic conditions, S100A8/A9 and hemin could already partially

activate platelet receptors, offering a possible explanation for the persistent pro-thrombotic risk in those patients. It is important to underline that in a pathological condition, it is very likely that multiple DAMPs (e.g. histones, mitochondrial DNA, HMGB1, cytokines, etc) will be released simultaneously, therefore they could increase their reciprocal effects or also compete for the same receptors as well as promote their reciprocal secretion from immune cells. It is also possible that the binding of one DAMP on platelets alters platelet receptor expression and distribution, altering the response to other DAMPs. Further research is warrant and, in this context, and the use of more developed methods including multiple variables, should be performed.

Another aspect that requires further research, is the possibility that both S100A8/A9 and heme are buffered in the circulation by proteins (such as albumin), which might prevent their unwanted activation. It is already well understood that heme is bound by albumin and hemopexin whilst the binding partner for S100A8/A9 acquire a conformational change in the plasma through calcium binding, leading to circulating S100A8/A9 in non-active form. It is important to explore the mechanisms described in the plasma of healthy donors and patients and to validate these mechanisms in relevant preclinical models.

Overall, the findings described in this thesis show good potential for further investigations into the following areas: *i)* exploring further the effect of specific DAMPs on platelet activation, aggregation and platelet phenotypes; *ii)* the importance of DAMPs not only as biomarkers but as therapeutical targets; *iii)* the unmet need of finding new molecules aimed at bridging the inflammation gap present in

thromboinflammation; *iv)* the need for clinicians to stratify patients affected by thrombotic diseases on the basis of inflammatory markers; *v)* the need to understand in depth intracellular pathways elicited by S100A8/A9 and heme and their acute vs chronic effect as well as their similarities and differences in sterile vs infectious inflammation; *vi*) understanding the possible crosstalk between platelet receptors activated by different DAMPs that are very likely present at the same time in the circulation of patients affected by thromboinflammation both *in vitro* and *in vivo*; *vii*) characterising the systemic effects of DAMPs, their potential role on megakaryocytes and their possible priming effect on platelets causing them to improperly respond to further inflammatory/thrombotic stimuli.

5.8 Conclusions

These results suggest that S100A8/A9 and heme are two potent prothrombotic DAMPs which can affect platelet function leading to distinct platelet activation profiles. Importantly, in both cases, platelet activation was not affected by inhibiting P2Y12 and COX-1, suggesting that DAMPs-induced platelet activation is refractory to anti-platelet drugs currently widely used in the clinical practice (**Figure 5.2**). Therefore, new therapeutic strategies aimed at targeting platelet pathways activated by specific DAMPs should be developed and implemented into the clinical practice.

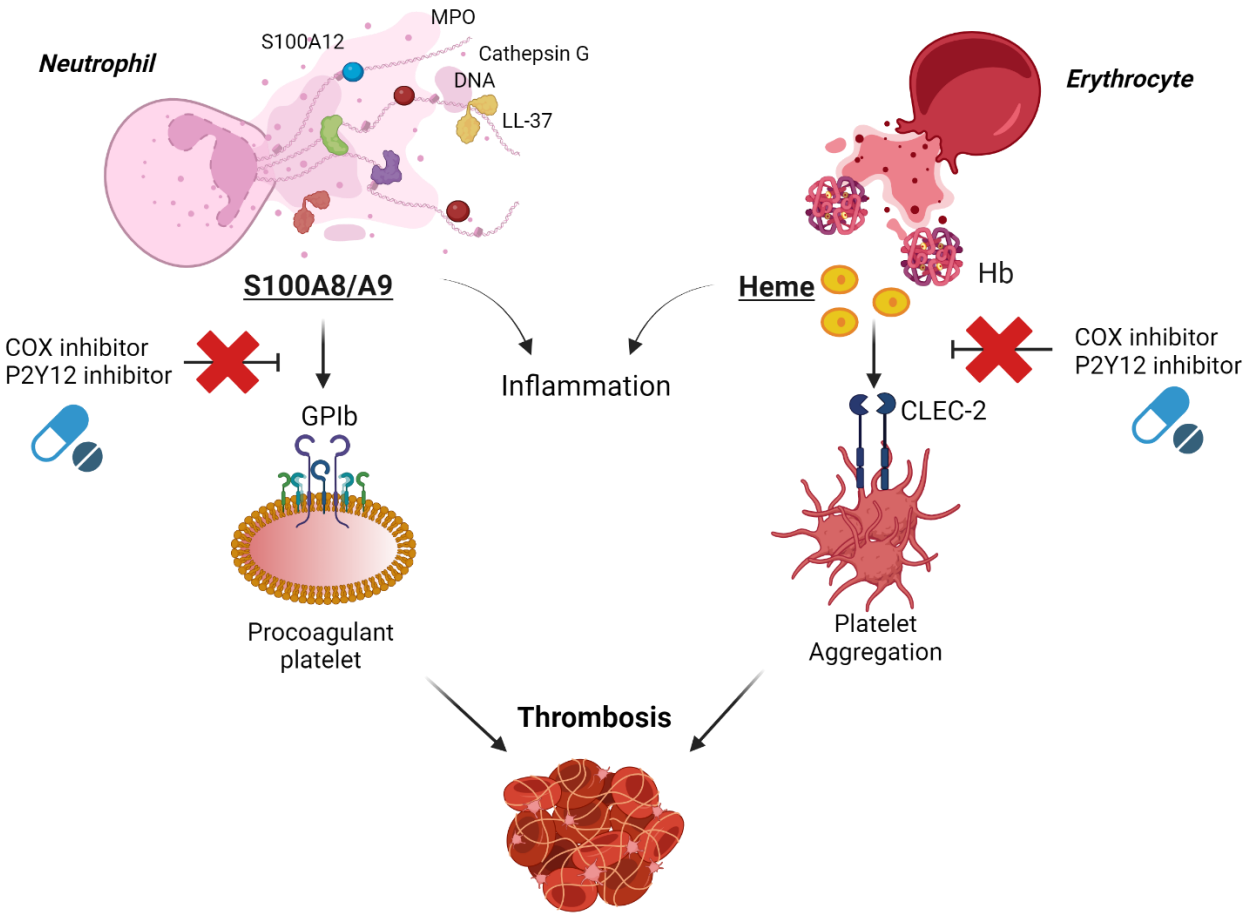


Figure 5.2 – S100A8/A9 and Heme induce platelet activation resistant to COX and P2Y12 inhibitors. S100A8/A9 released mainly by neutrophils, through GPIb receptor, induces the formation of procoagulant platelets which promote fibrin production. Differently, Heme, released by lysed erythrocytes, induces platelet aggregation at low dose. Both procoagulant platelets and pro-aggregatory platelets contribute to thrombus formation and thromboinflammation. Pathways activated by either S100A8/A9 or heme are resistant to commonly used anti-platelet drug such as COX and P2Y12 inhibitors.

S100A8/A9 and heme, had been already proposed as biomarkers of diseases to perform risk assessment, monitor response to treatment or predict prognosis. In this thesis, we identify a direct effect of these DAMPs on platelet activation, indicating their

role as platelet activators in thromboinflammation. This suggest they should be considered as therapeutical targets. Strategies aimed at lowering the effects of S100A8/A9 and heme should be aimed at either targeting their receptors or their levels in the circulation.

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APPENDIX 1

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THROMBOSIS AND HEMOSTASIS

S100A8/A9 drives the formation of procoagulant platelets through GPIbα

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KEY POINTS

- **S100A8/A9 plasma levels are increased in patients with COVID-19, and sustained high levels are associated with worse clinical outcome.**
- **S100A8/A9 induces the formation of procoagulant platelets through GPIbα supporting fibrin generation and immune-driven thrombosis.**

S100A8/A9, also known as "calprotectin" or "MRP8/14," is an alarmin primarily secreted by activated myeloid cells with antimicrobial, proinflammatory, and prothrombotic properties. Increased plasma levels of S100A8/A9 in thrombo-inflammatory diseases are associated with thrombotic complications. We assessed the presence of S100A8/A9 in the plasma and lung autopsies from patients with COVID-19 and investigated the molecular mechanism by which S100A8/A9 affects platelet function and thrombosis. S100A8/A9 plasma levels were increased in patients with COVID-19 and sustained high levels during hospitalization correlated with poor outcomes. Heterodimeric S100A8/A9 was mainly detected in neutrophils and deposited on the vessel wall in COVID-19 lung autopsies. Immobilization of S100A8/A9 with collagen accelerated the formation of a fibrin-rich network after perfusion of recalcified blood at venous shear. In vitro, platelets adhered and partially spread on S100A8/A9, leading to the formation of distinct populations of either P-selectin or phosphatidylserine (PS)-positive platelets. By using washed platelets, soluble S100A8/A9 induced PS exposure but failed to induce platelet aggregation, despite GPIIb/IIIa activation and alpha-granule secretion. We identified GPIbα as the receptor for S100A8/A9 on platelets inducing the formation of procoagulant platelets with a supporting role for CD36. The effect of S100A8/A9 on platelets was abolished by recombinant GPIbα ectodomain, platelets from a patient with Bernard-Soulier syndrome with GPIb-IX-V deficiency, and platelets from mice deficient in the extracellular domain of GPIbα. We identified the S100A8/A9-GPIbα axis as a novel targetable prothrombotic pathway inducing procoagulant platelets and fibrin formation, in particular in diseases associated with high levels of S100A8/A9, such as COVID-19.

Introduction

Platelets are crucial for hemostasis and are key drivers of pathogenic thrombosis both in sterile and infectious conditions.[1,2] Circulating activated platelets and platelet-leukocyte aggregates are increased in acute and chronic sterile thromboinflammatory diseases such as atherosclerosis,[3] deep vein thrombosis (DVT),[4] ischemic stroke,[5] myocardial infarction (MI),[6,7] and in infectious conditions such as sepsis,[8] influenza,[9] and COVID-19,[10] with the increase in these levels correlating with disease severity.[11-13] Moreover, procoagulant platelets and microvesicles (MVs), which support fibrin generation independent of platelet aggregation, are observed in trauma,[14] COVID-19,[15] coronary artery disease,[16,17] sepsis,[18,19] DVT, and stroke,[20-22] but the mechanisms triggering their formation are not well known.

Activation of neutrophils and neutrophil extracellular traps formation (NETosis) lead to the release of damage-associated molecular patterns (DAMPs) such as histones, myeloperoxidase (MPO), and S100A8/A9, which can promote inflammation and thrombosis.[23,24] Recent work has demonstrated that

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plasma levels of S100A8/A9, also known as "calprotectin" or "myeloid-related protein 8/14 (MRP-8/14)" are elevated in patients with COVID-19 and correlate with disease severity as well as thrombotic complications.[25-29] High S100A8/A9 levels are also observed in MI,[30-32] sepsis,[33] and DVT,[34] with S100A8/A9 being used as a biomarker for immune cell activation and inflammation.[35-37] S100A8 and S100A9 proteins belong to the 24 members of the multifunctional S100 family of cytoplasmic EF-hand helix-loop-helix Ca^{2+}-binding proteins which regulate Ca^{2+} balance, cell apoptosis, migration, proliferation, differentiation, energy metabolism, and inflammation.[38] Extracellular S100A8 and S100A9 and the heterodimer S100A8/A9 promote inflammation and endothelial activation.[39] The heterodimer S100A8/A9 is the predominant form, comprising up to 45% and ~5% of all cytosolic proteins in neutrophils and monocytes, respectively.[38,40,41] Three receptors have been described to mediate S100A8/A9 proinflammatory functions: the receptor for advanced glycation end products (RAGE), toll-like receptor-4 (TLR4), and the scavenger receptor CD36, which are expressed on different cells including platelets, immune cells, and endothelial cells.[42-45] Innate immune cell activation leads to the release of S100A8/A9 and its deposition on venules, supporting leukocyte recruitment and transmigration.[29] Moreover, S100A8/A9 was shown to bind to heparan sulfate glycosaminoglycans on endothelial cells[46] and to induce endothelial activation through CD36[47] and RAGE.[39] Therefore, both soluble and immobilized S100A8/A9 may contribute to inflammation and thrombosis,[27,34] with multiple receptors and mechanisms involved in thromboinflammatory diseases. We investigated the function of extracellular S100A8/A9 as a DAMP regulating platelet function and thrombosis. We show that S100A8/A9 induces the formation of procoagulant platelets through GPIbα, independently of the known receptors for S100A8/A9, TLR4, and RAGE. CD36 blockage partially reduced GPIIb/IIIa activation and phosphatidylserine (PS) exposure on platelets without alteration in P-selectin expression. We have identified the S100A8/A9-GPIbα axis as a novel prothrombotic pathway that triggers the formation of procoagulant platelets accelerating fibrin generation and thrombosis.

Materials and methods

Ethical approval

Ethical approval for collecting blood from healthy volunteers was granted by Birmingham University Internal Ethical Review (ERN_11-0175). Mouse experiments were performed in accordance with UK laws (Animal [Scientific Procedures] Act 1986) with approval of the local ethical committee and UK Home Office approval (PPL Pp9677279). Ethical approval for collecting postmortem tissues was granted by the North-East — Newcastle & North Tyneside 1 Research Ethics Committee (19/NE/0336). Collection of postmortem formalin-fixed and paraffin-embedded tissue was approved (IRAS: 197937) for tissue obtained via prospective consent postmortem and retrospective acquisition of tissue in which consent for use in research had already been obtained. Ethics for patient tissue were approved by the Health Research Authority with a National Health Service Research Ethics Committee. All necessary patient/participant written consent has been obtained, and the appropriate institutional forms have been archived. This research adheres to the tenets of the Declaration of Helsinki. Blood from patients with COVID-19 was collected under the ethical approval of Medical University of Vienna (EK1315/2020) as part of the Austrian Coronavirus Adaptive Clinical Trial (ACOVACT; ClinicalTrials.gov NCT04351724).

Plasma S100A8/A9 enzyme-linked immunosorbent assay (ELISA)

Plasma was collected by blood centrifugation for 10 minutes at 1000*g* (4°C) followed by 10 minutes of centrifugation at 10.000*g* at room temperature.[48] Plasma samples were stored at −80°C until use. Plasma levels of S100A8/A9 were measured using LEGEND MAX Human MRP8/14 (Calprotectin) ELISA Kit (BioLegend).

Immunofluorescence staining of lung sections

Human paraffin-embedded lung sections (6 μm) were processed and stained as previously described.[49] Antibodies against platelet CD42b, MPO, fibrin, S100A8/A9, and S100A9 (supplemental Table 1, available on the *Blood* website) were incubated overnight at 4°C followed by secondary conjugated antibodies. Lung autofluorescence was quenched using a commercial kit (Vector Laboratories) and slides mounted using ProLong Gold Antifade Mountant (Life Technologies). Sections were imaged using an Epifluorescent microscope or Zeiss Axioscan Z1 microscope and analyzed using ZEN software and ImageJ.

Recombinant S100A8/A9 production and purification

Commercial recombinant S100A8/A9 (BioLegend) and in-house–produced proteins were used. Recombinant human heterodimeric S100A8/A9 was produced as previously described with minor changes.[50] Protein production and purification are detailed in the supplemental Data.

Platelet preparation and functional assays

Human and mouse blood were collected in sodium citrate and ACD-A Anticoagulant Citrate Dextrose solution, respectively, as previously described.[51,52] Platelet preparation and functional assays (platelet aggregation and ATP generation, thrombin generation test, flow adhesion assay, platelet spreading, intracellular Ca^{2+} release) are detailed in the supplemental Data.

Data analysis

All data were presented as mean ± standard deviation (SD) unless stated otherwise. The logarithmic dose-binding curves were generated through 4-parameter nonlinear regression analysis with variable slopes. The statistical difference between groups was analyzed using either one-way or two-way analysis of variance (ANOVA) with multiple comparisons or Kruskal-Wallis test with multiple comparisons or as stated in the legends for Figures 1, 5, and 6 using Prism 7 (GraphPad Software Inc).

Results

Sustained high levels of S100A8/A9 in the plasma of patients with COVID-19 correlate with adverse outcome

Plasma S100A8/A9 levels are elevated in patients with COVID-19 and these levels correlate with thrombosis.[28,53,54] In a cohort of 87 patients with COVID-19,[55,56] plasma levels of S100A8/A9

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were measured by ELISA over the first week of study enrollment. Study inclusion occurred up to 72 hours after hospital admission. Patients were stratified on the basis of the clinical severity into uncomplicated (not requiring intensive care treatment) and complicated (intensive care unit [ICU] survivors and nonsurvivors). At study recruitment, the levels of S100A8/A9 were elevated in the plasma of all patients with COVID-19 compared with healthy controls (Figure 1A). Over the first 7 days of monitoring, the levels of S100A8/A9 remained high in patients with complicated COVID-19 but decreased in uncomplicated patients (Figure 1B-C). The levels of S100A8/A9 were significantly higher in nonsurvivors ranging between 10 and 40 μg/mL. These data show that sustained high levels of plasma S100A8/A9 correlated with COVID-19 disease severity.

Heterodimeric S100A8/A9 is detected in neutrophils and on the lung vessel walls of patients with COVID-19

S100A8/A9 release from activated immune cells leads to deposition on venule endothelial cells, supporting leukocyte recruitment and transmigration.[29] The presence and location of S100A8/A9 were assessed in lung autopsies from COVID-19 nonsurvivor patients (n = 8)[49] and control sections from age-matched patients (n = 3). By using an antibody recognizing the heterodimer S100A8/A9 but not S100A8 or S100A9 alone, S100A8/A9 was detected in neutrophils (MPO-positive cells) and on the vessel wall (Figure 1D-E). S100A8/A9 was also detected in a fraction of CD42b-positive cells in patients with COVID-19 but not in controls. Platelets were observed lining the endothelium as single platelets, as small aggregates positive for S100A9 and fibrin, or were integrated into large fibrin-rich thrombi (Figure 1D,F-G). Contrary to the antibody recognizing the dimeric form, staining with anti-S100A9 antibody revealed a strong colocalization between CD42b and S100A9 in COVID-19 lungs (Figure 1G). These results show an increase in soluble S100A8/A9 alongside its deposition on the vasculature in COVID-19 lungs.

Immobilized S100A8/A9 supports fibrin generation at venous shear rate Platelet-derived S100A8/A9 was shown to promote thrombosis partially via CD36.[27] Platelets from patients with COVID-19 are enriched in S100A8/A9 and platelet releasate induces endothelial cell activation via CD36[28] but the effect of extracellular S100A8/A9 on platelets is not well known. We assessed the effect of soluble and immobilized recombinant S100A8/A9 on fibrin generation in whole blood from healthy donors. Commercial and in-house–produced recombinant S100A8/A9 were used and characterized (supplemental Figure 1A-D). Both proteins are heterodimers and the S100A9 protein is phosphorylated at Thr113, which is essential for the proinflammatory functions of extracellular S100A8/A9[57] (supplemental Figure 1A-D). S100A8/A9 induces the secretion of tumor necrosis factor (TNF)-α from human monocytes in a TLR4-dependent manner as TAK-242 significantly reduced TNF-α levels in monocyte supernatant, showing the functionality of in-house–produced S100A8/A9 (supplemental Figure 1E). Addition of S100A8/A9 in platelet-rich plasma (PRP) isolated from healthy donors did not support thrombin generation in calibrated automated thrombography compared with PRP alone (supplemental Figure 2A-F). However, perfusion of whole blood over S100A8/A9-coated surfaces under recalcified conditions at venous shear rate (100 s^{-1}), induced platelet adhesion with local fibrin generation as assessed using anti-fibrin/fibrinogen antibody and early recruitment of PS-positive platelets detected by annexin-V binding (Figure 1H-J). Compared with S100A8/A9 or collagen alone, surface coated with S100A8/A9 in combination with collagen accelerated the recruitment of annexin-V–positive platelets and the formation of multiple fibrin-rich areas and widespread fibrin network, which significantly limited blood flow (Figure 1H-J). These results indicate that immobilized S100A8/A9 accelerates the formation of fibrin-rich thrombi at venous shear.

Recombinant S100A8/A9 induces human platelet activation without aggregation in vitro

It has been recently shown that arterial and venous thrombus formation was reduced in mice deficient in S100A8/A9.[27,34] To identify whether S100A8/A9 exerts a direct effect on platelets leading to pathogenic thrombosis, we assessed the effect of recombinant S100A8/A9 on platelet function in vitro. The incubation of human washed platelets with increasing concentrations of S100A8/A9 at ranges previously detected in thromboinflammatory diseases and COVID-19 patients[26,33,53,58,59] induced platelet activation as assessed by flow cytometry (Figure 2). S100A8/A9 induced a slow release of P-selectin from alpha-granules as measured using an anti–P-selectin antibody (Figure 2A-B, supplemental Figure 2G). Platelet activation by S100A8/A9 was not homogenous among healthy donors, with high responders reaching similar levels of platelet activation as for collagen-related peptide (CRP) and thrombin receptor-activating peptide 6 (TRAP-6), even at low doses of S100A8/A9 (10-20 μg/mL) (Figure 2B). The upregulation in P-selectin expression was associated with increased platelet-neutrophil aggregates in diluted whole blood after stimulation with S100A8/A9 (Figure 2C). S100A8/A9 induced GPIIb/IIIa activation as assessed by the binding of an anti-CD61/CD41 PAC-1 antibody, which recognizes the activated form of the integrin (Figure 2D). GPIIb/IIIa activation occurred quickly after the addition of S100A8/A9 compared with the slow upregulation of P-selectin (supplemental Figure 2H). Surprisingly, despite GPIIb/IIIa activation, S100A8/A9 did not induce platelet aggregation (Figure 2E-F) as assessed by light transmission aggregometry or dense granule release (Figure 2G-H). S100A8/A9 did not induce platelet aggregation in the presence of exogenous fibrinogen, suggesting that the absence of aggregation is not due to a defect in fibrinogen secretion but possibly an alteration in fibrinogen binding (Figure 2I-J). Indeed, addition of exogenous labeled fibrinogen to platelets in the presence of S100A8/A9 showed low binding capacity compared with CRP (Figure 2K-L). These results indicate that S100A8/A9 induces P-selectin upregulation and GPIIb/IIIa activation without platelet aggregation, suggesting a novel mechanism of platelet activation independent of platelet aggregation.

S100A8/A9 induces PS exposure on platelets and the release of PS-positive MVs Procoagulant platelets, which expose high levels of PS, have a very low capacity to aggregate with some procoagulant platelet populations sustaining the active form of GPIIb/IIIa.[60] Therefore, surface expression of PS on S100A8/A9-activated platelets was assessed by annexin-V binding using flow cytometry. S100A8/A9 induced PS exposure on the surface of platelets (Figure 3A-C), which occurred

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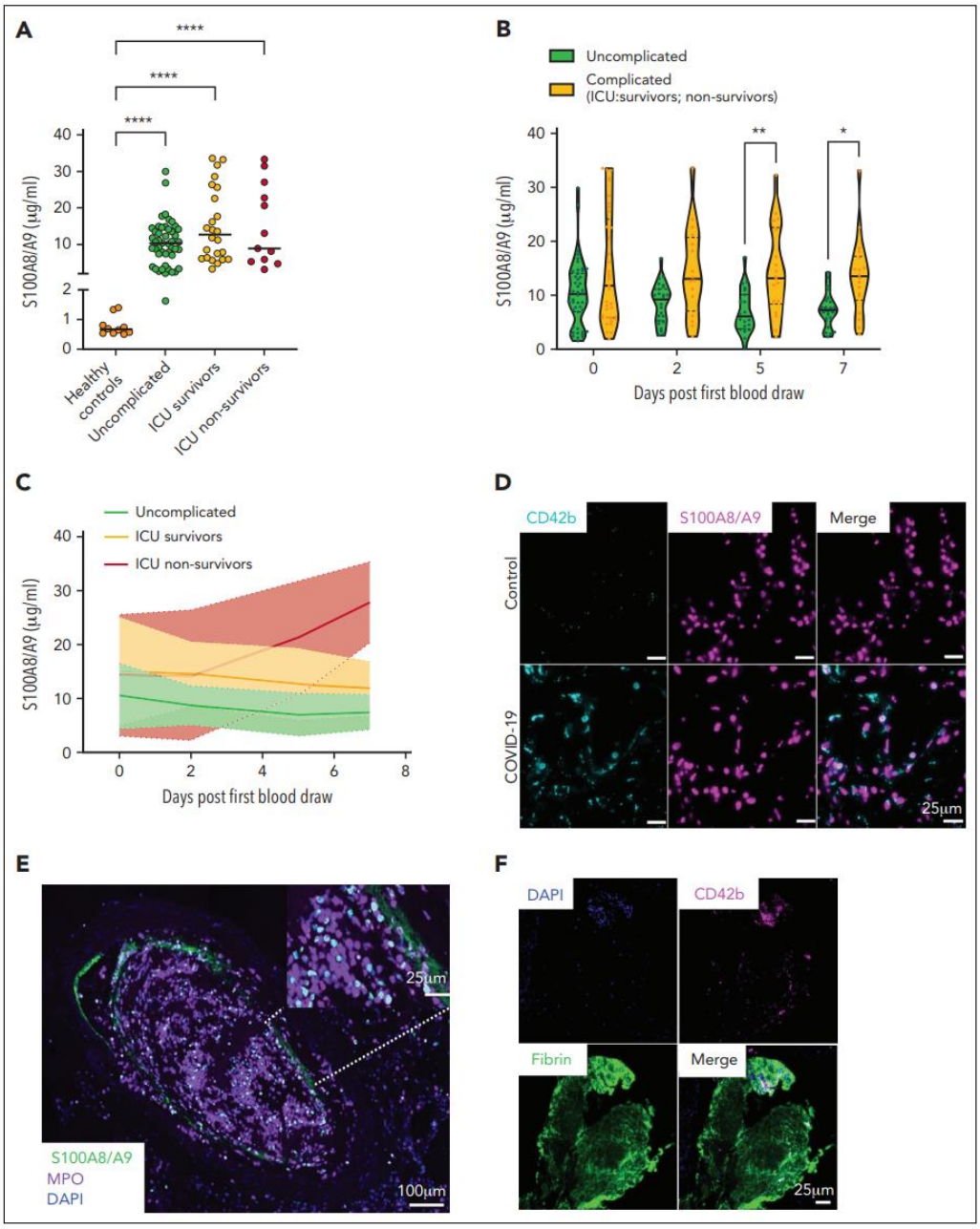


Figure 1. S100A8/A9 accelerates the recruitment of annexin-V–positive platelets and fibrin generation at a venous shear rate. (A-C) Plasma levels of S100A8/A9 were measured by ELISA in healthy donors and patients with uncomplicated and complicated (ICU survivors/nonsurvivors) COVID-19 over 7 consecutive days after inclusion in the study. (A) S100A8/A9 levels in the plasma of healthy donors (control, n = 10), uncomplicated COVID-19 patients (n = 48), ICU survivors (n = 26), and ICU nonsurvivors (n = 13) on the first day of patient inclusion in the study. (B-C) S100A8/A9 levels in the plasma over 7 consecutive days after patient recruitment. (D-G) Representative immunofluorescence staining of lung autopsies obtained from COVID-19 patients or age-matched control. Images were captured using Epi fluorescence microscope and slide scanner Axioscan Z1. (D) Platelet CD42b and heterodimeric S100A8/A9 staining in lung parenchyma and microcirculation in COVID-19 and control lung autopsies. (E) Staining for S100A8/A9, MPO, and nuclei (DAPI) of a large vessel in the lung of a patient with COVID-19 with thrombotic complications. (F) Staining for platelet CD42b, fibrin, and nuclei (DAPI) in large thrombi of a patient with COVID-19. (G) S100A9, CD42b, and fibrin staining in COVID-19 lung. Arrow shows platelet-fibrin microaggregates. (H) Whole blood under recalcified conditions was perfused at a venous shear rate (100 s^{-1}) over S100A8/A9 (40 µg/mL), collagen (100 µg/mL), or combination of S100A8/A9 and collagen-coated chambers. The

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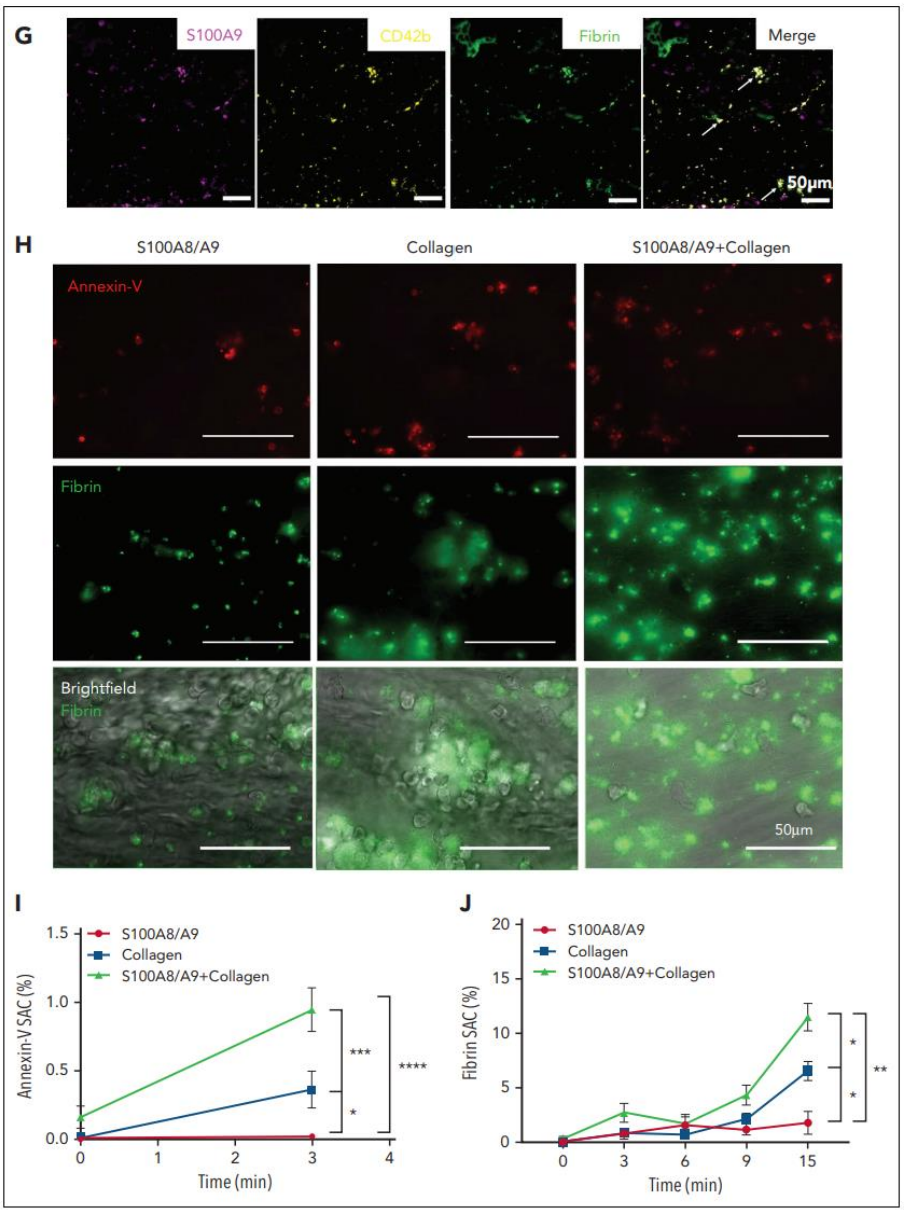


Figure 1 (continued) presence of PS-positive platelets and fibrin were assessed using annexin-V-Alexa Fluor-647 and FITC-anti-fibrinogen/fibrin antibody, respectively. (H) Representative images taken at 15 minutes. (I) Quantification of annexin-V signal (3 minutes) (n = 5) and (J) fibrin signal time course (0-15 minutes) (n = 5) using predefined semiautomated scripts in Fiji as detailed in the supplemental Methods (available on the *Blood* website). Results are shown as mean ± standard error of the mean (SEM). The statistical significance was analyzed using two-way ANOVA with Tukey's multiple comparison test between all groups. *$P < .05$, **$P < .005$, ***$P < .001$, ****$P < .0001$.

rapidly after the addition of S100A8/A9 (supplemental Figure 2I). S100A8/A9 also increased the level of PS on CD41-positive MVs (Figure 3D). Platelet activation is dependent on the presence of the heterodimer because recombinant S100A8 or S100A9 alone did not induce platelet activation (supplemental Figure 2J-L). Platelets adhered to and spread on immobilized S100A8/A9, whereas no significant platelet adhesion was observed on bovine serum albumin used as negative control (Figure 3E). Platelet adhesion on immobilized S100A8/A9 showed predominantly high expression of either PS or P-selectin

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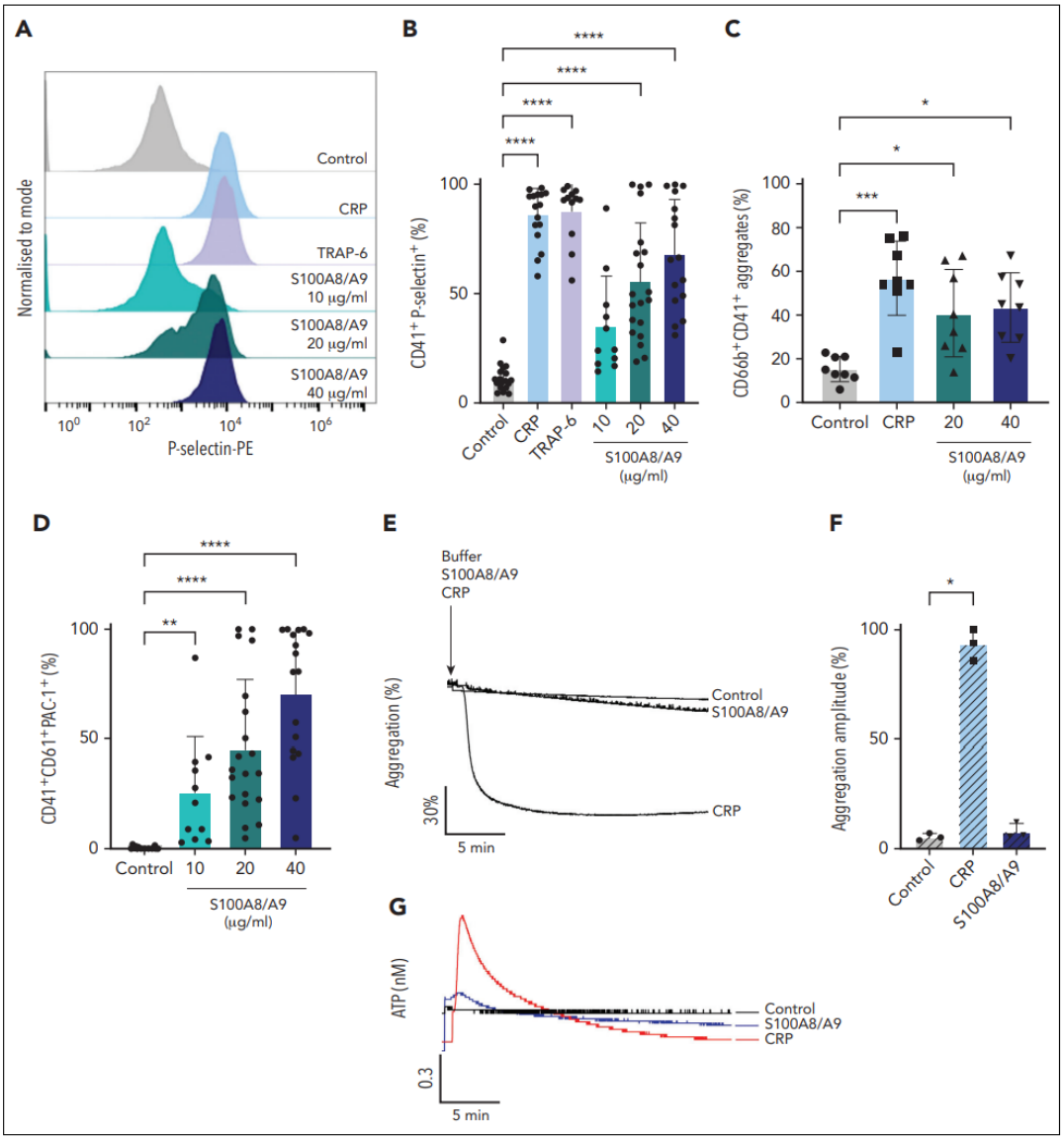


Figure 2. Recombinant S100A8/A9 induces human platelet activation in vitro. Human washed platelets (10^6 platelets/condition) were incubated with different concentrations of recombinant heterodimeric S100A8/A9 (10, 20, and 40 µg/mL) for 30 minutes at 37°C. (A-B) Platelet activation was determined by flow cytometry using anti–P-selectin antibody. (A) Representative plots from a healthy donor; histograms normalized to mode (each peak normalized to its mode for each condition). (B) Percentage of $CD41^+$P-selectin$^+$ platelets. (C) The percentage of platelet-neutrophil aggregates ($CD66b^+/CD41^+$) in whole blood after addition of S100A8/A9 for 30 minutes at 37°C (n = 7). Blood was diluted 1:5. (D) Anti-CD41/CD61 PAC-1 antibody (against activated GPIIb/IIIa) binding to platelets was assessed by flow cytometry and presented as percentage of $CD41^+$ platelets positive for PAC-1 (n = 17). (E-F) Washed platelets (2×10^8/mL) were incubated with S100A8/A9 (40 µg/mL) or CRP (10 µg/mL), and platelet aggregation was assessed for 20 minutes by light transmission aggregometry (n = 3). (G-H) Washed platelets (2×10^8/mL) were incubated with S100A8/A9 (40 µg/mL) or CRP (10 µg/mL), and ATP generation was assessed for 6 minutes with the CHRONO-LUME luciferin:luciferase assay kit from Chronolog (n = 3). (E,G) Representative traces. (I-J) Washed platelets (2×10^8/mL) were incubated with S100A8/A9 (20 µg/mL or 40 µg/mL) for 6 minutes under stirring condition followed by the addition of exogenous fibrinogen (200 µg/mL). Platelet aggregation was assessed for 6 minutes by light transmission aggregometry (n = 3). (K-L) Alexa-Fluor 488-labeled fibrinogen binding to platelets. (K) Representative plot for fibrinogen binding. (L) Percentage of platelets positive for fibrinogen-Alexa Fluor 488, CRP (10 µg/mL) and TRAP-6 (100 µM) were used as positive control. Data are shown as mean ± SD. The statistical significance was analyzed using ordinary one-way ANOVA. *P < .05, **P < .005, ***P < .001, ****P < .0001.

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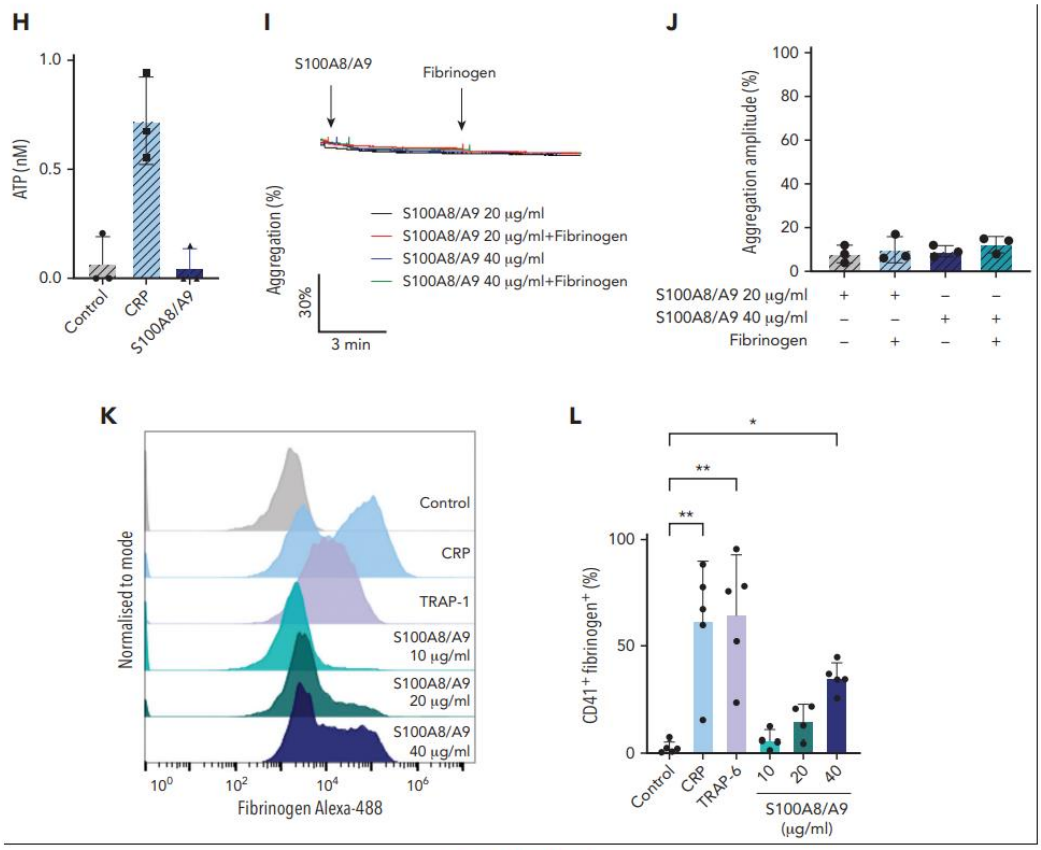


Figure 2. (continued)

on adherent platelets, with low expression of P-selectin on PS-positive platelets and vice versa (Figure 3F-G). Increasing the coating density of S100A8/A9 augmented the formation of PS-positive platelet and PS-positive MVs rather than P-selectin–positive platelets (Figure 3G). In addition, platelet adhesion on S100A8/A9 induced intracellular Ca^{2+} release (Figure 3H-I), with high Ca^{2+} signal observed in adherent but not spread platelets. These results indicate that S100A8/A9 induces intracellular Ca^{2+} release, PS exposure, and the release of PS-positive MVs, and that this activation is restricted to the heterodimeric form of S100A8/A9.

CD36 blockade and immunoreceptor tyrosine-based activation motif receptor inhibitors differentially reduce the effect of S100A8/A9 on platelets

S100A8/A9 has 3 known receptors (RAGE, TLR4, and CD36), which are all expressed on platelets.[61-63] To identify the functional receptor for S100A8/A9 mediating the formation of procoagulant and activated platelets, we first assessed the effect of inhibitors targeting TLR4, RAGE, and CD36 in human washed platelets. Inhibition of RAGE using Azeliragon or S100A9 binding to TLR4 using Paquinimod did not alter platelet activation by S100A8/A9 (Figure 4A-C). Moreover, inhibition of TLR-4, using the small molecule inhibitor TAK-242 or an antibody inhibiting S100A8/A9 binding to TLR4 and RAGE (S100A8/A9 Ab clone A1505B), also did not alter platelet activation or PS exposure (supplemental Figure 3A-C, supplemental Table 2). Blocking downstream signaling of CD36 using sulfosuccinimidyl oleate (SSO), which irreversibly blocks CD36, partially reduced GPIIb/IIIa activation and PS exposure without affecting P-selectin expression (Figure 4D-F). An antibody that blocks the oxidized low-density lipoprotein binding site to CD36 (CD36 blocking Ab) had no effect on platelet activation, suggesting the presence of distinct binding sites for S100A8/A9 and oxidized low-density lipoprotein on CD36 (supplemental Figure 3D-E). Further, inhibition of platelet immunoreceptor tyrosine-based activation motif receptors using inhibitors for Src (PP2), Syk (PRT-060318), or Btk (ibrutinib) did not alter the binding of anti-CD61/CD41 PAC-1 antibody or annexin-V (Figure 4G-I), while significantly reducing P-selectin expression

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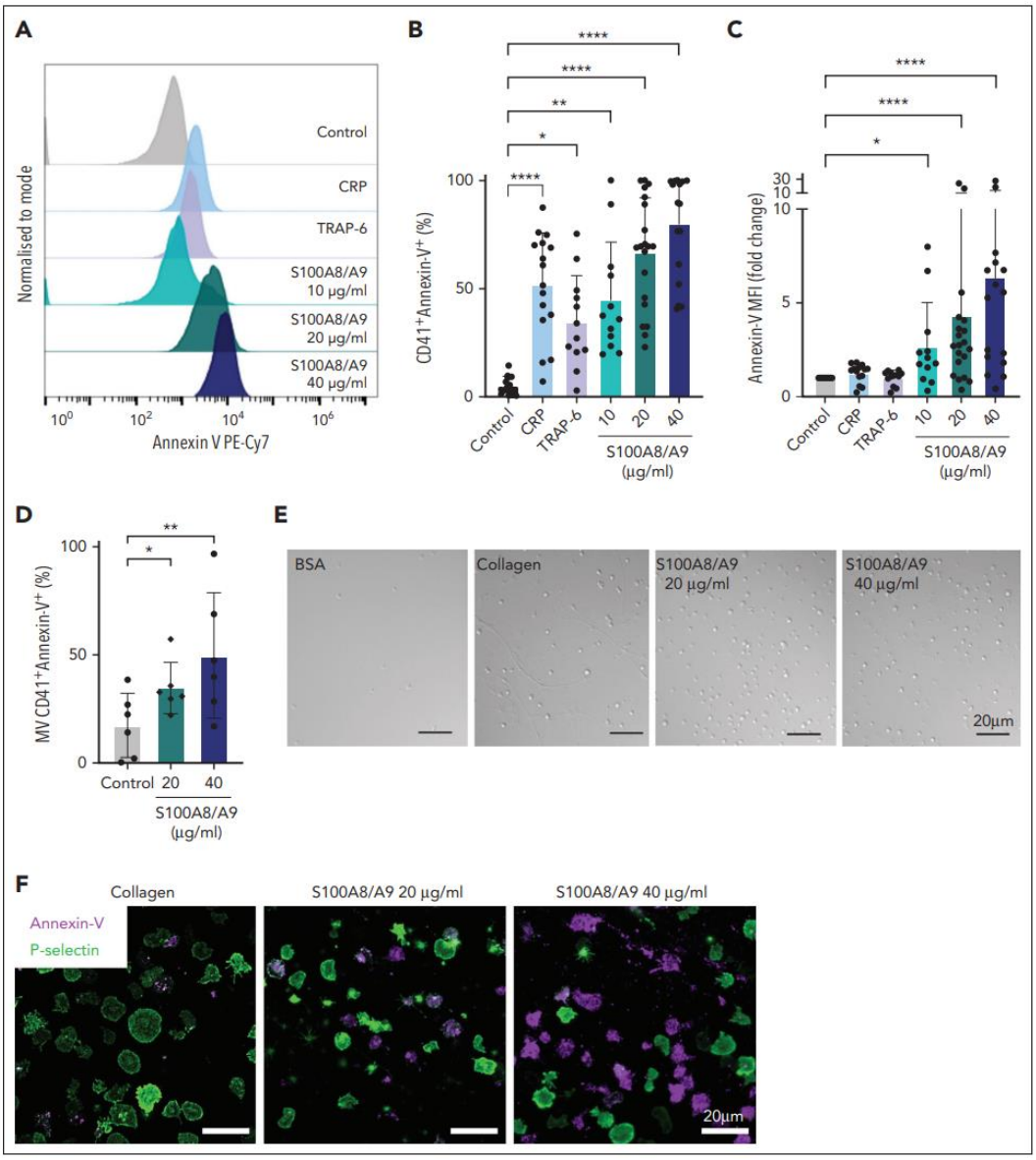


Figure 3. S100A8/A9 induces the formation of procoagulant platelets. Human washed platelets (10^6 platelets/condition) were incubated with different concentrations of S100A8/A9 (10, 20 and 40 µg/mL) for 30 minutes at 37°C. (A-C) PS exposure was determined by flow cytometry using PE-Cy7–labeled annexin-V (n = 17). CRP (10 µg/mL) and TRAP-6 (100 µM) were used as positive control. (A) Representative histogram for annexin-V staining. Representative histograms normalized to mode (each peak normalized to its mode for each condition). (B) Percentage of $CD41^+$annexin-V^+ platelets. (C) Fold change in the mean fluorescent intensity (MFI) of agonist-activated platelets over control. (D) The percentage of $CD41^+$MVs positive for annexin-V (n = 6). (E-F) Human washed platelets spreading on collagen or S100A8/A9. Representative differential interference contrast (DIC) images of spread platelets. (F) Representative annexin-V (magenta) and P-selectin (green) staining for adherent platelets. (G) Quantification of annexin-V–positive, P-selectin–positive, or annexin-V and P-selectin–double positive platelets adherent on collagen and S100A8/A9 (positive platelets/total platelets). The statistical significance was analyzed using two-way ANOVA with Tukey's multiple comparison test between all groups. (H-I) Assessment of intracellular Ca^{2+} release from platelets spread over surfaces coated with S100A8/A9 (20 and 40 µg/mL), fibrinogen, or bovine serum albumin assessed using BAPTA-1-AM Ca^{2+} sensitive dye. (H) Representative Ca^{2+} traces assessed using Oregon Green-488 BAPTA-1-AM. (I) Peak fluorescence at time point zero (F0/Fmax). Data are shown as mean ± SD. The statistical significance was analyzed using a nonparametric test (Kruskal-Wallis test). *P < .05, **P < .005, ***P < .001, ****P < .0001.

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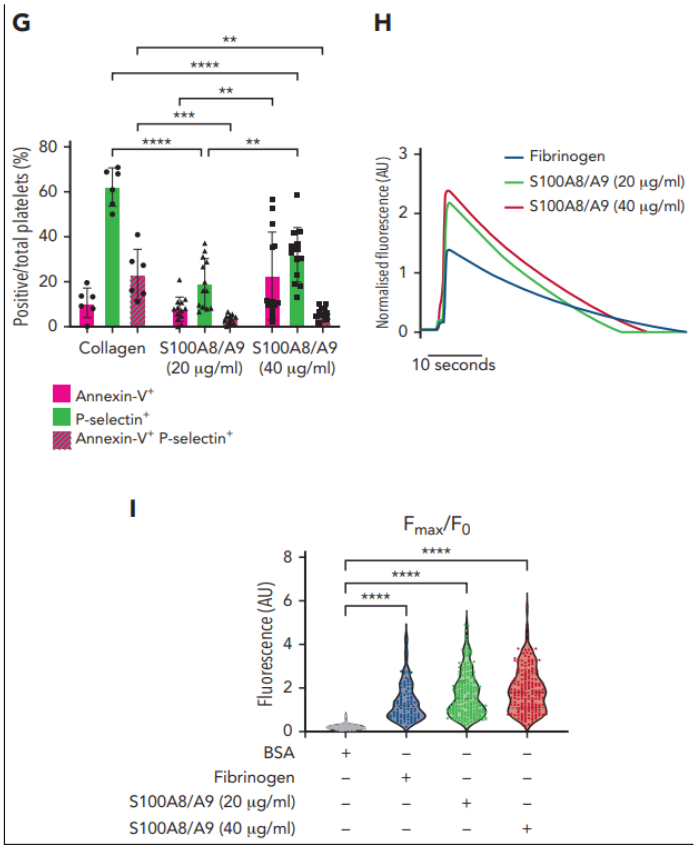


Figure 3. (continued)

on platelets (Figure 4H). Inhibition of Syk showed the highest inhibition of P-selectin expression, which was associated with a decrease in platelet-neutrophil aggregates in whole blood (supplemental Figure 3F). Inhibition of cyclo-oxygenase using indomethacin or P2Y12 using cangrelor did not alter platelet activation or PS exposure (supplemental Figure 3G-I). These results suggest that S100A8/A9-mediated P-selectin upregulation is regulated by Syk/Src, whereas GPIIb/IIIa activation and PS exposure are partially mediated through CD36.

Murine recombinant S100A8/A9 activates mouse platelets in a dose-dependent manner through GPIbα

As CLEC-2, GPVI, GPIb, and CD36 signal through Syk/Src,[64,65] we assessed the effect of S100A8/A9 on murine platelets deficient in CLEC-2, GPVI, CLEC-2/GPVI, or platelets deficient in the extracellular domain of GPIbα (IL4R/GPIbα−Tg mice). Mouse S100A8/A9 induced the activation of wild-type (WT) platelets in a dose-dependent manner as assessed using anti–P-selectin antibody and annexin-V binding in a similar manner as in human platelets (Figure 5A-B). Deletion of GPVI, CLEC-2, or both receptors, did not significantly alter P-selectin expression or annexin-V binding in response to S100A8/A9 compared with WT mice (Figure 5C-D). However, GPVI$^{-/-}$ and GPVI$^{-/-}$ CLEC-2$^{-/-}$ mice showed a bimodal pattern for P-selectin expression. S100A8/A9-induced mouse platelet activation was significantly reduced in platelets deficient in the extracellular domain of GPIbα (IL4R/GPIbα−Tg) as measured by P-selectin expression (Figure 5E-F), annexin-V binding (Figure 5G-H), and GPIIb/IIIa activation (Figure 5I-J). Compared with GPIIb/IIIa activation in human platelets, mouse S100A8/A9 induced modest integrin activation (Figure 5I-J). The partial reduction in annexin-V binding observed in IL4R/GPIbα−Tg platelets was completely blocked by the addition of the CD36 inhibitor SSO (Figure 5K). Collectively, these results indicate that GPIbα and CD36 are putative receptors for S100A8/A9 on mouse platelets.

Recombinant human GPIbα binds to S100A8/A9 and potentiated von Willebrand factor (VWF)-mediated platelet agglutination

To assess whether GPIbα is the main receptor for S100A8/A9 on human platelets or indirectly regulates S100A8/A9-mediated platelet activation, direct binding between GPIbα and S100A8/A9 was assessed by ELISA. S100A8/A9 binds to recombinant

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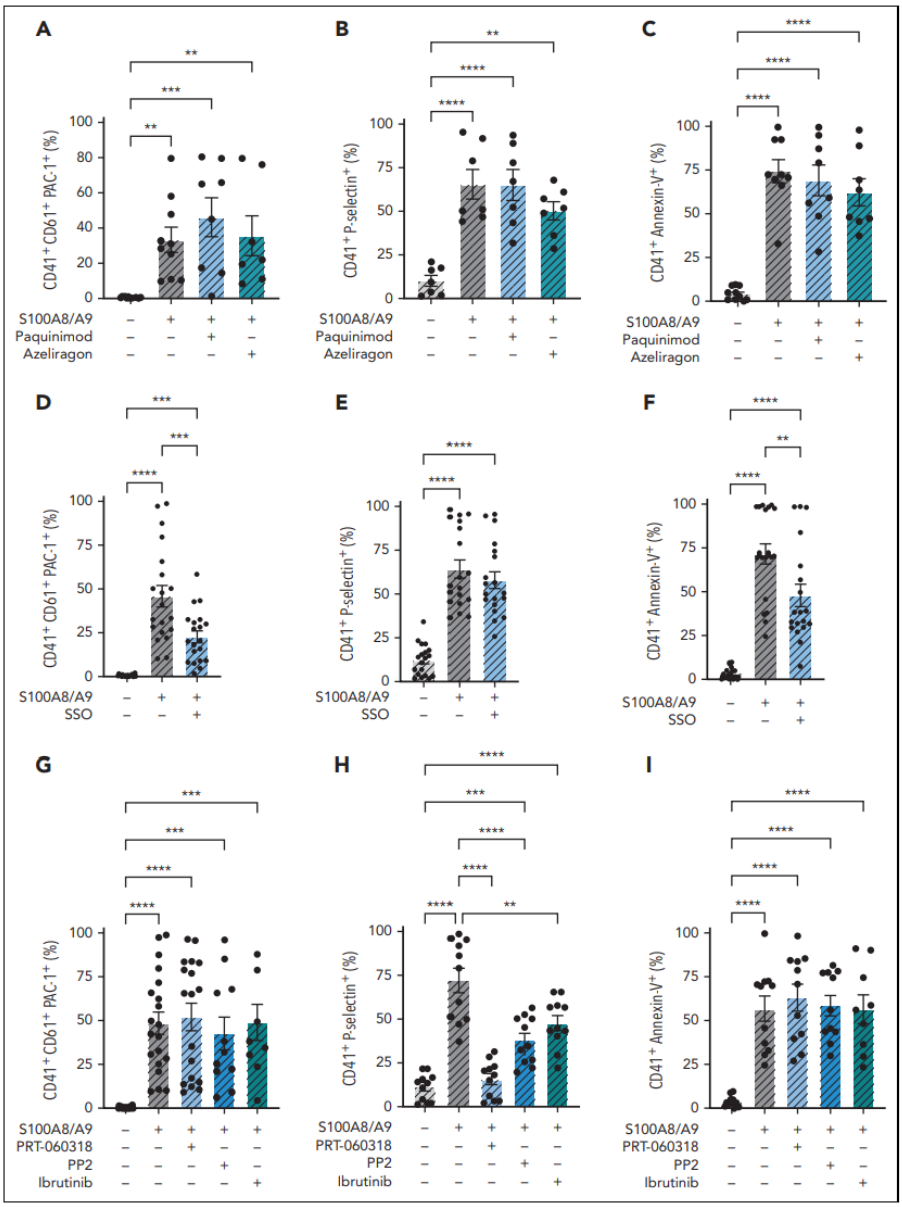


Figure 4. CD36 blockade partially decreases GPIIb/IIIa activation and PS exposure, whereas immunoreceptor tyrosine-based activation motif receptor inhibition reduces P-selectin expression. Human washed platelets (10^6 platelets/condition) were incubated with S100A8/A9 (20 μg/mL) with or without different inhibitors for 30 minutes at 37°C. Inhibitors were preincubated with platelets for 10 minutes before addition of S100A8/A9: Paquinimod (blocks S100A9 binding to TLR4) (10 μM), RAGE inhibitor Azeliragon (1 μM), CD36 inhibitor SSO (25 μM), Syk inhibitor PRT-060318 (10 μM), Src inhibitor PP2 (20 μM), and BTK inhibitor Ibrutinib (500 nM) were used. Platelet activation was determined by flow cytometry using (A,D,G) anti-CD41/CD61 PAC-1 antibody, (B,E,H) anti–P-selectin antibody, and (C,F,I) annexin-V binding. Data are shown as the percentage of platelets positive ($CD41^+$) for these markers. Data are shown as mean ± SD. Statistical significance was analyzed using ordinary one-way ANOVA. $^*P < .05$, $^{**}P < .005$, $^{***}P < .001$, $^{****}P < .0001$.

GPIbα with an EC50 of 130 ± 75 nM (Figure 6A). Furthermore, the effect of anti-GPIbα antibodies AK2, SZ2, and the peptide OS-1, which were previously described to block the interaction of GPIbα with VWF, on S100A8/A9-induced platelet activation was assessed as described above. SZ2 and AK2 antibodies, which recognize distinct epitopes on GPIbα located within the

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anionic/sulfated tyrosine region (Tyr2-Glu282) and in the first leucine-rich repeat (Leu36-Gln59), respectively, and OS-1 peptide, which allosterically inhibits GPIbα-VWF interaction, were tested at concentrations previously shown to inhibit VWF-mediated platelet agglutination[66-68] (supplemental Figure 4A-G, Figure 6B-D). AK2 and SZ2 significantly decreased PS exposure and GPIIb/IIIa activation, and this inhibition was further pronounced using a combination of both antibodies (Figure 6B-D, supplemental Figure E-G). OS-1 peptide[68] did not alter the effect of S100A8/A9 on platelets while inhibited VWF-induced platelet agglutination in the presence of ristocetin (supplemental Figure 4C-D,H-J). The effect of S100A8/A9 was not altered by increasing the concentrations of OS-1 (supplemental Figure 4H-J). Priming of platelets with S100A8/A9 did not induce platelet agglutination in the presence of VWF (Figure 6E-F), however, S100A8/A9 potentiated VWF-mediated platelet agglutination in the presence of ristocetin (Figure 6E,G). These results show that S100A8/A9 binds to GPIbα, overlapping with VWF-binding sites, and it potentiates VWF-dependent platelet agglutination.

GPIbα is the main receptor for S100A8/A9 on human platelets To confirm that GPIbα is the main receptor for S100A8/A9 on human platelets, platelet activation by S100A8/A9 was assessed in the presence of recombinant soluble GPIbα (His-17-Leu505) (rGPIbα) as a competitive strategy. Human washed platelets failed to respond to S100A8/A9 in the presence of equimolar concentrations of rGPIbα (1.7 μM) (Figure 7A-I). The inhibitory effect was observed for P-selectin upregulation (Figure 7A-C), PS exposure (Figure 7D-F), and GPIIb/IIIa activation (Figure 7G-H). Furthermore, rGPIbα inhibited the formation of PS-positive MV (Figure 7I).

The key role for GPIb was confirmed using platelets isolated from a patient with Bernard-Soulier syndrome (deficiency in GPIb-V-IX complex). The patient has a mutation in *GP9* gene resulting in a defect in GPIb/IX/V complex and the absence of GPIbα expression from platelets (detailed in Supplemental data). Platelets from this patient failed to respond to S100A8/A9, whereas the response to CRP was normal (Figure 7J-L). S100A8/A9 did not induce P-selectin expression, GPIIb/IIIa activation, or PS exposure (Figure 7J-L). These results confirm that GPIbα is the main receptor regulating S100A8/A9-mediated human and mouse platelet activation inducing P-selectin expression, GPIIb/IIIa activation, and the formation of procoagulant platelets and platelet-derived PS-positive MVs.

Discussion

In this study, we identified S100A8/A9-GPIbα axis as a novel mechanism triggering the formation of procoagulant platelets, independent of platelet aggregation. We show that (1) S100A8/A9 levels are increased in the plasma of patients with COVID-19 and that sustained high levels correlate with adverse outcome; (2) staining of lung autopsies from fatal COVID-19 cases showed a deposition of S100A8/A9 on lung vessel walls, in neutrophils, and a population of CD42b-positive cells; (3) immobilized recombinant heterodimeric S100A8/A9 accelerated fibrin generation at venous shear rate; (4) S100A8/A9 heterodimer but not S100A8 or S100A9 monomers/homodimers induced a slow alpha granule secretion, an increase in intracellular Ca^{2+} and a rapid exposure of PS; (5) S100A8/A9 failed to induce fibrinogen binding and platelet aggregation, despite GPIIb/IIIa activation; (6) S100A8/A9-mediated formation of procoagulant platelet occurred independently of S100A8/A9 classic receptors RAGE and TLR-4, but it was mediated by the platelet adhesion receptor GPIbα with a supporting role for CD36; and (7) S100A8/A9-mediated platelet activation is resistant to classic antiplatelet drug targeting cyclo-oxygenase and P2Y12.

Platelet activation and high levels of S100A8/A9 are observed in many thromboinflammatory diseases including MI, DVT, and infections such as COVID-19,[25,33,34,69,70] and these parameters correlate with thrombotic complications. The presence of procoagulant platelets with high cytosolic Ca^{2+} and PS levels were observed in patients with COVID-19, and these levels correlated with increased organ failure assessment score and D-dimer levels and were more pronounced in patients with thrombotic complications.[11,71] Moreover, procoagulant MVs are detected even in nonsevere forms of the disease.[72,73] The formation of procoagulant platelets in patients with COVID-19 is partially antibody driven;[71] however, alternative pathways might be involved, particularly in the early days after SARS-CoV-2 infection. In accordance to previous reports,[25,26,74] we found elevated levels of S100A8/A9 in patients with COVID-19. The levels of S100A8/A9 were higher in severely ill patients and sustained high levels of S100A8/A9 over the first week of monitoring were observed in nonsurvivors and associated with worse prognosis. In lung autopsies from fatal COVID-19 cases, S100A8/A9 was found mainly in neutrophils, a population of CD42b-positive cells, and on the vessel wall. NETosis, neutrophil necrosis, and platelet activation were shown to induce S100A8/A9 release, and these levels were further enriched in pathologic conditions.[28,75] Platelets isolated from COVID-19 patients are enriched in S100A8 and S100A9 mRNA and platelet releasate from COVID-19 patients induces endothelial cell activation in vitro in a CD36-dependent manner.[28] In our cohort, S100A9 was detected in platelets, whereas the presence of the S100A8/A9 heterodimer was less pronounced. It was recently shown that neutrophils transfer S100A8 and S100A9 to platelets during NETosis or necrosis and the abundance of S100A8/A9 in platelets depends on neutrophil count in patients with ST-elevation MI.[69] Whether platelets contain S100A8 and S100A9 as monomer/homodimers rather than heterodimers with distinct proinflammatory and prothrombotic functions requires further investigation.

Because S100A8/A9 deposition on venules supports immune cell recruitment and transmigration,[29] we assessed its capacity to induce platelet activation and thrombosis at venous shear rate. Although immobilized S100A8/A9 induced platelet adhesion, an increase in intracellular Ca^{2+}, the expression of P-selectin, PS-positive platelets, and MVs, S100A8/A9 alone did not support platelet aggregation and significant fibrin generation at venous shear rate. However, co-immobilization of S100A8/A9 with collagen accelerated fibrin generation in recalcified blood, which was associated with early recruitment of PS-positive platelets. This procoagulant effect was not observed in PRP, because addition of soluble S100A8/A9 in PRP did not support thrombin generation. These data suggest that immobilized S100A8/A9 rather than plasma-soluble S100A8/A9 represents a potent prothrombotic danger signal supporting thrombosis in healthy donors. It is tempting to speculate that S100A8/A9 deposition on the vasculature in

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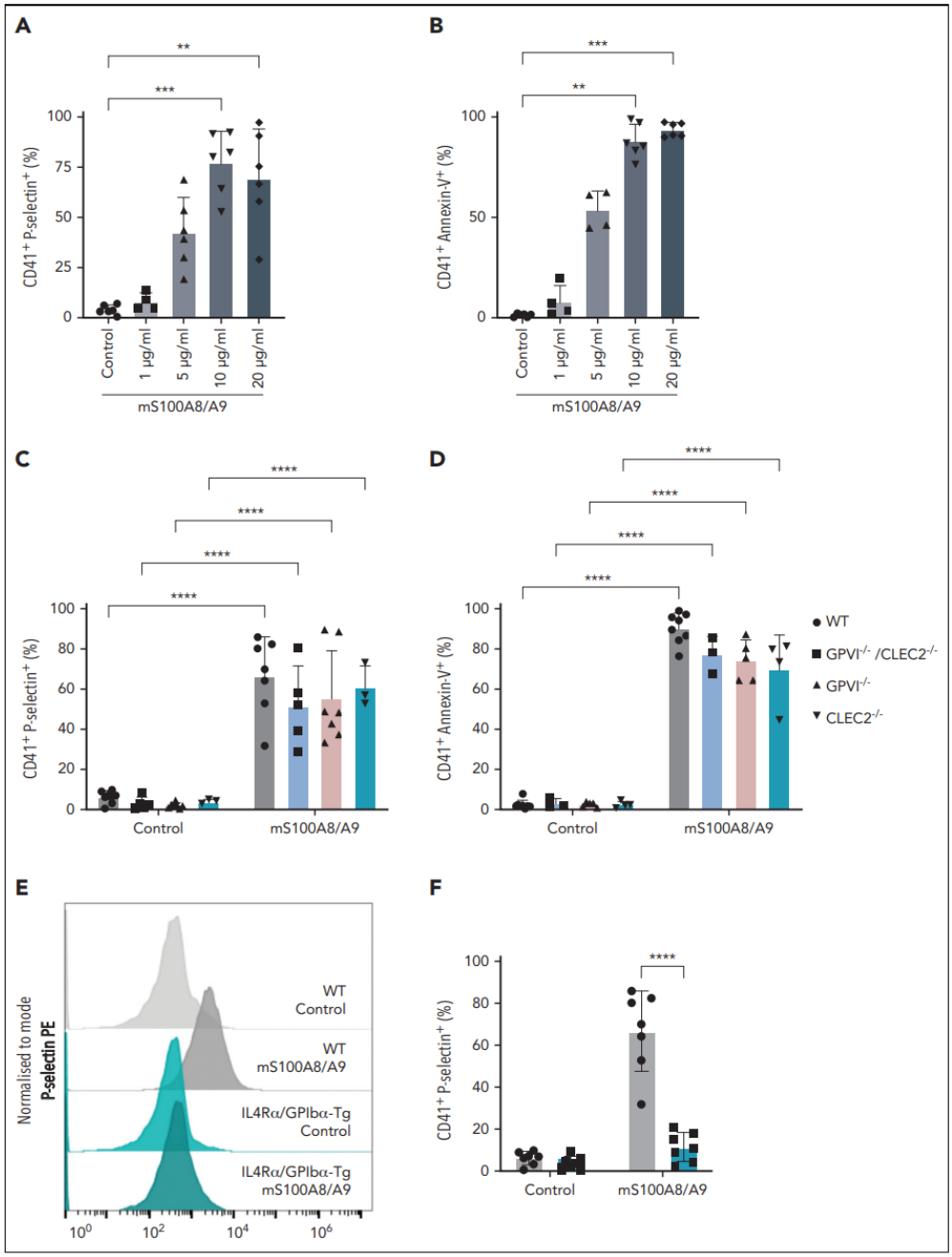


Figure 5. Murine S100A8/A9 activates mouse platelets through GPIbα and CD36. Washed murine platelets (10^6 platelets/condition) were incubated with different doses of recombinant mouse S100A8/A9 for 30 minutes at 37°C. Platelet activation was determined by flow cytometry using (A) anti–P-selectin antibody and (B) annexin-V binding. (C-K) Washed platelets isolated from WT, GPVI ($GPVI^{-/-}$), CLEC-2 ($CLEC\text{-}2^{-/-}$), GPVI/CLEC-2 knock-out ($GPVI^{-/-}/CLEC\text{-}2^{-/-}$), or $GPIb\alpha^{-/-}$ mice (IL4R/GPIbα–Tg mice) were incubated with S100A8/A9 (10 µg/mL) for 30 minutes at 37°C. The percentage of platelets positive for P-selectin, GPIIb/IIIa activation (JON/A), and annexin-V binding were

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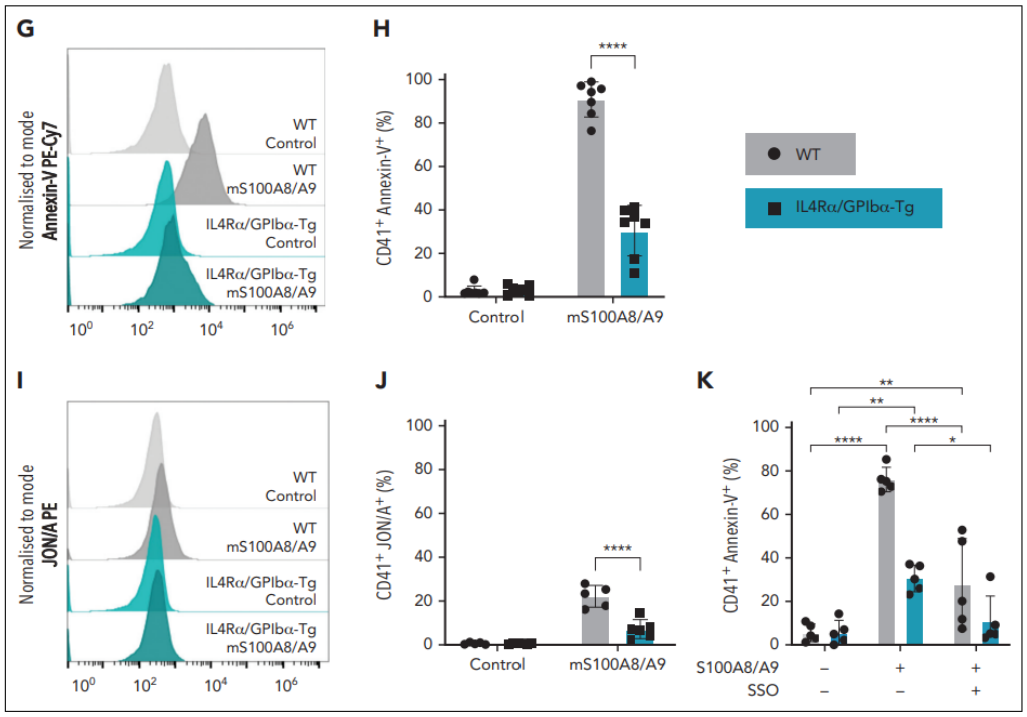


Figure 5 (continued) measured by flow cytometry. Data are shown as percentage platelets ($CD41^+$) positive for different markers. (K) Platelets isolated from WT or IL4R/GPIbα−Tg mice were pretreated with SSO (25 μM) before addition of S100A8/A9 (10 μg/mL) for 30 minutes at 37°C, and annexin-V binding was assessed by flow cytometry. The statistical significance was analyzed using nonparametric test (Kruskal-Wallis test) (A-B), ordinary two-way ANOVA with Sidak's multiple comparison test (C,D,F,H,J), and two-way ANOVA with Tukey's multiple comparison test (K). Data are shown as mean ± SD. $*P < .05$, $**P < .005$, $***P < .001$.

COVID-19 patients contributes to the formation of fibrin-rich thrombi.

The early recruitment of PS-positive platelets and subsequent fibrin generation on immobilized S100A8/A9 suggest the formation of procoagulant platelets. The formation of procoagulant platelets in vitro is classically studied after co-stimulation of platelets with collagen and thrombin or with Ca^{2+} ionophore A23187, which can lead to the formation of 2 types of procoagulant platelets, with distinct properties.[60] The first population is associated with a sustained level of intracellular Ca^{2+}, depolarized mitochondrial membrane, and inactivated GPIIb/IIIa with low capacity to aggregate. The second population retains mitochondrial membrane potential with intracellular Ca^{2+} levels returning to normal, active GPIIb/IIIa, and high aggregation properties.[60,76-79] S100A8/A9-induced procoagulant platelets share common features of both populations, which might be due to distinct signaling pathways downstream of S100A8/A9 binding to platelets. S100A8/A9, but not S100A8 or S100A9 alone, induced procoagulant platelets with activated GPIIb/IIIa. However, S100A8/A9-activated platelets failed to bind fibrinogen and to induce platelet aggregation, suggesting the presence of a novel prothrombotic mechanism associated with GPIIb/IIIa activation but independent of platelet aggregation. We further identified GPIbα as the main receptor for S100A8/A9 supporting the formation of procoagulant platelets, both in mice and humans. GPIbα has multiple extracellular agonists including VWF, thrombin, P-selectin,[80] and MAC-1,[81] and these interactions regulate thrombosis and inflammation.[82] Using antibodies recognizing epitopes on GPIbα involved in VWF binding, we further show that blocking Leu36-Gln59 region with AK2 antibody[66] or the anionic/sulfated tyrosine region (Tyr276-Glu282) with SZ2 antibody[67] decreased S100A8/A9-induced PS exposure and GPIIb/IIIa activation. These results suggest that S100A8/A9 effect on platelets is regulated by at least 2 different sites on GPIbα. It is possible that S100A8/A9 binding to these epitopes further affects the engagement of GPIbα with VWF, P-selectin, and MAC-1 altering immune cell activation and recruitment, independent of thrombosis. The anionic/sulfated region on GPIbα, with its high negative charge and sulfate groups, shares similarities with heparin, which can bind with high affinity to S100A8/A9.[46] Whether S100A8/A9 binding to this heparin-like sequence on GPIbα induces the formation of procoagulant platelets requires further investigation. S100A8/A9 binding to platelets further potentiates VWF-dependent platelet agglutination in the

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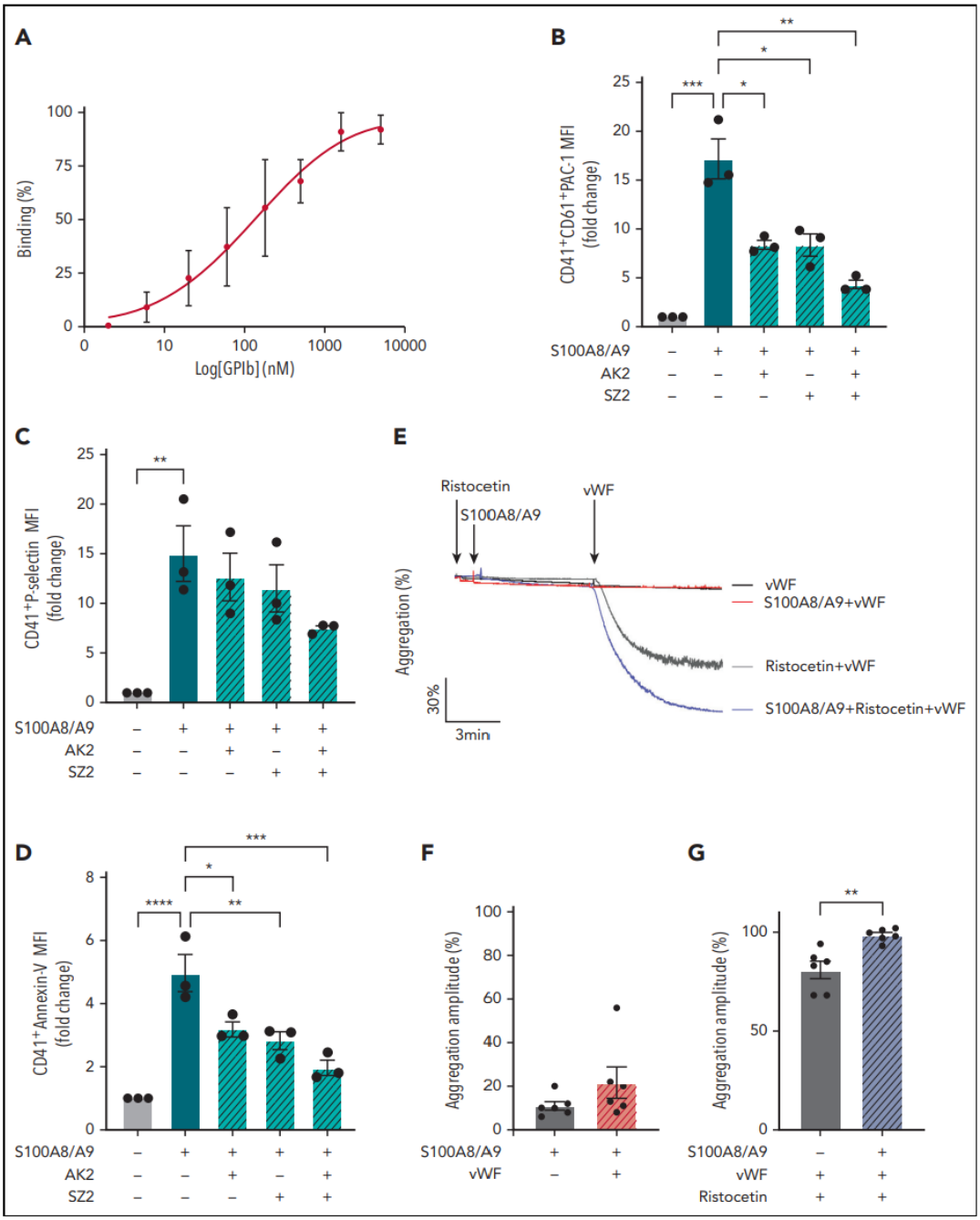


Figure 6. GPIbα binds to S100A8/A9 and increases VWF-dependent platelet agglutination. (A) Binding of rGPIbα to immobilized S100A8/A9. Data presented as mean ± SEM (n = 3). (B-D) Washed human platelets (10^6/condition) were preincubated with or without AK2 Ab (40 μg/mL), SZ2 (40 μg/mL), or both for 10 minutes at 37°C and then stimulated with S100A8/A9 (20 μg/mL) for 30 minutes at 37°C. Platelet activation was determined by flow cytometry using (B) anti-CD41/CD61 PAC-1 antibody, (C) anti–P-selectin antibody, and (D) annexin-V binding. Data are shown as MFI of treated platelets over control (unstimulated) for different markers. (E-G) Ristocetin-induced platelet aggregation was assessed by addition of VWF (1 μg/mL) and ristocetin (1.5 mg/mL) to washed platelets. Platelets were primed with S100A8/A9 and ristocetin for 6 minutes before addition of VWF. Platelet aggregation was monitored for 6 minutes after VWF addition (n = 6). (E) Representative trace. Data are shown as mean ± SEM. The statistical significance was analyzed using ordinary one-way ANOVA (B-D) and nonparametric Mann–Whitney *t* test (Kruskal-Wallis test) (F-G). **P* < .05, ***P* < .01, ****P*<.001, *****P* < .0001.

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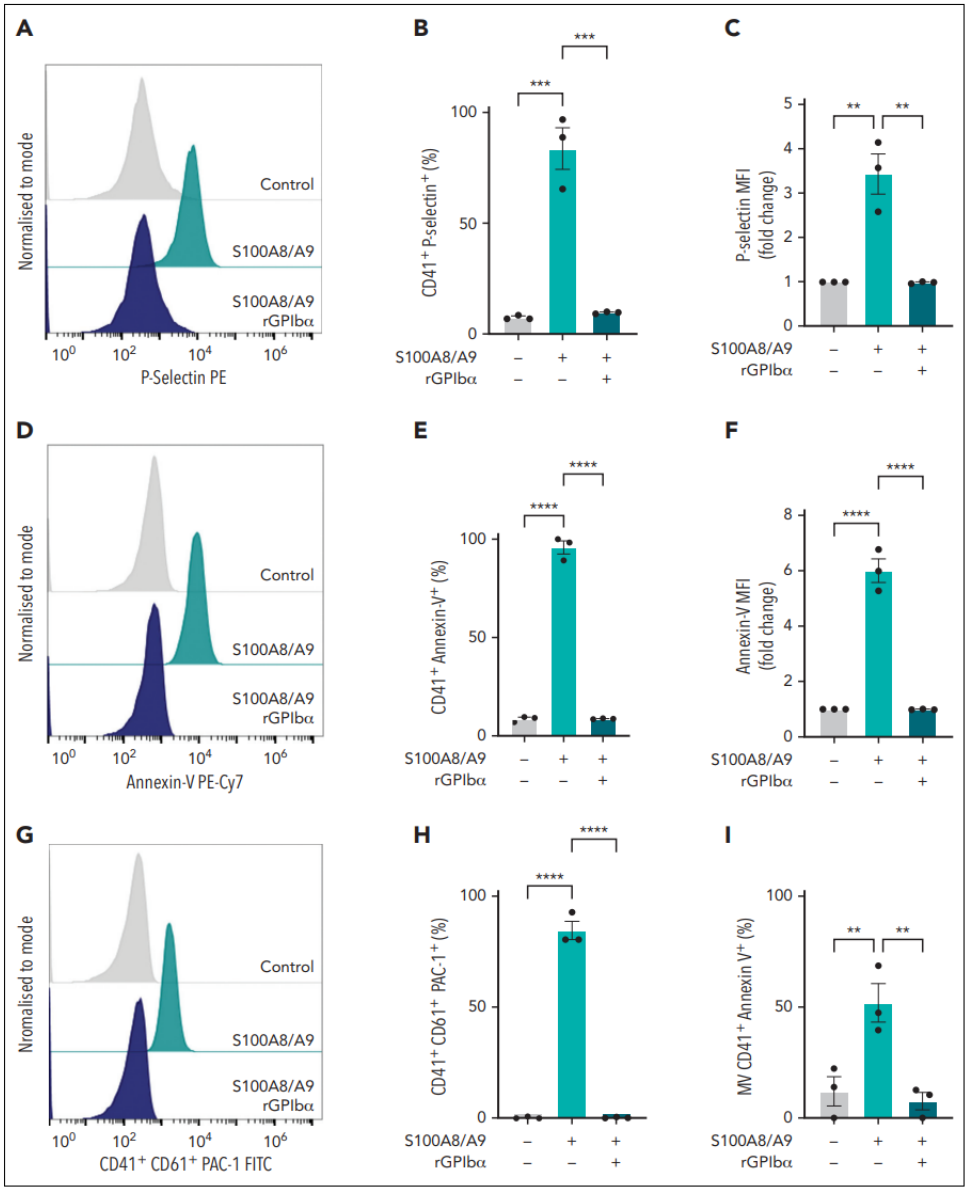


Figure 7. Recombinant human GPIbα blocks platelet response to S100A8/A9, whereas Bernard-Soulier syndrome platelets failed to respond to S100A8/A9. Washed human platelets (10^6 platelets/condition) were incubated with S100A8/A9 (40 μg/mL) in the absence or presence of recombinant human GPIbα (rGPIbα; 1.7 μM) for 30 minutes at 37°C (n = 3). Platelet activation was assessed by flow cytometry using (A-C) anti–P-selectin, (D-F) PS exposure using annexin-V, and (G-I) GPIIb/IIIa activation using PAC-1 antibody. Human washed platelets from a healthy donor or a Bernard-Soulier syndrome patient were incubated with S100A8/A9 (20 and 40 μg/mL) for 30 minutes at 37°C (J-L). CRP (10 μg/mL) was used as positive control. (J) GPIIb/IIIa activation, (K) P-selectin, and (L) PS exposure were measured. The statistical significance was analyzed using one-way ANOVA. Data presented as mean ± SEM. *$P < .05$, **$P < .005$, ***$P < .001$, ****$P < .0001$.

presence of ristocetin, suggesting synergy between S100A8/A9 and VWF. This can be further modulated by CD36 because blockade of the receptor with SSO reduces GPIIb/IIIa activation and PS exposure. Indeed, CD36 was previously shown to partially reduce S100A8/A9-induced thrombosis in mouse models of DVT, photochemical-induced carotid injury, and laser-induced injury to the cremaster microcirculation.[27,34] Our data support the contribution of CD36 to platelet GPIIb/IIIa

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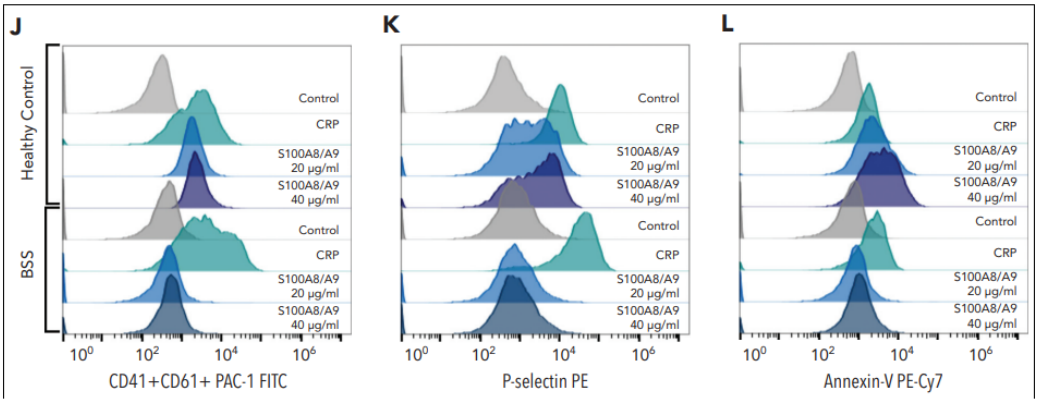


Figure 7. (continued)

activation and PS exposure without affecting P-selectin expression. It is likely that the initial interaction occurs through GPIbα with crosstalk between GPIbα and CD36, essential for platelet activation.[83]

In conclusion, our study identified for the first time the S100A8/A9-GPIbα axis as a novel immune-driven pathway for the formation of procoagulant platelets accelerating fibrin generation. Despite GPIIb/IIIa activation, platelets failed to bind to fibrinogen, suggesting that this mechanism of thrombosis is independent of platelet aggregation. Existing drugs can block distinct pathways, with Syk inhibitors reducing P-selectin expression on platelets and subsequent platelet-neutrophil aggregates. However, inhibition of GPIbα alone or in conjunction with CD36 is essential to limit PS exposure and GPIIb/IIIa activation. Because this novel mechanism is resistant to classic, emerging antiplatelet or anti-S100A8/A9 drugs targeting TLR4 and RAGE, this study supports the need for the development of novel therapies to limit the formation of procoagulant platelets in thromboinflammatory diseases associated with the release of S100A8/A9, such as COVID-19.

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Authorship

Contribution: M.C. designed and performed experiments, collected and analyzed data, and wrote the manuscript; W.C.S., G.P., M.J.S., J.P., J.C.C., J.B., J.S.R., Z.Z., and A.S. performed experiments and analyzed data; J.H.B. and A.O.K. analyzed data; M.P., N.S.P., P.L.R.N., P.H., A.J.I., G.E.R., S.P.W., and M.R.T. provided key reagents and contributed to data analysis; N.J.M. and A.A. provided key reagents and supported research analysis; J.R. designed research and experiments, performed experiments, collected and analyzed data, and wrote the manuscript; and all authors read and approved the paper.

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Footnotes

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APPENDIX 2

Heme induces human and mouse platelet activation through C-type-lectin-like receptor-2

Intravascular hemolysis is a serious complication developed in many severe infections such as malaria, sepsis,[1] typical hemolytic uremic syndrome[2] and congenital diseases such as sickle cell disease.[3] Hemolysis accompanied by thrombocytopenia, platelet activation, platelet-leukocyte aggregates, inflammation, vascular obstruction and organ damage. During hemolysis, red blood cell destruction leads to the release of molecules such as ADP, hemoglobin and heme which exert potent prothrombotic and proinflammatory actions.

Under physiological conditions, free heme is scavenged by the plasma protein hemopexin, and is subsequently catabolized by heme oxygenase-1 into carbon monoxide, biliverdin and ferrous iron (Fe^{2+}). However, acute or/and chronic hemolysis exhausts the scavenging system leading to an increase in free heme in the blood. Upon release, reduced heme is rapidly and spontaneously oxidized in the blood into ferric (Fe^{3+}) form, hemin, with increased levels observed in hemolytic diseases. It was recently shown that hemin induces human platelet activation and platelet death through ferroptosis, an intracellular iron-mediated cell death.[4] However, the mechanisms and receptors involved in platelet activation by hemin are not known.

In this study, we investigated hemin-mediated human and mouse platelet activation *in vitro*. An increase in labile (i.e., weakly bound and free) heme/hemin is detected in patients with hemolytic diseases with plasma concentrations ranging between 2 μM and 50 μM.[5,6] Similar concentrations of heme are detected in mice with chronic or acute hemolysis such as sickle cell mice or Escherichia coli-induced hemolysis.[1,7] In this study, we show that hemin (2-12 μM) induces rapid aggregation of human washed platelets, while aggregation is slower and reduced in magnitude at higher (≥ 25 μM) concentrations (Figure 1A, B). Platelet aggregation was associated with increased P-selectin expression and GPIIbIIIa activation as measured by flow cytometry (Figure 1C, D). High (≥25 μM) but not lower concentrations of hemin significantly increased phosphatidylserine (PS) exposure on platelets as assessed using Annexin V staining (Figure 1E). Moreover, high concentrations of hemin induced toxicity as measured by increased levels of lactate dehydrogenase (LDH) in the supernatant of activated platelets (Figure 1F). Recent data have shown that a high concentration of hemin (25 μM) triggers lipid peroxidation and human platelet death through ferroptosis but not apoptosis or necroptosis.[4] Whether the increase in PS exposure modulates the contribution of platelets to the coagulopathies observed in hemolytic diseases requires further investigation.

Hemin is known to bind to Toll-like receptor (TLR) TLR4 on endothelial cells resulting in endothelial cell activation including secretion of Weibel-Palade bodies.[3] However, blocking TLR4 signaling in platelets using TAK-242 did not alter activation by hemin, suggesting a TLR-4-independent pathway of activation (Figure 1H). At low concentrations, human platelet aggregation by hemin was blocked by inhibitors of Syk (PRT-060318), Src family kinases (PP2) and Btk (Ibrutinib), and partially by GPIIbIIIa inhibitor eptifibatide (Integrilin) (Figure 1G, H). These results suggest that hemin induces human platelet activation *via* an immunoreceptor-tyrosine-based activation-motif (ITAM) receptor-based pathway.[8] Indeed, low concentrations of hemin provoked phosphorylation of Syk and PLCγ2 (Figure 1I). In order to identify the receptor triggering platelet activation by hemin, we used recombinant dimeric forms of C-type-lectin-like receptor-2 (CLEC-2) and collagen receptor glycoprotein VI (GPVI) (hFc-CLEC-2 and hFc-GPVI, respectively) as a blocking strategy. Pre-incubation of hemin with hFc-CLEC-2 but not hFc-GPVI abolished platelet aggregation by hemin identifying CLEC-2 as the receptor mediating human platelet activation by hemin (Figure 1G, H). These results also suggest a direct interaction between hemin and CLEC-2. However, while we believe that the inhibition is likely the result of competition between recombinant and platelet CLEC-2, we cannot rule out that recombinant CLEC-2-hemin complex may interfere with CLEC-2 clustering thereby inhibiting platelet activation. Platelet aggregation was not altered by AYP1, 9, an antibody that blocks the interaction between CLEC-2 and podoplanin, providing indirect evidence that hemin binds to a distinct site on CLEC-2 to podoplanin (Figure 1H). Protoporphyrin IX, the precursor of heme, and cobalt hematoporphyrin were shown to bind to CLEC-2 and inhibit podoplanin-CLEC-2 interaction without inducing platelet activation.[10] The type of interactions by which different porphyrins selectively bind to CLEC-2 and induce platelet activation requires further investigation. Platelet aggregation mediated by low concentrations of hemin (6.25 μM) was not altered by cyclooxygenase (COX) (indomethacin) or P2Y12 (cangrelor) inhibitors, showing that, in contrast to podoplanin, secondary mediators are not required for hemin-mediated platelet activation (Figure 1G, H). This may reflect the ability of hemin to induce higher oligomers of CLEC-2 increasing signal transduction. Platelet aggregation does not depend on oxidative stress as hemin-mediated platelet aggregation was not altered by the antioxidant N-acetyl cysteine (NAC) (Figure 1H; Online Supplementary Figure S1). At higher concentrations (>25 μM), platelet aggregation was not inhibited by integrilin or inhibitors of Src, Syk and Btk, suggesting that higher concentrations of hemin induce agglutination (*Online Supplementary Figure S2*). Increasing the concentrations of inhibitors did not alter platelet agglutination/aggregation (*not shown*). The distinct mechanisms of platelet activation by low and high concentrations of hemin might be related to formation of hemin aggregates at high concentrations, which might exert different activities as compared to monomeric or dimeric hemin present at low concentrations.

Hemin induces mouse platelet aggregation, albeit at a slightly higher concentration (Figure 2A). Similar to human platelets, aggregation by low concentrations of hemin was inhibited by inhibitors of Src, Syk and Btk tyrosine kinases, recombinant mouse Fc-mCLEC-2 and GPIIbIIIa blockade (Figure 2B, C). In contrast, inhibitors of TLR4, COX and $P2Y_{12}$ had no effect on platelet aggregation by hemin (Figure 2C). Platelet aggregation by hemin was significantly reduced in CLEC-2-deficient platelets confirming a key role for CLEC-2 (Figure 2D, E). However, deletion of CLEC-2 did not inhibit platelet shape change suggesting that one or more other receptors support platelet activation by hemin in mice.

The fact that recombinant CLEC-2 inhibits hemin-mediated platelet aggregation suggests a direct interaction between hemin and CLEC-2. Using surface plasmon resonance-based technique, we demonstrated that hemin binds to both murine and human recombinant CLEC-2 with K_D values of ~200 nM in both cases (Figure 3A, C). Hemin binding to mouse CLEC-2 was characterized by kinetic rate association (k_a) and dissociation (k_d) constants of k_a=2.77±0.08×104 mol^{-1} s^{-1} and 5.76±0.09×10^{-3} s^{-1}.

Similar values of kinetic rate constants were determined for hemin binding to human CLEC-2, ka=2.65±0.09×10^4 mol^{-1} s^{-1} and kd=5.06±0.05×10^{-3} s^{-1}. The interaction of hemin with human and mouse CLEC-2 was further confirmed by UV-vis absorbance spectroscopy (Figure 3B, D). The differential absorbance spectra profiles revealed significant shifts of the spectra of hemin towards higher wavelength by human and mouse CLEC-2 demonstrating a direct binding. The differences in the spectral changes of hemin in the presence of human and mouse CLEC-2 can be explained by distinct residues coordinating heme's iron. The blue shift in the Soret region observed with mCLEC-2 suggests the involvement of sulfur containing amino acid (cysteine)[11] whereas the red shift observed with human CLEC-2 suggests histidine coordination.

Altogether, these results show that hemin is an endogenous agonist for CLEC-2 leading to platelet activa-

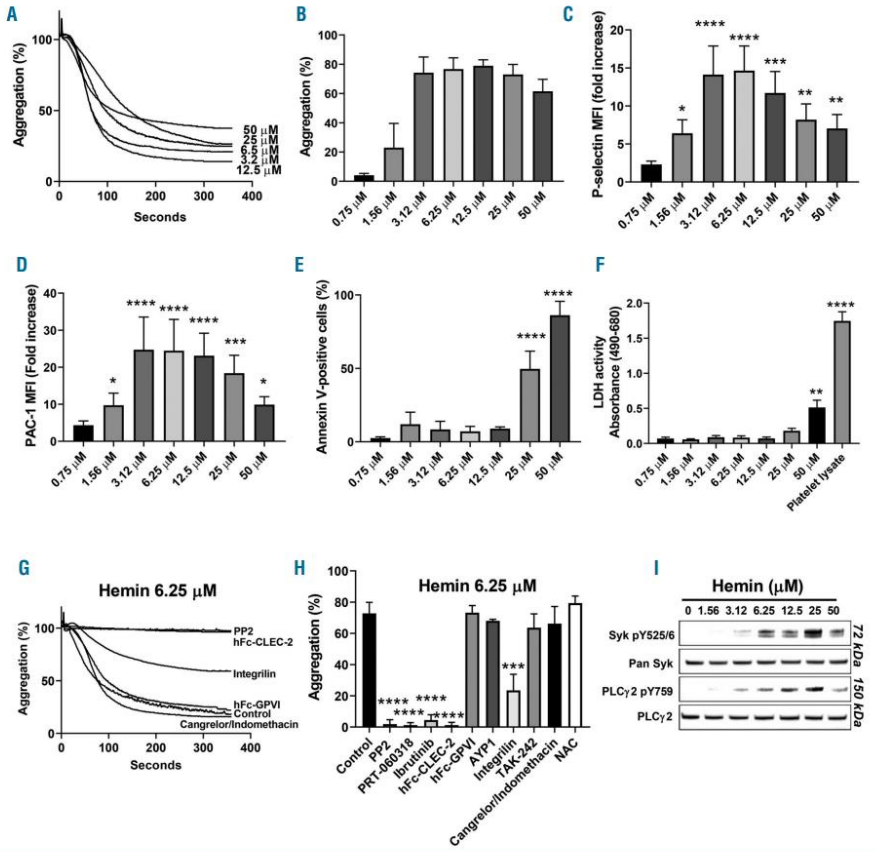


Figure 1. Hemin mediates human platelet activation through C-type-lectin-like receptor-2. (A, B) Human washed platelets (2x108/mL) were incubated with increasing concentrations of hemin in the presence of Ca^{2+} (2 mM). Platelet aggregation was assessed for 6 minutes (min) using light transmission aggregometry (n=5). (C, D) For flow cytometry analysis, 1x10^6 platelets were incubated with different concentrations of hemin for 20 min at 37°C. Platelet activation was determined by flow cytometry using (C) anti-P-selectin and (D) GPIIbIIIa PAC-1 antibodies (n=4). Data is shown as fold increase of median of fluorescence (MFI) of treated platelets over control (absence of hemin). (E) Phosphatidylserine exposure was assessed using Annexin V staining. (F) Lactate dehydrogenase (LDH) levels were measured in the supernatant following platelet aggregation (6 min). Platelet lysate was used to detect the total level of LDH in platelets. (G, H) Platelet aggregation by hemin was assessed using Btk inhibitor Ibrutinib (500 nM), Src family kinase inhibitor PP2 (20 µM), Syk inhibitor PRT-060318 (20 µM), TLR4 inhibitor TAK-242 (10 µM), $P2Y_{12}$ inhibitor Cangrelor (10 µM) or cyclooxygenase inhibitor indomethacin (10 µM). Platelets were pre-incubated with different inhibitors for 5 min at 37 °C prior to the addition of hemin. Dimeric C-type-lectin-like receptor-2 (CLEC-2) (hFc-CLEC-2, 20 µg/mL) or dimeric collagen receptor glycoprotein VI (GPVI) (hFc-GPVI, 20 µg/mL) were pre-incubated with hemin for 20 min at 37 °C prior to addition to platelets (n=5). (H) Histogram data are shown as mean ± standard deviation. (I) Protein levels of the phosphorylation of Syk and PLCγ2, and total Syk and PLCγ2 in platelet lysates were determined by western blotting after 6 min aggregation. Western blots are representative of five independent experiments. The statistical significance was analyzed using a one-way ANOVA with Tukey's multiple comparisons test using Prism 8 (GraphPad Software Inc, USA). Significance is shown compared to control (absence of hemin), *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

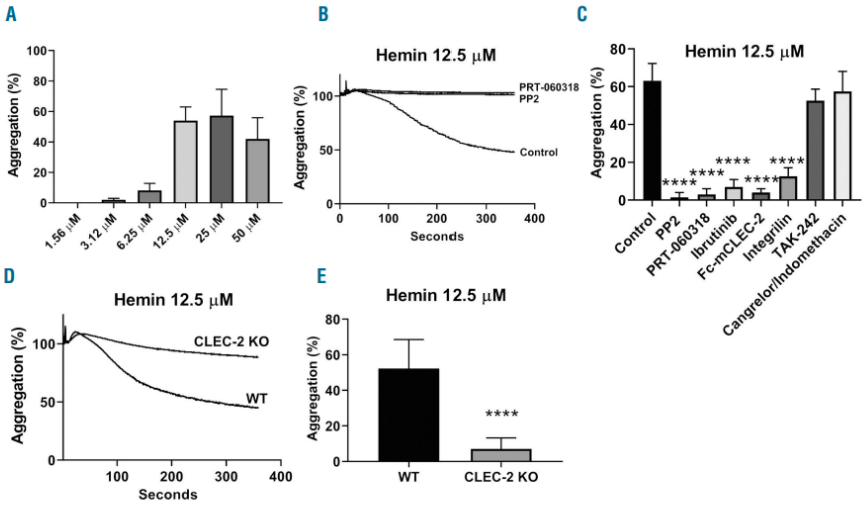


Figure 2. Hemin induces mouse platelet aggregation. (A) Mouse washed platelets ($2x10^8$/mL) were incubated with increasing concentrations of hemin in the presence of Ca^{2+} (2 mM). Platelet aggregation was assessed using light transmission aggregometry. (B, C) Hemin-mediated platelet aggregation (12.5 μM hemin) was assessed in the presence of Ibrutinib (2 μM), PP2 (20 μM), PRT-060318 (20 μM), TAK-242 (10 μM), Cangrelor (10 μM), recombinant mouse C-type-lectin-like receptor-2 (CLEC-2) (Fc-mCLEC-2, 10 μg/mL) (n=4). (D, E) Washed platelets from wild-type (WT) or Clec-2 deficient (Clec-1bfl/fl PF4cre, Clec-2 KO) were incubated with hemin (12.5 μM) in the presence of Ca^{2+} (2 mM) (n=5). (E) Histogram data are shown as mean ± standard deviation. The statistical significance was analyzed using a one-way ANOVA with Tukey's multiple comparisons test using Prism 8. Significance is shown compared to control ****P<0.0001.

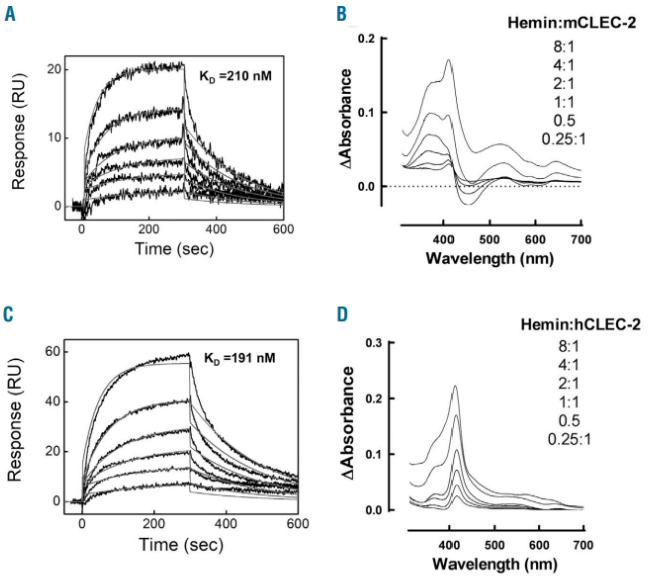


Figure 3. Hemin binds to mouse and human recombinant C-type-lectin-like receptor-2. (A, C) For surface plasma resonance, real-time interaction profiles were obtained after injection of increasing concentrations of hemin (19.5–625 nM) over recombinant mouse (A) and human (C) C-type-lectin-like receptor-2 (CLEC-2) immobilized on CM5 sensor chip. The association and dissociation phases were followed for 5 minutes, each. The black lines show the experimental data, the grey lines depict the fit obtained using Langmuir global analyses model. (B, D) For UV-vis absorbance spectroscopy, differential spectra were generated after titration of (B) mouse or (D) human dimeric CLEC-2 (2 μM) with increasing concentrations of hemin (0.5–16 μM). The differential spectra were obtained after subtraction of the spectra of hemin at a given concentration from the spectra of the same concentration of hemin in the presence of the protein. The measurements were done at 25°C in optical cell with 10 mm light path.

tion through activation of integrin GPIIbIIIa at low concentrations and agglutination at high concentrations. Recombinant CLEC-2 inhibits platelet activation by both mechanisms suggesting that this may represent a novel form of therapeutic intervention to inhibit platelet activation in hemolytic disease.

Ethical approval for collecting blood from healthy volunteers was granted by Birmingham University Internal Ethical Review (ERN_11-0175). Mice experiments were performed in accordance with UK laws (Animal [Scientific Procedures] Act 1986) with approval of the local ethical committee and UK Home Office approval (PPL P0e98D513).

Human and mouse washed platelets were prepared as previously described.[12,13] Platelet aggregation was assessed using light transmission aggregometry for 6 minutes (ChronoLog, Havertown, PA, USA). Fresh synthetic hemin solution was freshly prepared (Frontiers Scientific, USA) for each experiment. PP2 (Tocris/Biotechne Ltd), PRT-060318 (Caltag medsystems), indomethacin (Sigma-Aldrich), cangrelor (The Medicines Company), Ibrutinib (Stratech Scientific), N-Acetyl-L-cysteine (Sigma-Aldrich) and TAK-242 (Sigma-Aldrich) were preincubated for 5 minutes (min) at 37°C with platelets prior to addition of hemin. Human (h) and mouse (m) CLEC-2 and GPVI were produced as Fc fusion proteins (hFc-CLEC-2, hFc-GPVI, Fc-mCLEC-2) in human embryonic kidney HEK293 cells and purified using chromatography affinity. Recombinant human and mouse CLEC-2 were preincubated with hemin for 15 min at 37°C before addition to platelets. Following aggregation, platelets were centrifuged at 1,000g for 10 min and LDH was measured in the supernatant using CyQUANT™ LDH Cytotoxicity Assay (ThermoFisher Scientific). Clec-2 deficient mice (Clec-1bfl/fl PF4cre) and littermate controls were previously described.[14]

Platelet activation (106 platelets) by different concentrations of hemin (20 min at 37°C) was assessed by flow cytometry with antibodies against CD62P (PE/Cy5 anti-human CD62P antibody, biolegend) and activated GPIIbIIIa (Alexa Fluor® 647 anti-human CD41/CD61 antibody PAC1, Biolegend) using BD Accuri C6 Plus flow cytometer (BD Bioscience). For western blotting, Phospho-Syk (Tyr525/526) (C87C1) Rabbit mAb (#2710), Phospho-PLCγ2 (Tyr759) antibody (#3874), Syk antibody (#2712) and PLCγ2 antibody (#3872) were used (Cell signalling). Protein phosphorylation was assessed after 6 min of aggregation as previously described.[12]

The binding of human and mouse dimeric CLEC-2 to hemin was performed at 25°C and assessed using surface plasma resonance and UV-spectroscopy methods.[15]

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Contributions: JHB, MC performed experiments and analyzed data; YD, AS, EM generated reagents; LTR contributed to data interpretation; JDD contributed to research design, performed experiments and interpreted data; SPW contributed to research and data interpretation and provided reagents; JR designed and performed research, collected data, analyzed and interpreted data and wrote the manuscript. All authors reviewed and approved the manuscript.

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APPENDIX 3

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STATE OF THE ART

Novel mechanisms of thrombo-inflammation during infection: spotlight on neutrophil extracellular trap-mediated platelet activation

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Abstract

A state-of-the-art lecture titled "novel mechanisms of thrombo-inflammation during infection" was presented at the ISTH Congress in 2022. Platelet, neutrophil, and endothelial cell activation coordinate the development, progression, and resolution of thrombo-inflammatory events during infection. Activated platelets and neutrophil extracellular traps (NETs) are frequently observed in patients with sepsis and COVID-19, and high levels of NET-derived damage-associated molecular patterns (DAMPs) correlate with thrombotic complications. NET-associated DAMPs induce direct and indirect platelet activation, which in return potentiates neutrophil activation and NET formation. These coordinated interactions involve multiple receptors and signaling pathways contributing to vascular and organ damage exacerbating disease severity. This state-of-the-art review describes the main mechanisms by which platelets support NETosis and the key mechanisms by which NET-derived DAMPs trigger platelet activation and the formation of procoagulant platelets leading to thrombosis. We report how these DAMPs act through multiple receptors and signaling pathways differentially regulating cell activation and disease outcome, focusing on histones and S100A8/A9 and their contribution to the pathogenesis of sepsis and COVID-19. We further discuss the complexity of platelet activation during NETosis and the potential benefit of targeting selective or multiple NET-associated DAMPs to limit thrombo-inflammation during infection. Finally, we summarize relevant new data on this topic presented during the 2022 ISTH Congress.

KEYWORDS
DAMPs, histones, NETosis, platelets, S100A8/A9

Essentials

- Activated and procoagulant platelets and NETs are increased in patients with sepsis and COVID-19.
- S100A8/A9 and histones induce prothrombotic, procoagulant, and proinflammatory platelets.
- NET-associated DAMPs are targets in thrombo-inflammatory diseases.
- Finally, we summarize the related new research presented at the ISTH congress in 2022 in London.

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1 | INTRODUCTION

Neutrophils are key components of the innate immune system and the first immune cells recruited to the site of infection. They participate in host defense against pathogens through different mechanisms including pathogen phagocytosis, degranulation, and the release of neutrophil extracellular traps (NETs). Beyond their protective functions in trapping and killing pathogens, NETs can become pathogenic and contribute to organ damage and thrombotic complications in thrombo-inflammatory diseases including sepsis, COVID-19, deep vein thrombosis (DVT), sickle cell disease, stroke, and ischemia/reperfusion injury.

2 | MECHANISMS OF NET FORMATION: SUICIDAL VERSUS VITAL NETOSIS

The traditional concept that neutrophils are a short-lived homogeneous cell population was recently revised with the identification of multiple neutrophil phenotypes and functional states in healthy donors and patients with various diseases. The density gradient separation identified high-density neutrophils and low-density neutrophils (LDNs) populations, the latter isolated with the peripheral blood mononuclear cells. LDNs represent 2% to 10% of total neutrophils in healthy individuals, and these levels increase in pathological conditions, including in patients with autoimmune diseases (rheumatoid arthritis, systemic lupus erythematosus [1]), cancer, sepsis [2], and COVID-19 [3]. LDNs show enhanced proinflammatory phenotype, reactive oxygen species (ROS) production, and spontaneous NET formation [4]. In patients with COVID-19, the LDNs population was associated with coagulopathy and was found in a complex with highly activated platelets [5]. An expansion of this population is also observed in septic patients [6]. and mice, similarly characterized by a proinflammatory signature and increased NET formation [7].

NETs are web-like structures composed of decondensed chromatin fibers decorated with over 24 granular, cytoplasmic, and nuclear-derived antimicrobial proteins, peptides, and enzymes such as neutrophil elastase (NE), cathepsin G, myeloperoxidase (MPO), S100A8/A9, and histones [8,9]. Histones are grouped into 2 classes: lysine-rich (H1, H2A, H2B) and arginine-rich (H3 and H4) [9]. Two of each core histone (H2A, H2B, H3, and H4) are joined by a 'linker' histone (H1) to form the nucleosome and high-order structures.

NET formation or "NETosis," is a form of programmed cell death associated with the loss of membrane integrity and impaired neutrophil functions [8]. This form of NETosis, termed suicidal NETosis, can be triggered *in vitro* following the interaction of neutrophils with pathogens, immune complexes, calcium (Ca^{2+}) ionophore, inflammatory stimuli, some damage-associated molecular patterns (DAMPs), and by protein kinase C (PKC) activators such as phorbol-12-myristate-13-acetate (PMA) [8]. NETs are also observed *in vivo* in experimental models of infection and sterile inflammation and in patients with thrombo-inflammatory events. Whereas all these stimuli release proteolytically active NETs, different receptors and signaling pathways are involved in an agonist-dependent manner [10,11]. PMA, 1 of the most used inducers of NETosis *in vitro*, activates PKC leading to nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-dependent ROS generation, the selective release of NE from azurophilic granules into the cytosol, and subsequent translocation into the nucleus where H3 and H4 are cleaved. NE activity is also required for MPO translocation to the nucleus, which synergistically enhances the decondensation of chromatin, independent of MPO enzymatic activity [12]. The translocated NE and MPO quickly degrade the linker H; however, chromatin decondensation is primarily driven by the degradation of core histones, particularly late degradation of histone H4 [12,13]. Chromatin decondensation also depends on peptidylarginine deiminase 4 (PAD4), which induces the citrullination of H3 and H4 [14]. The citrullination of histones neutralizes the positive charge on arginine residues, increasing hydrophobicity, chromatin relaxation and disintegration of the nuclear envelope. Rupture of the nuclear membrane allows the mixing between nuclear DNA, granular, and cytosolic proteins followed by dissociation of the plasma membrane and the release of NETs into the extracellular space [12,15]. Subsequently, NETs can directly trap and kill pathogens and transfer antimicrobial peptides to macrophages enhancing their antimicrobial activity [16].

Alongside suicidal NETosis, another form of nonsuicidal NETosis, named as vital NETosis, has been described [17–19]. During vital NETosis, neutrophils are recruited to the site of infection in mice within 5 to 60 minutes and release NETs while maintaining cellular membrane integrity and preserving classical neutrophil functions [20]. Indeed, vital NETosis has been observed in neutrophils undergoing active phagocytosis without membrane disintegration [18–20]. Nuclear chromatin traffics from the nucleus to the extracellular space via vesicles in an oxidant-independent pathway liberating their DNA content following lysis [16]. Nuclear rupture and liberation of DNA in the cytoplasm can also occur during vital NETosis independent of vesicle release [19]. Vital NETosis occurs independently of NADPH activity and ROS generation and relies on increased intracellular Ca^{2+}. In a mouse model of *Staphylococcus aureus (S. aureus)*-induced skin infection [19]. breakdown of the nuclear envelope, chromatin decondensation, intracellular dispersion, and NETosis were shown to be dependent on Toll-like receptor (TLR) 2 and complement opsonization [19]. Lipopolysaccharide (LPS), a gram-negative bacterial stimulus, also induces rapid NET release *in vivo* through vital NETosis, which did not involve cell lysis and is mediated by TLR4 on platelets [17]. Priming of neutrophils with granulocyte macrophage-colony stimulating factor followed by short activation of TLR4 or complement C5a receptor induces another form of ROS-dependent vital NETosis associated with the released of mitochondrial but not nuclear DNA [21]. These NETs are able to kill the pathogen; however, whether protein content and thrombotic activity are comparable to NETs released by suicidal NETosis is not well known.

3 | ACTIVATED PLATELETS SUPPORT NETOSIS AND POTENTIATE THROMBO-INFLAMMATION

Platelets are key components of the hemostatic system limiting blood loss at the site of injury. During inflammation, platelets play a dual role by maintaining vascular integrity at the site of leukocyte transmigration while supporting immune cell recruitment and activation [22].

It is well established that platelets must first be activated to induce NETosis. Both classic platelet agonists (i.e., thrombin, ADP, collagen, and arachidonic acid) and several TLR ligands support platelet-driven NETosis, whereas resting platelets failed to induce NET formation [23,24]. Activated platelet-mediated NETosis was mostly shown to be independent of ROS, although under specific conditions, such as *S. aureus* infection, β-defensin 1 release from platelets supports ROS-dependent NETosis [25]. Whether platelets differentially alter the capacity of high-density neutrophils and LDNs to form NETs is not well known.

Multiple platelet receptors and soluble proteins regulate platelet-driven NETosis in humans and mice, with selective receptors involved in specific inflammatory or infectious environments [26]. CD62P (P-selectin) expression is essential for NETosis in mice [24,27]. as $CD62P^{-/-}$ platelets failed to support the release of NETs [27]. (Figure). Moreover, CD62P/P-selectin glycoprotein ligand 1 (PSGL-1) interaction was required for H3 citrullination and NET formation, and CD62P blocking aptamer inhibits NETosis in mice [27]. This interaction may however be limited by proteinase-release such as cathepsin G and NE, which can cleave PSGL-1 [28], possibly providing a negative feedback loop to dampen thrombo-inflammation. Interestingly, CD62P was shown to be dispensable for human platelet-mediated NETosis as shown *in vitro* following stimulation with the TRAP-6, gram-positive protein PAM3CSK4, LPS, or arachidonic acid [29,30]. but seems involved in heparin-induced thrombocytopenia (HIT) immune complexes-mediated NETosis in the presence of platelets [31]. Along CD62P, platelet high mobility group box 1 (HMGB1) was also shown to drive NETosis in both human and mice. Activated platelets present HMGB1 to neutrophils and commit them to autophagy and NET generation [26,29,32], independent of NADPH oxidase-mediated ROS production [33,34], through the interaction with the receptor of advanced glycation endproducts (RAGE) in mice and human [29,35,36]. Glycoprotein (GP) IIb/IIIa activation also supports NETosis in mice as targeting platelet activation with either aspirin or a GPIIb/IIIa inhibitor in an experimental model of transfusion-related acute lung injury decreased NET formation and lung injury; however, GPIIb/IIIa inhibition did not alter NETosis in human cells under static conditions [24,29,30], further supporting the presence of multiple mechanisms by which platelets support NETosis in humans and mice (Table 1, Figure). However, under flow conditions, von Willebrand factor (VWF)-GPIbα interaction was shown to prime human platelets leading to GPIIb/IIIa activation, its subsequent binding to SLC44A2 on neutrophils driving mechanosensitive-dependent NET formation [37]. Other platelet-derived soluble factors, including VWF [30], thromboxane A_2 (TXA_2) [30], platelet factor 4 (PF4), and CCL5 (RANTES) were also shown to support NETosis in human cells. Platelet PF4 and CCL5 also support NETosis in mice. In thrombin receptor-activating peptide (TRAP)-activated platelets, heterodimerization of platelets CCL5 and PF4 is required for NET formation and PF4 is required for NET formation in mice associated with neutrophil β2 integrin activation [38]. Beyond differences in neutrophil and platelet count, interspecies differences in both platelets and neutrophils could account for different mechanisms and receptors involved in NETosis. Indeed, human neutrophils (20%-80%) can undergo NETosis within 2 to 4 hours, whereas NET formation in mice, depending on the study, may require between 3 and 16 hours *in vitro* [39,116]. It is important to highlight that most mouse studies use bone marrow neutrophils [39], whereas peripheral blood is the preferred source of neutrophils in human studies. Other differences could be related to the experimental design (e.g., presence of serum, albumin) or also differences in protein content in both neutrophils and platelets between humans and mice. For example, mouse neutrophils lack α-defensins and azurocidin and have lower content in MPO [40]. and protein content in human and mice platelets exhibit significant differences both in membrane receptors and soluble proteins [41], possibly contributing to the differences in mechanisms observed between human and mice. Moreover, most human studies were performed in 2D systems lacking the cellular complexity and heterogeneity, limiting direct comparison with *in vivo* studies. The generation of organoids mimicking the complexity of human and mice tissues could overcome this limitation and provide a unique opportunity to study complex human diseases. Indeed, using a vascular organoid model, we have recently shown and presented at the state-of-the-art talk at ISTH Congress in 2022 that SARS-CoV-2 infection of pericytes alters their interaction with endothelial cells impairing vascular permeability and disrupting vessel architecture [42], highlighting the benefit of using complex organized 3D cellular models to mimic disease states and its relevance in studying infectious diseases.

Under inflammatory conditions, platelets, endothelial cells, and neutrophils cooperate to induce NET formation. Indeed, intracellular adhesion molecule-1 (ICAM-1) upregulated on endothelial cells binds β2 integrin (macrophage integrin-1 or Mac-1, CD11b/CD18) on neutrophils, which associated with platelet-derived CCL5 and PF4, potentiating NETosis [38]. PF4 was also shown to bind, stabilize, and compact NETs and to decrease the efficacy of DNase-1 [43]. Under flow conditions, LPS-stimulated human platelets support NETosis through lymphocyte function-associated antigen 1 (LFA-1 or CD11a/CD18), and deficiency in this receptor impairs NET formation and bacterial clearance following *Escherichia coli (E.coli)*-induced sepsis in mice [44]. Interestingly, LPS can induce both vital and suicidal NETosis, and this process is tightly regulated by the presence of platelets [45]. In the absence of platelets and serum, neutrophils are able to discriminate between different LPS serotypes. Among multiple LPS serotypes, LPS isolated from *E. coli O128:B12* and *P. aeruginosa serotype 10* induces potent suicidal NETosis *in vitro* through autophagy and in a ROS-dependent but TLR-4 independent manner [45]. Interestingly, LPS serotypes lose their selectivity in the presence of

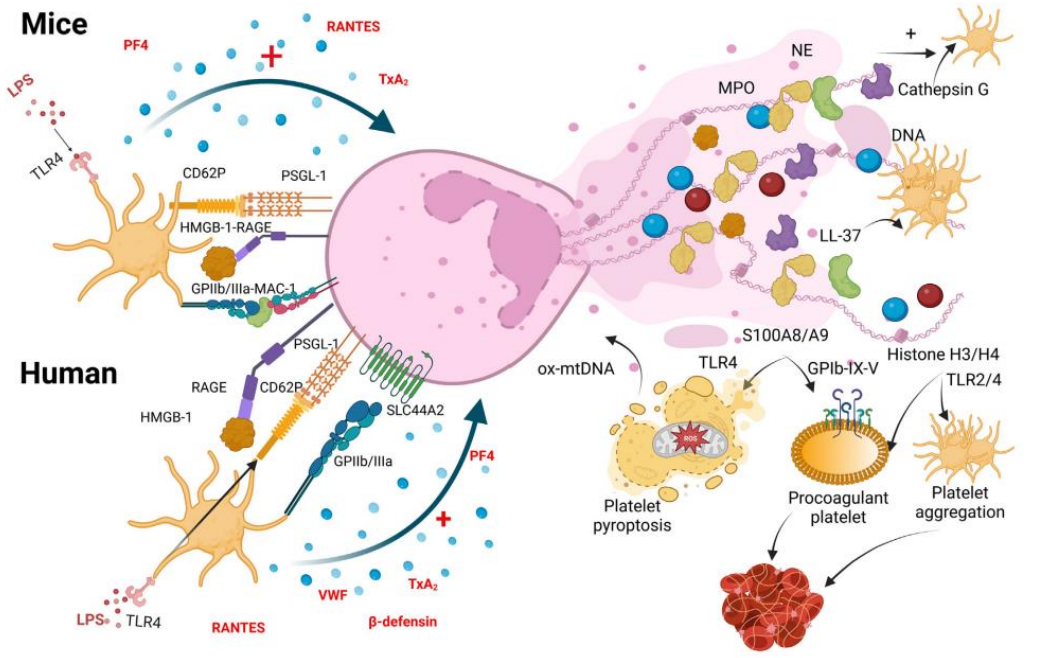


FIGURE Effect of NET-associated DAMPs on platelet activation. Platelet activation by pathogens or classical agonists induces platelet activation and supports platelet-dependent NETosis. Distinct receptors are involved in mice and humans and in response to different agonists *in vitro*, under flow conditions and *in vivo*. CD62P is a key driver of NETosis in mice *in vitro* and *in vivo* but not in humans *in vitro*. CD62P contributes to platelet-dependent vital NETosis in LPS-stimulated human platelets. Platelet HMGB1 supports NETosis through RAGE in humans and mice. GPIIb/IIIa mediates NETosis through MAC-1 in mice, whereas under flow conditions, VWF-GPIbα priming of human platelets triggers GPIIb/IIIa-dependent NETosis through SLC44A2. Platelet secretion of PF4, TxA_2, RANTES, and VWF also contributes to NETosis. Multiple NET-associated DAMPs trigger platelet activation and the release of proinflammatory molecules. Cathepsin G induces platelet aggregation, and this effect is amplified by neutrophil elastase. Histones, in particular H3 and H4, induce platelet aggregation alongside with the formation of procoagulant platelets and thrombin generation, mainly through TLR2/TLR4. S100A8/A9 supports the formation of procoagulant platelets through a mechanism involving GPIb-IX-V and induces platelet pyroptosis through TLR4 with the subsequent release of oxidized mitochondrial DNA (ox-mtDNA) supporting NETosis and amplifying thrombo-inflammation.

platelets that commit neutrophil to vital NETosis in a TLR-4- and CD62P-dependent manner. Therefore, despite the involvement of multiple regulatory factors, selective receptors, and interactions can play a primordial role in specific environments, with platelets able to switch NETosis from selective suicidal to promiscuous vital NETosis under specific conditions, making platelets an important target to reducing NETosis, in particular in the vasculature.

4 | DIRECT AND INDIRECT MECHANISMS OF NET-INDUCED PLATELET ACTIVATION

Not only do activated platelets trigger NETosis but NET release is also a potent source of prothrombotic molecules, which drive platelet activation and thrombotic complications. NET components recruit platelets under arterial and venous shear rates and act as a scaffold to support platelet aggregation, inducing alpha and dense granule secretion, phosphatidylserine (PS) exposure, and GPIIb/IIIa activation (Table 2). DNA, DNA-DAMP complexes, and free DAMPs contribute to thrombosis and platelet activation. Here, we highlight and discuss different mechanisms by which key NET-associated DAMPs induce platelet activation and thrombosis, focusing on histones, the alarming S100A8/A9, MPO, the proteinases elastase and cathepsin G and the antimicrobial peptide LL-37.

5 | HISTONES

Histones represent around 70% of NET-associated proteins, with H2A and H2B being the most abundant NET-bound histones [9]. These DAMPs exert powerful antimicrobial and cytotoxic functions through different mechanisms: lysine-rich H2A and H2B are able to penetrate the bacterial cell membrane and bind to bacterial DNA, whereas H1, H3, and H4 are capable of disrupting the membrane of bacteria [46].

TABLE 1 Major platelet receptors and proteins involved in NETosis in humans and mice. Platelets induce NETosis in humans and mice through different mechanisms involving cell–cell interactions and the release of soluble mediators.

HUMAN	PLATELET AGONIST	PLATELET RECEPTOR/PROTEIN	NEUTROPHIL RECEPTOR	REFERENCES
In vitro	TRAP-6	HMGB-1 TxA_2	RAGE	Maugeri et al. (29) Carestia et al. (59). Caudrillier et al. (24)
	AA	TxA_2, PF4, VWF GPIb	Mac-1	Carestia et al. (30)
	PAMCSK4	TLR2, PF4, VWF, TxA_2 GPIb	Mac-1	Carestia et al. (30)
	LPS	TLR4, PF4, TxA_2, VWF CD62P, TLR4 →	Mac-1 LFA-1 (flow condition) (Vital Netosis)	Carestia et al. (30) MdDonald et al. (44) Pieterse et al. (45)
	α-toxin	β-defensin-1		Kraemer et al. (25)
	HIT IC	CD62P		Perdomo et al. (31)
	GPIbα/VWF priming	Activated GPIIb/IIIa (flow condition)	SLC44A2	Constantinescu-Bercu et al. (37)
MOUSE	**PLATELET AGONIST/MOUSE MODEL**	**PLATELET RECEPTOR/PROTEIN**	**NEUTROPHIL RECEPTOR**	**REFERENCES**
In vitro		HMGB-1 CCL5, PF4	RAGE Mac-1	Maugeri et al. (29) Stark et al. (35) Rossaint et al. (38)
	Thrombin	CD62P	PSGL-1	Etulain et al. (27)
In vivo	*E. Coli*	TLR4	LFA-1 (CD11a/CD18)	Clark et al. (17) MdDonald et al. (44)
	Ventilator-induced lung injury TRALI	PF4, CCL5 TxA_2, GPIIb/IIIa	Mac-1 (CD11b/CD18)	Rossaint et al. (38) Caudrillier et al. (24)
	DVT	HMGB-1	RAGE	Stark et al. (35) Dyer et al. (36)

Once released into the extracellular space, histones bind to TLR2, 4, and 9 on various cells mediating cytotoxicity, the production of proinflammatory cytokines and thrombosis [47–49]. High level of histones, DNA-histone complexes, and citrullinated H3 are present in the serum of patients with severe trauma, sepsis [50], and COVID-19 [51], as well as during sterile inflammatory diseases such as DVT [52]. and stroke. These levels correlate with coagulopathy, disease severity, and mortality [53–55].

Histones are a key driver of thrombo-inflammation by directly activating platelets, endothelial cells, coagulation, and impairing fibrinolysis. The detrimental role of histones has been shown in several models of infection and sterile inflammation. For example, using an experimental model of sepsis, histones were shown to be major mediators of cell death [56]. Histone infusion also induces severe thrombocytopenia associated with micro- and macroplatelet aggregates in the lung [56]. The formation of microaggregates is independent of GPIIb/IIIa as β3 deficiency protects mice from histone-induced death but not from thrombocytopenia [56]. Histones bind to platelets and induce platelet activation and aggregation, Ca^{2+} influx, fibrinogen recruitment [57–59], and thrombin generation [58]. Among all histones, H3 and H4 are the most powerful platelet agonists and these interactions were shown to be potentiated by histone binding to DNA [58,59]. However, another study showed that the core histone structure and intact NETs failed to support coagulation, whereas recombinant human H3 and H4 trigger thrombin generation in platelet-dependent manner, a difference suggested to result from the neutralization of the binding capacities of histones by the negative charges on DNA [60]. Histone-induced thrombin generation was inhibited by antiplatelet drugs targeting $P2Y_{12}$, phosphodiesterase 3, and GPIIb/III, by heparin and activated protein C (APC); or by targeting TLR2 and TLR4 [58]. Histone-induced platelet activation depends on the phosphorylation of mitogen-activated protein kinases (MAPK) (e.g. extracellular stimuli-responsive kinase, phosphatidylinositol 3-kinase (PI3K)/Akt, p38, and Nuclear factor kappa-light-chain-enhancer of activated B cells (NF_KB)), which results in increased intracellular Ca^{2+}. Because of their positive charge, histones can bind and activate different platelet receptors in a charge-dependent manner, such as glycoprotein VI [61]. Another interesting feature of histones is their ability to induce procoagulant platelet formation [58], alongside the release of PS-positive microvesicles [51]. H4 induces platelet ballooning and supports coagulopathy in trauma patients [62]. and promotes intravascular coagulation and organ damage during sepsis [63]. Importantly, whereas TLR2 and TLR4 are implicated in histone-mediated cell death [47]. and support platelet aggregation and thrombin generation [58], deficiency in these receptors did not protect against histone-induced coagulopathy [64], suggesting the

TABLE 2 NET-associated DAMPs regulating platelet function. Receptors involved in platelet activation by NET-associated DAMPs and their effect on platelets. Toll-like receptors 2, 3, 4, 9 (TLR2, TLR3, TLR4, TLR9), Glycoprotein VI (GPVI), N-formyl peptide receptor 2 (FPR2/ALX), Protease activated receptor 4 (PAR4), and Transient receptor potential vanilloid 4 (TRPV4). Receptor for Advanced Glycation Endproducts (RAGE), Glycoprotein Ibα (GPIbα), Glycoprotein IIb/IIIa (GPIIb/IIIa), and oxidized mitochondrial DNA (ox-mtDNA).

INTRACELLULAR LOCALISATION	NEUTROPHIL PROTEIN	RECEPTORS	FUNCTIONAL RECEPTORS/BINDING PARTNERS ON PLATELETS	EFFECT ON PLATELETS	REFERENCE
Nuclear	Histones (H2A, H2B, H3, H4)	TLR2, TLR3, TLR4, TLR9, GPVI	TLR2, TLR4, GPVI	Platelet aggregation Increased Ca^{2+} influx Procoagulant platelets Increased thrombin generation	Traby et al. (51) Kutcher et al. (57) Semeraro et al. (58) Carestia et al. (59)
Granular	Cathelicidin (LL-37)	FPR2/ALX, GPVI	FPR2/ALX, GPVI	Platelet aggregation and secretion Ca^{2+} Influx	Salamah et al. (144) Pircer et al. (145) (146)
	Neutrophil elastase (NE)	PAR2, TRPV4, GPIIb/IIIa	GPIIb/IIIa	Enhances CG activity Increased fibrinogen binding and platelet aggregation	Selak et al. (136) Si-Tahar et al. (137)
	Cathepsin G (CG)	PAR4	PAR4	Platelet aggregation	Selak et al. (127)
	Myeloperoxidase (MPO)			Enhances agonist-induced platelet aggregation; inhibits procoagulant platelet formation	Kolarova et al. (121) Gorudko et al. (125) Beckmann et al. (126)
Cytoplasmic	S100 calcium-binding protein A8/A9 (S100A8/A9)	TLR4, RAGE, CD36	CD36, GPIbα TLR4	Platelet activation, procoagulant platelet formation, amplification of fibrin generation and platelet aggregation by VWF Gasdermin D-dependent platelet pyroptosis, release of ox-mtDNA, induction of NETosis	Colicchia et. (96) Su et al. (92)

involvement of multiple receptors/pathways in histone-mediated cytotoxicity and coagulopathy.

Histones can also indirectly induce platelet recruitment and activation through their interaction with endothelial cells and by altering the coagulation and fibrinolytic cascades. Under inflammatory and infectious conditions, circulating histones accumulate in the capillaries by binding to glycosaminoglycans (GAGs) within the glycocalyx, which acts as a polyanionic trap. Histones induce TLR-dependent endothelial cell activation [65,66]. and Weibel Palade body (WPB) exocytosis in a caspase-, Ca^{2+}-, and charge-dependent manner [67,68]. The release of ultra-large VWF-strings from WPB supports platelet capture through GPIbα [69]. Histones also directly bind to VWF [70], fibrinogen, and fibrin [71]. supporting a scaffold of highly prothrombotic and proinflammatory proteins associated with endothelial cells [72]. Moreover, histones, independent of the subtype, crosslink to fibrin, through covalent and noncovalent interactions and in an FXIIIa-dependent manner, impairing fibrinolysis [73]. The thrombo-inflammatory environment is amplified by the upregulation of tissue factor, vascular cell adhesion molecule-1 (VCAM-1), ICAM-1, and the downregulation [74]. of thrombomodulin, which in part, is mediated via TLR2 and TLR4 [75]. Activated endothelial cells can also induce NETosis and are susceptible to NETosis-induced cell death [76]. Therefore, NETs, platelets, and endothelial cells cooperate to sustain a thrombo-inflammatory milieu that exacerbates organ function.

Because of their potent prothrombotic, cytotoxic, and proinflammatory functions, extracellular histones are potential targets during sterile and infectious conditions. A variety of endogenous and pharmacological inhibitors have been shown to protect against inflammation and thrombosis caused by histones, including antibodies antihistone, APC [56], albumin [77], C-reactive protein [50,78], polysialic acid [24,79,80], thrombomodulin [81], and heparin [82]. Indeed, histone-induced platelet activation and aggregation [72], thrombocytopenia, and thrombosis in mice were partially inhibited by APC. The protective effect of APC is not solely dependent on its anticoagulant capacity as heparin lacking antithrombin binding inhibits histone cytotoxicity during sepsis [83]. Although APC decreased histone-induced cytotoxicity in a purified system, it did not decrease NET-induced cytotoxicity, indicating that the cytotoxicity of NET-bound histone is refractory to APC degradation [79]. Heparin also showed a beneficial effect in limiting NET-mediated NETosis. Heparin acts at many levels, for example, by removing histones from NETs, destabilizing the scaffold [72]. and by directly inhibiting potent DAMPs like histones and S100A8/A9. Indeed, heparin inhibits the prothrombotic effect of histones and reduces S100A8/A9 binding to heparan sulfate on endothelial cells, suggesting a potent role in heparin in limiting NETosis-associated platelet activation and thrombosis. Therapeutic doses of low molecular-weight heparin (LMWH) successfully inhibit the covalent interaction between fibrin and histone and restores fibrinolysis [73]. Interestingly, therapeutic doses of heparin increased the probability of survival in noncritically ill patients with COVID-19. [84]. The effect of the dose of heparin might be crucial to target histone as high concentrations of heparin successfully inhibit the effect of histones on platelets, whereas a low heparin:histone ratio (1:10) showed an opposite effect and increased histone binding [85]. Moreover, LMWH inhibits the ability of human platelets to support NETosis *in vitro* [29]. Therefore, heparin acts on different axis limiting thrombo-inflammation, independent of its anticoagulant functions.

6 | S100 A8/A9: POTENT PROINFLAMMATORY AND PROTHROMBOTIC DAMP

S100A8/A9, also known as calprotectin, represents the bulk of NET-bound cytosolic proteins [9]. and exerts potent antimicrobial, prothrombotic, and proinflammatory functions. Because of the low stability of monomeric S100A8 and S100A9, heterodimeric S100A8/A9 is the predominant form present in the plasma. Indeed, mice deficient in S100A9 lack S100A8 protein expression despite normal mRNA levels, showing the essential requirement of S100A9 for S100A8 stability [86]. These alarmins have a helix-loop-helix motif with charged amino acid residues, resulting in their high affinity for divalent ions, such as Ca^{2+} and Zinc (Zn^{2+}), inducing a conformational change in S100A8/A9.

Following NETosis, around 25% of neutrophil S100A8/A9 remains bound to NETs, whereas 25% is released as soluble protein. S100A8 and S100A9, also known as myeloid-related proteins (MRP) 8 and 14, are members of the S100 alarming family of proteins, representing ~45% and 5% of the neutrophil and monocyte cytoplasmic proteins, respectively. S100A8/A9 is released from neutrophils during NETosis but is also passively released during cell necrosis and death but not degranulation [8,9,87]. Activated endothelial cells, through the engagement of E-selectin with PSGL-1, also promote S100A8/A9 release from neutrophils, which in turn, binds in an autocrine manner to neutrophil TLR4 inducing β2 integrin activation [88]. The expression of S100A8/A9 in neutrophils is upregulated under chronic inflammatory conditions, including diabetes and acute coronary syndrome [89]. Interestingly, not only neutrophils and monocytes express and upregulate S100A8/A9, but platelets can also overexpress and release S100A8/A9 following activation. Indeed, platelets isolated from healthy donors were shown to express S100A8 and S100A9 by flow cytometry and electron microscopy [90]; however, proteomic analysis showed that platelets isolated from healthy donors didnot contain S100A8/A9 [91]. In the disease state, including in patients with ST-elevation myocardial infarction (STEMI [91]), systemic lupus erythematosus [90], sepsis [92], and COVID-19 [93], platelet S100A8/A9 expression is increased, although the amount released is considerably low compared with neutrophils [90,94]. One of the mechanisms by which platelets increase intracellular S100A8/A9 protein level is through transfer from neutrophils, which can subsequently be released following platelet activation [91]. S100A8/A9 gene expression is also increased by specific stimuli such as interferon alpha [90], suggesting the presence of multiple pathways regulating the platelet gene and protein expression of S100A8/A9. SARS-CoV-2 was also shown to upregulate S100A8 and S100A9 gene expression in megakaryocytes [93]. S100A8 and S100A9 gene expression is upregulated

in patients with COVID-19 [93,95], and platelet releasate induces endothelial activation through CD36 *in vitro* [93]. S100A9-positive platelets were also observed in lung autopsies from patients with fatal COVID-19, however the presence of the heterodimer was observed in only a fraction of these platelets [96]. It is likely that multiple mechanisms are involved in the expression, upregulation, and release of S100A8/A9 from platelets, however the mechanisms regulating this process and its functional relevance in the disease state are not well known.

Intracellularly, S100A8/A9 acts as Ca^{2+} sensor, regulates arachidonic acid metabolism and trafficking [97,98], cytoskeleton remodeling [99], phagocytosis and leukocyte migration. Extracellular S100A8/A9 exerts an antimicrobial activity, mainly by chelating divalent ions, such as Zn^{2+} and manganese (Mn^{2+}), limiting bacterial growth [100]. Purified S100A8/A9 inhibits the growth of multiple pathogens *in vitro* including *Salmonella Typhimurium (STm) and S. aureus* and its antimicrobial effect is increased by the presence of Ca^{2+}, which increases tetramerization and oligomerization [100]. However, the protective effect of S100A8/A9 *in vivo* is pathogen- and organ-dependent [100–102]. Indeed, S100A9 deficiency does not contribute to host defense during *STm* infection [103]. but increased the bacterial load in the liver but not in the heart during *S. aureus* infection [100,101]. The reduction in bacterial load in the heart was associated with increased NETosis [100,101]. and subsequent transfer of antimicrobial peptides to macrophages, increasing bacterial killing [16]. Therefore, tailored targeting of S100A8/A9 could confer protection in selective tissues during infection, subsequently protecting from organ damage and thrombosis.

Extracellular S100A8/A9 also exerts potent prothrombotic and proinflammatory functions, most of which are regulated by 3 receptors: TLR4, RAGE, and the scavenger receptor CD36. During inflammation, S100A8/A9 deposition within venules [104]. and large vessels [96]. supports immune cell recruitment and transmigration, contributing to vascular inflammation [104]. Similar to histones, S100A8/A9 released from transmigrating myeloid cells binds with high affinity to GAGs within the glycocalyx via the A9 subunit and this interaction is blocked by heparin, heparan sulfate and chondroitin sulfate [105]. Because of its potent proinflammatory functions, multiple strategies aiming to selectively target S100A8/A9 were developed. S100A9 blockers, Paquinimod and ABR-238901, which selectively inhibit its interaction with TLR4 and TLR4/RAGE, respectively, showed beneficial effects in experimental models of sepsis [92,106], myocardial infarction [107], and SARS-CoV-2 infection [108]. S100A8/A9 also exhibits some anti-inflammatory properties to mitigate tissue damage by suppressing NF-kB activation and reducing mRNA expression of molecules such as IL1β, IL-6, and TNFα, dampening inflammatory responses by clearing inflammatory cytokines from circulation *in vivo* [109].

Beyond its antimicrobial, pro- and anti-inflammatory properties, S100A8/A9 exerts potent prothrombotic functions by altering platelet and endothelial cell functions. S100A9 deficiency reduced photochemical injury–induced arterial thrombosis [110], laser-induced injury of the cremaster microcirculation, and attenuated DVT using stasis or stenosis models without significant alteration of hemostasis [111]. The prothrombotic effect of S100A8/A9 in mice was partially dependent on CD36 [110]. Targeting S100A9 using anti-S100A9 vaccine, which alters S100A8/A9-CD36 interaction, reduces thrombosis in middle cerebral artery occlusion stroke and carotid artery occlusion models [112]. Whether platelet or endothelial CD36 contributes to the prothrombotic effect of S100A8/A9 in different models is not fully elucidated. Therefore, it is likely that the pathological roles of S100A8/A9 depends on its interactions with multiple receptors which can be differentially engaged in a cell-, organ-, and disease-dependent manner.

S100A8/A9 prothrombotic effect depends on multiple interactions with endothelial cells, platelets, immune cells, and possibly other components of the hemostatic system. S100A8/A9 was shown to increase advanced glycation end products (AGEs)-treated human umbilical vascular endothelial cells (HUVECs) activation [113]. Interestingly, whereas ICAM-1, VCAM-1 and IL-6 secretion required HUVEC priming, monocyte chemoattractant protein-1 (MCP-1) levels were increased following treatment irrespective of HUVECs preactivation [113], implying the involvement of different receptors/pathways in S100A8/A9-induced endothelial cell activation. S100A8/A9 can also alter platelet function through different receptors, which can synergize the pathogenic effects. We have recently shown that S100A8/A9 triggers human- and mouse-washed platelet activation, the exposure of PS and CD62P on platelets, increases intracellular Ca^{2+} and supports fibrin generation in whole blood, all together hallmarks of a subset of procoagulant platelet [96], data presented at the state-of-the-art talk at ISTH congress in 2022. Despite GPIIb/IIIa activation, S100A8/A9 did not induce platelet aggregation, which is likely because of a low capacity to bind fibrinogen [114]. PS and CD62P expression as well as GPIIb/IIIa activation were significantly reduced in mouse platelets lacking the extracellular domain of GPIbα or in human platelets isolated from a patient with Bernard–Soulier syndrome, showing a central role for GPIbα in this mechanism. It was shown that Ca^{2+}-induced S100A8/A9 tetramerization inhibits its binding to TLR4, preventing excessive systemic effects [115]. However, platelet activation in washed platelets and whole blood was performed in the presence of Ca^{2+}, suggesting that heterodimeric and tetrameric S100A8/A9 can support this activation. S100A8/A9 is also able to support platelet aggregation independent of fibrin generation as priming of washed platelets with S100A8/A9 potentiates VWF-mediated platelet agglutination in the presence of ristocetin, suggesting that S100A8/A9 acts as an amplifier of platelet aggregation and fibrin generation [114]. The mechanism by which GPIbα, solely or in cooperation with other receptors, regulates this process is not known. S100A8/A9 interaction with platelets is not limited to the formation of procoagulant platelets. S100A8/A9 was also shown to induce gasdermin D-dependent pyroptosis in platelets through TLR4 [92]. Platelet pyroptosis correlates with plasma levels of S100A8/A9 during sepsis, and recombinant S100A8/A9 induces NLRP3 inflammasome and caspase-1 activation, which is inhibited by paquinimod. This activation is dependent on mitochondrial ROS and the release of oxidized mitochondrials from platelets, forming a positive feedback

loop to amplify NETosis and inflammation. S100A9 also triggers NET formation in a ROS-dependent manner via RAGE and TLR4 [116]. The release of S100A8/A9, in particular in disease associated with S100A8/A9 upregulation in platelets, could contribute to platelet-driven NETosis and amplifies the crosstalk between platelets and neutrophils. This thrombo-inflammatory environment involves different receptors, which can differentially alter platelet activation and function in the disease state.

7 | MPO: NEGATIVE AND POSITIVE REGULATORS OF PLATELET ACTIVATION

Following neutrophil stimulation, NADPH activation generates high amounts of superoxide leading to the release of hydrogen peroxide (H_2O_2). MPO is a heme-containing enzyme stored in azurophil granules, which catalyzes the reaction of H_2O_2 and halide (Cl^-, Br^-, I^-) to produce the major neutrophilic oxidant, hypochlorous acid (HOCl) [117]. MPO-H_2O_2-halide system induces rapid platelet secretion of serotonin, which is inhibited by blocking peroxidase, H_2O_2 degradation, Mg^{2+} ion chelation, or by inhibiting platelet metabolic activity [118]. During vascular inflammation, such as atherosclerosis [119] and sepsis [120], MPO increases endothelial dysfunction by limiting nitric oxide availability. Beyond its effect on the endothelium, MPO directly binds to platelet and enhances platelet aggregation in response to different agonists, independently of its enzymatic activity [121]. Binding of MPO to platelets also induces actin cytoskeleton reorganization and increases intracellular Ca^{2+} levels, and this effect is primarily observed with monomeric rather than dimeric MPO. Platelet priming was inhibited by the physiological inhibitor of MPO ceruloplasmin and by the modification of MPO by HOCl [122]. We and others have recently shown that heme activates human platelets through CLEC-2 [123] and glycoprotein VI [124]. It is conceivable that MPO, through heme-ITAM receptor interactions, further alters platelet function during NETosis, although this might be affected by the concentration of free heme available [125]. On the other hand, MPO and MPO/ H_2O_2 were shown to act as negative regulators of the procoagulant activity of activated platelets, and this effect was inhibited by lacthaderin, which specifically recognizes PS [126]. These data support a dual role for MPO in platelet activation, which can differentially alter thrombosis in disease states associated with both thrombotic and bleeding complications.

8 | NE AND CATHEPSIN G

NE and cathepsin G are serine proteases stored in the azurophilic granules of neutrophils participating in the intracellular degradation of pathogens. Beyond their antimicrobial functions, extracellular NE and cathepsin G alter platelet function and contribute to thrombosis [127]. Deficiency in NE and cathepsin G along with other serine proteases, as observed in the rare autosomal recessive disease Papillon-Lefèvre syndrome, does not induce marked immunodeficiency. These neutrophils cannot generate NETs in response to PMA but not Ca^{2+} ionophore, showing that these proteases are important for ROS-dependent NETosis [128,129].

Beyond their role in NETosis, cathepsin G and NE affect platelet function, thrombin generation, and thrombosis [130]. Pharmacological inhibition or genetic deficiency of cathepsin G decreases platelet accumulation and vessel occlusion in an $FeCl_3$-induced mesenteric arteriole injury model and reduces brain injury in a mouse model of stroke [131]. Cathepsin G is a key driver of neutrophil-dependent platelet activation and aggregation via protease-activated receptor 4 (PAR4) [131–133]. Ca^{2+} mobilization and secretion induced by low concentrations of cathepsin G are partially dependent on arachidonic acid metabolites and ADP, whereas stimulation by higher concentrations is independent of secondary mediators showing that cathepsin G is a strong inducer of platelet activation [127]. Cathepsin G blockade also partially reduced platelet aggregation induced by NETs [134]. Soluble cathepsin G is rapidly neutralized by proteases, whereas cathepsin G associated with the neutrophil membrane remains catalytically active, is resistant to proteases and plays an important role in neutrophil-dependent platelet activation. Indeed, the close contact between neutrophils and platelets allows cathepsin-mediated platelet aggregation, irrespective of the presence of antiproteinases [135].

NE, by itself, does not activate human platelets but it strongly enhances the weak aggregation induced by a threshold concentration of cathepsin G [136]. Mechanistically, NE proteolytically activates GPIIb/IIIa through the cleavage of the carboxyl terminus of the GPIIb subunit heavy chain, increasing fibrinogen binding [137]. The synergism between NE and cathepsin G requires the participation of ADP and TxA_2 [136], and preincubation of platelets with elastase increased the apparent affinity of cathepsin G binding to platelets. In a mouse model of air pollution-induced lung inflammation, a model associated with mild thrombogenicity, cathepsin G and NE can lyse red blood cell membrane proteins, induce the leakage of ADP into the plasma and platelet sensitization [138]. On the other hand, physiologic concentrations of NE were shown to specifically inhibit thrombin-induced platelet aggregation and ristocetin-induced agglutination of washed platelets in a time- and dose-dependent manner. It results in the cleavage of GPIbα and reduction in thrombin-binding sites on platelets [139], suggesting a possible negative feedback loop to limit platelet recruitment and formation of procoagulant platelets associated with NETosis. Despite the effect of NE on platelet activation *in vitro*, NE deficiency did not alter the thrombus formation in a mouse model of DVT [140]. Similar to other DAMPs, NE also activates endothelial cells and VWF release, which can indirectly support platelet recruitment and thrombosis [141]. NE is able to induce a biased PAR1 signaling distinct from thrombin activation of this receptor leading to $G\alpha_i$-coupled p42/44 MAPK signaling downstream of PAR1 activation, but not $G\alpha_q$-coupled Ca^{2+} signaling [142]. This noncanonical activation of PAR1 suppresses endothelial permeability induced by thrombin in HUVECs, suggesting a role of NE in the regulation of the endothelial barrier at sites of neutrophil activation and inflammation. Interestingly, in a nanotechnology-based drug delivery approach, NE was successfully used as a target for cell-specific delivery of selective drug, reducing its

potential side effects [143]. Therefore, NET-associated DAMPs are not only a direct target for therapies to limit thrombosis by reducing platelet and endothelial cell activation, but can also be used as platform for targeted delivery of drugs to specific sites and cells.

9 | CATHELICIDIN LL-37

LL-37 is a cathelicidin with direct antimicrobial and proinflammatory properties and its expression is upregulated during inflammation. Beyond these known functions, LL-37 enhances platelet aggregation and thrombus formation through formyl peptide receptor 2 (FPR2/ALX) [144]. LL-37 was shown to induce platelet aggregation in washed platelets but not in platelet-rich plasma, which involves GPVI and its downstream signaling through protein tyrosine kinases Src/Syk and phospholipase C [145], whereas very high concentrations inhibit platelet aggregation and thrombus formation [146]. LL-37 was observed in arterial thrombi isolated from patients with acute myocardial infarction and thrombi isolated ferric chloride-induced carotid injury in mice [145]. Interestingly, LL-37 induces CD62P, IL-1β, CD40L, and HMGB1 in platelets, platelet-neutrophil interactions and supports neutrophil extravasation without inducing GPIIb/IIIa activation and aggregation, suggesting an increase in the proinflammatory phenotype of platelets [145]. Platelets also contain LL-37 and release it upon activation by collagen, CRP-XL or TRAP-6. [144]. LL-37 could also enhance thrombosis by increasing factor Xa and thrombin activity [147].

Despite its prothrombotic effect, LL-37 was shown to be protective during bacterial sepsis. Indeed, in a model of polymicrobial infection (CLP), LL-37 improved survival in septic mice by decreasing macrophage pyroptosis and the release of IL-1β, increasing NET formation and the release of antimicrobial ectosomes improving sepsis outcome [148]. However, the beneficial effect of LL-37 might depend on the model used as bacterial clearance in the CLP model was shown to be independent of NETs [149]. LL-37 was also reported to inhibit LPS binding to endothelial cells and subsequent apoptosis in endotoxic shock models [150], suggesting that the antimicrobial and protective effect of LL-37 might outcome its prothrombotic functions.

10 | ISTH 2022 MEETING REPORT

Interesting novel data describing different mechanisms by which DAMPs contribute to thrombo-inflammatory diseases were presented at 2022 ISTH meeting in London. Targeting platelet-leukocyte interaction during sterile inflammation and infection was presented throughout multiple abstracts, further supporting the direct contribution of these complexes to disease severity. Multiple abstracts reported endothelial activation, NETosis, and impaired platelet function in patients with severe COVID-19. The increase in NET-associated DAMPs such as histones, MPO, MPO-DNA, and S100A8/A9 and impaired DNase activity in patients with severe COVID-19 was presented at the ISTH meeting. Basic science research provided important insights to the contribution of procoagulant platelets and platelet-derived DAMP HMGB1 to NETosis during experimental ischemic stroke [151,152], emphasizing the importance of targeting DAMPs and procoagulant platelets to limit thrombo-inflammation. An abstract showed a distinct transcriptomic signature for procoagulant platelets compared with activated nonprocoagulant platelets, with dynamin playing a key role in the formation of procoagulant platelets [153]. The ISTH 2022 meeting highlighted an increasing interest in studying the mechanisms by which activated and procoagulant platelets contribute to thrombo-inflammatory diseases, either directly or indirectly by regulating immune cell activation and behavior.

11 | FUTURE DIRECTIONS

A large amount of evidence shows the fundamental contribution of the reciprocal interaction between platelets and neutrophils in mediating inflammation and thrombosis. Degradation of NETs using DNase-1 was extensively assessed in different experimental models of inflammation and infection and promising results showed that DNase infusion in mice inhibits thrombosis in several models including DVT [52], stroke and sepsis. Systemic DNAse-1 treatment can also be associated with some limitations including the time of intervention-dependent beneficial effect [33,154]. and the release of toxic NET degradation products [43]. following NET cleavage. DNase-1 was recently shown to hydrolyze adenosine triphosphate (ATP) and adenosine diphosphate (ADP) into adenosine, inhibiting platelet aggregation and neutrophil activation, showing an off-target of DNase-1 beyond NET degradation [155]. Moreover, the efficacy of DNase-1 in dismantling NETs can be significantly altered by the presence of anti-DNase-1 auto-antibodies, as observed in patients with systemic lupus erythematosus, reducing DNase-1 access to NETs and inhibiting its activity [156]. It is important to note that DNase successfully degrades NETs but this treatment does not inhibit NET-associated DAMPs such as histones and S100A8/A9, both of which are major contributors to thrombo-inflammatory events. Beyond its proinflammatory properties, targeting S100A8/A9 prothrombotic activity is a promising target during sepsis and other diseases associated with NETosis such as COVID19, sepsis, DVT, and stroke. The fact that heparin or DNase, did not inhibit total NET-mediated platelet aggregation *in vitro* [134], supports the need to target multiple DAMPs to inhibit platelet activation [134], which can be differentially regulated in different disease states and affected organs. Understanding the relationship between NET-associated DAMP release, the expression and distribution of their receptors and its alteration is a disease state would allow to develop strategies to successfully target the detrimental effect of NETs to reduce organ damage. The enrichment in selective DAMPs such as S100A8/A9 in neutrophils and platelets from patients and experiments models of sepsis and COVID-19 suggests that inhibition of selective DAMPs such as S100A8/A9 and histones or simultaneous inhibition of different pathways regulating their effect on cells is a promising area of research that could substantially reduce thrombo-inflammation in a tailored manner without increasing bleeding complications.

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M.C., G.P., P.G., and J.R. wrote the manuscript.

RELATIONSHIP DISCLOSURE

There are no competing interests to disclose.

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