FUNCTIONS OF PLATELET MMRN1 IN ADHESION TO COLLAGEN

THE ROLE OF MULTIMERIN 1 (MMRN1) IN PLATELET ADHESION AND
CHARACTERIZATION OF ITS INTERACTIONS WITH FIBRILLAR COLLAGENS

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TITLE: The role of multimerin 1 (MMRN1) in platelet adhesion and characterization of its interactions with fibrillar collagens

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ABSTRACT

Multimerin 1 (human: MMRN1, mouse: Mmrn1) is a large homopolymeric glycoprotein that is synthesized and stored by platelets and endothelial cells until activation-induced release. MMRN1 is able to support platelet adhesion through mechanisms involving von Willebrand factor (VWF) and glycoprotein (GP)Ibα, and β$_3$ integrins on activated platelets, and it enhances platelet adhesion to fibrillar collagen, potentially by binding to putative MMRN1-specific GPAGPOGPX (where O is hydroxyproline and X is valine or glutamine) motifs in fibrillar collagens. Using mice with and without selective Mmrn1 deficiency, the goals of this thesis were: 1) further characterize the ability of Mmrn1 to enhance platelet adhesion to collagen, 2) explore the role of fluid shear stress in the ability of Mmrn1 to enhance platelet adhesion, and 3) test the specificity of the GPAGPOGPX motif for Mmrn1 and the ability of GPAGPOGPX to support or enhance platelet adhesion. Mmrn1-deficient (*Mmrn1$^{-/-}$*) mouse platelets showed impaired aggregate formation on fibrillar collagen surfaces under high (1500 s^{-1}) and low (300 s^{-1}) shear flow compared to wild-type (*Mmrn1$^{+/+}$*) mouse platelets, which was due to reduced initial adhesion and a slower rate of platelet accumulation onto collagen surfaces. Similarly, *Mmrn1$^{-/-}$* platelets formed smaller aggregates on immobilized recombinant (r)Vwf surfaces compared to wild-type platelets, and *Mmrn1$^{-/-}$* platelets had impaired adhesion and aggregate formation on immobilized murine fibrinogen, but not fibrin, when platelets were pre-activated to release Mmrn1. Type I fibrillar collagen was found to contain a variant of the GPAGPOGPX motif

(GPAGPOGPI), and GPAGPOGPX motifs supported adhesion of wild-type, but not *Mmrn1^{-/-}*, platelets. When presented with the VWF-binding GPRGQOGVMGFO motif and the integrin $\alpha_2\beta_1$-binding GFOGER motif present in fibrillar collagens, the GPAGPOGPX motifs synergistically enhanced platelet adhesion. These findings expand upon the known adhesive functions of platelet multimerin 1 and update knowledge of the motifs that support platelet adhesion to fibrillar collagens.

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LIST OF ABBREVIATIONS AND SYMBOLS

-/-	Homozygous null
+/-	Heterozygous null
+/+	Wild-type
α	Alpha
$\alpha_1(I)$	Type I collagen alpha-1 chain
$\alpha_2(I)$	Type I collagen alpha-2 chain
$\alpha_1(II)$	Type II collagen alpha-1 chain
$\alpha_1(III)$	Type III collagen alpha-1 chain
β	Beta
γ	Gamma (denotes shear rate)
λ	Lamda
τ	Tau (denotes shear stress)
μ	Mu (denotes viscosity or unit prefix "micro")
aa	Amino acid
ACD	Acid citrate dextrose
ADAMTS	A disintegrin-like and metalloprotease with thrombospondin type I repeats
ADP	Adenosine Diphosphate
APC	Activated protein C
ANOVA	Analysis of variance
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
BSS	Bernard-Soulier syndrome
C-terminal	Carboxyl-terminal
cDNA	Complimentary deoxyribonucleic acid
CD	Cluster of differentiation
COL	Collagen
CRP	Collagen-related peptide
Da	Dalton
DNA	Deoxyribonucleic acid
dyn	Dynes (unit of force)
ECMR	Extracellular matrix receptor
ECM	Extracellular matrix
ED	Extra domain
EDS	Ehlers-Danlos syndrome
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMILIN	Elastin microfibril interface-located protein
EMI	Elastin microfibril interface domain
FcR	Crystalizing fragment (Fc) receptor
FG	Fibrinogen (human)
Fg	Fibrinogen (mouse)

FN	Fibronectin (human)
Fn	Fibronectin (mouse)
Fmoc	Fluorenylmethyloxycarbonyl
F	Coagulation factor
GFP	Gel-filtered platelets
GP	Glycoprotein
GPS	Gray platelet syndrome
GT	Glanzmann thrombasthenia
HEK	Human embryonic kidney
IAP	Integrin-associated protein
Ig	Immunoglobulin
ITAM	Immunoreceptor tyrosine-based activation motif
k	Prefix "kilo"
LTA	Light transmission aggregometry
m	meter(s) or denotes the prefix "milli"
M	Molar
MA	Maximal aggregation
MIDAS	Metal ion-dependent adhesion site
MMRN1	Multimerin 1 (human)
Mmrn1	Multimerin 1 (mouse)
N-terminal	Amino-terminal
n	prefix "nano"
NO	Nitrous oxide
OI	Osteogenesis imperfecta
OCS	Open canalicular system
OD	Optical density
PAR	Protease-activated receptor
PCR	Polymerase chain reaction
PE	Phosphatidylethanolamine
PFA	Paraformaldehyde
PGI_2	Prostacyclin
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
PS	Phosphatidylserine
QPD	Quebec platelet disorder
RBC	Red blood cell
RGD	Arginine-glycine-aspartic acid tripeptide
R	Recombinant
rh	Recombinant human
s	Second(s)
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SNCA	α-synuclein (human)

Snca	α-synuclein (mouse)
SPD	Storage pool deficiency
TF	Tissue factor
TKO	Triple knockout
TTP	Thrombotic thrombocytopenic purpura
TRAP	Thrombin receptor-activating peptide
TSP-1	Thrombospondin-1 (human)
Tsp-1	Thrombospondin-1 (mouse)
TSR	Thrombospondin type repeat
TxA2	Thromboxane A2
U	Unit
ULVWF	Ultra-large von Willebrand factor
VLA	Very late antigen
VN	Vitronectin (human)
Vn	Vitronectin (mouse)
VTE	Venous thromboembolism
VWD	Von Willebrand disease
VWF	Von Willebrand factor (human)
Vwf	Von Willebrand factor (mouse)
WPB	Weibel-Palade body
X	Placeholder designation for an amino acid residue

CHAPTER 1: INTRODUCTION

1.1 THESIS OBJECTIVES

The objectives of this thesis were to further characterize the role of multimerin 1 (human: MMRN1, mouse: Mmrn1) in platelet adhesion, specifically through: 1) its interactions with fibrillar collagens, 2) identifying additional potential binding partners for MMRN1, and 3) testing the effects of fluid shear stress on MMRN1 adhesive functions. Previous studies have demonstrated that MMRN1 is capable of supporting platelet adhesion under a range of shear conditions,[1,2] contributes to platelet adhesion onto fibrillar collagens,[2–4] modulates thrombin generation and platelet factor V storage,[5–7] and contributes to thrombus formation *in vivo*.[3,4] The structure and known functions of MMRN1 will be discussed in the following subsections. Next, background on hemostasis and thrombosis, platelet adhesion, and major adhesive proteins and platelet receptors involved in platelet adhesion will be presented. Lastly, in light of this information, currently unanswered questions regarding MMRN1 adhesive functions will be presented and discussed, which form the rationale for the work included in this thesis.

1.1.1 Multimerin 1 structure, synthesis, and storage

Multimerin 1 is a member of the EMILIN/Multimerin family, which consists of four homotrimeric proteins encoded by separate genes that share an N-terminal elastin microfibril interface (EMI) domain, a central region predicted to contain coiled-coil structures, and a C-terminal globular C1q-like domain (*Figure 1*).[8–10] MMRN1 is unique from other EMILINs, which are synthesized by a variety of cell types and constitutively secreted into the elastic fiber system of connective tissues.[9] MMRN1 is synthesized by megakaryocytes and endothelial cells, and is packaged and stored within granules until activation-induced secretion.[10–13] MMRN1 is not detectable in plasma,[14–16] but studies of cultured endothelial cells *in vitro* show that secreted MMRN1 is incorporated into extracellular matrix (ECM) fibers,[17] suggesting MMRN1 may also be a component of the normal endothelial basement membrane.
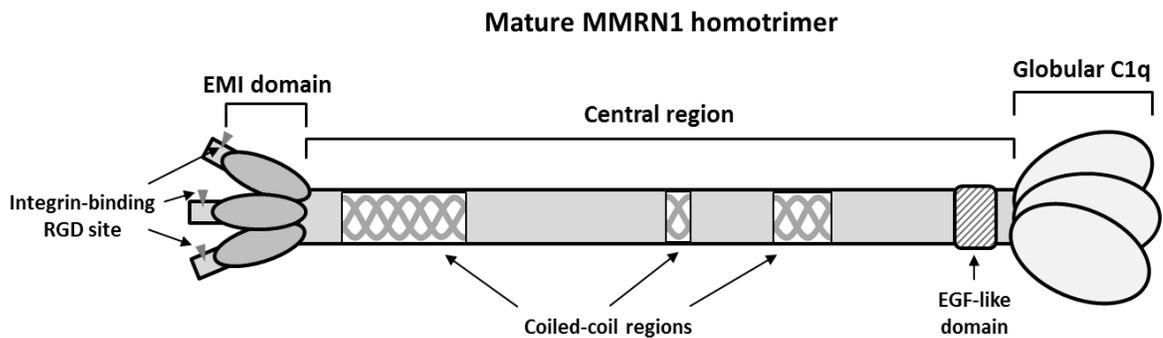
Figure 1: Structure of a native MMRN1 homotrimer. The EMI domain contains multiple free Cys residues that are likely involved in multimerization, whereas the gC1q domain is the potential locus of trimerization, similar to other gC1q-containing EMILINs.[9] Human MMRN1 and mouse Mmrn1 share the same domains and organization, except that Mmrn1 lacks the N-terminal RGD site. While *MMRN1*

synthesizes a prepropolypeptide that is 1228-aa long, the *Mmrn1* gene product is 1210-aa, and MMRN1 and Mmrn1 share 67% amino acid similarity.[18]

MMRN1 is synthesized as a 1228-aa polypeptide, denoted as preproMMRN1, that consists of a 19-aa N-terminal signal peptide and the 1209-aa proMMRN1 polypeptide.[10] The signal peptide is cleaved to produce proMMRN1, which undergoes extensive N-linked glycosylation and minimal O-linked glycosylation.[11] Together, N- and O-linked carbohydrates account for approximately one third of the molecular mass of the MMRN1 subunit.[16] proMMRN1 polypeptides form homotrimers through inter-chain disulfide linkages and subsequently form larger multimers that undergo further proteolytic processing to remove the propolypeptide domain.[19] MMRN1 multimers can range from a single 450 kDa homotrimer to millions of Da in size.[14,15] There are two distinct MMRN1 subunits stored within human platelets, denoted as p-155 and p-170 based on their size in kDa.[16] p-155 makes up the majority of human platelet MMRN1, whereas p-170 is a larger MMRN1 subunit present in small quantities within platelets, and it is cleaved from proMMRN1 closer to the N-terminus than p-155,[11] although the biological significance of the differences in p-155 and p-170 is presently unknown. MMRN1 subunits produced by endothelial cells are slightly larger, which likely reflects differences in proteolytic processing of synthesized polypeptides between endothelial cells and megakaryocytes/platelets.[12] Human MMRN1 and mouse Mmrn1 share 67% similarly, and a key difference is that Mmrn1 lacks the N-terminal integrin-binding RGD site.[18]

Within human platelet α-granules, MMRN1 is stored complexed to coagulation factor V (FV).[7] Approximately 25% of platelet FV is covalently bound to MMRN1 via disulfide linkage to the B-domain of FV, whereas the remaining FV in platelets is non-covalently bound to MMRN1.[20] Although the significance of these two types of FV-MMRN1 complexes is unclear, both forms can be converted to activated FV (FVa).[20] Following platelet activation, released MMRN1 binds to the external platelet membrane, and it is also found within the open canalicular system (OCS),[1,11] with the largest multimers remaining bound to the platelet surface and smaller multimers are released into the local milieu.[14] MMRN1 in endothelial cells is primarily stored in dense-core granules that are morphologically distinct from Weibel-Palade bodies (WPBs) that contain VWF and P-selectin, although small amounts of MMRN1 are present in WPBs.[17] It is interesting to consider that differential packaging and storage of MMRN1 versus VWF by endothelial cells could be of biologic importance. Following activation-induced secretion, endothelial cell MMRN1 stays on the external cell membrane, without detectable secretion into culture media.[17]

1.1.2 Multimerin 1 adhesive and procoagulant functions

MMRN1 is a ligand for VWF,[21] collagens I-III and VI,[2,22] FV,[5,23] phosphatidylserine (PS),[23] and $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_3$ on activated platelets.[1] MMRN1 binds to VWF through a two-site, two-step interaction with the VWF A1 and A3 domains, and VWF is required for platelets to adhere to MMRN1 under high shear flow (1500 s^{-1}).[2,21]

Similarly, GPIbα is required for platelets to adhere to MMRN1, but MMRN1 has no

detectable binding to GPIbα,[2] which suggests that VWF-GPIbα interactions and VWF-

MMRN1 binding mediate platelet adhesion to MMRN1 under high shear flow.

Exogenous MMRN1 enhances platelet adhesion to fibrillar collagen under high shear

flow through a mechanism that requires VWF,[2] and Mmrn1-deficient (*Mmrn1*^{-/-}) mice

have impaired platelet adhesion to collagen under high shear.[3,4] Under stasis and low

shear flow (≤ 150 s^{-1}), platelets adhere to MMRN1 through mechanisms involving $\alpha_{IIb}\beta_3$

and $\alpha_V\beta_3$, with less reliance on VWF-GPIbα binding.[1,2] Resting platelets do not adhere to

MMRN1 under stasis or flow,[1,2] which suggests that the high-affinity conformation or

receptor clustering of β_3 integrins triggered by platelet activation are required for

MMRN1 binding.

MMRN1 is able to modulate thrombin generation through its interactions with

FV, activated FV(a), and PS on the surface of activated platelets.[5] Within the platelet

cytosol, FV undergoes variable proteolytic cleavage of its B-domain, which results in a

variety of partially active forms of FV within platelets,[24] meaning that once released, FV

that was complexed to MMRN1 within platelet α-granules is capable of binding to the

external platelet membrane and promoting coagulation.[7,25] Diseases that result in

MMRN1 deficiency or impair MMRN1-FV binding are associated with impaired platelet

FV storage.[6,26,27] MMRN1 is hypothesized to affect thrombin generation: MMRN1 binds

to FV with high affinity and FVa shows less stable binding to MMRN1 than FV, which is

posited to facilitate FVa delivery onto the platelet surface.[7,23] MMRN1 also competitively

binds to the C1 and C2 domains of FV/FVa,[25] so that while FV or FVa are bound to MMRN1, FVa is prevented from binding to PS on the platelet surface to form the prothrombinase complex. In this regard, MMRN1 may regulate the rate of prothrombinase formation through its interactions with FVa.

1.1.3 Multimerin 1-deficient mice

Two different mouse models of Mmrn1 deficiency have been studied to date. C57BL/6J mice with a spontaneous tandem deletion of the adjacent genes *Mmrn1* and *Snca* (which encodes α-synuclein) were first studied in the absence of a model for selective Mmrn1 deficiency.[28] Double-deficient (*Mmrn1$^{-/-}$/Snca$^{-/-}$*) mice, which have a complete deletion of the *Mmrn1* gene, have normal bleeding times, but show impaired platelet-rich thrombus formation *in vivo* in FeCl$_3$-treated mesenteric arterioles and impaired platelet adhesion to Horm collagen *in vitro* that are corrected by adding back recombinant human (rh)MMRN1.[3] *Mmrn1$^{-/-}$/Snca$^{-/-}$* platelets also show enhanced α-granule release in response to thrombin compared to the wild-type, which is postulated to result from loss of α-synuclein.[29] Due to enhanced α-granule release shown by *Mmrn1$^{-/-}$/Snca$^{-/-}$* mice, the phenotype of *Mmrn1$^{-/-}$/Snca$^{-/-}$* is hypothesized to be less severe than mice with selective Mmrn1 deficiency.[3,4]

Selective Mmrn1-deficient (*Mmrn1$^{-/-}$*) mice were subsequently generated using the knockout-first allele strategy.[30] Adult mice heterozygous for the *Mmrn1* knockout-

first allele were purchased from the European Mutant Mouse Archive and used to generate mice with selective Mmrn1 deficiency. Generation of selective *Mmrn1*^{-/-} mice was performed as a collaborative effort between the laboratories of Dr. Catherine Hayward and Dr. Bradley Doble. In this model, exon 3 of *Mmrn1* was excised by Cre-Lox recombination, causing a frameshift mutation that is predicted to result in an aberrant transcript susceptible to nonsense-mediated decay.[30,31] *Mmrn1*^{-/-} mice are viable and fertile without any overt developmental abnormalities, spontaneous bleeding, or reduced survival, and have no detectable platelet Mmrn1.[4] Dr. D'Andra Parker characterized some phenotypic aspects of selective Mmrn1-deficient mice as part of her doctoral thesis work under the supervision of Dr. Catherine Hayward, which includes analyses of: complete blood cell counts; Western blotting for Mmrn1 in platelet lysates; plasma and platelet Vwf levels; expression of GpIbα, β_1 integrin, and β_3 integrins on resting platelets; P-selectin and activated $\alpha_{IIb}\beta_3$ expression on thrombin-activated platelets; bleeding time and blood loss; light transmission aggregometry (LTA) responses; and high shear platelet adhesion assays to Horm collagen and recombinant (r)Vwf.[4] Additionally, analyses of thrombus formation *in vivo* were performed by Dr. Yiming Wang in the laboratory of Dr. Heyu Ni as part of collaborative studies between the laboratories of Dr. Ni and Dr. Hayward.

Mmrn1^{-/-} and wild-type mice have similar blood cell counts, plasma and platelet Vwf levels, and resting platelet expression of GpIbα, β_1 integrin, and β_3 integrins. Notably, P-selectin expression of thrombin-activated platelets is similar between *Mmrn1*^{-/-}

and wild-type mice. LTA responses are similar between *Mmrn1*^{-/-} and wild-type platelets

using murine TRAP (500 µM; tested in PRP), thrombin (0.5 and 1.0 U/ml; tested in

GFP), ADP (10 and 20 µM; tested in PRP), and Horm collagen (5 and 10 µg/ml; tested in

PRP), but gel-filtered *Mmrn1*^{-/-} platelets show a significant defect in maximal aggregation

responses (20-30% reduction) to Horm collagen compared to wild-type platelets.

Additionally, *Mmrn1*^{-/-} platelets show impaired adhesion to Horm collagen under high

shear flow, but similar adhesion to rVwf compared to the wild-type. Lastly, *Mmrn1*^{-/-}

mice have normal bleeding times and wound blood loss, but impaired platelet adhesion,

thrombus formation, and vessel occlusion *in vivo* in $FeCl_3$-treated mesenteric arterioles.

Platelet aggregates formed by *Mmrn1*^{-/-} mice also show a tendency to break apart from

the apex of the developing thrombi, contributing to the delay in time until the first

thrombus greater than 20 µm and time until vessel occlusion. Notably, most other

knockout or transgenic mouse models tested using the $FeCl_3$-induced vessel injury model

show defects in one or two parameters (*Table 1*), whereas platelet adhesion and occlusive

thrombus formation are abnormal in *Mmrn1*^{-/-} mice from start to finish. These findings

suggest that Mmrn1 has a supportive role in platelet adhesion, thrombus formation, and

thrombus stability.

1.1.4 Multimerin 1 deficiency states in humans

To date, no selective qualitative or quantitative defect in MMRN1 has been

described in humans, but some disease states are associated with platelet MMRN1

deficiency: Quebec platelet disorder (QPD), gray platelet syndrome (GPS), and αδ-storage pool deficiency (SPD). QPD is an autosomal dominant disorder that results in delayed mucocutaneous bleeding due to a fibrinolytic defect.[32] QPD is caused by a duplication mutation that includes *PLAU*, which results in overexpression of *PLAU* by megakaryocytes and intra-platelet activation of the fibrinolytic cascade, leading to α-granule protein degradation, with loss of MMRN1 and FV.[6] GPS is characterized by a reduction or absence of platelet α-granules due to impaired retention of α-granule proteins caused by mutations affecting *NBEAL2*,[33–35] *GFI1B*,[36] or *GATA1*,[37] or acquired through unknown mechanisms.[38] Lastly, αδ-SPD is associated with reduced numbers of both α- and dense granules in platelets, causing a deficiency of their contents, which has been attributed in some cases to mutations in *GFI1B* or *IKZF5*.[39,40]

1.2 HEMOSTASIS AND THROMBOSIS

Hemostasis is the process through which animals with a closed circulatory system prevent blood loss from damaged or ruptured vessels. Hemostasis is regulated by the interaction of numerous cell types, proteins, and enzymes, and culminates in the formation of a hemostatic plug composed of platelets, erythrocytes, and plasma proteins within and around a polymeric fibrin meshwork.[41] The fibrin meshwork acts as a provisional scaffold to cease blood loss while allowing for the attachment and penetration of cells for remodeling of the damaged area. Impairments in hemostasis due to defects or deficiencies in any of the cells, receptors, plasma or granule proteins, and coagulation

factors involved in hemostatic plug formation lead to unchecked blood loss, which can result in spontaneous and/or severe hemorrhage if untreated.

The formation of a hemostatic plug normally does not occlude the injured portion of the blood vessel and blood continues to circulate following plug formation. Thrombosis is the pathological formation of a platelet-rich clot, or thrombus, within a vessel that can partially or entirely occlude a blood vessel. Thrombi have the potential to occlude vessels or dislodge from the vessel wall and occlude smaller vessels downstream. Depending on the site of thrombus deposition, vessel occlusion can result in, among other conditions, myocardial infarction, ischaemic stroke, or venous thromboembolism (VTE), which are leading causes of morbidity and mortality in North America.[42–44] The following sections describe the biological basis of hemostatic plug formation and thrombosis, the role of platelets in these processes, and the clearance of platelet-rich fibrin meshworks via fibrinolysis.

1.2.1 Blood coagulation and fibrinolysis

The development of a polymeric fibrin clot involves the interaction of numerous enzymes and non-enzymatic cofactors that culminate in the cleavage of soluble fibrinogen (FG) to fibrin by thrombin, which changes blood from a liquid to a gel.[41] This process is referred to as blood coagulation, and polymeric fibrin is a major constituent of coagulated blood.[45] Blood coagulation can occur via two pathways: extrinsic and

intrinsic,[46,47] which converge at the activation of coagulation factor X (FX). Activated

FX, FX(a), cleaves circulating prothrombin, the zymogen precursor of thrombin, to

thrombin.

The extrinsic pathway of blood coagulation involves the initiation of coagulation

by a protein normally "extrinsic" to blood. Tissue factor (TF) is a membrane-associated

lipoprotein that is present in most cells, including platelets, endothelial cells, and

monocytes.[48] TF is constitutively expressed on the membrane of fibroblasts and smooth

muscle cells in the deeper layers of the vessel wall,[49–52] and can be expressed on the

surface of platelets,[53–55] endothelial cells,[56–58] or monocytes in response to activation.[51,59]

When TF is exposed to flowing blood, either by exposure of flowing blood to TF-bearing

cells in the vessel wall or following activation of blood cells,[60] it binds to coagulation

factor VII (FVII),[61] and induces conformational change of FVII to an active form FVII(a).

The TF-FVIIa complex binds to coagulation factors IX (FIX) and (FX), subsequently

activating them to FIXa and FXa.[62] FIXa generates additional FXa, and FXa produces

small amounts of thrombin during what is referred to as the "initiation" phase of

coagulation.[41,63]

The intrinsic pathway is defined by all of the components necessary for

coagulation being present in blood. It is initiated following exposure of blood to

negatively charged surfaces (e.g. extracellular DNA, RNA, or polyphosphate – the latter

is released from platelet dense granules),[64] which triggers autodigestion of factor XII

(FXII) to its active form FXII(a).[65] FXIIa cleaves factor XI (FXI) to its active form FXI(a),[66,67] and FXIa triggers a cascade of enzymatic reactions that also leads to FXa and thrombin generation.[41] The intrinsic pathway is not independent of the extrinsic pathway. For example, following the initiation phase, thrombin proteolytically activates components of the intrinsic pathway: FVIII, FIX, and FXI, and thrombin can initiate the intrinsic pathway independent of FXIIa by cleavage of FXI.[68] The intrinsic pathway can also be initiated by autoactivation of FXI in contact with a negatively charged surface,[69] such as platelet polyphosphate.[70] The extrinsic pathway is considered to be the major initiation pathway of coagulation *in vivo*,[71–73] whereas the intrinsic pathway has been linked to thrombus growth and inflammation.[63]

The trace amounts of thrombin generated during the initiation phase cleave and activate circulating plasma FV to FVa and factor VIII (FVIII) to activated FVIII(a).[41] FVa and FVIIIa are non-enzymatic cofactors that greatly increase the generation of FXa by FIXa and thrombin by FXa, respectively.[74–76] It is possible that the partially activated FVa released from platelets provides the initial FVa for thrombin generation.[77,78] Formation of the FIXa/FVIIIa and FXa/FVa complexes results in a burst of thrombin generation, which is referred to as the "amplification phase".[63] The large quantities of thrombin cleave circulating plasma FG and FG released from platelet granules to fibrin monomers, which spontaneously aggregate into fibrils.[79,80] Thrombin also converts factor XIII (FXIII) to its active form FXIII(a),[81] and in the presence of fibrin (as a cofactor), FXIIIa laterally crosslinks and stabilizes fibrin fibrils.[82]

The generation of thrombin is tightly regulated to prevent inappropriate activation of coagulation and fibrin formation in flowing blood.[68] Thrombin generation is self-limiting, in that it cleaves and activates protein C, a major physiologic inhibitor of thrombin generation.[83] Activated protein C (APC) cleaves FVa and FVIIIa, which attenuates thrombin generation.[83] Additionally, heparin sulfate-containing proteoglycans in the vessel wall bind and activate antithrombin,[84] which binds and inactivates thrombin,[85] among other coagulation factors, and accounts for roughly 75% of physiologic thrombin inhibition.[86] The dissolution of fibrin clots, which is the final stage of hemostasis, is mediated by plasmin.[87] Plasmin circulates as its zymogen precursor, plasminogen, in plasma and becomes activated by tissue plasminogen activator (tPA) or urokinase plasminogen activator (uPA).[88,89]

Most coagulation reactions occur on biological surfaces.[90–94] For instance, the prothrombinase complex is composed of FXa and FVa bound to PS or PE that are exposed on the surface of activated platelets,[95] and binding to membrane lipids stabilizes FXa-FVa interactions.[96] More specifically, platelets are vital to blood coagulation and the formation of a fibrin-rich hemostatic plug because they: 1) provide a surface for coagulation reactions at the site of injury, 2) contain many procoagulant molecules that they release locally to support coagulation, and 3) localize coagulation reactions to the site of injury.[97] This is partly evidenced by the bleeding diatheses exhibited by patients with various forms of thrombocytopenia,[98–100] and, conversely, the effects of anti-platelet

drugs, which can significantly reduce the risk of thrombosis and thrombosis-related mortality.[101–105]

1.1.2 Platelets

Platelets are small, anucleate cells that circulate in flowing blood at a concentration of approximately 150 to 450 x 10^6 cells/ml in humans.[106] Human platelets circulate in a resting state in a discoid shape of approximately 1 to 2 µm in diameter.[107] Mouse platelets are more numerous and smaller; they circulate at concentrations ranging from 400 to 1600 x 10^6 cells/ml.[108] Platelets are unique to mammals: fish, birds, and reptiles possess analogous cells called thrombocytes that carry out similar functions.[109]

Platelets are formed by polyploid megakaryocytes (MKs) in bone marrow.[110] The current view of platelet formation is that MKs form proplatelet extensions composed of a thin cytoplasmic process dotted with platelet-sized blebs.[111–113] These proplatelet extensions protrude into the vascular sinuses of blood vessels in bone marrow,[114,115] where they fragment into platelets and enter circulation.[116,117] Recently, the lung microcirculation has also been identified as a major site of platelet production.[118] Platelets are constitutively formed and released into circulation at a rate of approximately 100 billion per day in humans,[119] where they circulate for up to 10 days in humans and about 5 days in mice.[120,121] Circulating platelets gradually decrease in size, undergo membrane changes, and have reduced hemostatic efficiency,[122,123] meaning that at any moment, a

mosaic population of platelets of different size and adhesive potential circulate within flowing blood.

Platelets contain a variety of organelles and many proteins, including proteins endocytosed from plasma and proteins that are endogenously synthesized by MKs, such as MMRN1.[7,11,13] Platelets are anucleate but are capable of some protein synthesis, since they contain mRNAs and ribosomes derived from MKs.[124] A defining feature of platelets is the presence of numerous α-granules and dense granules.[125,126] Alpha-granules are the site where most secreted proteins are stored in platelets and they are the most numerous organelle in platelets,[127,128] numbering from roughly 40 to over 100 per platelet, and account for roughly 10% of platelet volume.[129] Platelets release their α-granule contents following activation, and the platelet secretome ranges in estimates from around 300 to over 700 proteins,[130,131] which includes soluble plasma proteins (e.g. FG, fibronectin [FN], vitronectin [VN], thrombospondin-1 [TSP-1], VWF, and MMRN1) and membrane-bound proteins (e.g. integrin $\alpha_{IIb}\beta_3$, GPVI, GPIb-IX-V, and CD36). Within dense granules, platelets store ADP and polyphosphate, which modulate platelet activation and coagulation, respectively.[132,133] In addition to storing and releasing procoagulant and pro-adhesive molecules, platelets store and release a variety of pro-inflammatory molecules, chemotactic factors, and growth factors. Accordingly, emerging roles of platelets in inflammation,[134–136] immunity,[137–139] angiogenesis,[140–142] and cancer have been reported,[142–145] but are outside the scope of this thesis.

In laminar flow, platelets are pushed to the vessel wall by circulating erythrocytes, which allows platelets to constantly survey the integrity of the vessel wall surface for sites of damage.[146] The endothelial cells lining blood vessels and capillaries maintain circulating platelets in a resting state by constitutive release of nitrous oxide (NO) and prostacyclin (PGI_2),[147,148] expression of CD39,[149] and an extensive glycocalyx.[150–152] Once platelets encounter an area of vessel wall injury or endothelial cell activation, or leave the circulation through a severed vessel, they rapidly adhere and become activated. In wounds, this process is referred to as primary hemostasis.[97,153]

1.3 PLATELET ADHESION

Platelet adhesion is a critical initial step in hemostasis because localization of platelets at the site of vessel injury is absolutely required for platelets to fulfil their hemostatic functions.[97,144,154–156] Similarly, thrombus formation can be initiated by platelet adhesion.[146] Understanding the mechanisms of platelet adhesion is crucial to the development of treatment and prevention strategies in hemostatic and thrombotic disorders. This section will focus primarily on platelet adhesion in the hemostatic response to injury, although the mechanisms involved in platelet tethering and recruitment of additional circulating platelets onto a growing platelet aggregate are mostly conserved between hemostasis and thrombosis.[157,158] *Figure 2* outlines the general steps involved in platelet adhesion, from exposure of collagens in the vessel wall to formation of a stable, platelet-rich hemostatic plug. In recent years, a preliminary phase

referred to as the "protein wave" of hemostasis has been reported,[155,159–161] in which circulating plasma proteins that support platelet adhesion (e.g. FG, FN, and VWF) bind to the exposed extracellular matrix following vessel injury. These interactions result in an exposed vessel wall that is "hyper-adhesive", in that it contains numerous sites for additional pro-adhesive plasma proteins and circulating platelets to attach, and provides a provisional protein scaffold from which a polymeric fibrin meshwork can later be deposited.
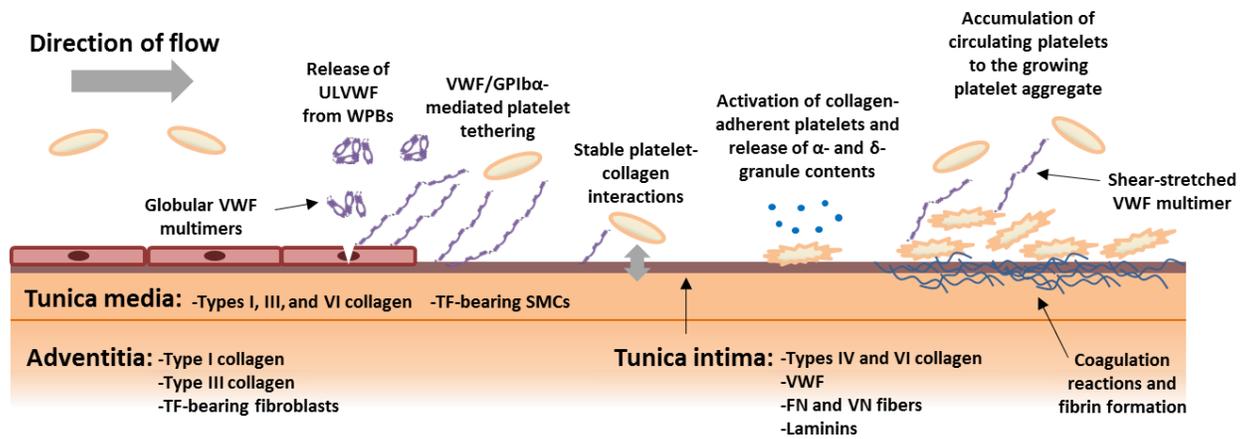
Figure 2: Overview of platelet adhesion following injury to the vessel wall under high shear flow. 1) Vessel injury exposes the subendothelium and deeper layers of the vessel wall to flowing blood, and ruptures or activates nearby endothelial cells lining the site of injury releasing ultra-large (UL)VWF and MMRN1. 2) Circulating plasma proteins, such as FG, FN, VWF, and VN bind to extracellular matrix components (e.g. collagens and laminins). Additionally, FN, VN, and VWF are constitutively present within the vessel wall. Together, these interactions prime the exposed vessel wall to support platelet adhesion. 3) Collagen- or EC-bound VWF multimers extend under shear into the vessel lumen, which transiently bind to GPIbα on circulating platelets. VWF/GPIbα binding decreases the velocity of platelets, allowing them to slowly translocate along the exposed vessel wall. 4) Platelets bind to exposed fibrillar collagens via integrin $\alpha_2\beta_1$ and GPVI and become activated. 5) Platelet activation triggers the generation and release of soluble agonists (ADP and TxA2), procoagulant molecules (FV and polyphosphate), and pro-adhesive molecules (i.e. VWF, FG, FN, VN, TSP-1, and MMRN1). Integrins $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ shift to a high-affinity conformation on activated

platelets, which enables ligation of additional proteins or motifs and stabilizes adhesion. 6) Additional platelets are recruited onto the activated platelet monolayer by VWF multimers bound to activated $\alpha_{IIb}\beta_3$ and become activated by the high local concentration of soluble agonists. Simultaneously, coagulation reactions occur on the surface of collagen- and thrombin-activated platelets at the interface of the vessel wall, resulting in fibrin meshwork formation.

Shear stress and shear rate affect the dynamics of platelet adhesion.[156,162,163] Wall shear stress (τ_W, referred to hereafter as "shear stress") refers to the force applied to the vessel wall as blood flows across the vessel wall through the lumen at a constant rate, taking into consideration the viscosity (μ) of the fluid, and is measured by units of force per area (dyn/cm^2). Wall shear rate (γ_W, referred to hereafter as "shear rate") refers to the rate of shearing deformation experienced by the vessel wall as blood flows across, expressed as reciprocal seconds (s^{-1}), which is a function of the size of the vessel lumen and the velocity of flowing blood. Given that vessel diameter and velocity both affect the shear rate experienced by the vessel wall, arteries and veins of different sizes and capillaries experience different shear rates. In humans, normal shear rates range from 20 to 200 s^{-1} in veins, 300 to 800 s^{-1} in medium and large arteries, and 500 to 5000 s^{-1} in small arteries.[159,164] Under pathological conditions, stenotic vessels can experience shear rates in excess of 40,000 s^{-1}.[165] An important consideration is that shear rates are not constant or uniform *in vivo*: blood flow through the heart into downstream vessels is pulsatile, meaning that the shear rate increases and decreases as blood is pushed through vessels by muscular contractions of the heart.[166] Additionally, shear rate changes based on vascular tone, which is locally controlled by smooth muscle contraction or dilation of the tunica media.[167] Shear gradients and microenvironments have also been observed in areas

where the vessel lumen is constricted or obstructed,[158,168,169] or where vessels bend and branch.[170] Murray's law posits that the circulatory system branches in such a way that minimizes the energy cost of pumping blood through a vessel.[171] It is shown that this tendency toward energy minimization affects shear rates; vessel bifurcations result in areas of low shear stress or high oscillatory shear (i.e., reversal of shear stress that occurs during pulsatile flow).[172] Additionally, vessel injury stimulates the localized release of vasoconstrictive molecules from endothelial cells, which increases the shear rate at and around the site of injury as the lumen decreases in diameter.[173] Therefore, it is important to note that fluid shear rates are complex and dynamic, and its categorization into high and low shear is an abstraction that is useful for descriptive and experimental purposes. Blood is a Non-Newtonian colloid,[156] but exhibits the properties of a Newtonian fluid, in that its shear stress and shear rate are linearly correlated. For this reason, it is treated as a Newtonian fluid in these calculations.

Under shear rates >500 s^{-1}, platelet receptors $\alpha_2\beta_1$, $\alpha_{IIb}\beta_3$, and GPVI are increasingly insufficient to support significant platelet adhesion to the exposed vessel wall.[174,175] VWF binding to the vessel wall and its shear-induced unfolding into long strings is required for platelet adhesion under high shear flow (ranging from ~500 to $>20,000$ s^{-1}).[174,176,177] The first point of contact between circulating platelets and the exposed subendothelium is generally considered to be immobilized VWF multimers extending into the lumen of the blood vessel,[178–181] which requires VWF to be bound to various proteins in the ECM. VWF multimers transiently bind to GPIbα on platelets,

which decreases the velocity of circulating platelets and allows them to slowly translocate along the exposed vessel wall.[174,178] Platelet translocation facilitates activation via platelet GPVI binding to fibrillar collagens in the vessel wall and stable adhesion to collagens and other matrix constituents by platelet integrins $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$, respectively.[174,175] Under low (<500 s^{-1}) shear flow, ligation of ECM components by platelet receptors $\alpha_2\beta_1$, $\alpha_{IIb}\beta_3$, and GPVI are sufficient to support stable adhesion at the site of injury.[174,175,182,183]

Following the initial deposition of platelets onto the exposed vessel wall, additional platelets begin aggregating onto the stably adherent monolayer of activated platelets. Primary adherent platelets are activated through multiple mechanisms. First, ligation of GPIbα by VWF stimulates intracellular Ca^{2+} mobilization and activation of integrin $\alpha_{IIb}\beta_3$,[184–186] and ligation of either $\alpha_2\beta_1$ or $\alpha_{IIb}\beta_3$ similarly trigger outside-in signalling in platelets that results in shape change and intracellular Ca^{2+} mobilization.[187–189] Fibrillar collagen is a potent agonist of platelet activation, and collagen binding to GPVI is a key initiator of platelet activation.[190] Additionally, platelets are further activated by trace amounts of thrombin generated by TF/FVIIa-activated FX at the site of injury. Full platelet activation by either collagen or thrombin triggers intracellular shape change, synthesis of thromboxane A_2 (TxA_2), and the localized release of α-granule contents, which include pro-adhesive proteins (e.g. VWF, FG, FN, TSP-1, VN, and MMRN1) and soluble platelet agonists (e.g. ADP and TxA_2).[191–196] Plasma and platelet VWF multimers stably bind to activated $\alpha_{IIb}\beta_3$ on the activated platelet surface, where they extend into the lumen to tether additional circulating platelets.[156,197–199] Captured

platelets become activated by the high local concentration of agonists and stably adhere to

a growing network of platelets and adhesive glycoproteins.[146,156,181,200] This process of

additional platelet recruitment continues until the site of injury is fully covered in a

platelet aggregate.[156,181,200] Simultaneous to platelet adhesion, coagulation reactions take

place on the surface of activated platelets, which results in a high local concentration of

thrombin and the formation of a polymeric fibrin meshwork.[201] The process of fibrin clot

formation occurring alongside platelet adhesion and activation is referred to as

"secondary hemostasis".[41] In addition to the self-limiting regulatory mechanisms of

coagulation, platelet adhesion is tightly regulated in order to prevent the aggregation stage

from continuing unchecked and occluding the vessel at the site of injury.[160,202] In

particular, roles in the regulation of the extent of platelet aggregate formation at sites of

injury have been reported for plasma FN, TSP-1, and VN, which are discussed in sections

1.2.1.3, *1.2.1.4*, and *1.2.1.5*, respectively.

1.3.1 Platelet adhesive proteins

The following sections focus on the roles of specific plasma, extracellular matrix,

and/or platelet/endothelial cell granule proteins in supporting platelet adhesion and

platelet aggregation (*Figure 3*). There are some striking structural and functional

similarities between many of the adhesive glycoproteins circulating in plasma and stored

within platelet and endothelial cell granules. FG, FN, TSP-1, VN, VWF, and MMRN1 are

multi-domain glycoproteins, which enables these proteins to interact with a variety of

ligands. FN, VN, TSP-1, VWF, and MMRN1 can self-associate to create large

homopolymers, and FG can also self-associate after thrombin-mediated conversion of FG

to fibrin, which has the potential to greatly increase the avidity of binding interactions.
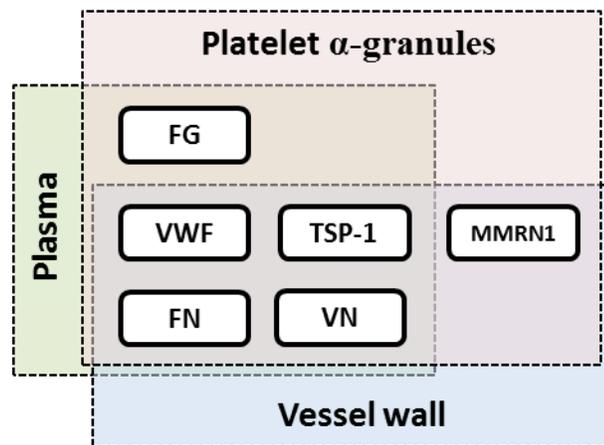
Figure 3: Distribution of select proteins within plasma, platelets α-granules, and the vessel wall that are known to support platelet adhesion and/or aggregation.

FG, FN, TSP-1, and VN are also able to bind to one another. It has been

postulated that adhesive plasma proteins, in particular FN, TSP-1, and VN, facilitate the

formation of large macromolecular complexes at sites of injury that enhance platelet

adhesion and aggregation.[203–206] Accordingly, a supporting, but non-essential role in

platelet adhesion and thrombus formation has been reported for FN, TSP-1, and VN.

Given their similarities to MMRN1, it is possible that MMRN1 similarly assists in the

formation of large, macromolecular complexes at sites of injury or a developing thrombus

to enhance platelet adhesion and stabilize platelet-platelet or platelet-matrix interactions.

Mice with Fn-,[207,208] Tsp-1-,[209] or Vn-deficiency[210] do not experience abnormal bleeding,

but some parameters of platelet adhesion and *in vivo* thrombus formation are impaired in these animals (*Table 1*), which is similar to observations of *Mmrn1*^{-/-} mice.[203,208,211,212] Additionally, Fg-, Vwf-, or combined Fg/Vwf-deficient mice are still able to aggregate *in vitro*[213] and form thrombi *in vivo* in response to experimentally-induced vessel injury,[211] which suggests other proteins, such as Fn, Tsp-1, Vn, and/or Mmrn1, support these processes in the absence of Fg and Vwf. Lastly, another functional similarity between FN, TSP-1, and VN is that they are demonstrated to play a dual role in platelet adhesion and platelet aggregation,[203–205] switching between supporting or inhibiting platelet aggregation based on their conformation or the presence of other plasma proteins.

Model	Background	Bleeding following tail transection	Ferric chloride mesenteric vessel injury model		
			Plt. Adhesion	Thrombus growth	Vessel occlusion
$\alpha_2^{-/-}$	C57BL/6J[183,214,215]	Normal[214]; Severe[183]*	Normal[215]	Delayed; frequent emboli[215]	Delayed; frequent emboli[215]
GpVI^{-/-}	C57BL/6J[216–218]; BalbC[183]	Slight[216]; Moderate[183]; Normal[218]	-	Delayed[217]†	Delayed[217]†
GpIbα^{-/-}	C57BL/6J[219,220]	Severe[219]	Abolished[220]‡	Abolished[220]‡	Abolished[220]‡
$\beta_3^{-/-}$	C57BL/6J[221]	Severe[221]	-	-	-
Vwf^{-/-}	C57BL/6J[211,212,222,223]	Severe[222,223]	Impaired[211,212,222,223]	Delayed[211,222]	Delayed/ absent[211,222]
Tsp-1^{-/-}	C57BL/6J[209,212,224]; Swiss[225]	Normal[209]	Normal[212]	Delayed[212]	Delayed[212]
Global Fn^{-/-}	C57BL/6J[226]	-	-	-	-
Cdtl. pFn^{-/-}	C57BL/6J[207,208]	Normal[207]	Normal[208]	Delayed[208]	Delayed[208]
Fg^{-/-}	129/CF-1[227]; C57BL/6J[211,227]	Severe[227]¶	Normal[211]	Normal[211]	Embolize and occlude downstream[211]
Vn^{-/-}	C57BL/6J[203,228,229]	Normal[228]	Normal[203]	Normal[203]	Delayed; frequent emboli[203]
Mmrn1^{-/-}	C57BL/6J[4]	Normal[4]	Impaired[4]	Delayed[4]	Delayed/absent[4]

Table 1: Phenotypic traits of transgenic and knockout mice for proteins involved in platelet adhesion, hemostasis, and thrombosis. Distal tip tail amputations used in bleeding studies vary from 1 to 6 mm from the end of the tail. Studies report similar concentrations and application of $FeCl_3$, and shear rate in mesenteric arterioles is estimated to be approximately 1400 s^{-1}.[222] *Tested using α_2-deficient mice of a mixed C57BL/6J and BalbC background compared to C57BL/6J or BalbC wild-type controls.

[†]Studies were performed using FcRγ-deficient mice, which have no detectable surface expression of GpVI. [‡]Studies were performed using GpIbα functional knockout mice, which are transgenic mice that express a chimeric form of GpIbα that does not bind Vwf. These mice do not have giant platelets characteristic of *GpIbα* knockout mice or Bernard-Soulier syndrome. [¶]Bleeding time for these animals was measured by amputation of the 5th digit. "-" indicates data not available or data that has not been reported.

1.3.1.1 Fibrinogen and fibrin

Fibrinogen (FG) is a large dimeric glycoprotein composed of three pairs of polypeptide chains: Aα, Bβ, and γ, which have a total mass of approximately 340-kDa.[230] The designation of polypeptide chains as a Latin and a Greek character is due to the thrombin-mediated cleavage of FG to fibrin.[231] Cleavage of FG by thrombin releases fibrinopeptides A and B leaving two αβγ fibrin monomers.[230] The AαBβγ FG dimer is approximately 45 nm in length and held together at a central nodule by inter-chain disulfide bonds.[232] The majority of FG is synthesized by hepatocytes in humans,[233] where it is constitutively secreted into circulation at a very high concentration ranging from 2 to 4 g/l in plasma in humans.[234] In adult C57BL/6J mice, Fg circulates in plasma at concentrations ranging from 1.6 to 2.4 g/l.[235–237] Plasma FG is also uptaken into MKs and platelets and stored within α-granules through a mechanism involving FG binding to $\alpha_{IIb}\beta_3$.[238]

FG is a ligand for FN,[239,240] TSP-1,[241,242] fibrin,[243] and $\alpha_v\beta_3$,[244] but its interaction with $\alpha_{IIb}\beta_3$ has been the most studied, leading to $\alpha_{IIb}\beta_3$ being referred to as the "fibrinogen receptor".[245] FG binds to $\alpha_{IIb}\beta_3$ on activated platelets with a K_D of approximately 100

nM,[246] which is almost 100-fold less than the concentration of FG in plasma. These data suggest that the FG-binding site on $\alpha_{IIb}\beta_3$ is likely immediately occupied by FG following platelet activation. FG binding to $\alpha_{IIb}\beta_3$ is critical for platelet aggregation under low shear stress.[247] FG binding to $\alpha_{IIb}\beta_3$ involves the KQAGDV sequence in the FG γ-chain and its dimeric structure allows FG to crosslink platelets by each FG γ-chain engaging a single copy of $\alpha_{IIb}\beta_3$ on adjacent platelets.[248] The human FG dimer contains two pairs of integrin-binding RGD sequences (residues Aα 95-97 and Aα 572-574), however, these RGD motifs are not required for FG-mediated platelet aggregation.[249,250] RGDS peptides are able to inhibit $\alpha_{IIb}\beta_3$ binding to FG *in vitro* presumably because they can compete with the FG γ-chain KQAGDV motif for ligation of $\alpha_{IIb}\beta_3$.[251] These motifs are shown to be necessary for clot retraction, suggesting they may be essential for the interaction of $\alpha_{IIb}\beta_3$ with fibrin.[252–255]

FG is critically important to hemostasis. FG levels are a major predictor of blood loss and death following hemorrhage from surgery or severe trauma,[256–260] and FG concentrate is a commonly used intervention to prevent post-surgical or trauma-induced severe bleeding.[261,262] Human patients with congenital or acquired afibrinogenemia suffer from spontaneous bleeding and severe bleeding after minor trauma.[263] Conversely, elevated FG levels (4.0 to 4.9 g/l) are associated with a greater risk of thrombosis.[264] Mice with fibrinogen deficiency (*Fg*^{-/-}) are viable and fertile, but have reduced survival due to spontaneous intraabdominal hemorrhage (*Table 1*).[211,227] Whole blood from *Fg*^{-/-} mice fails to clot either spontaneously or in response to thrombin, and *Fg*^{-/-} mice experience

severe bleeding in response to injury.[227] PRP from $Fg^{-/-}$ mice also fails to aggregate in response to ADP.[227] In response to FeCl$_3$ treatment of mesenteric arterioles, $Fg^{-/-}$ mice show normal platelet adhesion and thrombus development, but their thrombi are unstable and embolize downstream of the initiation of thrombus growth.[211]

Immobilized FG is capable of supporting platelet adhesion through a mechanism that requires $\alpha_{IIb}\beta_3$ under shear rates up to 1000 s^{-1}.[174] Platelets immediately arrest onto FG surfaces under shear rates of approximately 600 to 900 s^{-1}, with platelets typically adhering as a monolayer.[265] Maximal adhesion to FG is reported for shear rates between 50 and 250 s^{-1}.[174,265] Platelet adhesion to FG has been observed under shear rates up to 2000 s^{-1}, but very few platelets adhere to FG-coated surfaces under these conditions.[174,265] Additionally, platelet $\alpha_{IIb}\beta_3$ binding to immobilized FG is very stable: real-time analyses of platelet adhesion to FG report it is 90% irreversible.[174] It is postulated that the slow rate of bond formation between FG and $\alpha_{IIb}\beta_3$ is insufficient to support platelet adhesion under high shear rates, and that platelet GpIbα binding to immobilized VWF potentiates stable platelet adhesion under high shear by reducing the velocity of flowing platelets, which enables immobilized FG to bind activated $\alpha_{IIb}\beta_3$ on circulating platelets.[181]

Platelet adhesion to immobilized FG and fibrin are shown to be roughly identical,[174] but some differences in the mechanisms of platelet adhesion to FG versus fibrin have been described. Monoclonal anti-$\alpha_{IIb}\beta_3$ antibodies or the snake venom halysin, which blocks FG binding to $\alpha_{IIb}\beta_3$, block platelet adhesion to both immobilized FG and fibrin, whereas RGD peptides are only effective to partially inhibit platelet adhesion to

FG and do not inhibit adhesion to fibrin.[266] Similar to the effect of RGDS peptides on platelet aggregation, it is postulated that RGDS peptides compete for ligation of $\alpha_{IIb}\beta_3$ with the KQAGDV motif in circulating FG, rather than inhibiting RGD-dependent binding of FG to $\alpha_{IIb}\beta_3$. In regard to platelet adhesion to fibrin, differences in ultrastructure between immobilized FG and three-dimensional fibrin meshworks likely play a significant role.

Following cleavage of FG to fibrin by thrombin, fibrin monomers rapidly self-associate into larger polymers. Fibrin polymerization is initiated by cleavage of fibrinopeptides A and B by thrombin,[81] which exposes fibrin-binding sites in the $\alpha\beta\gamma$ fibrin monomer, allowing monomers to self-associate. Fibrin monomers bind one another in a half-staggered manner, adding additional monomers longitudinally to form large two-stranded oligomers.[267,268] Once oligomers reach 0.5 µm in length, they are considered protofibrils,[79,80] which are capable of associating laterally into fibrin fibers. Fibers begin branching to produce a three-dimensional network of fibrin fibers,[269,270] although the mechanisms of fibrin branching are not well-understood. The biological requirements of fibrin networks are that they must be rigid enough to stop bleeding, but simultaneously allow the penetration of cells for wound healing and tissue remodelling.

Fibrin binds numerous other proteins, including activated coagulation factor XIII (FXIIIa),[81] FN,[239] FG,[243] VWF,[271] β_3 integrins on activated platelets,[272] $\alpha_V\beta_3$ and ICAM-1 on activated endothelial cells,[273] and $\alpha_M\beta_2$ on activated leukocytes.[274] Collectively, these

interactions enable platelets and leukocytes to stably adhere to growing fibrin meshworks, which is essential for hemostasis as well as wound healing by attracting leukocytes to areas of vessel injury. FXIIIa stabilizes fibrin networks by catalyzing the formation of covalent lysine bonds between fibrin molecules and crosslinking other proteins to the fibrin network, such as FN, FG, and VWF.[271] FN affects fibrin fiber density and diameter in clots following crosslinkage to fibrin by FXIIIa, and is postulated to contribute to wound healing.[275] FG binding to fibrin monomers is hypothesized to be a regulatory mechanism that inhibits polymerization of small amounts of fibrin generated in flowing blood.[243] Additionally, a "platelet-trapping" phenomenon has been described, in which polymeric fibrin meshworks mechanically trap circulating platelets.[276] This process requires thrombin to produce fibrin and activate platelets, but not $\alpha_{IIb}\beta_3$. Thrombin can bind GPIb, PAR-1, or PAR-4 to activate platelets, which facilitates release of platelet FV, FV-mediated amplification of thrombin generation, and substantial fibrin generation. In the presence of lotrafiban, which blocks FG and VWF binding to $\alpha_{IIb}\beta_3$, platelets are able to aggregate, and this aggregation is dependent on fibrin polymerization by thrombin.[276]

Recently, fibrin has been shown to bind GPVI on human and mouse platelets, and fibrin binding to GPVI is capable of inducing outside-in signalling resulting in tyrosine phosphorylation.[277,278] A low level of FcR γ-chain phosphorylation has also been shown following platelet GPVI binding to immobilized FG, compared to other agonists such as collagen.[279] Although platelet activation is not required for platelets to adhere to FG,[265] GPVI activation by FG is involved in subsequent platelet spreading on immobilized FG.

GPVI-deficient platelets fail to generate extensive lamellipodial sheets on immobilized FG.[279] Platelet activation has long been known to be required for platelet spreading on FG,[195,280] and, interestingly, ligation of activated $\alpha_{IIb}\beta_3$ has been reported to trigger outside-in signalling similar to ligation of GPVI, inducing Ca^{2+} mobilization in platelets.[188,189] FG binding to $\alpha_{IIb}\beta_3$ similarly triggers Ca^{2+} mobilization through outside-in signalling.[281,282] Although fibrin binds to $\alpha_{IIb}\beta_3$, it is not yet known if fibrin similarly induces outside-in signalling via $\alpha_{IIb}\beta_3$.

1.3.1.2 Fibronectin

Fibronectin (human: FN; mouse: Fn) is a homodimeric glycoprotein composed of two 230- to 250-kDa subunits that are connected by a pair of disulfide bonds near their C-termini.[275,283–285] Each subunit is composed of repeating modules: 12 type I, 2 type II, 15 type III modules, two additional type III modules referred to as extra domains (ED)-A and ED-B, and a variable region. The FN monomer is encoded by a single gene and alternative splicing of *FN* pre-mRNA results in up to 20 FN variants in humans, which vary in abundance depending on tissue and cell type. FN isoforms are divided into two categories: 1) the soluble, inactive form in plasma (pFN) that does not contain ED-A or ED-B, and 2) the insoluble cellular form (cFN) that contains at least one ED.[160] cFN is a more heterogeneous group of isoforms with cell type-specific splicing of *FN* pre-mRNA, which are synthesized and deposited locally into extracellular matrices.[286,287] Plasma FN is synthesized exclusively by hepatocytes in humans and mice and constitutively released

into circulation.[288] pFN circulates in a compact, inactive conformation[289] at a concentration of roughly 100 to 300 μg/ml in humans and 580 μg/ml in mice,[290] and also accounts for up to 50% of FN content in some murine tissues.[291] pFN is endocytosed and packaged into platelet α-granules through a mechanism involving $\alpha_{IIb}\beta_3$ and accounts for roughly 80% of Fn content in mouse platelets.[207] The latter portion of platelet Fn is made up of cFn, which is likely of megakaryocyte origin.[207]

The active form of FN is in a fibrillar conformation, in which FN molecules self-assemble into fibers ranging in diameter from 10 nm to micrometers in width, and lengths up to tens of micrometers.[292] The initiation of FN fiber formation by platelets is not well-understood, but has been studied in the context of fibroblasts. After FN binds to integrin $\alpha_5\beta_1$ on the cell surface, $\alpha_5\beta_1$ translocates along the plasma membrane, which mechanically stretches FN dimers to expose cryptic binding sites in type III modules that enable pFN binding, and cells can actively add FN molecules to FN fibers.[291] FN matrix fibers are held together by intermolecular, non-covalent bonds that stabilize these structures in an insoluble state, and FN fibers are believed to act as a provisional scaffold during wound healing.[293]

FN binds to integrins $\alpha_{IIb}\beta_3$,[294,295] $\alpha_V\beta_3$,[296] and $\alpha_5\beta_1$[297] on platelets, fibrillar collagens I and III,[298] FG,[298] fibrin,[298] and TSP-1.[299] Platelet adhesion to FN is mediated by platelet integrins binding to the RGD sequence in the tenth type III module of FN.[300] Immobilized FN supports platelet adhesion under stasis and shear up to 1300 s^{-1}, with

maximal adhesion observed at 300 s^{-1}.[301] Platelet adhesion to FN under stasis or flow involves RGD-dependent binding to $\alpha_5\beta_1$ and $\alpha_{IIb}\beta_3$. Antibody blockade of $\alpha_5\beta_1$ reduces adhesion to FN by roughly 50%, whereas blockade of $\alpha_{IIb}\beta_3$ completely abolishes platelet adhesion to FN.[301] Similarly, Glanzmann thrombasthenia (GT) platelets, which lack $\alpha_{IIb}\beta_3$, do not adhere to immobilized FN under flow.[301] Platelet adhesion to immobilized FN on its own is a relatively weak interaction compared to platelet adhesion onto fibrillar collagen or cultured subendothelial matrix, and platelets are more easily detached from FN under increased shear stress compared to their detachment from VWF or collagen surfaces.[302] Additionally, FN binding to $\alpha_{IIb}\beta_3$ requires platelet activation,[295] which induces a shift to the high-affinity conformation of $\alpha_{IIb}\beta_3$.

A supporting role of FN has been reported in platelet adhesion to fibrillar collagens or to fibrin. Platelet adhesion to type I or III fibrillar collagen is significantly reduced when platelets are re-suspended in FN-free plasma under a range of shear rates (490, 800, and 1300 s^{-1}),[303,304] and pFN enhances adhesion and aggregate size of washed platelets onto collagen under high shear flow (1250 s^{-1}) in a dose-dependent manner.[305] In regard to fibrin, pFN becomes tethered to the α-chain of fibrin in a reaction catalysed by thrombin-activated FXIII,[306] and pFN crosslinked to immobilized fibrin matrices enhances platelet adhesion and aggregate formation compared to fibrin matrices alone. It is postulated that the presence of pFN stabilizes platelet thrombi and enhances platelet cohesion.[206] Activated platelets adherent to immobilized FN, fibrin, or type I collagen are

also able to assemble FN into fibers, whereas platelets adherent to immobilized FG, VN, or VWF suppress pFN assembly.[305,307,308]

Until recently, the ability of FN to modulate platelet aggregation was controversial. pFN binds to thrombin-activated, but not ADP-activated, platelets.[309] Monoclonal anti-FN antibodies inhibit platelet aggregation by thrombin or collagen, but exogenous pFN inhibits thrombin- or collagen-induced platelet aggregation.[310] The adhesive properties of pFN are considered to depend on the conformation of FN, whether it is present as soluble dimers, insoluble fibers, or crosslinked to fibrin. Consequently, a dual role has been proposed for pFN in hemostasis and thrombosis: pFN can switch from an inhibitory role in platelet adhesion and platelet aggregation to a supportive role in the presence of fibrin.[204]

Fibrin is required for pFn dimers to support platelet aggregation, and pFn inhibits platelet aggregation in response to agonists or conditions that do not induce fibrin formation.[160,311] It is postulated that the close proximity of RGD loops on a FN dimer restricts the ability of soluble FN to bind $\alpha_{IIb}\beta_3$ on adjacent platelets simultaneously, and soluble FN binding to $\alpha_{IIb}\beta_3$ reduces the availability of binding sites for FG, which inhibits platelet aggregation.[202] A similar inhibitory mechanism has been proposed for plasma VN monomers,[204] which also inhibit platelet aggregation,[203] presumably through occupancy of $\alpha_{IIb}\beta_3$ on activated platelets. Fibrin acts as a bridging protein that allows soluble FN to bridge platelets by crosslinking to fibrin monomers. This is further

evidenced by studies of triple-deficient $Vwf^{-/-}/Fg^{-/-}/pFn^{-/-}$ (TKO) mice.[204] Coupled with

the absence of Fg and Vwf, TRAP- or collagen-induced platelet aggregation is enhanced

in TKO mice compared to double-deficient $Vwf^{-/-}/Fg^{-/-}$ controls. Similarly, TKO mice

have enhanced platelet adhesion, faster thrombus formation, increased thrombus stability,

and shorter vessel occlusion times in response to $FeCl_3$-induced vessel injury *in vivo*

compared to $Vwf^{-/-}/Fg^{-/-}$ mice. It is postulated that *in vivo*, pFn crosslinked to fibrin in the

core of thrombi supports platelet aggregation and clot stability, while soluble pFN at the

periphery of a growing thrombus inhibits further platelet accumulation to limit thrombus

size and prevents vessel occlusion.[159,160,202,311] The switch between inhibitory and

supporting roles is suggested to require either self-association of pFN into fibers or

crosslinking to fibrin or other matrix proteins.

To date, a single family has been described with partial FN deficiency, but they

have normal hemostasis and platelet function.[312] Complete deficiency of FN has not been

reported, and global *Fn* inactivation is embryonic lethal in mice,[226] which appears to

reflect important roles in embryonic development. Targeted inactivation of *Fn* expression

by hepatocytes has been used to generate $pFn^{-/-}$ mice,[207] which develop normally, but

have almost no detectable plasma Fn and approximately 80% reduction in platelet Fn

following tissue-specific inactivation of *Fn*. $pFn^{-/-}$ mice have no bleeding diathesis, but it

is unclear how much the pool of cFn remaining in platelets contributes to hemostasis.

Notably, cFN, which contains ED-A, is more active in initiating fiber assembly and

incorporating into thrombi.[313,314] In response to $FeCl_3$-induced vessel injury, platelet

adhesion is normal in the mesenteric arterioles of *pFn*^{-/-} mice, but aggregate formation is significantly delayed, and as a result, vessel occlusion times are delayed compared to wild-type mice.[208] However, thrombi in *pFn*^{-/-} mice are stable and stay attached at the site of injury.

1.3.1.3 von Willebrand factor

Von Willebrand factor (VWF) is a large homopolymeric glycoprotein assembled from dimers of two 250 kDa subunits that are encoded by the VWF gene. VWF subunits are synthesized as pre-pro-VWF, which consists of an N-terminal signal peptide, a propeptide, and the mature 2050-aa VWF subunit. The mature VWF subunit consists of four repeating domain structures classified as "A", "B", "C", and "D", which are organized as D'-D3-A1-A2-A3-D4-C1-C2-C3-C4-C5-C6-CK. Mature VWF monomers assemble into dimers, which subsequently form N-terminal-linked multimers through inter-chain disulfide bonds that can range from 0.5 to 20 MDa in size.[315,316] Although VWF shares some similarities with MMRN1 in that it is a large, multi-domain glycoprotein that forms large, disulfide-linked multimers, VWF and MMRN1 are structurally unrelated.[10]

VWF is synthesized by megakaryocytes and endothelial cells,[317–319] and is present in plasma, within platelet and endothelial cell granules, and in the basement membrane of endothelial cells. VWF multimers circulate in plasma at approximately 10 µg/ml in

humans in a compact, globular conformation.[320] The inactive, globular conformation of

plasma VWF conceals the collagen- and GPIbα-binding sites within the A1 and A3

domains.[321–323] As plasma VWF self-associates into larger multimers, shear forces cause

the large multimers to begin tumbling and unfolding, which induces unfolding of the

cryptic VWF A domains. Upon exposure of VWF A domains in flowing blood,

circulating ADAMTS13 cleaves a peptidyl bond in the VWF A2 domain, which reduces

VWF multimer size, subsequently regulating both multimer size and conformation.[324–327]

ADAMTS13 also regulates VWF multimer size at sites of vessel injury by cleaving ultra-

large (UL)VWF multimers released by activated endothelial cells and platelets.

VWF binds to collagens I, III, IV,[328] and VI,[329] GPIbα, $\alpha_{IIb}\beta_3$, fibrin,[330] TSP-1,

MMRN1, and FVIII. Through its numerous interactions, VWF contributes to hemostasis

and thrombosis in multiple ways. VWF acts as a carrier protein for FVIII in plasma that

protects it from proteolytic degradation and can deliver FVIII to sites of vessel injury.

VWF also participates in platelet-endothelial cell and leukocyte-endothelial cell

interactions, which contribute to inflammatory and angiogenic processes. VWF can be

crosslinked to fibrin by FXIIIa, which is suggested to enhance clot stability.[271] Lastly,

VWF contributes to platelet adhesion and thrombus formation through its ability to bind

platelet GPIbα to localize circulating platelets at sites of injury or onto developing

thrombi under high shear flow.

VWF multimers bound to the vessel wall, either to activated endothelial cells or the exposed subendothelium, are considered the critical ligand for initial platelet adhesion. VWF binding to GPIbα has a fast on-rate that enables transient binding of platelets under conditions of high shear flow,[331] whereas interactions between platelet integrins (e.g. $\alpha_{IIb}\beta_3$, $\alpha_2\beta_1$, and $\alpha_5\beta_1$) and other surface-bound extracellular matrix proteins (e.g. FG, fibrin, laminins, collagens, and MMRN1) are not sufficient to support platelet tethering under high shear flow. Platelet adhesion to VWF occurs under a range of shear rates, and the morphology of platelet aggregates on VWF surfaces is typically strings of adherent platelets. Additionally, VWF is able to mediate platelet adhesion to fibrillar collagens, TSP-1, fibrin, and MMRN1 under shear rates that trigger unfolding of VWF multimers (approximately $\geq$500 s^{-1}). Platelet adhesion to collagen under high shear flow requires VWF for platelets to significantly adhere and aggregate onto collagen surfaces.[302,303] Adhesion of human platelets lacking VWF or Vwf-deficient mouse platelets to collagen is abolished under high shear flow.[332] VWF also enhances platelet adhesion and aggregate formation onto MMRN1-coated surfaces under high shear flow,[21] and VWF is required for MMRN1 to enhance platelet adhesion to fibrillar collagen.[2] Platelet adhesion to TSP-1 surfaces involves VWF and GPIbα under high shear flow, but TSP-1 is considered a counter receptor for GPIbα in the absence of VWF, and is capable of supporting platelet adhesion under high shear flow through a mechanism involving GPIbα but not VWF.

Following initial platelet adhesion, large VWF multimers are released from adherent activated platelets, which stably bind through RGD-dependent interactions to activated $\alpha_{IIb}\beta_3$.[333] Platelet-bound VWF multimers continue tethering additional circulating platelets to contribute to platelet aggregate growth.[200] VWF multimers also crosslink $\alpha_{IIb}\beta_3$ on adjacent activated platelets, which enhances the stability of platelet aggregates.[333] As the diameter of the vessel lumen decreases following thrombus formation, the shear rate at the apex of the thrombus significantly increases. VWF is capable of supporting platelet aggregation under extremely high shear ($\geq 15,000$ s^{-1}) independent of FG and $\alpha_{IIb}\beta_3$,[177,247] which may play a role in vessel occlusion or thrombus formation in stenotic vessels. Vwf-deficient ($Vwf^{-/-}$) mice fail to form occlusive thrombi due to a channel that forms at the apex of the thrombus that fails to fully occlude.

Quantitative or qualitative defects in VWF cause a variety of bleeding symptoms based on the type of defect, which are collectively referred to as von Willebrand disease (VWD).[334] VWD is divided into types based on the type and severity of the defect. Type 1 VWD is defined as a partial quantitative defect in structurally and functionally normal VWF. Type 2 VWD refers to disorders in which VWF is functionally abnormal, and is further subdivided based on the type of functional defect. Type 3 VWD is the most severe, in which there is near or complete loss of VWF in plasma and cells and, consequently, a reduction of plasma FVIII. Type 3 VWD patients suffer from severe mucocutaneous bleeding and prolonged challenge-related bleeding.[335] $Vwf^{-/-}$ mice, which are essentially a model of type 3 VWD, develop normally, but have reduced survival due

to spontaneous bleeding.[211,222] In response to injury, $Vwf^{-/-}$ mice have severely prolonged bleeding compared to wild-type mice.[222,223,332] In response to $FeCl_3$-induced injury of mesenteric arterioles, $Vwf^{-/-}$ mice have impaired platelet adhesion, thrombus formation, and vessel occlusion compared to the wild-type.[211,222]

1.3.1.4 Thrombospondin-1

Thrombospondin-1 (TSP-1) is a large homotrimeric glycoprotein of approximately 450 kDa. Its domain structure contains an oligomerization domain that is responsible for trimerization, a VWF C domain that shares high homology with FN type 1 modules, numerous thrombospondin type repeats (TSRs), and two EGF-like domains.[336] TSP-1 is synthesized by endothelial cells, smooth muscle cells, and fibroblasts.[337] Consequently, TSP-1 is present in the vessel wall matrix and constitutively secreted into plasma by endothelial cells. Platelets do not synthesize TSP-1, but uptake TSP-1 from plasma, which accounts for roughly 20 to 30% of protein content in α-granules.[338] Normally, TSP-1 concentrations in plasma range from 0.1 to 0.3 µg/ml. Following platelet activation and α-granule secretion, localized concentrations of TSP-1 increase roughly 100-fold.[339]

Given its complex multi-domain structure, TSP-1 is known to bind to numerous plasma and/or ECM proteins and platelet receptors: integrins $\alpha_V\beta_3$ and $\alpha_2\beta_1$,[340–342] GPIV (CD36), integrin-associated protein (IAP; CD47), GPIbα, FG,[242,343] fibrin,[343] VWF,[315]

FN,[344] laminins,[345] and collagens.[346] TSP-1 may directly interact with $\alpha_{IIb}\beta_3$, but many studies report conflicting results.[340,347–350] In an isolated system, TSP-1 fails to trigger aggregation of $\alpha_{IIb}\beta_3$-coated beads,[205] suggesting other proteins are required to mediate this interaction.

TSP-1 is shown to have a non-essential, but supporting role in platelet adhesion and platelet aggregation through its interactions with FG. Pre-treatment with anti-TSP-1 Fab fragments inhibit platelet aggregation in response to collagen and thrombin.[241,351] Anti-TSP-1 Fab fragments decrease the size and number of large aggregates, resulting in more small aggregates and non-aggregated single platelets. TSP-1 binds and crosslinks $\alpha_{IIb}\beta_3$-bound FG, which decreases the distance required for cell-cell interactions, increases collision frequency, and localizes additional adhesive proteins that support platelet adhesion.[205] TSP-1 can also switch to an inhibitory role in platelet aggregation by binding to soluble FG, which blocks FG binding to $\alpha_{IIb}\beta_3$ on platelets.[205] Additionally, TSP-1-coated beads co-aggregate, suggesting that TSP-1 bound to the cell surface is also capable of crosslinking with TSP-1 on adjacent cells. Further, TSP-1 enhances platelet aggregation by blocking the inhibitory effects of NO through its interactions with CD36 and IAP.[352]

TSP-1 supports platelet adhesion under stasis and shear rates up to 4000 s^{-1} through multiple mechanisms. Platelet adhesion to immobilized TSP-1 increases with shear up to rates of approximately 1800 s^{-1}, after which platelet adhesion to TSP-1

gradually declines with increasing shear.[299,353] Under high shear flow, monoclonal anti-$\alpha_{IIb}\beta_3$, -$\alpha_v\beta_3$, and -IAP antibodies do not inhibit platelet adhesion to TSP-1. Antibodies that block CD36 binding or VWF binding to GPIbα partially inhibit platelet adhesion to TSP-1, whereas monoclonal antibodies that block GPIbα binding abolish platelet adhesion to TSP-1. Additionally, type 3 VWD platelets adhere normally to TSP-1 under high shear, which shows that GPIbα supports platelet adhesion to TSP-1 under high shear flow independent of VWF. These data are consistent with *in vivo* observations that *GpIb$\alpha^{-/-}$* mice exhibit a more severe impairment in thrombus formation compared to *Vwf$^{-/-}$* mice. Additionally, TSP-1 binds collagens I-VI and contributes to platelet adhesion to immobilized fibrillar collagen surfaces under flow through its interactions with CD36, which enhance platelet activation, PE expression, and thrombus stability on collagen.[346,354,355] TSP-1 binding to IAP is also shown to enhance platelet activation and aggregation by collagen.[356]

TSP-1 contributes to hemostatic plug formation and thrombosis through multiple mechanisms. TSP-1 binds to thrombin,[357,358] plasminogen,[359] and TFPI,[360] which affect fibrin formation and thrombin-induced platelet activation. TSP-1 interactions with fibrin accelerate the rate of fibrin formation, decrease fibrin fiber diameter and increases the number of fibrin fibers in fibrin networks, and may regulate branching during network formation.[361] TSP-1 also contributes to platelet recruitment to sites of injury or growing thrombi through its interactions with VWF. TSP-1 binds to the A3 domain of VWF and blocks ADAMTS-13 mediated cleavage of the adjacent VWF A2 domain.[225,362] In this

regard, TSP-1 facilitates the maintenance of large VWF multimers at the site of injury, which are more effective at tethering circulating platelets. TSP-1 binding to VWF is also postulated to stabilize platelet adhesion to VWF. *In vivo*, large Vwf multimers released by endothelial cells are more rapidly degraded by Adamts13 in *Tsp1*^{-/-} mice compared to the wild-type.[363]

Tsp-1^{-/-} mice are viable and fertile, but exhibit abnormalities in the lungs and thoracic spine,[209] consistent with the high expression levels of TSP-1 in pulmonary tissues. Consequently, *Tsp-1*^{-/-} mice experience diffuse alveolar hemorrhage, but bleeding in response to injury is similar between *Tsp-1*^{-/-} and wild-type mice. *Tsp-1*^{-/-} platelets aggregate normally in response to thrombin, ADP, TxA_2 analogue, or collagen.[209,225] In response to $FeCl_3$-induced vessel injury, *Tsp-1*^{-/-} have a slower rate of thrombus growth and delayed vessel occlusion due to impaired thrombus stability and frequent embolization. Impaired thrombus formation in *Tsp-1*^{-/-} mice is attributed to loss of the TSP-1/CD36 signalling axis, given that *Tsp-1*^{-/-} and *CD36*^{-/-} mice have a similar phenotype.[354] Although TSP-1 is argued to be a counter-receptor for GPIbα in the absence of Vwf, double-deficient *Tsp-1*^{-/-}/*Vwf*^{-/-} mice have a similar phenotype to *Vwf*^{-/-} mice in response to $FeCl_3$-induced vessel injury,[212,364] suggesting that Tsp-1 is not the only counter receptor for GPIbα.

1.3.1.5 Vitronectin

Vitronectin (VN) is a 75 kDa glycoprotein synthesized by hepatocytes that is present in plasma, platelet α-granules,[365] and the extracellular matrix of blood vessels.[366–368] VN circulates in plasma at a concentration ranging from 200 to 500 µg/ml in humans.[369,370] Plasma VN circulates as soluble monomers that are constitutively uptaken and stored by MKs and platelets in α-granules.[371] VN exists in two conformations, which regulate its ability to bind ligands and support platelet adhesion and aggregation. Plasma VN monomers have a compact conformation in which their integrin-binding RGD sites are not accessible.[372] VN in platelet α-granules and the ECM self-associate into multimers that are able to bind integrins.[372,373] Additionally, ligand binding can induce conformational change of plasma VN monomers that enables VN to multimerizes and exposes binding sites for platelet integrins,[374] and a small portion (~2%) of the plasma VN pool is present in the active, integrin-binding conformation.

VN binds to $\alpha_V\beta_3$ and $\alpha_{IIb}\beta_3$ on platelets,[375] fibrin,[376] fibrillar collagens types I and III,[377] PAI-1,[378] plasminogen, and the thrombin-antithrombin complex. Platelet adhesion to VN requires platelet activation and involves $\alpha_V\beta_3$ and $\alpha_{IIb}\beta_3$. Resting platelets do not adhere to VN under stasis or flow.[265,379] The physiologic role of platelet adhesion to VN on its own is unclear, but VN incorporated into fibrin surfaces enhances platelet adhesion and aggregation onto fibrin through homotypic interactions between platelet-bound VN and fibrin-bound VN.[380] Following platelet activation and α-granule secretion, the local

VN concentration increases approximately 200-fold, with about 50% of VN remaining bound to the platelet surface.[381] Further, VN is co-localized with fibrin *in vivo* and stabilizes thrombi through its interactions with PAI-1.[382–384] Binding to PAI-1 or thrombin-antithrombin induces conformational change of plasma VN monomers to their active, integrin-binding form.[374,385–387]

Given the conformation-dependent functions of VN, studies have reported conflicting results for the role of VN in platelet aggregation. Monoclonal anti-VN antibodies inhibit platelet aggregation *in vitro*,[380,388] whereas VN is also shown to inhibit platelet aggregation through competition with FG and VWF for binding to $\alpha_{IIb}\beta_3$.[229,389] Conflicting results have also been reported for aggregation of Vn-deficient (*Vn$^{-/-}$*) mouse platelets. *Vn$^{-/-}$* mice develop normally and do not experience spontaneous bleeding or prolonged bleeding in response to injury.[210,229] Initially, *Vn$^{-/-}$* platelets were reported to have normal ADP-induced aggregation, but accelerated thrombin-induced aggregation compared to the wild-type.[229] Later analysis showed that thrombin-induced platelet aggregation is abolished in *Vn$^{-/-}$* platelets, whereas ADP-induced of *Vn$^{-/-}$* platelets is enhanced compared to the wild-type.[203] This discrepancy is reported to result from differences in experimental design and measurement type.

Vn$^{-/-}$ mice were first reported to show significantly shorter vessel occlusion times *in vivo* in response to FeCl$_3$-induced vessel injury compared to wild-type mice,[229] which suggests that Vn normally inhibits thrombus formation *in vivo*. Subsequent analyses of

thrombus formation *in vivo* following FeCl$_3$-induced vessel injury have demonstrated that thrombus growth and vessel occlusion are delayed in *Vn$^{-/-}$* mice due to frequent dissolution of thrombi, embolization, and vessel re-opening.[203] Similarly, PAI-1-deficient or double-deficient *PAI-1$^{-/-}$/Vn$^{-/-}$* mice develop unstable thrombi in response to FeCl$_3$-induced vessel injury.[390,391] Following laser-induced vessel injury, fibrin content is lower and unstable in thrombi formed by *Vn$^{-/-}$* mice compared to the wild-type.[203] These studies demonstrate a crucial role of Vn and PAI-1 in supporting thrombus stability. Additionally, it is hypothesized that Vn multimers form macromolecular complexes with other adhesive proteins to increase the avidity of platelet-platelet interactions during thrombus formation to further stabilize thrombi.[203]

1.3.2 Platelet adhesive receptors

Transmembrane receptor complexes on the platelet surface are the means through which platelets interact with other cells and the extracellular environment via inside-out signalling, outside-in signalling, or both. In particular, some platelet receptors have been demonstrated to play crucial roles in platelet adhesion and aggregation, such as integrins $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$, the GPVI-FcRγ complex, and the GPIb/IX/V complex. A supporting, but non-essential role has also been described for $\alpha_V\beta_3$, which is expressed on the platelet surface in very low numbers and can bind FG,[244] VN,[392] FN,[296] and MMRN1.[1,2] The relative abundance of these receptors on resting human and mouse platelets is presented in *Figure 4*. Although the functions of various receptors discussed below are not limited

to platelet adhesion and aggregation, the following sections will focus on the mechanisms through which these receptors contribute to these processes.
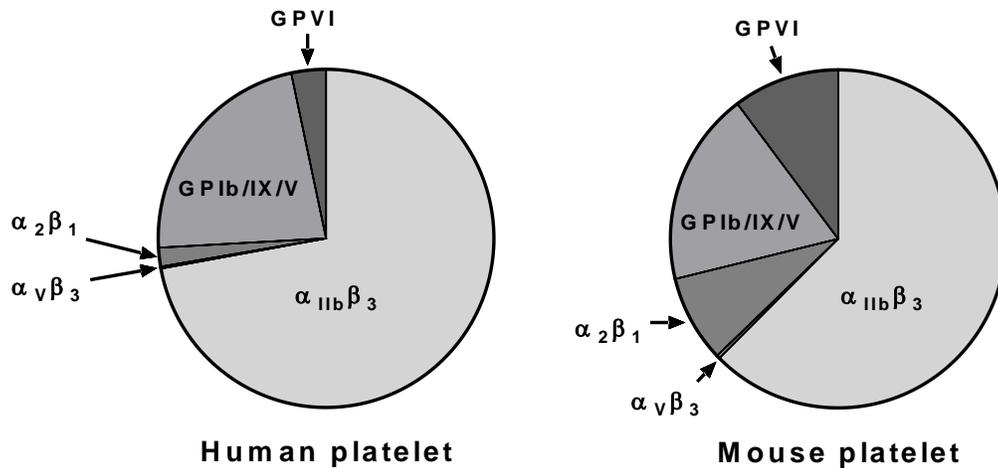
Figure 4: Estimated relative abundance of major receptors on resting human and mouse platelets.

1.3.2.1 Alpha-IIb Beta-3

Integrin $\alpha_{IIb}\beta_3$ (sometimes called GPIIb/IIIa) is a heterodimeric transmembrane receptor complex expressed primarily by MKs and platelets,[393,394] but it is also present on mast cells[395,396] and some tumour cells.[397] In MKs and platelets, the α_{IIb} and β_3 subunits are constitutively synthesized by MKs and are present as a heterodimer on the cell membrane and within α-granules.[398] On the cell surface, the $\alpha_{IIb}\beta_3$ complex is capable of outside-in signalling and supporting cell adhesion to extracellular matrix proteins.[399] There are approximately 80,000 copies of $\alpha_{IIb}\beta_3$ present on the membrane of resting

human platelets and approximately 120,000 copies on a single mouse platelet.[400–403]

These numbers increase by approximately 30 to 50% following platelet activation,[404,405]

wherein $\alpha_{IIb}\beta_3$ stored within α-granules is incorporated into the cell membrane during

granule secretion.

Integrin $\alpha_{IIb}\beta_3$ binds to FG,[406–408] FN,[309,409] VN,[375] VWF,[410] and MMRN1,[1,2] but

platelet activation affects the affinity and avidity of these interactions. On resting

platelets, $\alpha_{IIb}\beta_3$ is present in an inactive conformation, in which a genu in each

extracellular portion of the α_{IIb} and β_3 subunits allows $\alpha_{IIb}\beta_3$ to fold into a bent

conformation.[411] Platelet activation by agonists such as thrombin, collagen, ADP, or

VWF triggers $\alpha_{IIb}\beta_3$ to shift to an extended, high-affinity conformation through inside-out

signalling. Platelet activation also induces clustering of $\alpha_{IIb}\beta_3$ on the platelet surface,

which increases the avidity of $\alpha_{IIb}\beta_3$-mediated ligand binding.[412–414]

Integrin $\alpha_{IIb}\beta_3$ is essential for stable platelet adhesion and aggregate formation.

Platelets irreversibly adhere to FG or VWF via activated $\alpha_{IIb}\beta_3$,[174] which stabilizes

primary adhesion and subsequent platelet aggregation. The only known exceptions are: 1)

conditions of elevated shear ($>15,000$ s^{-1}) in which reversible platelet aggregation can

occur independent of $\alpha_{IIb}\beta_3$ and platelet activation or 2) mechanical trapping of platelets

within a fibrin meshwork that can occur independent of $\alpha_{IIb}\beta_3$.[177,247,276] Resting and

activated platelets adhere to FG or fibrin via $\alpha_{IIb}\beta_3$ under conditions of stasis and flow,

and platelet activation significantly enhances platelet adhesion and aggregate formation

onto immobilized FG or fibrin surfaces.[174] Resting platelets do not significantly adhere to FN- or VN-coated surfaces under flow, but platelet activation allows platelets to stably adhere to FN or VN via $\alpha_{IIb}\beta_3$ and other minor receptors ($\alpha_5\beta_1$ and $\alpha_v\beta_3$, respectively) to form aggregates.[265] Similarly, platelet adhesion to MMRN1 under stasis and low shear flow is mediated by $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ and requires platelet activation.[2]

A critical role of $\alpha_{IIb}\beta_3$ in platelet aggregation onto fibrillar collagen under high shear flow has been demonstrated for both human and mouse platelets. Monoclonal anti-$\alpha_{IIb}\beta_3$ antibodies eliminates mouse platelet aggregate formation on fibrillar collagen under high shear flow (1300 s^{-1}),[183] and treatment with peptidomimetic lotrafiban, which blocks the FG/Fg and VWF/Vwf binding site on $\alpha_{IIb}\beta_3$, abolishes human or mouse platelet aggregate formation on fibrillar collagen without affecting primary adhesion onto collagen or activation by collagen.[415] Additionally, RGDS peptides inhibit platelet aggregate formation onto collagen under high shear flow (1500 s^{-1}), which suggests the involvement of integrin-binding RGD motifs in platelet aggregate formation on collagen surfaces.

Hereditary or acquired qualitative or quantitative defects in $\alpha_{IIb}\beta_3$ are referred to as Glanzmann thrombasthenia (GT), which are the cause of severe bleeding symptoms in human patients.[416] Notably, patients with defects in β_3 affect both $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$, whereas defects in the α_{IIb} subunit only affect $\alpha_{IIb}\beta_3$.[417] Aggregation of GT platelets is severely impaired (typically absent) in response to ADP, thrombin, collagen, and

epinephrine, which further demonstrates the critical role of $\alpha_{IIb}\beta_3$ in platelet aggregation at low shear. GT platelets agglutinate but do not aggregate in response to ristocetin, which also highlights the ability of VWF to mediate platelet agglutination under conditions sufficient to expose the GPIbα-binding VWF A1 domain. Beta-3-deficient mice develop normally, but have a severe bleeding phenotype that is similar to GT in human patients.[221] Beta-3-deficient mice exhibit severe bleeding in response to injury and reduced survival due to spontaneous hemorrhage. Additionally, β_3-deficient mice are protected from thrombosis in a large arteries following treatment with $FeCl_3$ and systemic intravascular thrombosis induced by a variety of agonists.[418]

1.3.2.2 Alpha-2 beta-1

Integrin $\alpha_2\beta_1$ is a transmembrane heterodimeric receptor complex composed of the α_2 and β_1 subunits, which associate via disulfide linkage on the surface of cells. Alpha-2 beta-1 is one of four known integrins capable of direct binding to collagen.[419,420] The α_2 subunit contains a highly conserved inserted (I)-domain that shares homology with the VWF A domain and is responsible for collagen binding.[420–422] The I-domain contains a metal ion-dependent adhesion site (MIDAS) that requires either Mg^{2+} or Mn^{2+} to form a complex with collagen.[422,423]

Integrin $\alpha_2\beta_1$ is expressed on the surface of MKs, platelets, endothelial cells, fibroblasts, and lymphocytes. In contexts other than hemostasis, $\alpha_2\beta_1$ is referred to as

either very late antigen (VLA)-1 or extracellular matrix receptor (ECMR)-II.[424–426] Endothelial $\alpha_2\beta_1$ anchors endothelial cells to collagens and laminins in the subendothelial basement membrane, and may contribute to sprouting angiogenesis and wound healing.[427–429] On platelets, $\alpha_2\beta_1$ is present at approximately 1000 to 3000 copies per platelet and functions primarily as a receptor for various collagens and laminins.[430] Mouse platelets express substantially greater numbers of $\alpha_2\beta_1$, with approximately 16,000 copies per platelet.[403] On resting platelets, $\alpha_2\beta_1$ is present in a low affinity conformation in which the I-domain is inaccessible, but is still capable of binding some high-affinity ligands.[431] Following platelet activation by GPVI or protease-activated receptors (PARs)-1 and -4, inside-out signalling triggers $\alpha_2\beta_1$ to shift to a high-affinity conformation in which the I-domain is exposed.[432]

Integrin $\alpha_2\beta_1$ binds to types I-VI and XI collagen and laminins in the extracellular matrix.[433–435] More specifically, $\alpha_2\beta_1$ preferentially binds types I, II, III, and V collagen, and integrin activation is not required for $\alpha_2\beta_1$ to bind types I, II, and III collagen. Binding to types IV and VI collagen and laminins requires platelet activation to trigger the high-affinity conformation of $\alpha_2\beta_1$. Under stasis and low shear flow, $\alpha_2\beta_1$ is sufficient to support significant and stable platelet adhesion to fibrillar collagen.[183] Platelet activation enhances $\alpha_2\beta_1$-mediated platelet adhesion to fibrillar collagen, but is not required.[436] Under conditions of elevated shear stress, VWF binding to collagen to tether circulating platelets is required for significant platelet adhesion to fibrillar collagen.[332] In the absence

of collagen-bound VWF multimers, platelets adhere individually or in much smaller, nodular aggregates under high shear flow.[175]

Defects in $\alpha_2\beta_1$ have been reported in human patients that are associated with prolonged bleeding, failure of platelets to aggregate in response to collagen, and failure of platelets to adhere to collagen.[437,438] Alpha-2-deficient mice develop normally without hemostatic complications,[214,439] despite the ubiquitous expression of $\alpha_2\beta_1$ on fibroblasts and various epithelial cell types. Alpha-2-deficient mice bred on a C57BL/6J background do not experience bleeding,[214] but α_2-deficient mice of a mixed C57BL/6J/BalbC background show severe bleeding in response to injury.[183] Adhesion of α_2-deficient platelets to collagen is abolished under low shear, where VWF is not involved, and severely impaired under high shear flow.[183,214,215] Alpha-2-deficient mice also show a delayed aggregation response to collagen,[214,439] which is postulated to arise from the loss of stable adhesion to collagen that facilitates collagen-induced platelet activation by GPVI. In response to $FeCl_3$-induced vessel injury, thrombus growth and vessel occlusion are impaired in α_2-deficient compared to wild-type mice.[215] This has been attributed to a role of $\alpha_2\beta_1$ in enhancing TxA_2 release within growing thrombi, rather than defects in platelet adhesion to collagen. Similarly, in response to photochemical injury, α_2-deficient mice have delayed thrombus growth and vessel occlusion compared to the wild-type.[440]

1.3.2.3 Glycoprotein VI

Glycoprotein VI (GPVI) is a transmembrane immunoreceptor that is exclusively expressed by MKs and platelets.[441] It is present on the surface of human platelets in similar numbers as $\alpha_2\beta_1$ (~1000 to 4000 copies per platelet),[430] whereas mouse platelets express significantly greater numbers of GPVI.[403] GPVI is part of the immunoglobulin (Ig) superfamily and contains two Ig-like domains that are responsible for ligand binding via an immunoreceptor tyrosine-based activation motif (ITAM). GPVI is present in disulfide-linkage with FcRγ on the platelet surface. GPVI is relatively small (62 kDa) and lacks a significant intracellular domain. GPVI linkage to FcRγ is required for GPVI expression on the platelet surface and the extensive intracellular domain of FcRγ is critical for signal transduction of the GPVI/FcRγ complex.[442–444] FcRγ is a homodimer and a copy of GPVI is linked to each FcRγ monomer so that GPVI is present as a dimer on the platelet surface.[445,446] Clustering of GPVI/FcRγ complexes also appears to be necessary for collagen binding.[447]

GPVI binds to collagens I-VI,[441,448] laminins,[449,450] FG,[279] and fibrin.[451] GPVI binding to fibrillar collagens I and III induces full activation of platelets, including PS exposure, blebbing, and granule secretion.[452] GPVI binding to laminin, FG, or fibrin does not induce full platelet activation. GPVI binding to laminin may be involved in platelet spreading,[450] whereas a role of GPVI-fibrin interactions in promoting thrombin generation and thrombus stability has been reported.[451] Ligation of the dimeric

GPVI/FcRγ complex that crosslinks the Ig-like domains of GPVI results in receptor triggering.[453,454] Ligands capable of binding GPVI but not crosslinking GPVI Ig-like domains do not trigger full platelet activation.[441] Collagen fibers are able to crosslink GPVI Ig-like domains, whereas ligands such as laminins and fibrin do not.

GPVI deficiency has been reported in several human patients with mild bleeding symptoms.[455–460] Platelets from GPVI-deficient patients fail to aggregate in response to collagen, but have normal aggregation responses to thrombin, ADP, and ristocetin.[455,456,459,460] Additionally, platelets from some GPVI-deficient patients fail to adhere to collagen,[455] while platelets from other patients show impaired adhesion to collagen.[460,461] GpVI-deficient ($GpVI^{-/-}$) mice bred on a C57BL/6J background have a mild prolongation of bleeding in response to injury,[216,218] and $GpVI^{-/-}$ BalbC mice exhibit a moderate bleeding phenotype.[183] $GpVI^{-/-}$ platelets do not aggregate in response to collagen and adhesion of $GpVI^{-/-}$ platelets to collagen is significantly impaired under a wide range of shear rates (400 to 2600 s^{-1}).[183,216,462] Similarly, GpVI-deficiency significantly impairs thrombus formation in response to FeCl$_3$-induced injury of small and large arteries,[217,463,464] ligation injury,[465] or intravenous collagen injection.[440] Thrombus growth and vessel occlusion are delayed in $GpVI^{-/-}$ mice due to thrombus instability and frequent embolization. The role of GPVI in FeCl$_3$-induced vessel injury is posited to involve GPVI binding to FG and fibrin to promote thrombin generation and platelet recruitment in addition to its collagen binding functions.[451]

1.3.2.4 Glycoprotein Ib/IX/V complex

The glycoprotein Ib/IX/V complex (GPIb/IX/V) consists of four subunits encoded by four separate genes: GPIbα, GPIbβ, GPIX, and GPV, which is expressed on the surface of endothelial cells,[466] MKs, and platelets.[467] Approximately 25,000 copies of the GPIb/IX/V complex are present on a single resting human platelets and approximately 36,000 copies on a single resting mouse platelet.[220,403] In addition to its role as an adhesive receptor complex, GPIb-IX-V is a structural component of the platelet cytoskeleton that links the platelet membrane to actin filaments.[468] GPIbα is the most-studied subunit, which contains the binding sites for VWF, TSP-1,[469] P-selectin,[470] thrombin,[471,472] and $\alpha_M\beta_2$.[473,474] GPIbα mediates platelet adhesion to activated endothelial cells and activated leukocytes by binding P-selectin and Mac-1, respectively. GPIbα also supports platelet adhesion to endothelial cells and platelet-platelet interactions by binding to the A1 domain of shear-stretched VWF multimers.[475] In the absence of VWF, GPIbα is able to support platelet adhesion and thrombus formation *in vivo*,[220] suggesting that other ligands in the vessel wall or other receptors may contribute to GPIbα-mediated thrombus formation. Additionally, a role of GPV as a collagen receptor has also been reported, but its biological significance has not been explored.[476] Ligation of the GPIb/IX/V complex is able to induce intracellular signals leading to Ca^{2+} mobilization, cytoskeletal rearrangement, and activation of $\alpha_{IIb}\beta_3$.[477,478]

Mutations in the GPIbα, GPIbβ, or GPIX subunits of GPIb/IX/V that result in functional loss of VWF binding to GPIb/IX/V give rise to Bernard-Soulier syndrome (BSS):[479] a heterogeneous group of diseases characterized by giant, spherical platelets, mild thrombocytopenia, inability for platelets to aggregate in response to ristocetin, and pronounced bleeding symptoms in human patients.[480] *GpIbα$^{-/-}$* mice similarly exhibit giant platelets, thrombocytopenia, and pronounced bleeding symptoms.[219] Giant platelets characteristic of BSS are postulated to result from the loss GPIb/IX/V-mediated linkage of the cell membrane and actin cytoskeleton of MKs and platelets. Mice expressing a chimeric form of GPIbα that lacks the extracellular ligand-binding domain have structurally normal platelets without thrombocytopenia, but exhibit severe bleeding and severely impaired thrombus formation *in vivo*.[220]

1.4 THE COLLAGEN FAMILY

The collagen family consists of 28 known glycoproteins encoded by at least 45 distinct genes that are grouped together based on domain structure homology.[481–485] Collagens are characterized by the presence of a collagenous (COL) domain, which is a rod-like triple-helical motif composed of GXX' repeats (where X is frequently occupied by proline and X' is frequently occupied by hydroxyproline) of variable length.[483,485] All collagens are trimers composed of three α-chains.[481–484] The α-chains of each collagen type are encoded by separate genes, and only α-chains from the same collagen type associate into trimers.[484,486] The collagen superfamily is subdivided into two broad types

based on the relative length of their COL domain and their ability to self-assemble into fibers: fibrillar and non-fibrillar collagens.[484,486,487] Each collagen is assigned a Roman numeral and each α-chain is assigned an Arabic numeral based on their order of discovery.[484]

The distribution and amount of collagen types in blood vessels varies between the layers of the vessel wall as well as between veins and arteries.[488–491] Within blood vessels, fibrillar collagens I, III, V, and VIII are mostly present within the tunica media and the adventitia,[488–491] but thin, sparse type I and III collagen fibrils have been detected within the tunica intima (*Table 2*).[489,490] The thickness of the outer layers of the vessel wall, and consequently the amount of collagen, varies depending on the size of the blood vessel and due to the requirement of a thick muscular medial layer in arteries, but not in veins, to modulate vascular tone.[167,491] Smooth muscle cells synthesize the majority of collagens in the tunica media, and fibroblasts and macrophages synthesize collagens in the adventitia.[484] The tunica intima of veins, arteries, and capillaries contains non-fibrillar collagens IV, VI, and VIII in the endothelial cell basement membrane,[481,491,492] which provides a surface for attachment of endothelial cells.[488] Type VI collagen is also synthesized by smooth muscle cells in the elastic laminae of the tunica media,[491] which assists in the integration of matrix components with cells and one another.[481,488] The following sections will discuss the major fibrillar and non-fibrillar collagens in the vessel wall and their contributions to platelet adhesion, hemostasis, and thrombosis.

	Collagen type	Location in the vessel wall	Estimated abundance	Known functions
Fibrillar	I	Media Adventitia	Very high	Tensile strength;[493,494] load resistance;[493,494] platelet adhesion and activation[190,419,430]
	III	Media Adventitia	Low	Elasticity;[493] regulation of fiber size;[495,496] platelet adhesion and activation[190,419,430]
	V	Intima Media Adventitia	Very low	Regulation of collagen assembly[484,497,498]
Non-fibrillar	IV	Intima Media	Moderate	Cell attachment;[499] membrane flexibility[488]
	VI	Intima Media	Moderate	Integration of ECM components and cells[500]
	VIII	Intima Media	Moderate	Compressive strength;[488] angiogenesis[501]

Table 2: Tissue distribution, estimated abundance, and functions of major fibrillar and non-fibrillar collagens present in the vessel wall. This table provides estimates on the tissue distribution of collagens at the level of different layers of the vessel wall, which does not account for whether or not these collagens are cell-associated or fibril-associated, and how their distributions vary within each layer itself.[488,492]

1.4.1 Fibrillar collagen structure, synthesis, and assembly

The common feature of fibrillar collagens is that they contain a long, uninterrupted COL domain, which imparts rigidity and high tensile strength to collagen molecules.[484,486] Approximately 30% of proteins in the human body are collagens,[482] and of that, over 90% of the total collagen content in the human body is estimated to be fibrillar type I collagen.[502] Type I collagen is a heterotrimer composed of two α_1(I) and one α_2(I) chain, and has a ubiquitous structural role in a variety of tissues, including: skin,

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bone, dentin, tendon, blood vessels, cornea, and lungs.[493,494] It is synthesized by a wide range of cell types as procollagen,[485] which includes N- and C-terminal telopeptides that are important for triple helix assembly and regulation of collagen fibril formation.[487,503]

Types I, II, and III fibrillar collagens are highly conserved, but their relative abundance and tissue distributions vary. While type I collagen is the most abundant and most ubiquitous,[485] the distribution of type II collagen is limited to cartilage.[504] Type III collagen is often present in small quantities in tissues alongside type I or II collagen, and is thought to play a role in the regulation of collagen fiber diameter.[495,496] In particular, type III collagen is present in large quantities within the blood vessel wall,[489–491] which is postulated to be because type III collagen is more elastic than type I collagen and its presence would allow for greater extensibility of blood vessels.[488,505] Types V and XI fibrillar collagens are also present in small quantities within collagen fiber networks, and are presumed to function primarily in the regulation of fibril assembly.[495,497,498,506,507]

The synthesis, structure, and assembly of fibrillar collagens have been most studied in regard to type I collagen, but it is postulated that fibrillar collagens II and III assemble into triple helices through similar processes.[487,508] Following translation, each procollagen α-chain spontaneously assembles into a left-handed helix that lacks intra-chain hydrogen bonds.[487,503,508] The structure is most similar to a polyproline-II helix, or sometimes called a polyglycine II helix,[509–512] in which the pitch of the α-chain helix roughly corresponds to a single GXX' triplet (three residues).[512,513] Interactions between

the non-collagenous C-terminal globular domains of each α-chain potentiate their assembly into a right-handed triple superhelix that trimerizes from the C- to the N-terminus.[487,503,508] The tight packaging of α-chains into the triple helix requires the α-chains to stagger by a single residue, and every third residue is placed near the common helical axis.[513] Steric constraints require that glycine sits at the centre position within the triple helix because it is the smallest amino acid and almost entirely lacks a side chain.[513] Substitution of glycine residues at any position in the α-chain causes kinks in the triple helix that have downstream effects on triple helix assembly,[514] glycosylation of procollagen molecules, and triple helix stability.[503,515] Missense mutations of glycine residues in types I or III collagen are the cause of systemic connective tissue disorders that range in severity from mild to embryonic lethal,[516,517] and are discussed in more detail in *section 1.3.5*. Glycosylation and hydroxylation of the procollagen molecule occurs following triple helix assembly,[503,508] and the formation of inter-chain hydrogen bonds by hydroxylated proline residues stabilizes the triple helix.[487,503]

Triple-helical type I procollagen molecules are secreted into extracellular space,[487,503] wherein their N- and C-terminal propeptides prevent their assembly into fibrils. Cleavage of the N- and C-terminal propeptides by ADAMTS2 and bone morphogenetic protein (BMP)-1, respectively,[518,519] in the extracellular environment results in mature collagen molecules of approximately 1000-aa and 300 nm in length, which are able to spontaneously assemble into larger ultrastructures.[481,487] Collagen monomers associate end-to-end to form long strands, which subsequently aggregate

laterally into 5-mer bundles of monomer strands called fibrils.[515] Laterally adjacent

monomers overlap by 234 residues, which results in a 67-nm D-period that is the basic

repeated structure of a collagen fibril.[515] Collagen fibrils assemble into a larger unit called

a fiber, which is the native conformation of fibrillar collagens I, II, and III *in vivo*.[481,515]

1.4.2 Non-fibrillar collagens IV, VI, and VIII

The majority of collagens assemble into polymeric structures, including non-

fibrillar collagens, but non-fibrillar types IV, VI, and VIII collagen form different types of

ultrastructural networks that serve different functions than fibrillar collagens.[484,487,515]

Type IV collagen molecules are typically heterotrimers consisting of two α_1(IV)

and one α_2(IV) chain, which associate with one another via their C-terminal globular head

into three dimensional networks.[481,499] Type IV collagen is a major component of the

basement membranes of many cell types, including the endothelial cell basement

membrane.[484,487,489,490] Type IV collagen molecules contain interruptions within their

COL domain, which impart flexibility into the collagen molecule. The flexibility of type

IV collagen networks imparts elasticity and extensibility to basement membranes.[488,499]

Type VI collagen is a heterotrimer mostly often expressed as an α_1(VI), α_2(VI),

and α_3(VI) heterotrimer,[520] but some tissues express isoforms containing α_4(VI), α_5(VI),

and α_6(VI) chains in various combinations of trimers.[481,484] Type VI collagen forms

beaded filamentous microfibrils that connect different components of the basement membrane,[521,522] and type VI collagen is abundant within the endothelial cell basement membrane.[481,484] Within the tunica media, type VI collagen microfibrils mediate smooth muscle cell interactions with elastin fibers.[500] Similar to fibrillar collagens, type VI collagen assembly begins intracellularly, but once type VI α-chains have formed a triple-helical collagen monomer, they aggregate into tetramers before being secreted into extracellular space, where the tetramers associate end-to-end into beaded filaments.[521,522] The filaments then aggregate laterally into beaded microfibrils,[522] which are composite structures that integrate many different molecules within the basement membrane of cells.[523] The tissue distribution of type VI collagen is fairly ubiquitous,[524] and its primary known function is to integrate components of extracellular matrices to one another and to cells.[500]

Type VIII collagen is present as either a homotrimer of α_1(VIII) chains or as a heterotrimer of α_1(VIII) and α_2(VIII) chains that is also an abundant component of the basement membrane of endothelial cells.[481,525,526] Type VIII collagen similarly forms three dimensional networks that are postulated to stabilize the basement membrane and play a role in angiogenesis.[525] Type VIII collagen was originally referred to as "endothelial collagen" because it is abundantly produced and deposited into the tunica intima,[527] but it has since been shown to be produced by other cells.[528] Type VIII collagen molecules consist of a short, rod-like COL with a globular domain at both the C- and N-termini.[481] These globular domains associate with one another into a hexagonal lattice

structure. Type VIII collagen is considered to be important for the resistance of compressive forces,[528] but has also been shown to be synthesized by sprouting endothelial cells, suggesting it may contribute to angiogenesis.[529]

1.4.3 Collagens in hemostasis and thrombosis

Collagens have a dual role in hemostasis and thrombosis.[419,492] First, they provide an insoluble scaffold for the attachment of cells and other matrix constituents,[530] which facilitates and maintains a closed system of blood circulation.[492] Second, fibrillar and non-fibrillar collagens within the vessel directly support platelet adhesion and platelet activation.[430,531] Collagens are normally sequestered from flowing blood. Following blood vessel injury, flowing blood is exposed to high local concentrations of collagens in the layers of the vessel wall and connective tissues around vessels and in the tissues that blood is exposed to in a wound.[419,492]

Fibrillar collagens are the only known proteins that can support significant platelet adhesion and full platelet activation.[419,492] Fibrillar collagens are potent agonists of platelet activation that can induce platelet aggregation and fibrin formation.[190] Similar to thrombin-activated platelets, collagen-activated platelets express negatively charged phospholipids on their surface and release α-granule contents, which supports the assembly of the prothrombinase complex on the platelet surface, leading to thrombin generation and then fibrin formation.[190] Platelets adherent to fibrillar collagen spread,

form extensive lamellipodia, exhibit bleb formation, and release procoagulant TF-bearing microparticles.[419]

Type I-IV collagens are considered the "platelet reactive" collagens due to their ability to support platelet adhesion and induce platelet aggregate formation under low and high shear rates.[302] Platelets are also capable of adhering to types VI-VIII collagen, but platelets do not aggregate onto these collagens, and only adhere as a monolayer under shear rates lower than approximately 800 s^{-1}.[532] Notably, although platelets can adhere to and aggregate onto type IV collagen, type IV collagen binding to platelet GPVI does not induce full platelet activation.[533] Fibrillar collagens I and III in the vessel wall are considered the primary mediators of collagen-induced platelet activation.[190,441,492,531]

Collagen receptors on platelets have both adhesive and signaling functions. Accordingly, platelet adhesion to collagen is a dual process that supports both platelet adhesion and activation.[419,531] The classic "two-site, two-step" model of platelet adhesion to collagen states that platelets adhere to collagen via $\alpha_2\beta_1$ and are activated by GPVI binding to collagen.[430,531] These two processes involve distinct receptors and discrete recognition sites in the triple-helical domain of fibrillar collagens.[430,531,534] However, outside-in signalling following ligation of $\alpha_2\beta_1$ by collagen has been reported,[535–538] suggesting that $\alpha_2\beta_1$ may participate to some degree in platelet activation. Similarly, platelet GPVI is capable of supporting platelet adhesion to collagen in the absence of

$\alpha_2\beta_1$.[539] Therefore, platelet adhesion and activation by collagen should not be considered mutually exclusive processes, each mediated by a single receptor.

Presently, there are three major "adhesive axes" of platelet adhesion to collagen that have been described: $\alpha_2\beta_1$-mediated platelet adhesion, GPVI-mediated platelet activation that supports some adhesion and enhances $\alpha_2\beta_1$-mediated adhesion, and VWF-mediated platelet adhesion.[175] VWF is sometimes referred to as a collagen receptor due to its ability to mediate platelet adhesion to collagen by binding to collagens and GPIbα on platelets. The primary function of VWF-mediated platelet adhesion to collagen is to facilitate direct platelet-collagen interactions by tethering circulating platelets and allowing them to slowly translocate across the collagen surface under high shear flow.

The use of fragmentation methods (i.e. pepsin digestion or cyanogen bromide hydrolysis) and peptide libraries have been instrumental in identifying the regions in fibrillar collagens that bind various ligands.[540–544] In particular, Collagen Toolkits are peptide libraries that have been used to characterize the precise motifs and key residues in homotrimeric fibrillar collagens that bind VWF, $\alpha_2\beta_1$, and GPVI.[436,545–547] Collagen Toolkits are sets of synthetic triple-helical peptides that take advantage of the inherent properties of fibrillar collagen molecules (*Figure 5*). Given the repeated rod-like structure of the COL domain, its sequence can be segmented into short peptides, which, due to the repeating GXX' motif of the COL domain, causes these peptides to spontaneously assemble into α-helices and subsequently trimerize into a triple helix that recapitulates the

native conformation of the COL domain of a fibrillar collagen monomer.[548] Stable triple

helix formation is facilitated and maintained by the use of (GPP)$_5$ host sequences, which

do not support platelet adhesion or platelet activation.[548] The following sections discuss

the various recognition motifs for platelet collagen receptors and their importance in

hemostasis and thrombosis. The current scope of recognition motifs in types I and III

fibrillar collagen that support platelet adhesion or activation are presented in *Figure 6*, not

including MMRN1-binding sequences in fibrillar collagens presented later in this thesis.

As part of collaborative studies between the laboratories of Dr. Catherine Hayward and

Dr. Richard Farndale, Dr. Subia Tasneem performed analyses of MMRN1 binding to

Collagen Toolkit peptides, which identified a GPAGPOGPX motif (where O is

hydroxyproline and X is occupied by either valine or glutamine) that is postulated to be

the recognition motif in types II and III fibrillar collagen for MMRN1. These experiments

are the foundation for some of the work included in this thesis and are discussed in more

detail in *section 1.5*.
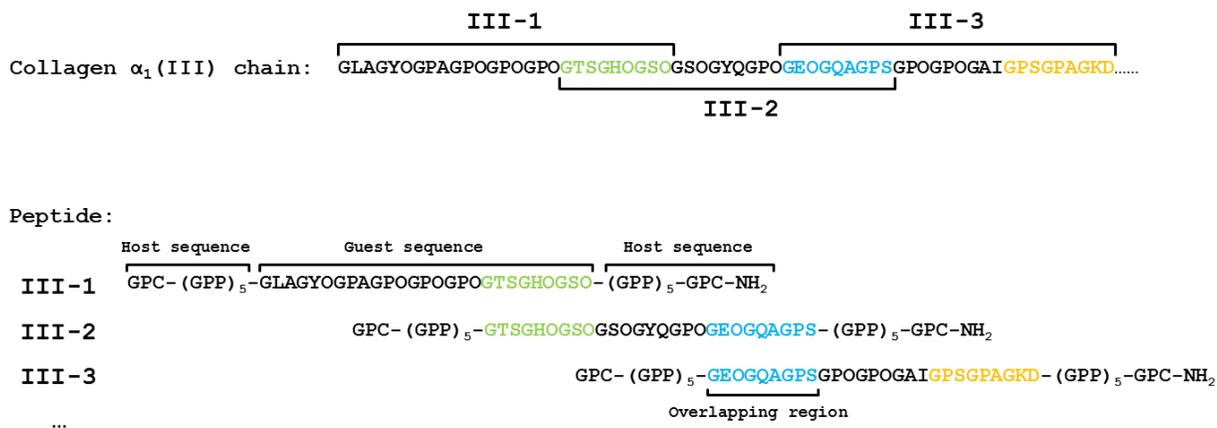
Figure 5: Structure of Collagen Toolkit III peptides. The primary sequence of COL
α$_1$(III) is divided into a set of 57 peptides containing a 27-aa guest sequence within inert

(GPP)$_5$ sequences. Each guest sequence overlaps with the guest sequence of adjacent peptides by 9-aa to account for the possibility of splitting recognition motifs at the junction of two sequences. The (GPP)$_5$ host sequences flanking each end of the guest sequence do not support platelet adhesion or activation, but facilitate the formation of a stable triple helix. The cysteine residue on the N- and C-termini of each peptide allows them to crosslink to one another.[549]
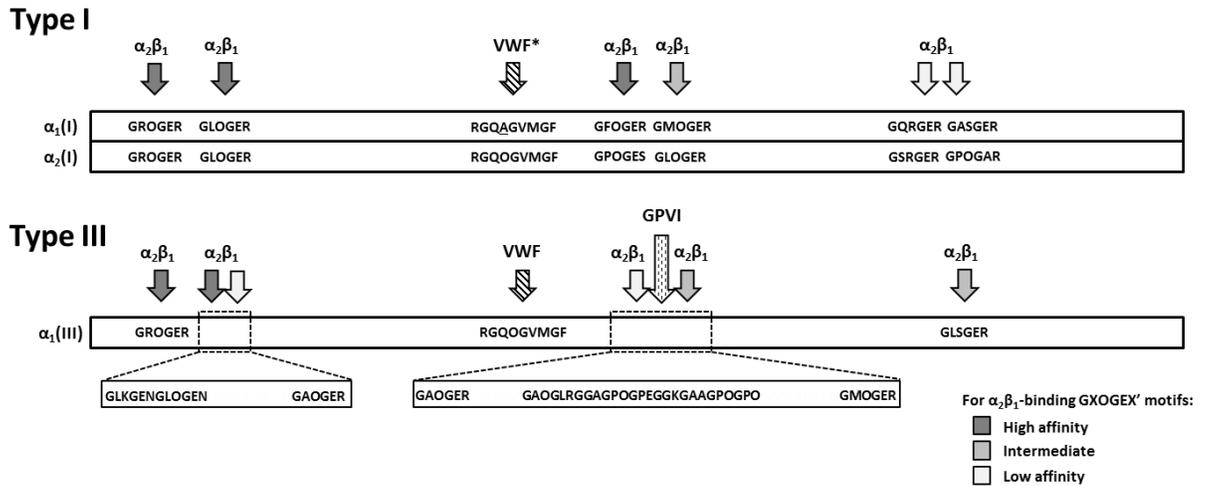
Figure 6: Recognition motifs in types I and III fibrillar collagens for α₂β₁, GPVI, and VWF. Arrows are spatially aligned based on their location in the primary sequence of COL α₁(I), COL α₂(I), and COL α₁(III), demonstrating the spatial distinctions between various motifs. *Postulated to be a VWF-binding sequence,[545] but has not been verified using a heterotrimeric peptide.

1.4.3.1 VWF recognition motifs in fibrillar collagens

VWF binds to types I and III fibrillar collagen via its A3 domain, and a minor role has been attributed to the A1 domain in VWF binding to collagen.[550] Binding of VWF to platelets is required for their adhesion to fibrillar collagen under high shear flow, and monoclonal anti-VWF or anti-GpIb antibodies completely abolish platelet adhesion onto

collagen. Conversely, mice with defective VWF-collagen binding are protected from

thrombosis *in vivo*.[551] VWF binding to collagen can occur via direct interaction between

collagen and the VWF A3 domain or by self-association of circulating plasma or platelet

VWF onto collagen-bound VWF.[552]

VWF binds to the GPRGQOGVMGF sequence in type III collagen (*Figure 6*),

and the binding affinity of VWF for GPRQOGVMGF is similar to the affinity of VWF

for full-length type III collagen, suggesting that this motif is the only sequence in fibrillar

collagen with which VWF interacts.[545] The GPRGQOGVMGF sequence is capable of

supporting platelet tethering and translocation, but not stable adhesion under shear rates

between 1000 and 3000 s^{-1},[175] consistent with the requirement of high shear stress to

reveal cryptic binding sites in the VWF A1 and A3 domains as well as the requirement of

$\alpha_2\beta_1$ for stable adhesion onto collagen. Under low shear flow (100 to 300 s^{-1}),

GPRGQOGVMGF synergistically enhances platelet adhesion when co-presented with the

$\alpha_2\beta_1$-binding GFOGER motif and collagen-related peptide (CRP), which activates

platelets via GPVI.[175] The GPRGQOGVMGF sequence in α_1(III) is mostly conserved in

the collagen α_1(I) chain, except for the hydroxyproline residue in the 5th position, but the

missing hydroxyproline is present at the corresponding 5th position in the α_2(I) chain,

which is predicted to enable VWF binding at this locus.[548] A non-triple-helical derivative

of peptide III-23 flanked by (GAP)$_5$ host sequences does not bind VWF nor platelets,

indicating that triple helical conformation is required for the recognition of III-23 by

VWF.[545] VWF multimer size also affects its ability to bind fibrillar collagen. ULVWF

multimers preferentially bind collagen compared to smaller VWF multimers,[553] which is likely due to multivalent binding between multiple VWF monomers within a multimer and multiple collagen molecules within a fibril. Accordingly, washed platelets are able to adhere to GPRGQOGVMGF under stasis,[545] consistent with the ability of ULVWF multimers released by platelets or endothelial cells to bind collagen in the absence of shear.

Although VWF co-localizes with type VI collagen in the subendothelium and mediates platelet adhesion to type IV or VI collagen, the interaction of VWF with non-fibrillar type IV and type VI collagens differs from its interactions with fibrillar collagens I and III. Types IV and VI collagen do not possess the GPRGQOGVMGF motif, and VWF binding to types IV and VI collagen requires the VWF A1 domain, but not the VWF A3 domain.[328,329] At present, the functions of VWF binding to types IV and VI collagen are not well characterized.

1.4.3.2 Alpha-2 beta-1 recognition motifs in fibrillar collagens

Platelet integrin $\alpha_2\beta_1$ is the only known receptor on platelets that is capable of supporting stable platelet adhesion to collagen by binding directly to collagen.[419] Alpha-2 beta-1 on resting or activated platelets binds with high affinity to the GFOGER sequence in COL α_1(I). The GFOGER motif was initially identified as the recognition motif for $\alpha_2\beta_1$ in type I fibrillar collagen using cyanogen bromide hydrolysed type I collagen

fragments.[540] Following the development of synthetic triple-helical collagen mimetic peptides and Collagen Toolkits, additional $\alpha_2\beta_1$-binding motifs have been identified in types I and III fibrillar collagen (*Figure 6*). The consensus recognition motif in collagens for $\alpha_2\beta_1$ is GXOGEX',[548] and it is present in various forms in types I and III collagens. GFOGER, GROGER, GLOGER, and GLOGEN are high-affinity motifs that can support adhesion of resting or activated platelets.[540,541,546] Additional motifs, such as GLKGEN, GLSGER, GMOGER, GAOGER, GASGER, and GQRGER bind to $\alpha_2\beta_1$ and support platelet adhesion, but only following platelet activation when $\alpha_2\beta_1$ is in an active conformation.[436,546] The low affinity GXOGEX' motifs in collagens are considered to be important in stabilizing platelet adhesion to collagen following activation of platelets and $\alpha_2\beta_1$.[436] High-affinity GXOGEX' motifs are likely essential to initial adhesion of platelets to collagen when $\alpha_2\beta_1$ is present in an inactive conformation on the platelet surface. Interestingly, the spatial relationship between GXOGEX' motifs is conserved between COL α_1(I) and COL α_1(III), suggesting that the distribution of recognition motifs in the COL domain may be important for $\alpha_2\beta_1$ function.

1.4.3.3 GPVI recognition motifs in fibrillar collagens

Monomeric GPVI binds to the GPO motif in the triple-helical COL domain. Given that GPVI is present as a dimer on the platelet surface, each copy of GPVI must bind to a respective GPO triplet in collagen to trigger receptor signalling. A single GPO triplet is insufficient to induce platelet activation via GPVI.[453] The Ig-like domains of

adjacent copies of GPVI are separated by a gap roughly equivalent to three amino acids in length.[441] The smallest GPVI recognition motif in fibrillar collagens is two GPO triplets, either directly adjacent or separated by four GPP repeats. Collagen Toolkit peptide III-30 contains multiple GPO triplets within its 27-aa guest sequence, and is able to bind and activate platelets via GPVI.[547] This region of type III collagen is consider the primary interaction site for GPVI (*Figure 6*). Similar GPO-rich regions are posited to be responsible for platelet activation by type I fibrillar collagen but have not been investigated in depth.

Fibrillar ultrastructure of collagen molecules is required for platelet activation via GPVI. Preparations of collagen that are either non-fibrillar or produce non-fibrillar fragments (i.e. cyanogen bromide treatment) support GPVI binding, but do not activate platelets.[240,554] Similarly, type IV collagen contains many GPO triplets, but does not trigger platelet activation via GPVI because it lacks the quaternary structure of fibrillar collagens.

1.4.4 Fibrillar collagen-related disease states

The Ehlers-Danlos syndromes (EDS) are a heterogeneous group of hereditary connective tissue disorders characterized in part by excessive bruising, hematomas, and increased bleeding despite normal findings for clinical tests of hemostasis.[516,555,556] There are currently six characterized subtypes of EDS,[516] which are linked to causative

mutations in genes encoding the α-chains of fibrillar collagens or enzymes involved in post-translational modification of collagen molecules. The conventional explanation of EDS-associated bleeding symptoms is that defects in collagen molecules lead to structural abnormalities in collagen fibers, resulting in fragile vessels that are prone to rupture.[557] In particular, type IV EDS, also called "vascular EDS", generally arises from missense mutations of glycine residues within the α_1 chain of type III collagen, which causes structural defects in blood vessels. Rupture of major arteries, the bowel, or gravid uteri are features of vascular EDS, along with fragile skin that splits easily in response to minor trauma. Over 250 different vascular EDS mutations have been described,[558] the majority of which are point mutations leading to substitutions for glycine in the triple helical domain of type III collagen. Interestingly, a mutation in the MMRN1-binding GPAGPOGPQ sequence (G852C) is associated with type IV EDS, but its potential effects on platelet adhesion are unclear. The possible effects of collagen mutations on MMRN1 binding and platelet adhesion are discussed in more detail in *section 4.5.3*. In mice, global knockout of *Col3a1* results in 90% perinatal mortality, with a reduced lifespan in surviving homozygotes, usually due to spontaneous vascular rupture. Instead, mice heterozygous for *Col3a1* deletion have been used as a model for EDS that recapitulates many key features of vascular EDS in humans.[559]

Osteogenesis imperfecta, also called "brittle bone disease", is a heterogeneous group of genetic disorders characterized by susceptibility to fracture that varies in severity from mild (type I) to embryonic lethal (type II). Dominantly inherited forms account for

90% of people with OI result from heterogeneous mutations in *COL1A1* or *COL1A2*.[517] The mildest forms of OI generally result in the loss of mRNA from one *COL1A1* allele, whereas severe forms can result from substitution of glycine residues within the COL domain of *COL1A1* or *COL1A2*. OI can sometimes result in a bleeding phenotype that is similar to features of EDS, which is suggested to be caused by defective collagen fibers and vessel fragility. It is currently not known what the specific effects of *COL1A1* and *COL1A2* mutations are on platelet adhesion.

1.5 UNANSWERED QUESTIONS REGARDING MMRN1 ADHESIVE FUNCTIONS

Given what is presently known about MMRN1 adhesive functions and platelet adhesion and platelet aggregation, there are numerous unanswered questions regarding the role of MMRN1/Mmrn1 in platelet adhesion. In particular, the observation that the adhesion of *Mmrn1*^{-/-} platelets to fibrillar collagen is impaired raised questions regarding the mechanism through which Mmrn1 contributes to platelet adhesion to collagen. It has been argued that the specific roles of ligands involved in platelet adhesion to collagen cannot be resolved by analyses of surface coverage alone.[560] It was previously noted that *Mmrn1*^{-/-} platelets showed a tendency toward forming smaller aggregates onto collagen under high shear flow (1500 s^{-1}),[4] but a standardized method for quantification of adherent platelet aggregates is not established for this assay. Additionally, the dynamics of the defect in platelet adhesion and aggregate formation onto collagen have not been explored. Endpoint analyses of platelet adhesion are not sensitive to differentiate between

defects in initial platelet tethering onto collagen, rate of aggregate growth, or aggregate stability. It is also unclear if this defect in platelet adhesion to collagen is present under low shear flow, in which VWF is not essential for platelets to significantly aggregate onto fibrillar collagen.

As described in *section 1.3.2.1*, a critical role of $\alpha_{IIb}\beta_3$ in platelet aggregation onto fibrillar collagen under high shear flow has been demonstrated for both human and mouse platelets. MMRN1 is a ligand for β_3 integrins on activated platelets, and Mmrn1 may contribute to platelet aggregate formation on fibrillar collagens through interactions with $\alpha_{IIb}\beta_3$ on activated mouse platelets. However, Mmrn1 lacks the N-terminal RGD site present in MMRN1,[18] meaning it is unclear if Mmrn1 binds β_3 integrins. It is possible that Mmrn1 may interact with $\alpha_{IIb}\beta_3$ through RGD-independent mechanisms, similar to FG.

Alternatively, Mmrn1 could crosslink $\alpha_{IIb}\beta_3$-bound adhesive proteins to indirectly support platelet aggregate formation, similar to TSP-1 and multimeric VN released from platelets. Given the complex, multi-domain structure of MMRN1/Mmrn1 and its ability to form large multimers, it may act as a bridging protein by binding FG on activated platelets to support platelet cohesion through interactions with platelet-bound FG. Potential defects in Fg-dependent platelet adhesion in the absence of Mmrn1 may also partly explain the antithrombotic phenotype of *Mmrn1$^{-/-}$* mice in response to FeCl$_3$ arteriolar injury, as Fg-deficient mice similarly show impaired thrombus formation, stability, and vessel occlusion in this model.[211]

Another possible binding partner for MMRN1/Mmrn1 is FN. FN supports platelet adhesion via RGD-dependent binding to $\alpha_5\beta_1$ and activated $\alpha_{IIb}\beta_3$, and MMRN1 may assist in FN-mediated platelet adhesion or possibly compete for ligation of activated $\alpha_{IIb}\beta_3$. MMRN1 could potentially act as a bridging protein that would enhance the ability of platelet FN to crosslink platelets or increase the avidity of platelet-platelet interactions by binding to $\alpha_{IIb}\beta_3$-bound FN.

Observations from studies of $FeCl_3$-treated mesenteric arterioles raised questions regarding the ability of Mmrn1 to support platelet adhesion, aggregation, and thrombus formation *in vivo*. Firstly, the role of collagen in $FeCl_3$-induced vessel injury is controversial.[561,562] Some evidence suggests that types IV and VI collagen in the endothelial basal lamina are exposed following exposure to $FeCl_3$,[211,563] but $FeCl_3$-induced vessel injury has not been shown to induce exposure of fibrillar collagens deeper within the vessel wall. Despite this, platelet adhesion and thrombus formation are significantly impaired in the arterioles of *Mmrn1$^{-/-}$* mice treated with $FeCl_3$.[4] The connection between impaired adhesion of *Mmrn1$^{-/-}$* platelets to fibrillar collagen *in vitro* and impaired thrombus formation in mesenteric arterioles *in vivo* is uncertain, but suggests other mechanisms are involved. For example, α_2-deficient mice have delayed thrombus formation with frequent embolization in $FeCl_3$-treated mesenteric arterioles, which is attributed to a reduction in TxA_2 formation, not impaired adhesion to collagens.[215]

Although MMRN1 modulates FV storage and thrombin generation, in the context of platelet adhesion, Mmrn1 binding to FG and FN to enhance or stabilize platelet adhesion may contribute to $FeCl_3$-induced thrombus formation. This would provide a link between impaired aggregate formation on collagen *in vitro* and impaired thrombus formation *in vivo* observed in *Mmrn1*^{-/-} mice. Another particularly interesting potential Mmrn1 binding partner is fibrin, which is generated in significant quantities in the $FeCl_3$-induced vessel injury model. The possibility for Mmrn1 to modulate platelet adhesion and thrombus formation *in vivo* through interactions with fibrin would represent an additional mechanism through which Mmrn1 contributes to platelet thrombus formation, and help reconcile *in vitro* and *in vivo* observations of *Mmrn1*^{-/-} mice.

The LTA responses of *Mmrn1*^{-/-} mice raised questions regarding how they relate to *in vitro* analyses of platelet adhesion to collagen. *Mmrn1*^{-/-} mouse platelets had normal collagen-induced aggregation when tested in PRP, but not using GFP, which is postulated to be due to the presence of other adhesive plasma proteins masking the contribution of Mmrn1.[4] However, in analyses of platelet adhesion to collagen under high shear flow using whole blood, *Mmrn1*^{-/-} mice had significantly impaired platelet adhesion to collagen. This could be due to 1) differences in shear rate between the models or 2) differences in how platelets are exposed to collagen (i.e. surface-bound versus soluble agonist). Testing the ability of *Mmrn1*^{-/-} and wild-type mouse platelets in whole blood in response to collagen would clarify if using PRP or gel-filtered platelets alters the ability

of *Mmrn1*$^{-/-}$ and wild-type platelets to aggregate in response to collagen compared to platelets in whole blood.

Presently, *in vitro* and *in vivo* analyses of *Mmrn1*$^{-/-}$ mice have focused on high shear flow (1500 s^{-1}), excluding LTA experiments. Immobilized MMRN1 can support adhesion of activated platelets under much higher shear rates (tested to a maximum of 6000 s^{-1}), and fluid shear stress is known to affect MMRN1 adhesive functions.[2] It is intriguing to consider that Mmrn1 could contribute to platelet aggregation under elevated shear rates, such as at the apex of a developing thrombus, which would provide evidence for a potential mechanism through which Mmrn1 contributes to thrombus growth and vessel occlusion. As described in *section 1.3.1.2*, VWF is a key mediator of platelet aggregation under elevated shear, and *Vwf*$^{-/-}$ mice fail to form occlusive thrombi due to small luminal channels that never fully occlude. Given that MMRN1 binds VWF, Mmrn1 could similarly bind Vwf to enhance Vwf-mediated platelet aggregation under elevated shear.

Lastly, there are unanswered questions regarding the ability of MMRN1 to interact with collagens. Previous work by our laboratory has identified a MMRN1-binding GPAGPOGPX sequence in types II and III fibrillar collagens (*outlined in section 1.4.3*), but it is unknown if a similar GPAGPOGPX sequence that binds MMRN1 is present in type I collagen, which is also a ligand for MMRN1. The GPAGPOGPX sequence is spatially and structurally discrete from other recognition motifs in collagen

that support platelet adhesion, but the specificity of GPAGPOGPX for MMRN1/Mmrn1 and its ability to support mouse platelet adhesion have not been evaluated. The ability of MMRN1 to enhance platelet adhesion to fibrillar collagen is intriguing, and could involve MMRN1-mediated platelet-collagen interactions that synergize with other receptors and proteins to enhance platelet adhesion. However, the ability of the MMRN1-binding GPAGPOGPX sequence to enhance platelet adhesion under stasis and flow when presented with other recognition motifs in fibrillar collagens has not been evaluated.

1.5.1 Hypothesis

Based on the considerations described above, I hypothesize that:

Mmrn1 contributes to platelet adhesion and aggregate formation by binding to adhesive proteins in plasma and platelets, such as fibrin(ogen) or fibronectin, contributes to platelet adhesion and aggregation under elevated shear stress, and enhances collagen-dependent platelet adhesion through a mechanism involving Mmrn1-specific motifs in fibrillar collagens.

1.5.2 Experimental aims

To test this hypothesis, my specific experimental aims were:

1. Characterize the ability of Mmrn1 to modulate aggregate formation onto fibrillar collagen and determine the dynamics of impaired platelet adhesion to fibrillar collagen shown by $Mmrn1^{-/-}$ mice, using analyses of:

    a. Aggregate size of collagen- or Vwf-adherent $Mmrn1^{-/-}$ and wild-type mouse platelets

    b. Real-time adhesion of $Mmrn1^{-/-}$ and wild-type mouse platelets to collagen under high shear flow

    c. Adhesion of $Mmrn1^{-/-}$ and wild-type mouse platelets to Horm collagen under low shear flow

    d. Collagen-induced aggregation of $Mmrn1^{-/-}$ and wild-type platelets in whole blood

2. Test the ability of MMRN1 to bind FG, fibrin, and FN, and assess the ability of Mmrn1 to augment platelet adhesion to FG, fibrin, and FN, using:

    a. Solid-phase MMRN1 binding assays to FG, fibrin, and FN

    b. Assays of static and low shear adhesion of $Mmrn1^{-/-}$ and wild-type platelets to murine Fg, fibrin, and Fn

3. Characterize the role of fluid shear stress in the ability of Mmrn1 to enhance platelet adhesion and aggregate formation.

    a. Assays of shear-induced aggregation of *Mmrn1*$^{-/-}$ and wild-type mouse platelets

4. Identify additional GPAGPOGPX-like sequences in type I fibrillar collagen and test their ability to bind MMRN1 and modulate platelet adhesion, using:

    a. *In silico* analysis of amino acid sequences of type I collagen α-chains

    b. Solid-phase MMRN1 binding assays to triple-helical collagen mimetic peptides

    c. Static and high shear adhesion assays using *Mmrn1*$^{-/-}$ and wild-type platelet and triple-helical collagen peptides

    d. Static adhesion assays using resting and activated human platelets and triple-helical collagen peptides

CHAPTER 2: MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Reagents and supplies

Reagents and supplies included: prostaglandin E_1 (PGE_1, Sigma-Aldrich Canada, Oakville, ON, CA), CNBr-activated Sepharose™ 4B (GE Healthcare Bio-Sciences AB, Uppsala, SE), Colorburst™ Blue TMB/peroxide color developing peroxidase substrate (ALerCHEK, Inc., Portland, ME, USA), BioTrace™ NT pure nitrocellulose blotting membrane (Pall Life Sciences, Pensacola, FL, USA), Immobilon™ Western Chemiluminscent horseradish peroxidase (HRP) substrate (Thermo Fisher Scientific, Inc., Waltham, MA, USA), ProLong™ Gold antifade reagent (Invitrogen Canada Inc., Burlington, ON, CA), ketamine (Narketan; Vetoquinol, Lavaltrie, QC, CA), atropine (Arto-SA, Rafter8, Calgary, AB, Canada), xylazine (Rompun™; BayerDVM, Shawnee Mission, KS, USA), polyethylene tubing (PE10; Becton Dickinson and Company, Sparks, MD, USA), 75x25x1 mm single-frosted glass microscope slides (VWR$^®$ VistaVision™; VWR International, Radnor, PA, USA), 22x22 mm glass micro cover slides (VWR International, Radnor, PA, USA), Extract-N-Amp™ Tissue Polymerase Chain Reaction (PCR) Kit (Sigma-Aldrich Canada, Oakville, ON, CA), Phe-Pro-Arg-chloromethylketone (PPACK; Haematologic Technologies Inc., Essex Junction, VT, USA), thrombin (Sigma-Aldrich Canada, Oakville, ON,CA), mouse Par4 amide (GYPGKF-NH_2; Bachem

Americas, Inc., Torrance, CA, USA), phosphatase substrate (p-Nitrophenyl phosphate; Sigma-Aldrich Canada, Oakville, ON, CA), C-Chip DHC-N01 disposable hemocytomer (Incyto, Seonggeo-eup, Seobuk-gu Cheonan-si, Chungnam-do, SK), microtitre plates (Costar® 3695; Corning, Inc., Kennebunk, ME, USA; Immulon® 2 HB; Thermo Scientific, Rochester, NY, USA), braided silk non-absorbable suture (SP115; Surgical Specialties Corporation™, Westwood, MA, USA), Precision Plus Protein™ dual color standard (Bio-Rad Laboratories [Canada] Ltd., Mississauga, ON, CA), plastic cuvettes (Chrono-Log Corp., Havertown, PA, USA), siliconized stir bars (Chrono-Log Corp., Havertown, PA, USA), 100bp DNA ladder RTU (ready-to-use; GeneDireX, Inc.), Vena8Fluoro+ Biochips (Cellix Ltd., Dublin, IE), 3.2% sodium citrate (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), heparin (Sandoz Canada, Boucherville, QC,CA), 2-Mercaptoethanol (Sigma-Aldrich Canada, Oakville, ON, CA) , 3,3'-dihexyloxacarbocyanine iodide (DiOC6[3]; Enzo Life Sciences Inc., Farmingdale, NY, USA), Tween® 20 detergent (Sigma-Aldrich Canada, Oakville, ON, CA), Triton X-100 detergent (Sigma-Aldrich Canada, Oakville, ON, CA), AccuStart™ II GelTrack PCR SuperMix (Quanta Biosciences, Inc., Beverly, MA, USA), and EDTA (Fisher Chemical™, Thermo Fisher Scientific, Inc., Waltham, MA, USA).

2.1.2 Antibody sources

Antibodies against multimerin 1 included: rabbit polyclonal anti-MMRN1 (sc-367225; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) raised against a MMRN1 C-terminal fragment (residues 1051-1228) that recognizes mouse Mmrn1; in-house prepared

monoclonal mouse anti-MMRN1 (JS-1), that recognizes an epitope in the C-terminal region of human MMRN1 (residues 961-1139);[10] and in-house prepared polyclonal rabbit anti-MMRN1 (P155).[15]

HRP-conjugated secondary antibodies used in Western blotting and ELISA included: donkey anti-mouse immunoglobulin G (IgG) and donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA).

Fluorescently-labelled antibodies against mouse platelet proteins included: phycoerythrin (PE)-labelled hamster anti-mouse β_3 (CD61; BD Biosciences, Mississauga, ON, CA); fluorescein isothiocyanate (FITC)-labelled rat anti-mouse GPIbα (CD42b; Emfret Analytics GmbH & Co. KG, Eibelstadt, Germany); and FITC-labelled rat anti-mouse P-selectin (CD62P; Emfret Analytics GmbH & Co. KG, Eibelstadt, Germany).

Monoclonal anti-mouse β_3 blocking antibody (9D2) was a gift from Dr. Heyu Ni.

2.1.3 Purified proteins

2.1.3.1 Recombinant human multimerin 1

Recombinant human (rh) MMRN1 was prepared by culture of human embryonic kidney (HEK) 293 cells that were stably transfected with the cDNA for prepromultimerin 1in pCMV5 (pCMV5-multimerin).[12] rhMMRN1 was affinity purified from cell culture media using JS-1 conjugated Sepharose beads as described.[1,20] Captured rhMMRN1 was eluted using 3.5 M $MgCl_2$, and desalted and concentrated by two centrifugations. The

concentration of affinity purified rhMMRN1 was evaluated by enzyme-linked immunosorbent assays (ELISA) via comparison to pooled normal platelet lysate as described,[1,21,27] where 1 unit (U) of MMRN1 is defined as the amount of MMRN1 in 10^9 pooled (*n*=20) normal platelets (estimated to be equivalent to 18.5 µg).[6,20] Costar® 96-well half-volume microtitre plates were coated using 0.5 µg/well JS-1 in carbonate buffer (14.2 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) overnight at 4°C. JS-1-bound MMRN1 was detected using 1:1000 polyclonal rabbit antisera against MMRN1 (P-155) followed by 1:2000 HRP-conjugated donkey anti-rabbit IgG. Subunit size of purified rhMMRN1 was assessed by reduced sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 6% polyacrylamide gel followed by transfer to a nitrocellulose membrane and immunoblotting. Nitrocellulose membranes were probed for rhMMRN1 using 1:5000 JS-1 followed by 1:25,000 HRP-conjugated donkey anti-mouse IgG. Silver staining of polyacrylamide gels to assess purity of rhMMRN1 was performed to assess purity of rhMMRN1 preparations as described.[1,21]

2.1.3.2 Triple-helical collagen mimetic peptides

Triple-helical collagen mimetic peptides were provided by the laboratory of Dr. Richard Farndale and were synthesized by Dr. Dominique Bihan and Dr. Arek Bonna. Collagen Toolkit and other triple-helical collagen peptides were generated using Fmoc solid-phase peptide synthesis and a host-guest strategy as described.[564] Although type II collagen is not found in the vessel wall, sequences from Toolkit II were included in these

analyses because it is homotrimeric and has a high degree of homology with type I collagen (75% identity between the COL domains of $\alpha_1[I]$ and $\alpha_1[II]$). It is presently not feasible to generate heterotrimeric triple-helical collagen peptides so a peptide library for the triple-helical (COL) domain of type I collagen is not available. Triple-helical collagen mimetic peptides used in this thesis are described in *Table 3*. CRP is a potent platelet GPVI agonist, which was used to activate human and mouse platelets in some experiments. GFOGER is a high-affinity ligand for platelet integrin $\alpha_2\beta_1$, and was used to measure $\alpha_2\beta_1$-dependent adhesion of Mmrn1-deficient platelets or to facilitate stable adhesion when co-presented on a surface with other collagen peptides. Collagen Toolkit peptide III-23, which contains the high-affinity VWF-binding motif GPRGQOGVMGF, was used as a ligand for platelet and plasma VWF. The inert triple-helical peptide GPP, which does not support platelet activation, platelet adhesion, VWF binding, or MMRN1 binding, was used as a negative control peptide.

Peptide	Amino acid sequence			Ligand	Function	Location
	C-terminal Host	Guest sequence	N-terminal Host			
DB304	GPC(GPP)₅	GPAGPOGPV	(GPP)₅GPC	MMRN1	-	α₁(III)
CC13	GPC(GPP)₅	GGSGPAGPOGPQGVK	(GPP)₅GPC	MMRN1	-	α₁(III)
AB-24	GPC(GPP)₅	GPAGPOGFQ	(GPP)₅GPC	-	-	α₂(I)
AB-25	GPC(GPP)₅	GPAGSOGFQ	(GPP)₅GPC	-	-	α₁(I)
AB-30	GPC(GPP)₅	GPAGPOGPI	(GPP)₅GPC	-	-	α₁(I)
III-23	GPC(GPP)₅	GPOGPSGPRGQOGVMGFOGPKGNDGAO	(GPP)₅GPC	VWF	VWF binding	α₁(III)
GFOGER	GPC(GPP)₅	GFOGER	(GPP)₅GPC	α₂β₁	Platelet adhesion	α₁(I)
CRP	GCO	(GPO)₁₀	GOG	GPVI	Platelet activation	N/A
GPP	GPC(GPP)₅	(GPP)₁₀	(GPP)₅GPC	None known	Inert	N/A

Table 3: Triple-helical collagen mimetic peptides used in this thesis. Underlined
sections of guest sequences indicate the recognition sequence for each ligand, or residues
in the GPAGPOGP(V/Q) motif that are conserved in variant peptides AB-24, AB-25, and
AB-30. "-" indicates data not available.

2.1.3.3 Other protein sources

Other proteins used in experiments included: human fibrinogen (Haematologic

Technologies Inc., Essex Junction, VT, USA), human fibronectin (Haematologic

Technologies Inc., Essex Junction, VT, USA), mouse fibrinogen (Fg; Molecular

Innovations, Novi, MI, USA), mouse fibronectin (Fn; Molecular Innovations, Novi, MI,

USA), Horm collagen reagent (Helena Laboratories, Beaumont, TX, USA), Cultrex® rat

collagen I (R&D Systems, Minneapolis, MN, USA), and bovine serum albumin (BSA; Sigma-Aldrich Canada, Oakville, ON, CA).

2.2 METHODS

2.2.1 Helsinki Declaration on human research

The study was conducted in accordance with the requirements of the revised Helsinki Declaration on human research and the Hamilton Integrated Research Ethics Board (HiREB). Blood samples from human participants were obtained with written informed consent.

2.2.2 Animal handling

The animal studies (including the generation and maintenance of mouse colonies, and experiments with mouse tissues) were conducted with the approval of the McMaster University Animal Research Ethics Board (Animal Utilization Protocol 13-02-02, which was subsequently replaced by 16-10-38) and in accordance with the standards set by the Canadian Council on Animal Care. Mice were interbred and housed in the vivaria at the Central Animal Facility within the McMaster University Medical Centre. Animals were housed in rooms with 12-hour dark-light cycles. Breeders and weanlings were housed within sterile ventilated caging before being transferred to specific-pathogen-free non-

sterile caging for general housing. Mice were fed a diet of sterile water and irradiated

regular chow *ad libitum* (18% protein rodent diet, Teklad Global Diets, Madison, WI,

USA). Gender- and age-mixed wild-type (*Mmrn1$^{+/+}$*) or Mmrn1-deficient (*Mmrn1$^{-/-}$*)

mice were used in all experiments to obtain representative sample populations. Where

possible, experimenters were blinded to the genotype of the mice. Mice not used for

experiments or retired breeders were either provided to the Discards program at the

Central Animal Facility or euthanized by CO_2 followed by cervical dislocation to confirm

death.

2.2.3 Generation of multimerin 1-deficient mice

Mmrn1-deficient mice were generated using the "knockout-first allele" strategy.[30]

Adult mice heterozygous for the *Mmrn1* knockout-first allele were purchased from the

European Mutant Mouse Archive (EMMA, EM:05337) and crossed with transgenic Flp

mice (B6;J-Tg(ACTFLPe)9205Dym/J) to remove the Frt site flanked *lacZ* and *Neo*

regions of the gene-trap cassette, resulting in mice with a floxed third exon of *Mmrn1*.

Offspring were then crossed with transgenic global cre mice (B6.C-Tg(CMV-cre)1Cgn/J)

to remove the third exon of *Mmrn1*. The resultant exon 3 (E3)-deleted heterozygous mice

were crossed with wild-type (C57BL/6J) mice to remove the flp and cre transgenes.

Breeding and crossing of mouse strains to generate Mmrn1-deficient mice was carried out

by Dr. Subia Tasneem, as part of collaborative studies between the laboratories of Dr.

Bradley Doble and Dr. Catherine Hayward. The frameshift mutation caused by deletion

of *Mmrn1* E3 is predicted to trigger nonsense-mediated decay of the truncated mutant transcript. Mmrn1-deficient mice have no detectable platelet Mmrn1 by Western blotting, which was performed by Dr. D'Andra Parker.

2.2.4 Genotyping of experimental mice

To confirm genotypes of experimental mice, DNA was extracted from either mouse tails (0.5 to 1 cm of distal tip) or ear notches using Extract-N-AMP™ Tissue PCR Kit according to the manufacturer's protocol, followed by PCR using the oligonucleotide primers: forward (5'-TCTTTCTCTCCCTCTCACCCT-3') and reverse (5'-GGCTCCTTTCATTCACAGCC-3'). PCR fragments were resolved on a 2% agarose gel. The PCR products for wild-type and Mmrn1-deficient mice were approximately 1038 base pairs (bp) and 336 bp, respectively. Heterozygous null mice displayed bands at both 1038 bp and 336 bp.

2.2.5 Mouse blood collection

Approximately 700 to 900 µl of arterial blood was collected by cannulation of the carotid artery from mice anesthetized with125 µg/kg ketamine supplemented with 12.5 µg/g xylazine for analgesia and 0.25 µg/g atropine to prevent bradycardia during the procedure.

2.2.6 Assays of platelet adhesion under shear

Mouse platelet adhesion was evaluated similar to methods described.[2,21] For flow experiments, blood was perfused through Vena8Fluoro+ microcapillary perfusion chambers (Cellix Ltd., Dublin, IE) at a constant rate using a Cellix Mirus 1.1 Nanopump controlled by VenaFlux™ software (Cellix Ltd., Dublin, IE). Shear stress τ (dyne/cm^2) and shear rate γ (s^{-1}) were calculated automatically by VenaFlux™ software based on the estimated viscosity of whole blood samples (μ = 0.045 dynes/cm^2) and the geometry of microcapillaries (channel width: 0.04 cm, channel height: 0.01 cm).

For analyses of endpoint adhesion of fluorescently-labelled platelets, Vena8Fluoro+ microcapillary channels were coated overnight at 4°C in a humidified box using 100 µg/ml Horm collagen (coated using 0.01 M acetic acid), 10 U/ml recombinant murine Vwf (coated using PBS), or 100 µg/ml mouse fibrinogen or mouse fibronectin (coated using carbonate buffer). In some channels, immobilized mouse fibrinogen was converted to fibrin *in situ* using 200 mU/ml human thrombin for 15 minutes before channels were washed with Tris-buffered saline (TBS) containing 10 U/ml heparin, similar to methods described. Prior to perfusion, microcapillary channels were blocked for one hour at 37°C in a humidified box using HEPES-buffered Tyrode's solution (137 mM NaCl, 2 mM KCl, 0.3 mM NaH$_2$PO$_4$, 1 mM MgCl$_2$, 2 mM CaCl$_2$, 5.5 mM glucose, 5 mM HEPES, 12 mM NaHCO$_3$) containing 5% (m/v) BSA. For analyses of platelet adhesion to Horm collagen, whole blood was collected into 93 µM (50 µg/ml) PPACK

and 2 U/ml heparin. For analyses of platelet adhesion to rVwf, fibrinogen, fibrin, and

fibronectin, whole blood was collected into 93 µM PPACK. Platelets were labelled using

4 µM DiOC6(3) for 10 minutes at 37°C before blood was perfused through

microcapillary channels (Horm collagen: 1500 s^{-1}, 3 minutes; rVwf: 1500 s^{-1}, 5 minutes;

mouse fibrinogen, fibrin, and fibronectin: 300 s^{-1}, 3 minutes). In some experiments,

platelets were pre-activated using 30 µM murine TRAP for 5 minutes at 37°C. Channels

were subsequently washed using HEPES-buffered Tyrode's solution containing 1 U/ml

heparin to remove non-adherent platelets. Experiments to measure endpoint adhesion to

Horm collagen or rVwf under high shear flow (1500 s^{-1}) were carried out by Dr. D'Andra

Parker, as part of her doctoral thesis work under the supervision of Dr. Catherine

Hayward. Additional analyses of the data were performed as part of this thesis to evaluate

aggregate sizes.

For real-time adhesion experiments, whole blood was collected into 10 U/ml

heparin as described[3] and labelled using 4 µM DiOC6(3) for 10 minutes at 37°C.

Perfusions were similarly performed using a Cellix Mirus 1.1 Nanopump controlled by

VenaFlux™ software. Images were captured every 50 ms using a Zeiss Axiovert S 100

inverted epifluorescence microscope coupled to a VS4-1845 image intensifier assembly

(Video Scope International Ltd., Sterling, VA, USA) and a Hamamatsu C9300 digital

camera (Hamamatsu Corporation USA, Bridgewater, NJ, USA), controlled by SlideBook

6 software (Intelligent Imaging Innovations, Inc., Denver, CO, USA). Focus was set to

the bottom surface of the microcapillary and capture was initiated before collagen-coated

microcapillaries were perfused for 5 minutes under high shear flow (1500 s^{-1}). Data were

analyzed by placing a 450x450 pixel (equivalent to ~45 mm^2) mask over the centre of the

microcapillary. Background fluorescence was determined by measuring the fluorescent

signal before blood was perfused, which was subtracted from each image to correct for

background fluorescence intensity. Mean, sum, and maximum fluorescence intensity at

each 100 ms time-point were calculated automatically using the Mask Statistics tool.

Erroneous spikes in fluorescence due to platelets or small aggregates passing directly

through the frame, but outside of the focal plane, were removed manually after plotting

data. Data are expressed as the sum of fluorescence intensities in a single, representative

experiment.

2.2.7 Quantification of platelet adhesion and aggregate size

A total of 15 images were captured of endpoint platelet adhesion in

microcapillaries coated with Horm collagen using a x40 objective or 10 images using a

x20 objective for rVwf, fibrinogen, fibrin, fibronectin, and collagen peptides, accounting

for ~10% of the total surface area of microcapillary channels. Percent surface area

covered by platelets was estimated in each image, similar to methods described,[2,3,21] using

ImageJ (National Institutes of Health, Bethesda, MD, USA) to obtain a mean. Number

and size of captured platelet aggregates were estimated using representative regions

(Horm collagen, 40x objective: ~60 mm^2 square, 1020x1020 pixels; rVwf, fibrinogen,

fibrin, fibronectin, and collagen peptides, 20x objective: ~60 mm^2 square, 515x515

pixels) at the center of microcapillary channels, avoiding areas out of focus. Grayscale images were converted to binary using the Threshold tool before separating features using the Watershed tool and quantification using the Analyze Particles tool. As the size of platelet aggregates on Horm collagen varied greatly (~1 to 40,000 μm^2), data were binned and frequency plots were used to evaluate data. As the size of platelet aggregates captured onto rVwf, fibrinogen, fibrin, fibronectin, and collagen peptides were more uniform (~1 to 5000 μm^2), mean feature size per image was used to evaluate data.

2.2.8 Assays of collagen-induced platelet aggregation in whole blood

Collagen-induced aggregation of mouse whole blood was evaluated by impedance aggregometry using a Chrono-Log® Model 700 Whole Blood/Optical Lumi-Aggregometer and Aggro/Link®8 software (Chrono-log Corporation, Haverton, PA, USA), according to the manufacturer's recommended settings for whole blood, which was collected into 1:10 (v/v) 3.2% sodium citrate and diluted 1:1 (v/v) with 0.9% NaCl. Samples were warmed to 37°C under constant stirring (1200 rpm) and once the baseline measurement had stabilized, 5 or 10 μg/ml Horm collagen was added. Aggregation responses were measured up to 15 minutes and reported in Ω of electrical resistance.

2.2.9 Assays of shear-induced platelet aggregation

Shear-induced platelet aggregation was evaluated using a HAAKE Mars rheometer (Thermo Electron Corporation, Waltham, MA, USA) fitted with a C35/0.5° Ti (35 mm diameter) cone. Shear stress τ and shear rate γ were calculated automatically by HAAKE RheoWin 3 software. Approximately 900 µl of whole blood was collected in 1:10 (v/v) 3.2% sodium citrate (1 ml final), and split into four autologous samples that were exposed to different shear rates: 0, 5000, 10000, or 15000 s^{-1}. Samples were supplemented with 5 mM $CaCl_2$ and 93 µM PPACK (to promote aggregation and prevent clotting, respectively) immediately before loading 220 µl onto the plate of the rheometer. Because pilot studies indicated that platelets in citrate-anticoagulated whole blood failed to significantly aggregate in response to shear, and PPACK-anticoagulated blood showed some degree of platelet aggregation in unsheared (resting) samples, blood was collected in citrate and then recalcified immediately prior to exposure to shear. Recalcified whole blood was sheared by the rotating cone in a 0.3 mm gap for 1 minute at room temperature before fixation using 1:4 (v/v) 0.625% paraformaldehyde (PFA; 0.5% final). Fixed platelets were labelled in the dark using either 1:8.3 (v/v) of anti-mouse GpIbα (CD42b, Emfret Analytics, Eibelstadt, Germany) for 15 minutes for flow cytometry or labelled with 4 µM DiOC6(3) for fluorescence microscopy. Samples for flow cytometry were diluted 1:200 (v/v) in PBS and BD™ Liquid Counting Beads (165 beads/µl, Becton, Dickinson and Company, BD Biosciences, San Jose, CA, USA) were added by gentle pipetting before samples were analyzed by collecting 50,000 events with a Beckman

Coulter® Epics® XL-MCL™ flow cytometer and Expo32™ software (Beckman Coulter, Brea, CA, USA).

Collected events were sequentially gated for FITC-positive single platelets using FlowJo™ 10 (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) based on forward and side scatter characteristics. PE-positive counting beads were gated separately to determine the number of collected beads/sample so that platelet concentration could be estimated as follows:

$$platelets/ml = \frac{single\ platelet\ events}{bead\ events} \text{ x } \frac{bead\ events}{\text{µ}l} \text{ x } 1000 \text{ x } dilution\ factor$$

Recalcified resting samples were also analyzed to determine the baseline platelet count. Percent loss of platelets at each shear rate was determined by calculating the % reduction of platelet concentration in sheared samples relative to the untreated sample. Aggregate size of platelets in samples subjected to shear was determined by fluorescence microscopy with comparison to untreated samples. DiOC6(3)-labelled fixed platelets were deposited as a monolayer onto 75x25x1 mm VWR® VistaVision™ glass slides using the Shandon™ Cytospin® 3 Cell Preparation System according to the manufacturer's instructions (1000 rpm for 5 minutes at room temperature, Thermo Fisher Scientific, Waltham, MA, USA). Slides were allowed to dry for 30 minutes in the dark before 1-2 drops of ProLong™ Gold anti-fade reagent was added and slides were covered with a 22x22 mm glass coverslip. Images of platelet aggregates were captured using a

Zeiss Axiovert 200 inverted epifluorescent microscope coupled to an AxioCam MRc and Axiovision software (Carl Zeiss Canada Ltd., Toronto, CA).

2.2.10 Protein binding assays

Solid-phase protein binding assays were used to assess rhMMRN1 binding to immobilized collagen, fibrinogen, fibrin, fibronectin, triple-helical collagen peptides, or bovine serum albumin (BSA, negative control) similar to methods described, with the following modifications: wells of Immulon® 2 HB flat-bottom plates were coated overnight at 4°C with 1 µg/well of protein or peptide. Collagen, Collagen Toolkit peptides, and triple-helical collagen peptides were coated using 0.01 M acetic acid. Fibrinogen and fibronectin were coated using carbonate buffer (14.2 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6). In some wells, bound fibrinogen was converted to fibrin *in situ* using 200 mU/ml of human thrombin for 15 minutes as described. Wells were blocked for one hour using 2% BSA in tris- (collagen and collagen peptides) or phosphate- (other proteins) buffered saline with 0.05% Tween® 20. Bound rhMMRN1 was detected using 0.14 µg/well of JS-1 followed by 0.04 µg/well of HRP-conjugated donkey anti-mouse IgG and then 3,3',5,5'-tetramethylbenzidine (TMB). The reaction was stopped after 10 minutes using 1 M H_2SO_4 and absorbance was measured at 450 nm using a Sunrise™ microplate absorbance reader and Magellan™ software (Tecan Austria GmbH, Grödig, AU). rhMMRN1 binding was tested in triplicate within each experiment to obtain a mean

and data are expressed as the mean optical density (OD) at a wavelength of 450 nm ±

standard deviation (SD).

2.2.11 *In silico* analyses of collagen amino acid sequences

Amino acid sequences for collagen were obtained from the Universal Protein

Knowledgebase (UniProtKB), which obtains sequence data from translated coding

sequences submitted to the EMBL-Bank/GenBank/DDJ nucleotide sequences resources

(International Nucleotide Sequence Database Collaboration). Alignments, sequence

similarity, and sequence identity were determined using Clustal Omega software.

2.2.12 Assays of static platelet adhesion using human or mouse platelets

Human and mouse blood for assays of static platelet adhesion were collected into

1:10 (v/v) acid citrate dextrose (ACD) anticoagulant. ACD-anticoagulated mouse blood

was supplemented with 500 µl of platelet wash buffer (2.35 mM citric acid, 5.5 mM D-

glucose, 128 mM NaCl, 4.26 mM $Na_2HPO_4 \cdot H2O$, 7.46 mM $NaH_2PO_4 \cdot H2O$, 4.77 mM

trisodium citrate dihydrate, 0.35% (m/v) BSA, 3 µM PGE_1, pH 6.5) before blood was

centrifuged to obtain platelet-rich plasma (PRP) diluted in buffer (260 x g, 5 minutes,

room temperature). The remaining blood was supplemented once more with 500 µl of

platelet wash buffer and centrifuged a second time to collect the remaining platelets in

buffer (260 x g, 5 minutes, room temperature). Platelets were pelleted and washed twice

using platelet wash buffer before final resuspension in adhesion buffer (50 mM Tris, 140

mM NaCl, 2 mM $MgCl_2$, 25 µM $CaCl_2$, 0.1% BSA, pH 7.4) at a concentration of 1.8 x

10^8 platelets/ml. Platelet concentration for final resuspension was estimated using a C-

Chip DHC-N01 disposable hemocytometer and a Zeiss Axiovert 25 inverted light

microscope fitted with a x10 objective lens (Carl Zeiss Canada Ltd., Toronto, CA).

Mouse platelets in buffer were left to rest at room temperature for 20 minutes to allow the

effects of inhibitors to wear off as described.[423,540,545] For some experiments, mouse

platelets were pre-activated using either 10 µg/ml CRP or murine TRAP (AYPGKF-NH_2)

for 5 minutes at 37°C to induce Mmrn1 release and activate platelet integrins. In

experiments using monoclonal anti-mouse β_3 blocking antibody 9D2, platelets were

simultaneously incubated with 10 µg/ml CRP and 10 µg/ml of antibody 9D2 for 5

minutes at 37°C.

ACD-anticoagulated human blood (20 ml/donor) was collected from the

antecubital vein of general population controls, after obtaining written informed consent,

then centrifuged (140 x g, 15 minutes, room temperature) to obtain PRP. PRP was

supplemented with platelet wash buffer before pelleting and washing the platelets twice

(740 x g, 10 minutes, room temperature) using platelet wash buffer. Pelleted platelets

were resuspended at 1.6 x 10^8 platelets/ml in adhesion buffer and similarly left to rest for

20 minutes at room temperature. Human platelet concentration was estimated using a

Coulter AcT diff Hematology Analyzer (Beckman Coulter, Inc., Brea, CA, USA) for final

resuspension. Where indicated, platelets were pre-activated using 10 µg/ml CRP (5 minutes, 37°C).

Static platelet adhesion was evaluated colourimetrically as described,[423,436,540,545,565] using wells pre-coated overnight at 4°C with 1 µg/well of: Horm collagen, rhMMRN1, mouse fibrinogen, mouse fibronectin, or collagen peptides. In experiments in which two or more collagen peptides were coated onto the well surface, 0.5 µg/well of each peptide was used for two peptides or 0.33 µg/well of each peptide for three peptides. In some experiments, MMRN1-binding GPAGPOGPX peptides were tested for platelet adhesion using a range of molarities (0.02 to 4 mM/well). Horm collagen and collagen peptides were coated using 0.1 M acetic acid, and rhMMRN1, mouse fibrinogen and mouse fibronectin were coated using carbonate buffer. In some wells, immobilized mouse fibrinogen was converted to fibrin *in situ* using 200 mU/ml of human thrombin, similar to methods described.[174] Wells were blocked for one hour using 5% BSA in TBS before 50 µl of platelet suspension was added to each well. After one hour, wells were washed to remove non-adherent platelets and the remaining adherent platelets were lysed using lysis buffer (30 mM citric acid, 70 mM trisodium citrate, 0.1% Triton™ X-100) containing 5 mM p-nitrophenyl phosphate. After one hour, the reaction was stopped by 2 M NaOH and absorbance was measured at 405 nm using a Sunrise™ microplate absorbance reader and Magellan™ software. Platelet adhesion was tested in triplicate within each experiment to obtain a mean, and data are expressed as the mean OD at a wavelength of 405 nm ± SEM.

2.2.13 Assays of platelet adhesion under high shear using collagen peptides

Mouse blood was collected into 1:10 (v/v) ACD and similarly supplemented with buffer and centrifuged twice (260 x g, 5 minutes, room temperature) to obtain PRP diluted in buffer before final resuspension at a concentration of 3 x 10^8 platelets/ml in adhesion buffer. The remaining red blood cells (RBCs) were pelleted and washed twice (1000 x g, 4 minutes, room temperature) using RBC washing buffer (140 mM Na_2CO_3, 35 mM $NaHCO_3$, 100 mu/ml apyrase).

Vena8 Fluoro+ microcapillary perfusion chambers were coated overnight at 4°C using 1 µg/channel of triple-helical collagen peptide dissolved in 0.01 M acetic acid. Chambers were coated with combinations of two triple-helical peptides, maintaining a total peptide amount of 1 µg/channel, as described previously.[175] In channels where only one adhesive peptide was presented, GPP (0.5 µg/channel) was added to maintain the total peptide amount at 1 µg/channel. Channels were blocked with 5% (m/v) BSA in adhesion buffer for 30 minutes prior to the experiment.

Platelets were labelled using 4 µM DiOC6(3) for 10 minutes at 37°C before pre-activation with 10 µg/ml of CRP for 5 minutes at 37°C. The platelet suspension was subsequently gently mixed with 1:1.2 (v/v) pelleted autologous washed RBCs immediately prior to perfusion. Each channel was perfused at a constant shear rate using a Cellix Mirus 1.1 Nanopump controlled by VenaFlux™ software. Channels were

subsequently washed with HEPES-buffered Tyrode's solution containing 1 U/ml heparin to remove non-adherent platelets before imaging.

2.3 STATISTICAL ANALYSES

Two-tailed Student's t-tests (paired or independent) were used for comparisons of two groups of data with normal distributions. Mann-Whitney U tests were used to evaluate data with non-normal distributions. Normality was determined visually by plotting data to assess spread and skewness and using the Shapiro-Wilk test. One-way or repeated measures ANOVA were used to evaluate data with more than two groups. In all single comparisons, $\alpha < 0.05$ was considered significant. Bonferroni correction was used for *post hoc* multiple comparisons where $k \leq 6$ or the Holm-Sidak method where $k > 6$, with $\alpha < 0.05$ considered significant for each family of comparisons. Statistical tests were performed using Prism 6 (GraphPad Software, San Diego, CA, USA). Data are reported as the mean $\pm$ standard error of the mean (SEM), unless otherwise stated.

CHAPTER 3: RESULTS

3.1 MULTIMERIN 1-DEFICIENT MICE

Some aspects of the phenotype of selective Mmrn1-deficient ($Mmrn1^{-/-}$) mice were characterized by Dr. D'Andra Parker as part of her doctoral thesis work at McMaster University under the supervision of Dr. Catherine Hayward and are outlined in *section 1.1.3*.

Similar to previous observations, mice homozygous for the E3-deleted *Mmrn1* allele ($Mmrn1^{-/-}$) used in these studies were viable and fertile without overt developmental abnormalities, spontaneous bleeding, or reduced survival. Each mouse used in experiments was confirmed to possess the predicted P1-P2 fragment size based on their *Mmrn1* genotype, consistent with previous analyses of this strain (*Figure 7*).
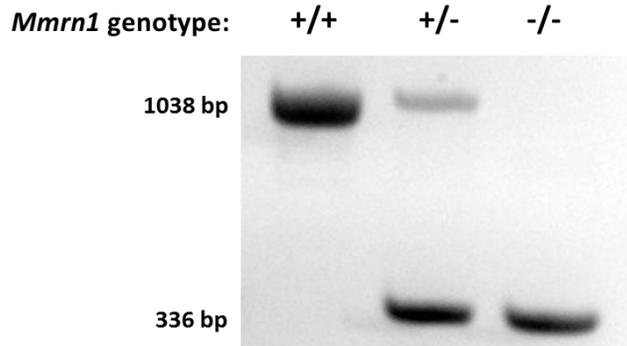
Figure 7: Electrophoretic mobility of P1-P2 fragments from *Mmrn1*⁺/⁺, *Mmrn1*⁺/⁻, and *Mmrn1*⁻/⁻ mice. The 1038 bp *Mmrn1* P1-P2 fragment represents the intact allele, and the 336 bp P1-P2 fragment represents the E3-deleted *Mmrn1* allele. Heterozygous mice show a darker band for the 336 bp fragment because smaller sequences are favoured in PCR, which results in greater amplification of the 336 bp P1-P2 fragment relative to the wild-type allele.

3.2 AGGREGATE FORMATION OF WILD-TYPE AND *Mmrn1*⁻/⁻ MOUSE

PLATELETS ON HORM COLLAGEN AND VWF UNDER HIGH SHEAR FLOW

Experiments measuring endpoint platelet adhesion to Horm collagen or recombinant (r)Vwf under high shear flow (1500 s⁻¹) were performed by Dr. D'Andra Parker (*as described in section 1.1.3*). I expanded upon these analyses by developing a method to quantify the size and number of fluorescently-labelled platelet aggregates, and I performed additional experiments to clarify the mechanism of the defect in platelet adhesion to Horm collagen exhibited by *Mmrn1*⁻/⁻ mice.

Mmrn1⁻/⁻ mice formed significantly smaller aggregates onto collagen at the end of perfusions (p≤0.004, *Figure 8*). Because MMRN1 is a ligand for VWF[21] and enhances

platelet adhesion to Horm collagen under high shear flow (1500 s^{-1}) through a mechanism involving both VWF and GPIbα,[2] platelet aggregate formation on rVwf surfaces was similarly tested. On average, *Mmrn1*$^{-/-}$ platelets formed significantly smaller aggregates compared to wild-type platelets ($p < 0.0001$, *Figure 9A*). The similar adhesion ($p = 0.7$) but smaller size of platelet aggregates ($p < 0.0001$, *Figure 9B*) adherent to rVwf for *Mmrn1*$^{-/-}$ platelets was also observed in experiments using washed platelets reconstituted in washed red blood cells (RBCs) to rVwf.
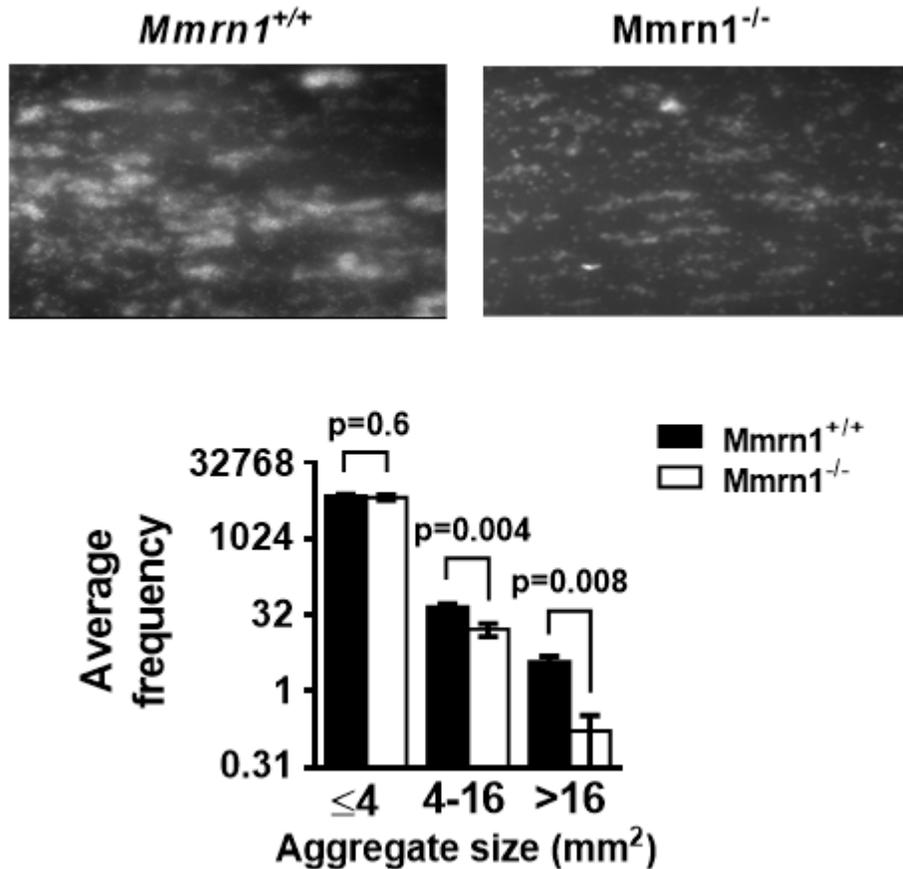
Figure 8: Aggregate size of wild-type versus *Mmrn1*$^{-/-}$ mouse platelets adherent to Horm collagen under high shear flow (1500 s^{-1}). (A) Representative images (original magnification x40) showing adhesion of wild-type and *Mmrn1*$^{-/-}$ platelets tested in whole blood to assess adhesion to Horm collagen, and (B) quantitative analysis of average frequency of different-sized platelet aggregates captured (*n* = 7 mice/group) ± SEM.
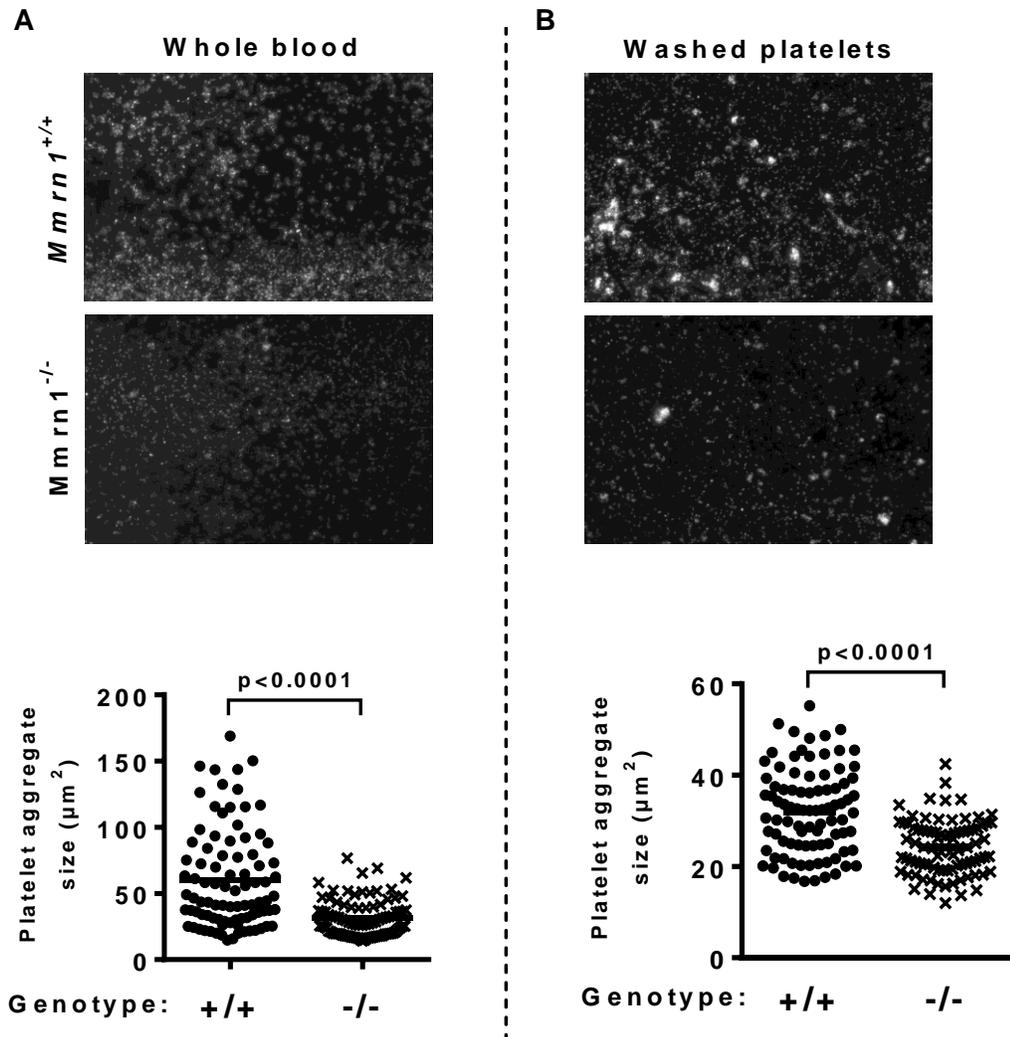
Figure 9: Aggregate size of wild-type versus *Mmrn1$^{-/-}$* mouse platelets adherent to recombinant (r)Vwf under high shear flow (1500 s^{-1}). Representative images (original magnification x20) showing adhesion of wild-type and *Mmrn1$^{-/-}$* platelets in (A) whole blood or (B) as preparations of washed platelets reconstituted with washed RBCs. Quantitative data shown compares the average frequency of different-sized platelet aggregates captured onto rVwf (*n* = 6 mice/group) ± SEM. Symbols indicate *Mmrn1* genotype (circles: *Mmrn1$^{+/+}$*; x's: *Mmrn1$^{-/-}$*).

It was unclear if the defect in endpoint adhesion to Horm collagen observed for *Mmrn1*$^{-/-}$ platelets was due to: 1) impaired platelet tethering and/or primary adhesion, 2) slower aggregate growth mediated via platelet-platelet interactions, 3) aggregate instability or embolization during perfusions, or a combination of multiple factors. I performed assays of platelet adhesion under high shear flow in real-time to measure the rate and amount of platelet accumulation onto immobilized Horm collagen. *Mmrn1*$^{-/-}$ platelets showed decreased primary adhesion to Horm collagen (*Figure 10A,B*, as indicated by a lower initial spike in fluorescence), and a slower rate of accumulation of fluorescent platelets during the 5-minute perfusion (*Figure 10A,B*).
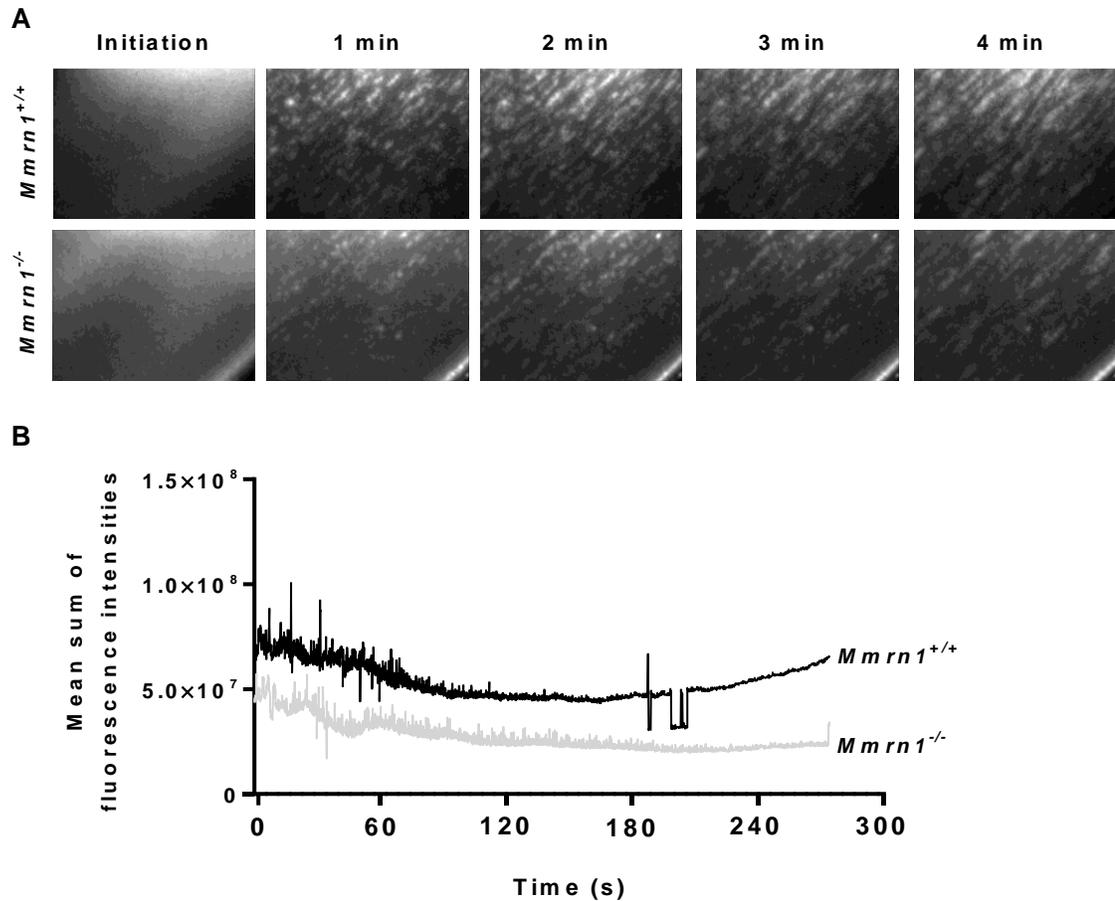
Figure 10: Real-time adhesion of wild-type versus *Mmrn1^{-/-}* mouse platelets to Horm collagen under high shear flow (1500 s⁻¹). (A) Representative time series images and (B) quantitative analyses of platelet adhesion to Horm collagen. The mean sum of fluorescence intensities, captured every 50 ms in a 450x450 pixel field at the centre of the microcapillary, was calculated for each biological replicate. The data are presented as the mean for each group (*n* = 3 mice/group). Time zero denotes induction of DiOC6(3)-labelled platelets in whole blood into the collagen-coated microcapillary, which spikes due to initial focus blur. The time at which blood was inducted into the microcapillary during the recording time was manually aligned for each replicate.

Platelet adhesion was also tested under low shear flow (300 s^{-1}) to determine if the effects of Mmrn1 loss on adhesion and aggregate formation on fibrillar collagen required high shear flow. *Mmrn1*$^{-/-}$ platelets formed visually smaller aggregates compared to wild-type platelets under low shear flow, although the morphology of captured aggregates (which extended through multiple planes of focus) for both wild-type and Mmrn1 deficient platelets prevented similar quantitative analysis of captured 2D images (*Figure 11*). Interestingly, long fluorescent strings were visible following perfusion of collagen with PPACK-anticoagulated wild-type mouse blood, which appeared to extend into the lumen of the chamber. These strings were not present in chambers perfused with *Mmrn1*$^{-/-}$ blood, and the morphology of *Mmrn1*$^{-/-}$ platelet aggregates on collagen was more nodular.
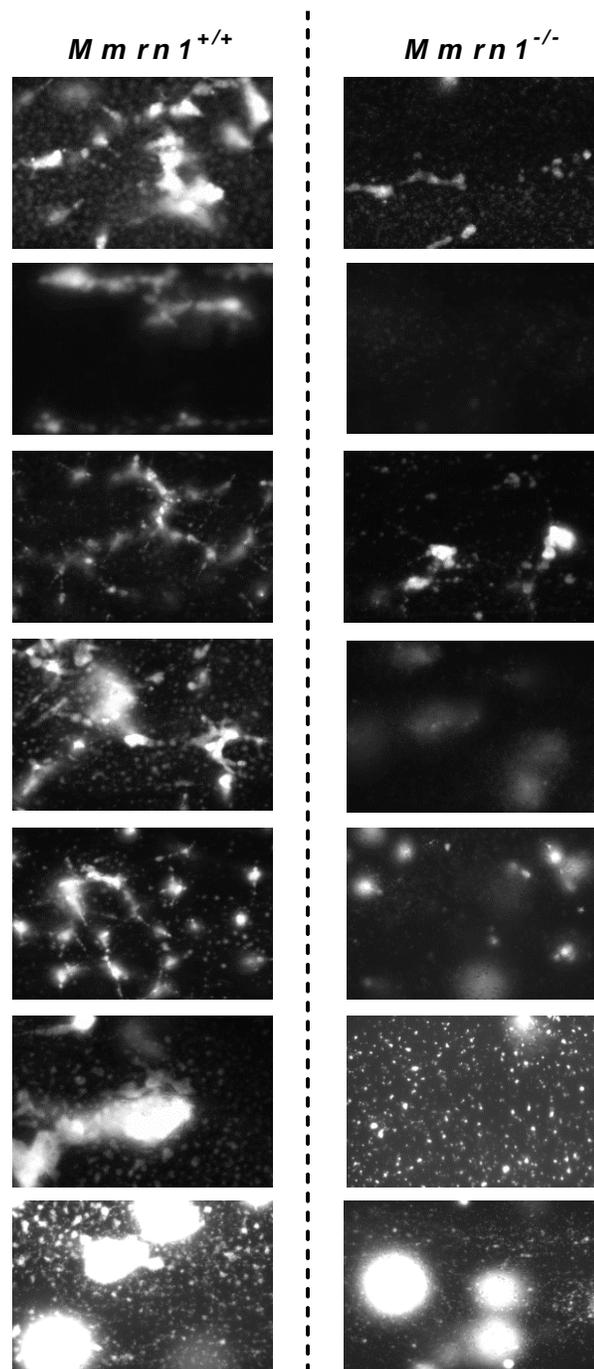
Figure 11: Adhesion of wild-type versus *Mmrn1*^{-/-} mouse platelets to Horm collagen under low shear flow (300 s⁻¹). Representative images showing adhesion of wild-type (left) and *Mmrn1*^{-/-} (right) platelets for each biological replicate tested ($n = 7$ mice/group) after focusing the image at the chamber surface.

3.3 ADHESION OF WILD-TYPE AND *Mmrn1*$^{-/-}$ MOUSE PLATELETS TO FIBRINOGEN, FIBRIN, AND FIBRONECTIN

I investigated the possibility that Mmrn1 affects aggregate formation on collagen and rVwf by influencing stable platelet-platelet interactions involving fibrinogen and/or fibronectin. First, I performed binding assays to determine if MMRN1 is a ligand for fibrinogen and/or fibronectin, before proceeding to whole blood perfusion assays to test the impact of Mmrn1 loss on platelet adhesion to immobilized fibrinogen or fibronectin. Fibrin was also included in these analyses to further elucidate the mechanism of impaired thrombus formation in FeCl$_3$-injured vessels of *Mmrn1*$^{-/-}$ mice.

rhMMRN1 showed dose-dependent binding to human fibrinogen, fibrin, and fibronectin FN that was significantly greater than non-specific binding to BSA ($p<0.05$, *Figure 12*). Under static conditions, activated *Mmrn1*$^{-/-}$ and wild-type showed similar adhesion to immobilized murine fibrinogen (Fg, $p=0.32$), fibrin ($p=0.14$), and murine fibronectin (Fn, $p=0.32$, *Figure 13*).
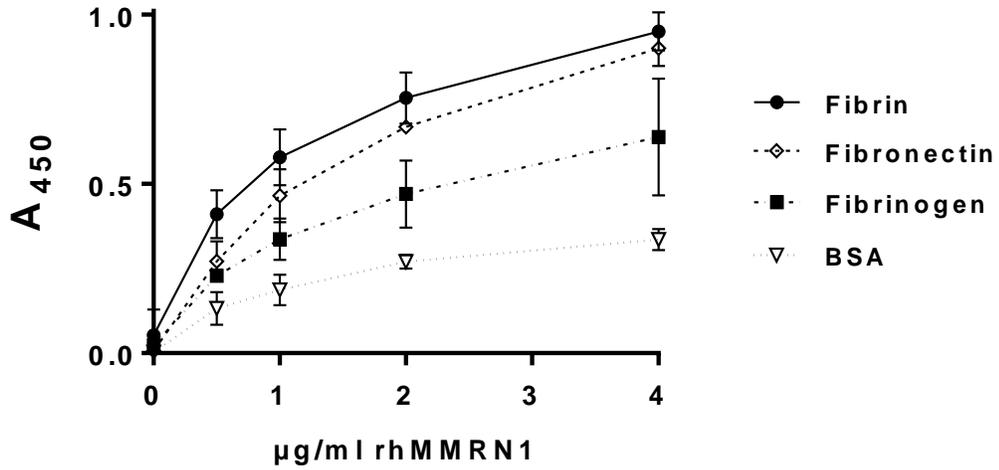
Figure 12: rhMMRN1 binding to human FG, fibrin, or FN. Absorbance values are reported as the mean of three independent experiments ± SD. Within each experiment, determinations were performed in triplicate to obtain a mean.
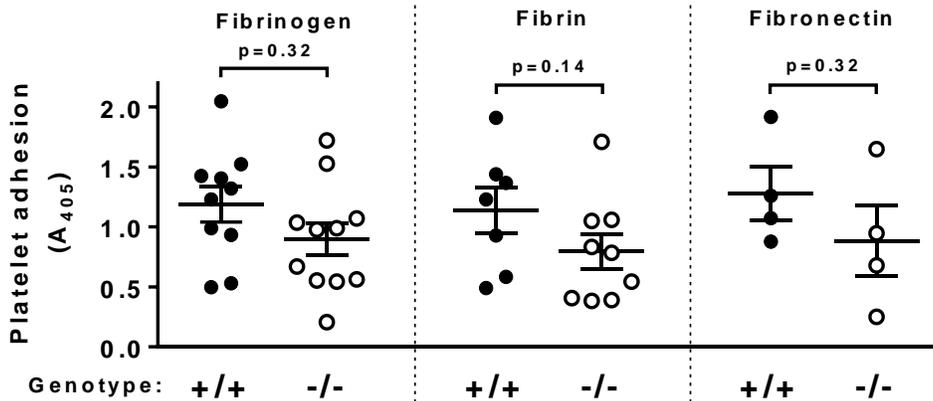
Figure 13: Static adhesion of wild-type versus *Mmrn1^-/-* CRP-activated mouse platelets to murine Fg, fibrin, and Fn. Data are shown as mean absorbance measured in each sample, each tested in triplicate with each protein to obtain a mean. Washed wild-type and Mmrn1-deficient platelets were pre-activated with 10 µg/ml CRP to induce release of platelet α-granule proteins, including Mmrn1, and to activate platelet integrins. Symbols indicate *Mmrn1* genotype (closed: *Mmrn1^+/+^*; open: *Mmrn1^-/-^*).

Under low shear flow (300 s^{-1}), untreated *Mmrn1*$^{-/-}$ and wild-type platelets in whole blood showed similar surface coverage (p=0.31, *Figure 14*) and aggregate formation (p=0.2, *Figure 15A*) onto immobilized Fg, however, significantly fewer *Mmrn1*$^{-/-}$ platelets than wild-type platelets adhered to Fg (p<0.0001, *Figure 15B*). When platelets were pre-activated with 30 µM murine TRAP (AYPGKF-NH$_2$) to induce the release of α-granule contents and pre-activate platelet integrins, the surface coverage of *Mmrn1*$^{-/-}$ platelets was significantly reduced compared to wild-type platelets (p=0.03, *Figure 14B*). TRAP-activated *Mmrn1*$^{-/-}$ platelets also formed smaller aggregates on Fg compared to activated wild-type platelets (p=0.007, *Figure 15A*). Lastly, significantly fewer *Mmrn1*$^{-/-}$ platelet aggregates adhered to Fg compared to wild-type platelets (p=0.03, *Figure 15B*).

A   Low shear (300 s^{-1}) adhesion to fibrinogen

B
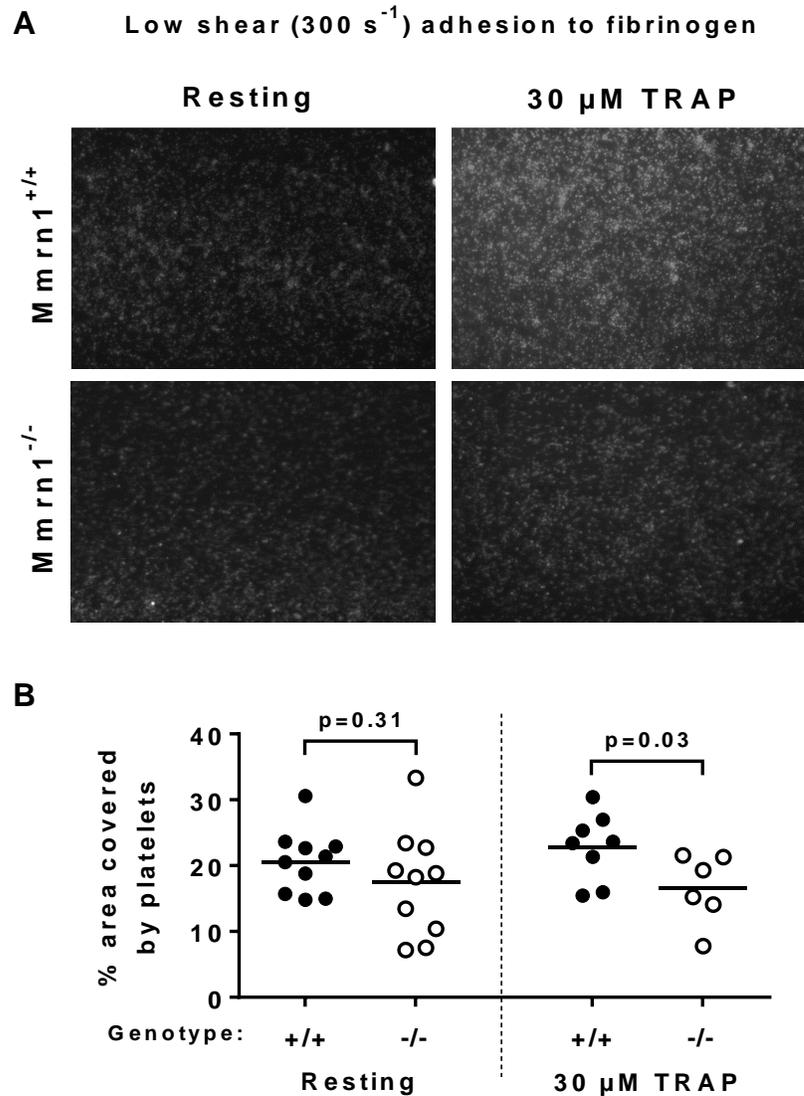
Figure 14: Wild-type versus *Mmrn1$^{-/-}$* mouse platelet adhesion to immobilized murine fibrinogen under low shear flow (300 s^{-1}). (A) Representative images (original magnification x20) and (B) quantitative analyses of % surface area covered by platelets. PPACK-anticoagulated whole blood samples were perfused over fibrinogen for 3 minutes under constant shear and subsequently washed to remove non-adherent platelets. Data represent the mean % area covered (*n*=10 images per mouse) for each biological replicate and bars indicate sample mean. Symbols in panel B indicate *Mmrn1* genotype (closed: *Mmrn1$^{+/+}$*; open: *Mmrn1$^{-/-}$*).
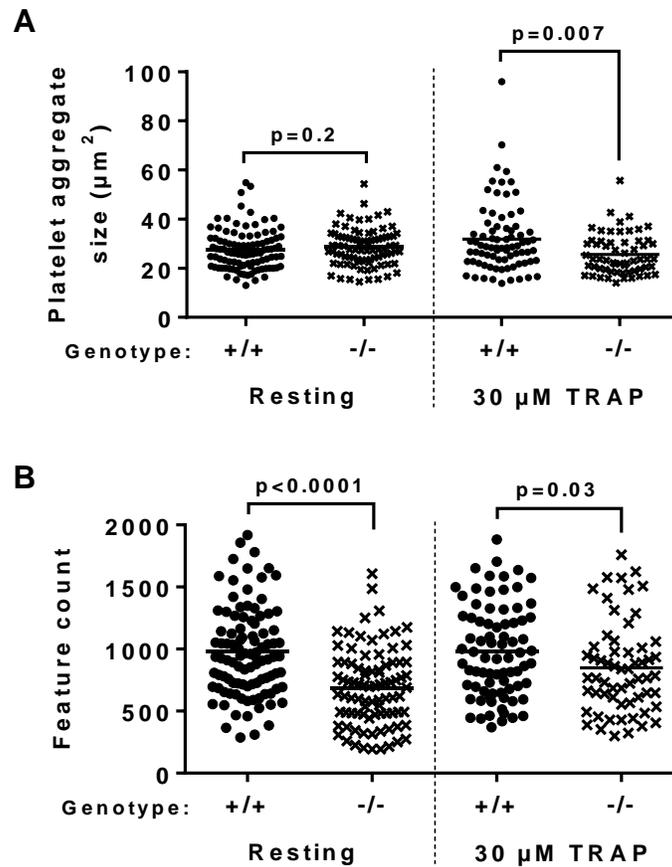
Figure 15: Wild-type versus *Mmrn1*$^{-/-}$ mouse platelet aggregate size and feature count captured onto immobilized murine fibrinogen under low shear flow (300 s^{-1}). Data indicate (A) the mean feature size and (B) the feature count in each image captured for wild-type resting (*n*=10 mice) and TRAP-activated (*n*=8 mice) and for *Mmrn1*$^{-/-}$ resting (*n*=10 mice) and TRAP-activated (*n*=6 mice) samples. Symbols indicate *Mmrn1* genotype (circles: *Mmrn1*$^{+/+}$; x's: *Mmrn1*$^{-/-}$).

Further experiments indicated that *Mmrn1*$^{-/-}$ and wild-type platelets in whole blood showed similar adhesion (p=0.84, *Figure 16*), aggregate formation (p=0.16, *Figure 17A*), and numbers of platelet features (p=0.73, *Figure 17B*) when perfused over immobilized murine fibrin. The pre-activation of platelets with 30 µM TRAP did not significantly alter these parameters (respective p values: adhesion: 0.41, *Figure 16B*; aggregate 0.18, *Figure 17A*; platelet features 0.72, *Figure 17B*).
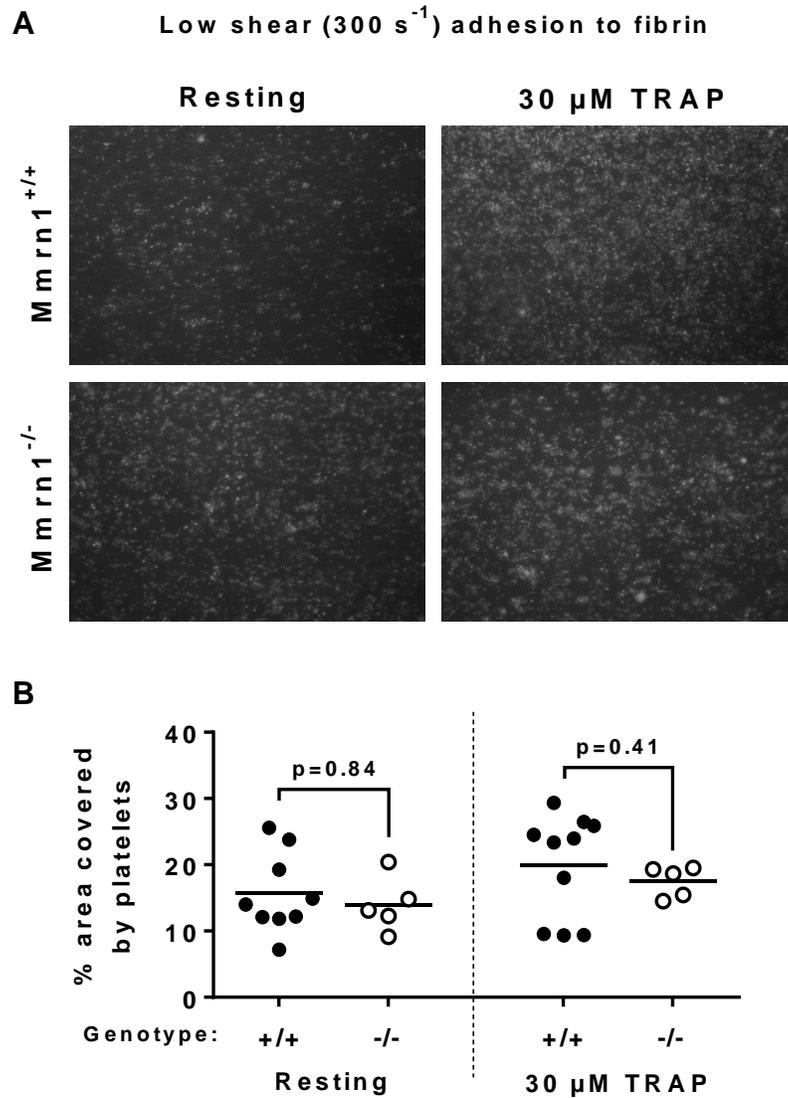
Figure 16: Wild-type versus *Mmrn1-/-* mouse platelet adhesion to immobilized murine fibrin under low shear flow (300 s⁻¹). (A) Representative images (original magnification x20) and (B) quantitative analyses of % surface area covered by platelets. PPACK-anticoagulated whole blood samples were perfused over fibrin for 3 minutes under constant shear and subsequently washed to remove non-adherent platelets. Data represent the mean % area covered by platelets (*n*=10 images per mouse) for each biological replicate and bars indicate sample mean. Symbols in panel B indicate *Mmrn1* genotype (closed: *Mmrn1+/+*; open: *Mmrn1-/-*).
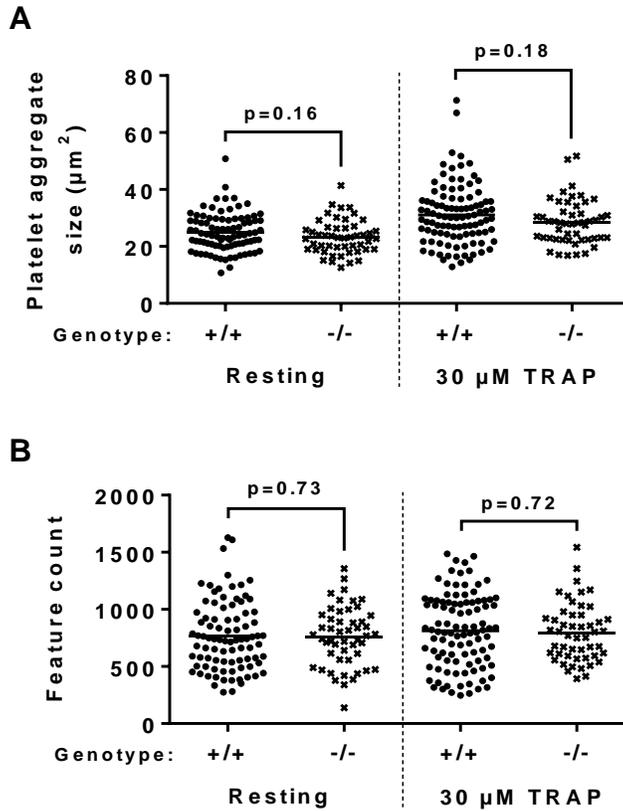
Figure 17: Wild-type versus *Mmrn1*$^{-/-}$ mouse platelet aggregate size and feature count captured onto immobilized murine fibrin under low shear flow (300 s^{-1}). Data indicate (A) the mean feature size and (B) the feature count in each image captured for wild-type resting (*n*=9 mice) and TRAP-activated (*n*=10 mice) and for *Mmrn1*$^{-/-}$ resting (*n*=5 mice) and TRAP-activated (*n*=5 mice) samples. Symbols indicate *Mmrn1* genotype (circles: *Mmrn1*$^{+/+}$; x's: *Mmrn1*$^{-/-}$).

Labelled mouse platelets in whole blood showed less adhesion to immobilized murine fibronectin under low shear flow (300 s^{-1}) compared to fibrinogen- or fibrin-coated surfaces, and adhesion of resting or TRAP-activated platelets to fibronectin was similar between *Mmrn1*$^{-/-}$ and wild-type samples (p=0.69 and p=0.62, respectively, *Figure 18*). Platelets tended to adhere individually, and did not form larger aggregates on

fibronectin surfaces following TRAP activation. Resting $Mmrn1^{-/-}$ platelets formed significantly larger aggregates on fibronectin compared to wild-type platelets (p=0.03), and TRAP-activated $Mmrn1^{-/-}$ platelets formed significantly smaller aggregates on fibronectin compared to wild-type platelets (p=0.03, *Figure 19A*). However, the mean differences in aggregate size were negligible: 4.5 μm^2 for resting platelets and 0.78 μm^2 for TRAP-activated platelets. Feature counts were similar between $Mmrn1^{-/-}$ and wild-type samples for resting (p=0.64) and TRAP-activated (p=0.92) platelets (*Figure 19B*). Pre-activation with TRAP did not significantly increase the number of adherent $Mmrn1^{-/-}$ (p=0.48) or wild-type (p=0.23) platelets.

A Low shear (300 s⁻¹) adhesion to fibronectin
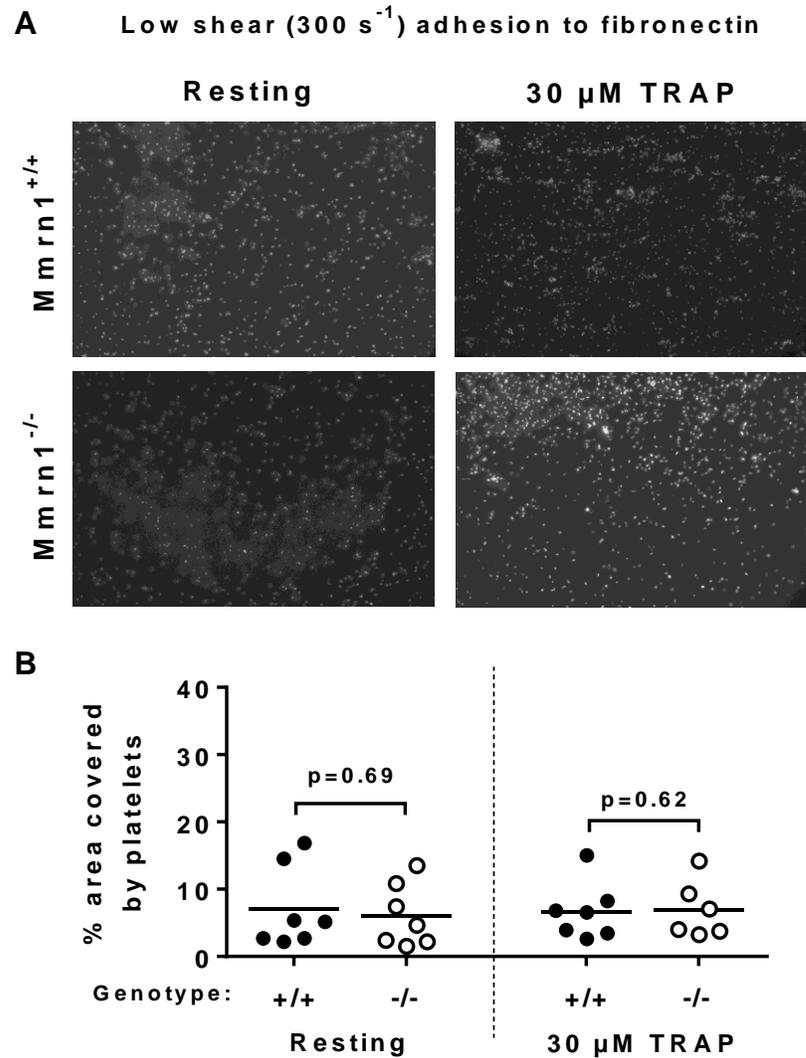
Figure 18: Wild-type versus *Mmrn1⁻ᐟ⁻* mouse platelet adhesion to immobilized murine fibronectin under low shear flow (300 s⁻¹). (A) Representative images (original magnification x20) and (B) quantitative analyses of % surface area covered by platelets. PPACK-anticoagulated whole blood samples were perfused over fibronectin for 3 minutes under constant shear and subsequently washed to remove non-adherent platelets. Data represent the mean % area covered by platelets (*n*=10 images per mouse) for each biological replicate and bars indicate sample mean. Symbols in panel B indicate *Mmrn1* genotype (closed: *Mmrn1⁺ᐟ⁺*; open: *Mmrn1⁻ᐟ⁻*).
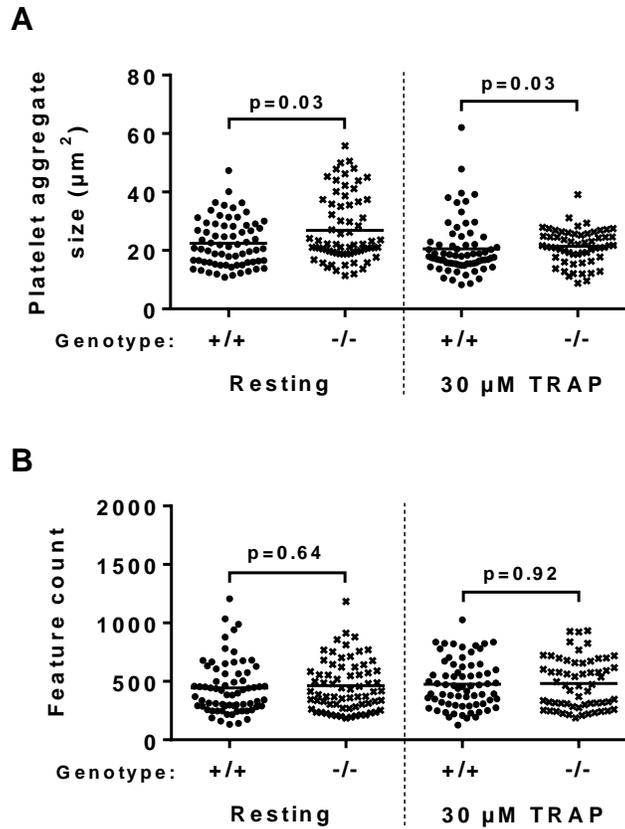
Figure 19: Wild-type versus *Mmrn1-/-* mouse platelet aggregate size and feature count captured onto immobilized murine fibronectin under low shear flow (300 s^{-1}). Data indicate (A) the mean feature size and (B) the feature count in each image captured for wild-type resting and TRAP-activated (*n*=7 mice) and for *Mmrn1-/-* resting (*n*=7 mice) and TRAP-activated (*n*=6 mice) samples. Symbols indicate *Mmrn1* genotype (circles: *Mmrn1+/+*; x's: *Mmrn1-/-*).

3.4 EFFECT OF INTEGRIN ALPHA-IIB BETA-3 ON STATIC ADHESION OF ACTIVATED WILD-TYPE AND *Mmrn1*^{-/-} MOUSE PLATELETS

I performed assays of static platelet adhesion with and without anti-mouse β_3 blocking antibody 9D2 to determine if mouse platelet adhesion to Mmrn1 is mediated by β_3 integrins. In the absence of platelet Mmrn1, activated *Mmrn1*^{-/-} and wild-type platelets showed similar adhesion to immobilized rhMMRN1 (p=0.49), indicating that platelet Mmrn1 is not essential for this adhesion. Antibody 9D2 inhibited adhesion of both *Mmrn1*^{-/-} and wild-type platelets to rhMMRN1, by 35.7±5.3% and 45.1±3.5%, respectively (p=0.15, *Figure 20*). 9D2 similarly inhibited the static adhesion of *Mmrn1*^{-/-} versus wild-type mice to Fg (% inhibition for *Mmrn1*^{-/-} vs. *Mmrn1*^{+/+}: 77±7.5 vs. 67.4±3.7, p=0.3), fibrin (% inhibition for *Mmrn1*^{-/-} vs. *Mmrn1*^{+/+}: 75.7±7.6 vs. 63.7±2.0, p=0.17), and Fn (% inhibition for *Mmrn1*^{-/-} vs. *Mmrn1*^{+/+}: 68.6±5.0 vs. 63±3.3, p=0.39), which were tested as positive controls for β_3-dependent adhesion. These data suggest that under these conditions, Mmrn1-mediated adhesion involves other, unidentified ligands. Additionally, inhibition of static platelet adhesion to rhMMRN1 did not increase with increasing amount of blocking antibody (*Figure 21*), indicating that 9D2 binding to platelets was saturable at the concentration tested to induce β_3 inhibition.
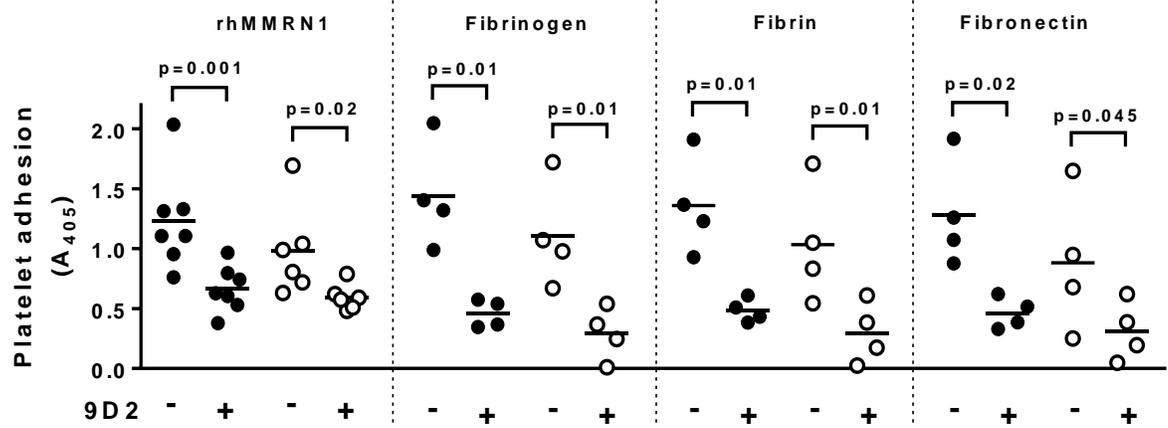
Figure 20: Effect of anti-mouse β3 blocking antibody 9D2 on static adhesion of wild-type versus *Mmrn1*-/- CRP-activated mouse platelets. Data are shown as mean absorbance of each biological replicate, each tested in triplicate for each protein to obtain a mean. Platelets were pre-activated with CRP, in order to induce Mmrn1 release, and incubated with antibody 9D2 (5 minutes, 37°C) to block β3-mediated adhesion. Symbols indicate the *Mmrn1* genotype (solid: +/+; open: -/-).
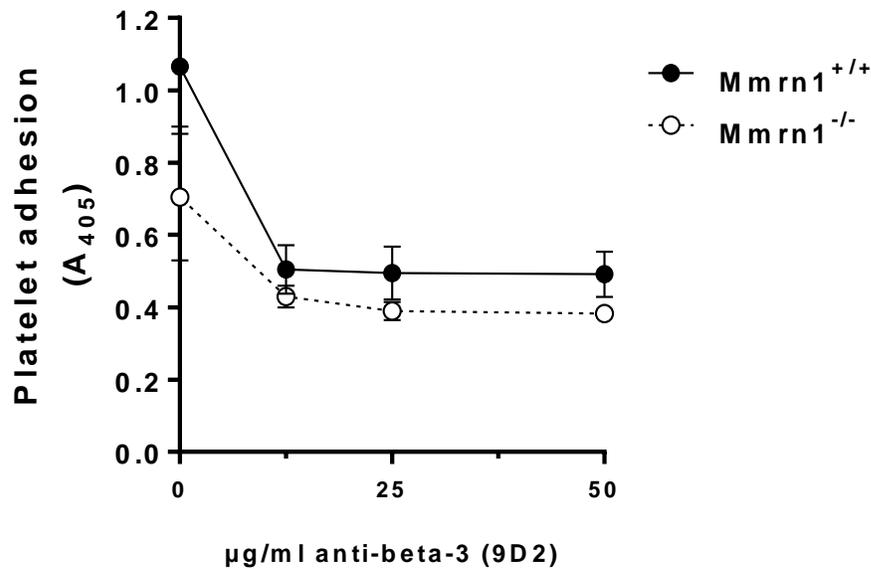
Figure 21: Effect of 9D2 concentration on static adhesion of wild-type versus *Mmrn1^{-/-}* CRP-activated mouse platelets to immobilized rhMMRN1. Data are shown as mean absorbance of *n*=3 mice/genotype ± SEM. Within each experiment, determinations were performed in triplicate to obtain a mean. Symbols indicate the *Mmrn1* genotype (solid: +/+; open: -/-).

3.5 COLLAGEN-INDUCED AGGREGATION OF WILD-TYPE AND *Mmrn1^{-/-}*

PLATELETS IN WHOLE BLOOD

I performed assays of collagen-induced platelet aggregation to determine if Mmrn1 contributes to collagen-induced platelet aggregation in whole blood samples. *Mmrn1^{-/-}* and wild-type mice showed similar aggregation profiles and similar maximal aggregation responses to 5 µg/ml (p=0.48) or 10 µg/ml (p=0.36) Horm collagen (*Figure 22*).
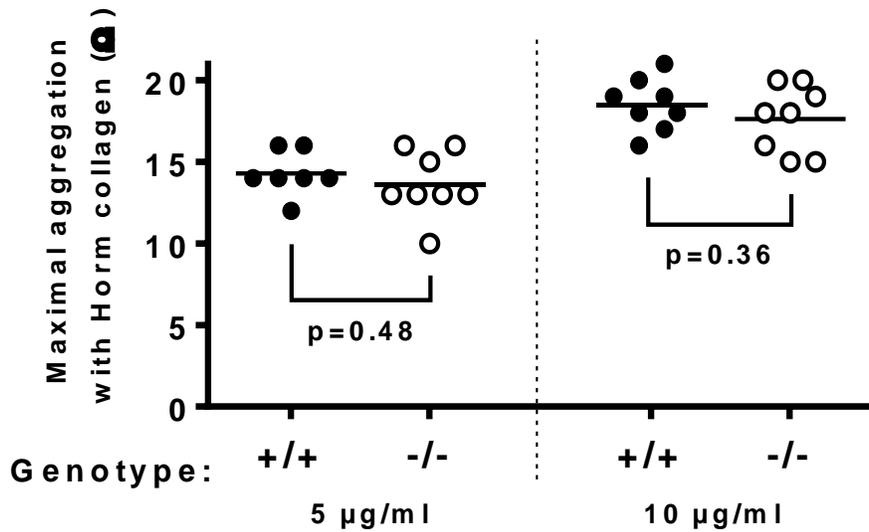
Figure 22: Aggregation responses of wild-type versus *Mmrn1⁻ᐟ⁻* mouse platelets tested in whole blood to Horm collagen. Maximal aggregation responses of mouse platelets to Horm collagen measured by electrical impedance (Ω) (*n*=7-8 mice/genotype). Symbols indicate Mmrn1 genotype (closed: *Mmrn1⁺ᐟ⁺*; open: *Mmrn1⁻ᐟ⁻*).

3.6 SHEAR-INDUCED AGGREGATION OF WILD-TYPE AND *Mmrn1⁻ᐟ⁻* MOUSE PLATELETS

I tested whether Mmrn1 loss influences platelet aggregate formation under high and pathological levels of mechanical shear stress using assays of shear-induced platelet aggregation in whole blood. Samples from wild-type and *Mmrn1⁻ᐟ⁻* mice showed similar platelet counts in resting whole blood samples (*Mmrn1⁺ᐟ⁺* vs. *Mmrn1⁻ᐟ⁻* ± SEM: 638 ± 119 vs. 652 ± 115, p=0.94). With both types of platelets, the detection of CD42b+ single platelets decreased incrementally with exposure to increasing shear rate (*Figure 23*). The % loss of single platelets, relative to the resting samples, was not significantly different

between *Mmrn1$^{-/-}$* and wild-type platelets at 5000 s^{-1} (p=0.81), 10000 s^{-1} (p=0.6), or

15000 s^{-1} (p=0.94, *Figure 24B*). Fluorescent microscopic analyses of SIPA similarly

showed that *Mmrn1$^{-/-}$* or wild-type platelets in whole blood formed aggregates with

similar morphology, which, on average, increased in size with increasing shear rate

(*Figure 24C*). To test SIPA in combination with collagen-induced platelet aggregation,

samples in some experiments were sheared immediately after pre-treatment with varying

concentrations of Horm collagen or CRP, but pre-activation coupled with shear stress

caused samples to clot, which made retrieval for analysis unfeasible.
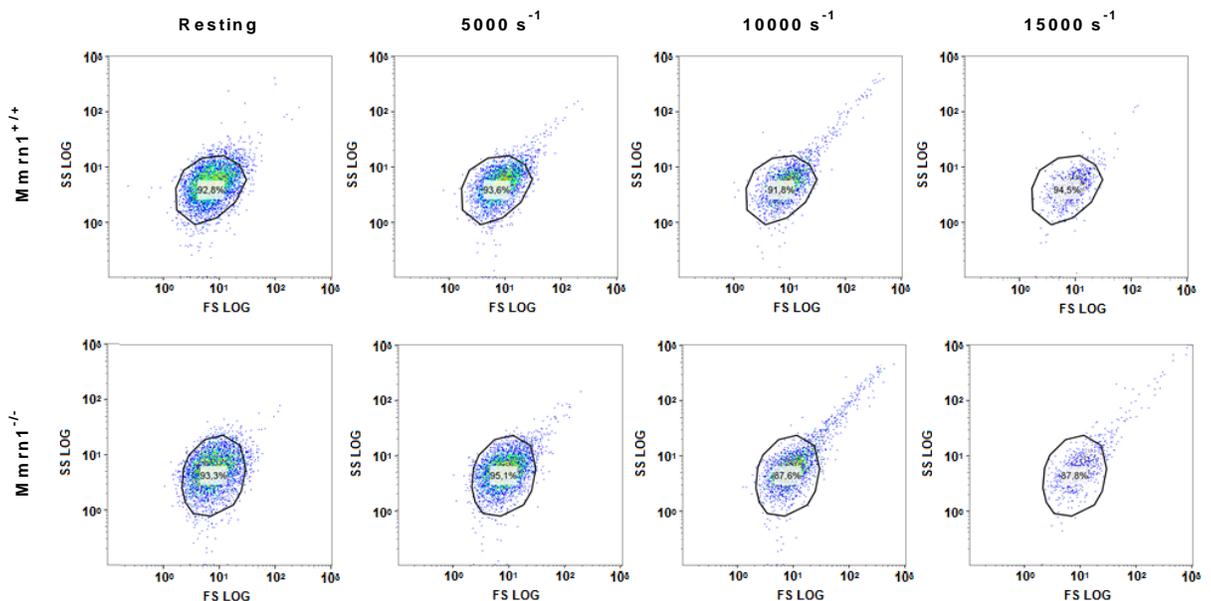
Figure 23: Representative scatterplots showing the effects of shear on wild-type versus *Mmrn1$^{-/-}$* mouse platelets detected by flow cytometry. A total of 50,000 events were collected and gated for FITC-positive events using autologous unlabelled samples.
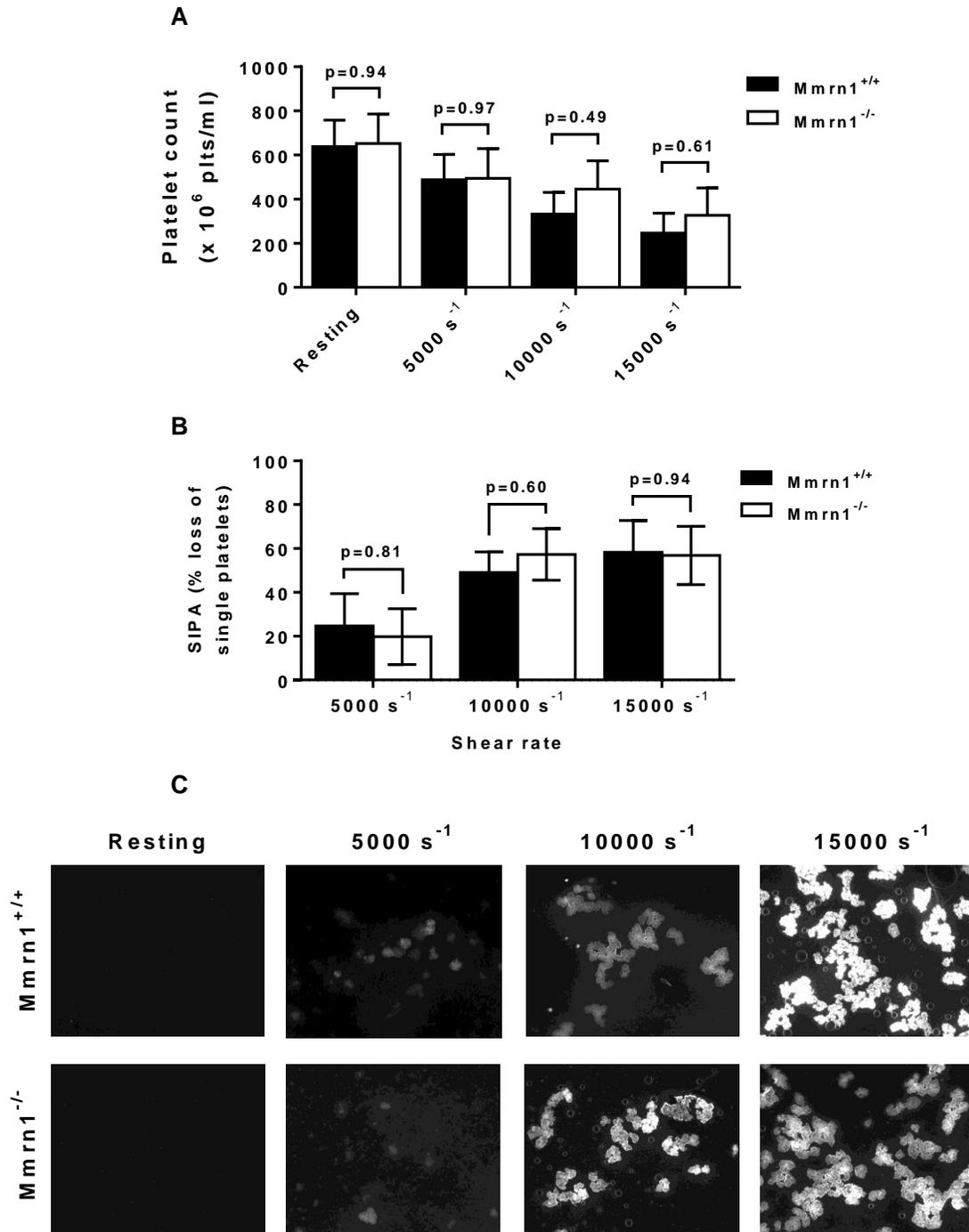
Figure 24: Aggregation of wild-type versus *Mmrn1^-/-^* mouse platelets in whole blood in response to variable shear rates. Shear-induced aggregation of mouse platelets shown by (A) platelet counts and (B) % loss of single platelets in whole blood samples.

Bars and whiskers represent the mean and SEM, respectively. (C) Representative images showing aggregate size of fixed, DiOC6(3)-labelled platelets following exposure to variable shear rates. In panels A,B, bars and whiskers represent the mean and SEM, respectively (n=8 mice/group).

3.7 *IN SILICO* ANALYSIS OF COLLAGEN AMINO ACID SEQUENCES AND MMRN1 BINDING TO VARIANT SEQUENCES OF GPAGPOGPX

I performed *in silico* searches for candidate sequences type I collagen to test for MMRN1 binding. Searches indicated that the GPAGPOGPX sequence is unique to fibrillar collagens: it is not present in any other human or mouse proteins, and both GPAGPOGPV in type II collagen and GPAGPOGPQ in type III collagen are highly conserved (*Table 4*). Many GPAGPOGPX-like sequences were present in peptides from Toolkits II and III, which narrowed the search for potential recognition motifs in the α-chains of type I collagen. Although there is high homology between the COL domains of fibrillar collagens I, II, and III, alignments indicated that the GPAGPOGPV locus (helix residues 151 to 159) from type II collagen is poorly conserved in collagens I and III. In total, three highly conserved GPAGPOGPX-like sequences were identified in type I collagen: GPAGPOGPI at helix residues 667 to 675 in D-period 3 of the α_1(I) triple helix, and GPAGSOGFQ in D-period 2 at residues 457 to 465 that aligns with a conserved GPAGPOGFQ sequence in α_2(I) (*Table 4*). In particular, GPAGPOGPI in collagen I overlaps in sequence alignments with GPAGPOGPQ (helix residues 682 to 690) in collagen III, which has a 9-residue extension at its N-terminus, offsetting helix numbering. Additionally, substitution of the hydrophobic residue valine for isoleucine is a conservative mutation,[566] further suggesting that GPAGPOGPI could support MMRN1 binding.

```
Collagen alpha-1(I) chain: GPAGPOGPI

UniProtKB ID:
P02452              Human (H. sapiens)           842 GPAGPAGPPGPIGNV
P11087              Mouse (M. musculus)          831 GPAGPAGPPGPIGNV
P02454              Rat (R. norvegicus)          831 GPAGPAGPPGPIGNV
P02453              Cow (B. taurus)              841 GPAGPAGPPGPIGNV
Q9XSJ7              Dog (C. lupus familiaris)    838 GPAGPTGPPGPIGNV
                                                     ***** *********


Collagen alpha-1(I) chain: GPAGSOGFQ

UniProtKB ID:
P02452              Human (H. sapiens)           631 GEQGPAGSPGFQGLP
P11087              Mouse (M. musculus)          631 GEQGPAGSPGFQGLP
P02454              Rat (R. norvegicus)          631 GEQGPAGSPGFQGLP
P02453              Cow (B. taurus)              630 GEQGPAGSPGFQGLP
Q9XSJ7              Dog (C. lupus familiaris)    628 GEQGPAGSPGFQGLP
                                                     ***************


Collagen alpha-2(I) chain: GP(A/P)GPOGFQ

UniProtKB ID:
P08123              Human (H. sapiens)           543 GEQGPPGPPGFQGLP
Q01149              Mouse (M. musculus)          549 GEQGPAGPPGFQGLP
P02466              Rat (R. norvegicus)          549 GEQGPAGPPGFQGLP
P02465              Cow (B. taurus)              541 GEQGPAGPPGFQGLP
O46392              Dog (C. lupus familiaris)    543 GEQGPAGPPGFQGLP
                                                     ***** *********


Collagen alpha-1(II) chain: GPAGPOGPV

UniProtKB ID:
P02458              Human (H. sapiens)           348 GQPGPAGPPGPVGPA
P28481              Mouse (M. musculus)          348 GQPGPAGPPGPVGPA
P05539              Rat (R. norvegicus)          280 GQPGPAGPPGPVGPA
P02459              Cow (B. taurus)              348 GQPGPAGPPGPVGPA
                                                     ***************


Collagen alpha-1(III) chain: GPAGPOGPQ

UniProtKB ID:
P02461              Human (H. sapiens)           845 GGSGPAGPPGPQGVK
P08121              Mouse (M. musculus)          844 GSSGPAGPPGPQGVK
P13941              Rat (R. norvegicus)          844 GGSGPAGPPGPQGVK
P04258              Cow (B. taurus)              689 GGSGPAGPPGPQGVK
                                                     * *************
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Table 4: Conservation of GPAGPOGPX sequences in types I, II, and III fibrillar collagens. Sequences were retrieved from UniProtKB. Sequence alignment was performed using the Align tool and sequence similarity was calculated automatically by UniProtKB.

These three variant sequences were synthesized as homotrimeric triple-helical peptides and tested for rhMMRN1 binding. GPAGPOGPI supported rhMMRN1 binding significantly more than inert control peptide GPP (p=0.0002), whereas GPAGPOGFQ and GPAGSOGFQ did not (p=0.12 and p=0.52, respectively, *Figure 25*).
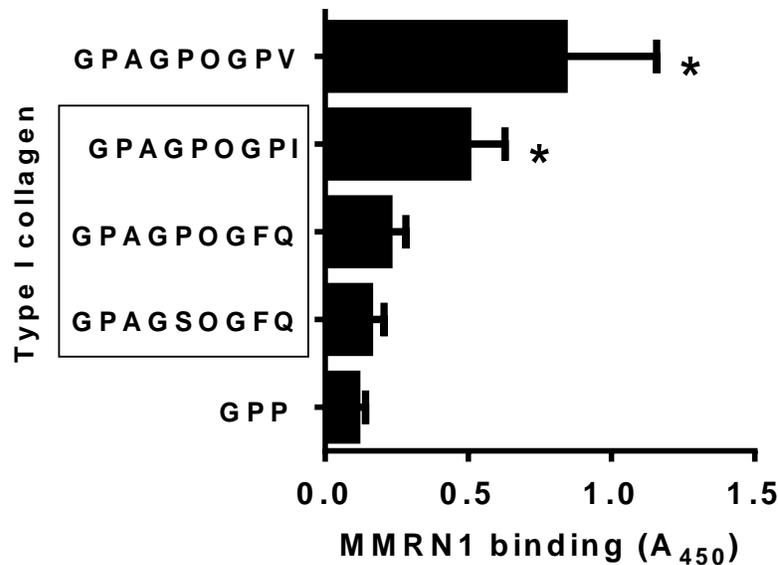
Figure 25: rhMMRN1 binding to homotrimeric triple-helical peptides derived from GPAGPOGPX-like motifs in the alpha-chains of type I collagen. Bars and whiskers represent the mean and SD of three independent experiments, respectively, performed with a single preparation of rhMMRN1. Within each experiment, determinations were performed in triplicate to obtain a mean. Asterisks (*) indicate statistical significance compared to GPP, using multiple paired *t*-tests and the Bonferroni correction, where number of comparisons $k = 4$ and $\alpha = 0.0125$ in each comparison. A total of 4 preparations of rhMMRN1 were tested, each yielding equivalent results (data not shown).

3.8 STATIC ADHESION OF ACTIVATED WILD-TYPE AND *Mmrn1*$^{-/-}$ PLATELETS

TO GPAGPOGPX, GFOGER, AND FULL-LENGTH FIBRILLAR COLLAGEN

The GPAGPOGPX motifs that bound rhMMRN1 are fully conserved in murine

collagens, and mouse Mmrn1 shares 67% sequence similarity with human MMRN1. I

tested the specificity of these peptides for Mmrn1 using assays of static adhesion of wild-

type and *Mmrn1*$^{-/-}$ platelets, pre-activated with CRP to induce Mmrn1 release and

activation of platelet integrins. Wild-type platelets showed dose-dependent adhesion to

GPAGPOGPQ or GPAGPOGPI, unlike *Mmrn1*$^{-/-}$ platelets, which showed minimal

adhesion to GPAGPOGPX peptides ($p<0.05$, *Figure 26A,B*). Additionally, CRP-activated

Mmrn1$^{-/-}$ platelets also showed impaired platelet adhesion to collagen (tested as a positive

control for maximal adhesion) under stasis compared to wild-type platelets ($p=0.03$,

Figure 26C). Despite this, additional testing showed that the ability of *Mmrn1*$^{-/-}$ platelets

to adhere to GFOGER was not impaired ($p=0.49$, *Figure 27*).
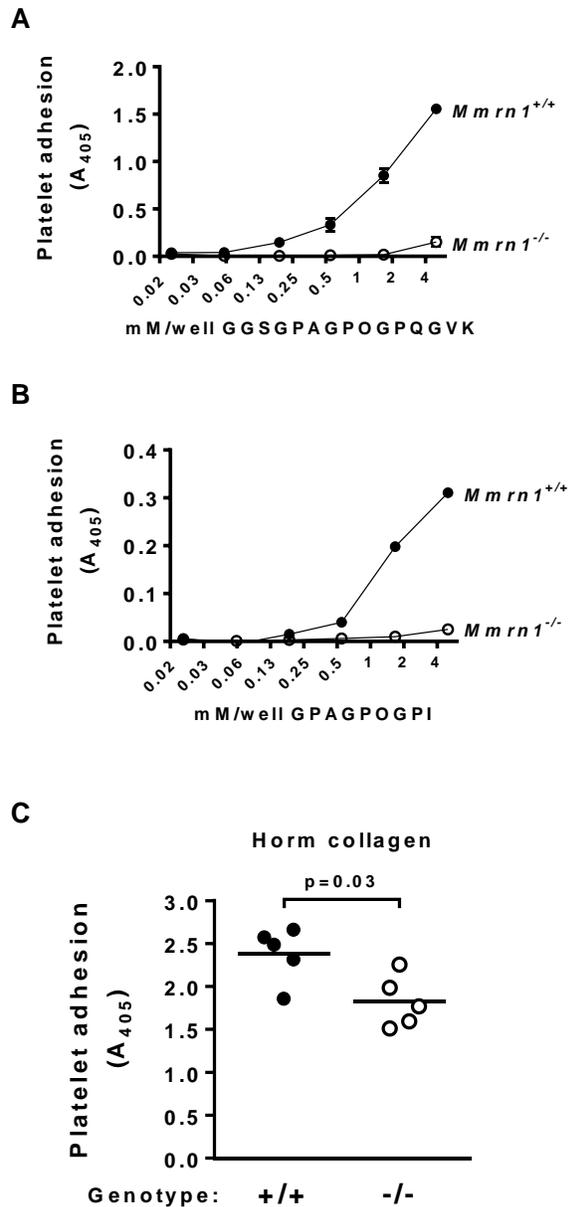
Figure 26: Static adhesion of wild-type versus *Mmrn1*-/- CRP-activated mouse platelets to triple-helical collagen mimetic peptides or Horm collagen. (A) Specificity of MMRN1-binding peptide GGSGPAGPOGPQGVK for wild-type (n = 4 mice) or *Mmrn1*-/- (n = 3 mice) platelets. (B) Specificity of MMRN1-binding peptide GPAGPOGPI for wild-type or *Mmrn1*-/- (n = 3 mice/genotype) platelets. (C) Adhesion of CRP-activated wild-type (n =5) and *Mmrn1*-/- (n = 5) to Horm collagen. In panels A,B absorbance values are corrected for non-specific adhesion by subtracting adhesion to GPP

at each concentration tested. In panels A-C, symbols indicate *Mmrn1* genotype (closed: *Mmrn1*$^{+/+}$; open: *Mmrn1*$^{-/-}$).
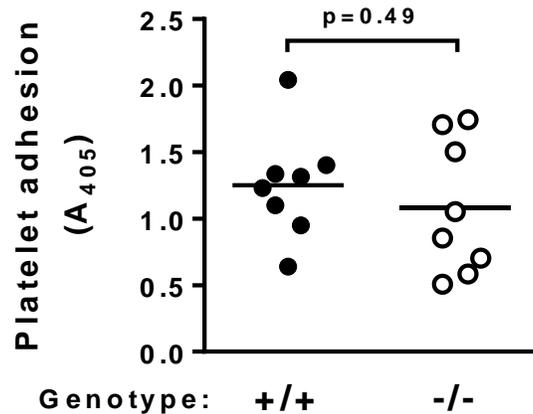
Figure 27: Static adhesion of CRP-activated mouse platelets to α2β1-binding peptide GFOGER. Data show the mean absorbance value of each biological replicate (n = 8 mice/group). Within each experiment, determinations were performed in triplicate to obtain a mean. Symbols indicate *Mmrn1* genotype (closed: *Mmrn1*$^{+/+}$; open: *Mmrn1*$^{-/-}$).

3.9 ADHESION OF WILD-TYPE AND *Mmrn1*$^{-/-}$ MOUSE PLATELETS TO TRIPLE-

HELICAL COLLAGEN PEPTIDES UNDER HIGH SHEAR FLOW (1500 s^{-1})

I performed assays of platelet adhesion with washed, CRP-activated mouse

platelets to test the effects of GPAGPOGPX on adhesion of wild-type platelets under high

shear flow (1500 s^{-1}). Presented on its own, GPAGPOGPQ was insufficient to support

platelet adhesion (*Figure 28A*). On surfaces co-coated with GFOGER and GPAGPOGPQ,

the presence of GPAGPOGPQ increased size of captured wild-type (p<0.0001), but not

Mmrn1$^{-/-}$ (p=0.06), platelet aggregates compared to surfaces co-coated with GFOGER

and GPP (*Figure 28B*), providing further evidence that adhesion to GPAGPOGPQ

requires Mmrn1. On surfaces coated with GFOGER and GPP, *Mmrn1*[-/-] platelets formed smaller aggregates than wild-type platelets (p <0.001), suggesting that Mmrn1 also supports GPAGPOGPX-independent platelet-platelet interactions. In order to recapitulate the conditions of platelet adhesion to fibrillar collagen *in vivo*, experiments were performed in which wild-type or *Mmrn1*[-/-] anticoagulated whole blood was perfused over surfaces coated with combinations of collagen peptides, including CRP to activate platelets (data not shown). Under these conditions, mouse platelets failed to adhere to peptide-coated surfaces, even when peptide coating concentration was increased or if blood was collected into PPACK or citrate. This may reflect the presence of molecules and pathways in murine whole blood that normally inhibit platelet adhesion to collagen, or that fibrillar ultrastructure could be required for resting mouse platelets in whole blood to adhere to collagen.
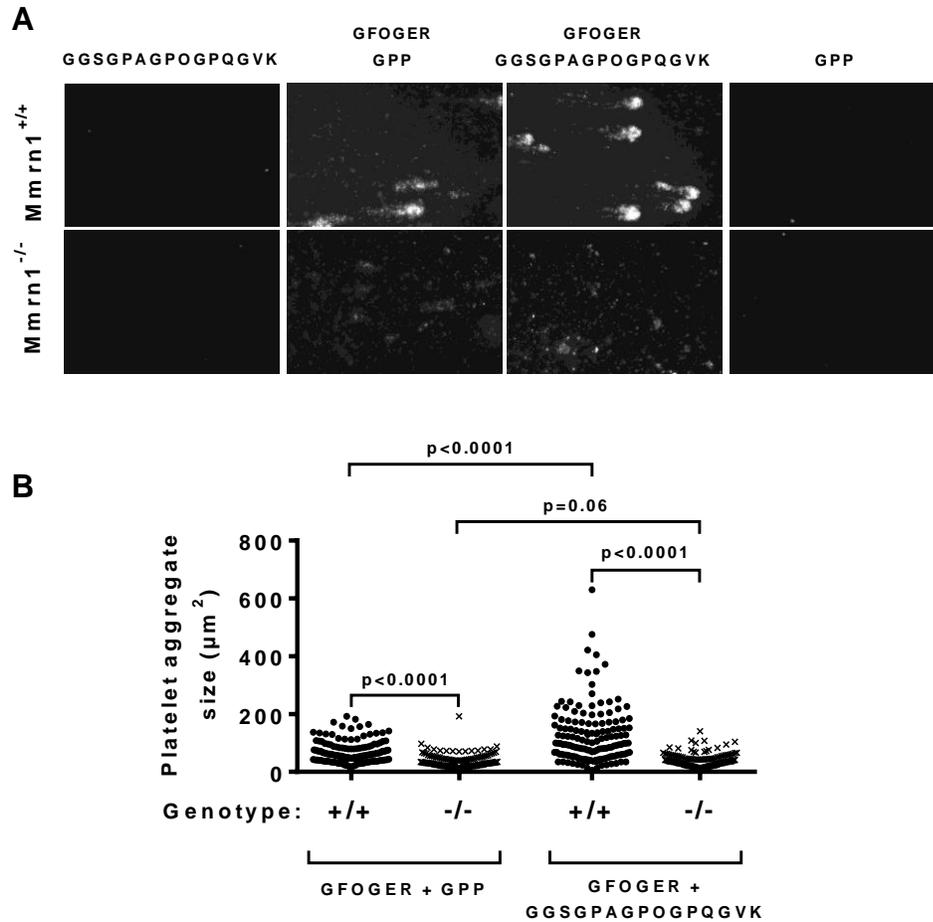
Figure 28: Adhesion of washed, CRP-activated mouse platelets to triple-helical collagen mimetic peptides under high shear flow (1500 s⁻¹). (A) Representative images (original magnification x20) and (B) quantitative analyses of wild-type and *Mmrn1⁻/⁻* platelet aggregates captured onto immobilized triple-helical collagen peptides. In panel B, data represent the mean feature size in each captured image ($n = 15$ images per experiment, $n = 5$ mice/group).

3.10 STATIC ADHESION OF HUMAN PLATELETS TO TRIPLE-HELICAL COLLAGEN PEPTIDES

I performed static adhesion assays to characterize the ability of GPAGPOGPX peptides to support or enhance adhesion of human platelets. When presented alone, GPAGPOGPX peptides did not support significant adhesion of CRP-activated human platelets (*Figure 29A*). When co-presented with the $\alpha_2\beta_1$-binding peptide GFOGER, GPAGPOGPX peptides significantly enhanced human platelet adhesion compared to GFOGER + GPP ($p<0.002$), similar to GFOGER co-presented with the VWF-binding peptide III-23 (*Figure 29B*). In comparison, co-presentation of III-23 with GPAGPOGPX peptides did not support significant adhesion of activated human platelets compared to III-23 + GPP (*Figure 29B*). When presented together, GFOGER, III-23, and GPAGPOGPX peptides synergistically enhanced adhesion of activated human platelets (*Figure 29C*). The enhancing effect of GPAGPOGPX peptides co-presented with GFOGER was significantly increased by platelet activation to induce MMRN1 release ($p<0.007$, *Figure 30A*). The effect of platelet activation was similar whether human platelets were pre-treated with CRP or exposed to CRP coated in wells ($p>0.24$, *Figure 30B*).
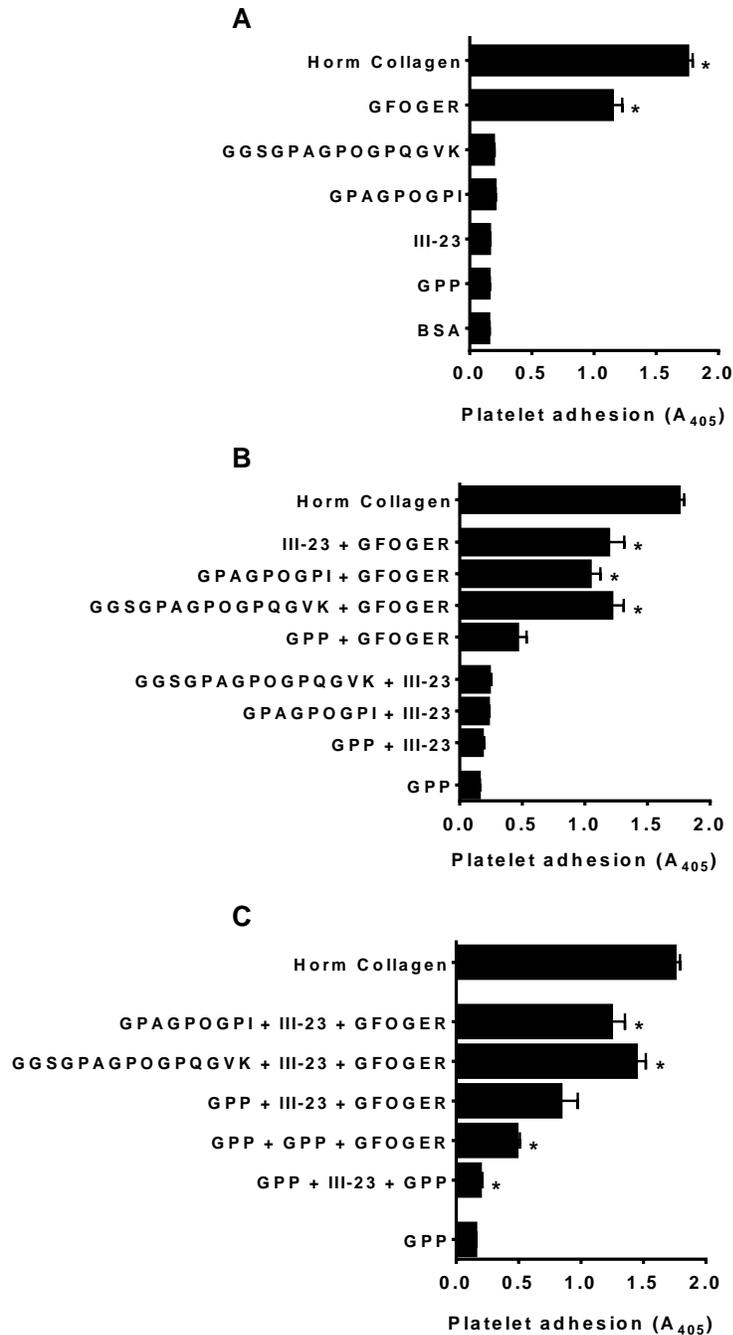
Figure 29: Synergistic effects of triple-helical peptide combinations on static adhesion of CRP-activated human platelets. Bars and whiskers represent the mean absorbance and SEM, respectively ($n = 3$ healthy controls). Asterisks (*) indicate statistical significance using multiple paired t-tests and the Bonferroni correction for

family-wise error. In panel A, statistical comparisons are relative to GPP. In panel B, statistical comparisons are relative to GPP + GFOGER (top) or GPP + III-23 (bottom). In panel C, statistical comparisons are relative to GPP + III-23 + GFOGER.
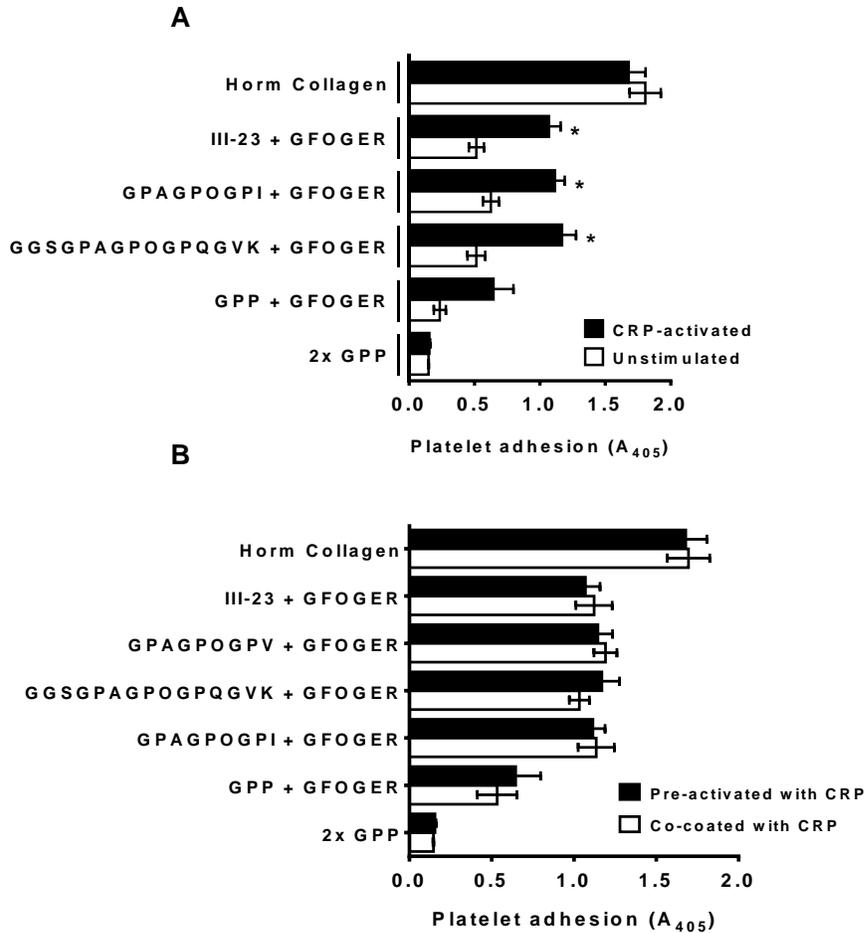
Figure 30: Effect of CRP activation on static adhesion of human platelets. Bars and whiskers represent the mean absorbance and SEM, respectively (n =3 healthy controls). Asterisks (*) indicate statistical significance between CRP-activated and unstimulated samples using multiple paired t-tests and the Bonferroni correction for family-wise error ($k = 5$, $\alpha = 0.01$ in each comparison). In panel A, comparison of adhesion of CRP-activated versus unstimulated platelets to GPP + GFOGER approached, but did not reach, significance (p=0.07).

CHAPTER FOUR: DISCUSSION

4.1 SUMMARY OF THESIS WORK AND KEY OBSERVATIONS

This thesis aimed to further characterize the role of MMRN1/Mmrn1 in platelet adhesion and platelet aggregation, specifically through its interactions with fibrillar collagens, identifying additional potential binding partners for MMRN1, testing the effects of fluid shear stress on MMRN1 adhesive functions, identifying novel MMRN1-binding motifs in type I collagen, and assess the specificity and functions of MMRN1-binding GPAGPOGPX sequences in platelet adhesion. The main findings of this thesis were that $Mmrn1^{-/-}$ platelets had impaired aggregate formation on Horm collagen under high shear flow (1500 s^{-1}), which was due in part to reduced initial adhesion and a slower rate of platelet accumulation onto collagen. Additionally, $Mmrn1^{-/-}$ platelets formed significantly smaller aggregates on immobilized rVwf under high shear flow (1500 s^{-1}), regardless of whether or not plasma Vwf was present. Impaired adhesion of $Mmrn1^{-/-}$ platelets to Horm collagen was also observed under low shear flow (300 s^{-1}) and stasis, suggesting that Mmrn1 could enhance platelet adhesion to collagen through additional, Vwf-independent mechanisms.

rhMMRN1 was found to be a ligand for human FG, fibrin, and FN. Additionally, $Mmrn1^{-/-}$ platelets had significantly impaired adhesion and aggregate formation onto murine Fg, but not fibrin, under low shear flow (300 s^{-1}), tested after platelets were pre-

activated to expose α-granule contents and activate platelet integrins. Contrary to expectations, activated $Mmrn1^{-/-}$ platelets had similar adhesion and aggregate formation onto Fn. Wild-type and $Mmrn1^{-/-}$ platelets were found to adhere to rhMMRN1 through mechanisms involving, but not requiring, $β_3$ integrins, suggesting the involvement of other receptors and/or proteins. Although MMRN1 is a ligand for VWF, platelet aggregation under elevated shear stress, which is sufficient to induce α-granule release, was similar between $Mmrn1^{-/-}$ and wild-type platelets at all shear rates tested, indicating that high-shear induced platelet aggregation is Vwf- but not Mmrn1-dependent.

Fibrillar type I collagen was found to contain a conserved GPAGPOGPI sequence that binds rhMMRN1 and aligns with the MMRN1-binding GPAGPOGPQ sequence in type III collagen. Both GPAGPOGPI and GPAGPOGPQ show high specificity for Mmrn1 as wild-type but not $Mmrn1^{-/-}$ platelets adhered to this motif under stasis and high shear flow (1500 s^{-1}). GPAGPOGPX peptides enhanced static adhesion of human platelets when co-presented with $α_2β_1$-binding peptide GFOGER or GFOGER and VWF-binding peptide III-23 together, which was significantly enhanced by platelet activation, which is required to release MMRN1 from α-granules.

The following sections discuss the proposed roles of MMRN1/Mmrn1 in platelet adhesion to collagen and present an updated model of the recognition motifs in collagen that support platelet adhesion or activation in light of these findings. Next, the strengths

and limitations of the approaches and techniques used in this thesis will be considered, along with potential future experiments to address questions raised by this thesis.

4.2 CONTRIBUTIONS OF MULTIMERIN 1 IN PLATELET ADHESION TO COLLAGEN

4.2.1 Potential roles of Mmrn1 in primary platelet adhesion and platelet tethering onto fibrillar collagen

As described in *section 1.4.3*, platelet adhesion to fibrillar collagen under high shear flow requires VWF to bind to the collagen surface, which tethers circulating platelets and reduces their velocity to facilitate stable adhesion onto collagen. Although MMRN1 (which is not detectable in normal plasma) is sequestered within platelet and endothelial cell granules until activation-induced release, with platelet activation at sites of injury, the local concentrations of MMRN1 likely rise so that it can participate with other ligands in platelet attachment. Vessel injury that results in activation or rupture of endothelial cells would also release MMRN1 locally, and it is difficult to exclude that *in vivo*, a small amount of MMRN1 is constitutively secreted into the subendothelium after biosynthesis by endothelial cells.[1,17] In these ways, MMRN1 may contribute to the initial "protein wave" of hemostasis that primes the site of injury for adhesion prior to initial platelet attachment. Additionally, in thrombotic diseases in which endothelial cells are

pathologically activated, MMRN1 would similarly be exposed to flowing blood by endothelial cells prior to platelet activation.

One important consideration is that *in vitro* perfusion experiments using resting platelets lack any initial presence of Mmrn1 on the adhesive substrate before contact with platelets. In experiments testing adhesion of resting murine platelets to Horm collagen under high shear flow, Mmrn1 is likely only released from α-granules following contact with collagen. While the ligation of VWF to GPIbα is able to induce activation of $α_{IIb}β_3$ and Ca^{2+} mobilization in platelets, it is insufficient to induce α-granule release from platelets.[186,567] Collagen-activated platelets would express Mmrn1 on their surface and may also release some Mmrn1 downstream of the site of attachment. Moreover, at sites of injury, there are likely multiple triggers to platelet activation. Thus, it is quite possible that Mmrn1 contributes to both early and later steps of platelet adhesion onto collagen. *Figure 31* outlines potential mechanisms for the role of MMRN1/Mmrn1 in primary platelet adhesion onto collagen under high shear flow based on previous data and observations described in this thesis.
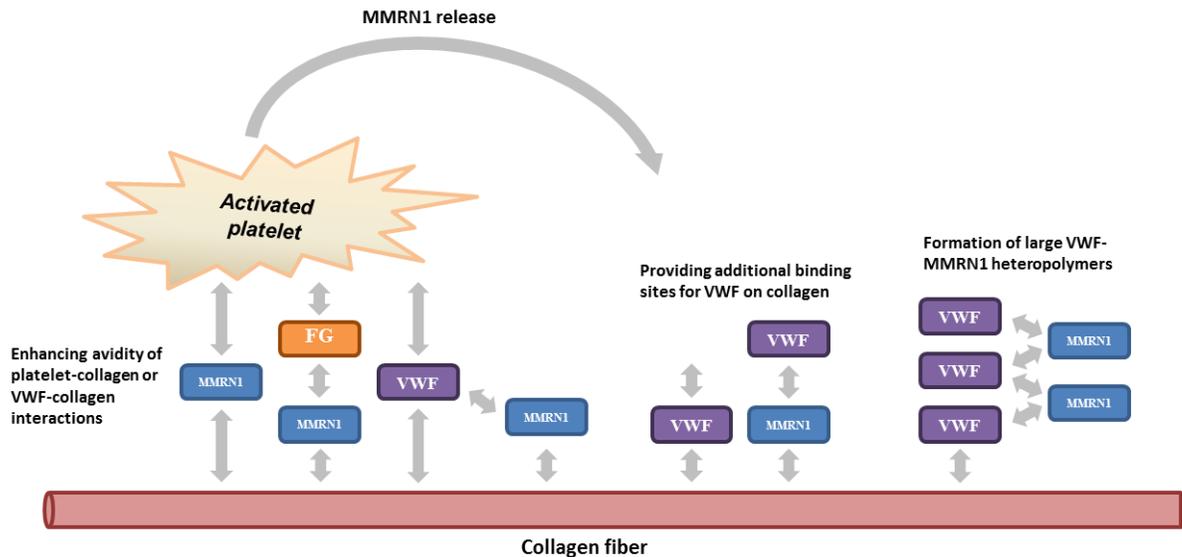
Figure 31: Potential mechanisms through which MMRN1/Mmrn1 enhances primary platelet adhesion to fibrillar collagen under high shear flow.

Mmrn1 that remains bound to the platelet surface following platelet activation could bind to GPAGPOGPX motifs in collagen fibers to stabilize platelet adhesion to collagen. Each type I or III collagen molecule contains a single, discrete VWF-binding GPRGQOGVMGF that is distinct from the MMRN1-binding GPAGPOGP(I/Q) motif. Mmrn1 binding to collagen could enhance the avidity of Vwf binding to collagen by providing additional bonds between Vwf and the collagen molecule to stabilize Vwf-collagen interactions. Additionally, Mmrn1 could mediate the binding of $\alpha_{IIb}\beta_3$-bound Fg on the activated platelet surface to collagen fibers to further stabilize platelet adhesion.

A second possible mechanism is that Mmrn1 released from the platelet surface could enhance Vwf-dependent tethering onto collagen by increasing the availability of

Vwf-binding sites on collagen fibrils downstream of attachment. Mmrn1 released into circulation by stably-adherent platelets would be able to bind GPAGPOGPX motifs in fibrillar collagen molecules downstream to essentially double the availability of Vwf-binding sites on collagen fibrils. This idea is particularly intriguing because the collagen-binding and MMRN1-binding regions of VWF involve the same domains: VWF A1 and VWF A3, meaning that MMRN1-bound VWF would be anchored in a similar way as collagen-bound VWF. Further, it is already shown that plasma VWF multimers can self-associate onto collagen-bound VWF through homotypic interactions to enhance platelet adhesion to collagen.[552] Although MMRN1 is not in direct competition for the same binding sequence on a single collagen molecule as other ligands, the steric parameters of VWF and MMRN1 binding to collagen are unclear. Both VWF and MMRN1 form large homopolymers, which can exceed millions of Daltons in size.[11,13,18,568,569] In particular, VWF multimers can reach up to 15 µm in length,[570] whereas a mature human type I or III fibrillar collagen molecule is approximately 300 nm in length. Large VWF and MMRN1 homopolymers likely engage multiple copies of their respective recognition motifs on a series of crosslinked, end-to-end collagen molecules within a collagen fibril. While it is possible that large MMRN1 homopolymers may be able to block VWF-binding to collagen or *vice versa*, the observations described in this thesis suggest that Vwf and Mmrn1 cooperatively enhance platelet adhesion to collagen. Previous studies also indicate that adding exogenous rhMMRN1 enhances platelet adhesion to Horm collagen,[2] further suggesting that VWF and MMRN1 homopolymers cooperatively support platelet adhesion to collagen. Collagen fibrils are quarter-staggered 5-mers of end-to-end

crosslinked collagen molecules that aggregate into bundles of fibrils, which would present an abundance of binding sites for both VWF and MMRN1 multimers, potentially rendering competition for collagen binding moot. *In vivo*, the stoichiometry of collagen, VWF, and MMRN1 is likely to be highly variable, dependent on a number of factors, including: the tissue in which the hemostatic or thrombotic response is occurring (i.e. collagen-rich or collagen-poor), the localized platelet concentration (the amount of platelet MMRN1 available for release), and the platelet activation state (how much platelet MMRN1 is released). Based on these considerations, it is difficult to conclude whether MMRN1 and VWF cooperatively or competitively support platelet adhesion to collagen in some or all situations, or if competition would be irrelevant due to a molar excess of collagen in most collagen-containing tissues. Fluid-phase competition assays could be useful for understanding how MMRN1, VWF, and $\alpha_2\beta_1$ cooperatively enhance platelet adhesion to collagen, but would be complicated to perform and interpret given that MMRN1 and VWF bind to one another. Instead, assays where varying concentrations of rhMMRN1 or BSA are incubated with or perfused over Horm collagen before perfusion with fluorescently-labelled VWF could be used to determine if MMRN1 can enhance VWF binding to collagen fibers.

The idea that adhesive plasma or platelet proteins bind one another to form large, macromolecular complexes has been proposed as a possible explanation for the supportive role of multimeric platelet VN,[203] TSP-1,[205] and insoluble (crosslinked) plasma FN[204,206,208] in platelet aggregate formation, as well as in platelet adhesion to

collagen.[303,304] It is possible that Mmrn1 either bound to the platelet surface or released into circulation could bind to shear-stretched Vwf multimers to form larger, heteropolymers to enhance platelet tethering and adhesion. Larger heteropolymers composed of Vwf and Mmrn1 would likely extend further into the lumen, which would increase the probability and frequency of encountering circulating platelets. Similar to Tsp-1, Mmrn1 may also bind to the A1 and A3 domains of Vwf to block cleavage of immobilized, shear-stretched Vwf multimers by Adamts13. This would facilitate the formation and maintenance of ultra-large Vwf multimers on the exposed collagen surface, which are more effective at supporting platelet adhesion.[571] As outlined in *section 1.2.3*, TSP-1 competitively binds to the VWF A2 and A3 domains to block VWF A2 cleavage by ADAMTS13,[362] and *Tsp-1*[-/-] mice show rapid degradation of ultra-large Vwf multimers released from endothelial cells following photochemical injury *in vivo*, which is restored by adding back recombinant TSP-1 or anti-ADAMTS13-antibodies.[225] Mmrn1 may function in a similar way to inhibit the regulation of Vwf multimer size by Adamts13. This possibility is discussed further in *section 4.5.1*. Another important consideration is the potential role of MMRN1 in thrombotic thrombocytopenic purpura (TTP), in which quantitative or qualitative defects in ADAMTS13 impair the regulation of plasma VWF multimer size, resulting in increased platelet adhesion to ULVWF multimers that underlie the microangiopathy of TTP. Given that MMRN1 binds to the VWF A1 and A3 domains, and the ability of Mmrn1 to contribute to platelet aggregate formation onto Vwf under flow, this raises the possibility that MMRN1/Mmrn1 could be

involved in the pathogenesis of TTP if it alters VWF cleavage by ADAMTS13 or the stability of platelet aggregates adherent to ULVWF multimers.

Lastly, it is interesting to consider that differently-sized Mmrn1 multimers could fulfil different adhesive functions. For example, larger MMRN1 multimers preferentially stay bound to the platelet surface, whereas smaller multimers tend to be released into the milieu,[15] which may have biological significance. Large, platelet-bound MMRN1 multimers could locally enhance or stabilize platelet adhesion, whereas smaller MMRN1 multimers might be more important for facilitating primary adhesion of additional platelets downstream of MMRN1 release.

The mechanisms through which Mmrn1 contributes to primary platelet adhesion under low shear likely differ in some respects because platelet adhesion and aggregate formation on collagen can occur independent of VWF under shear rates of approximately ≤ 1000 s^{-1}.[175] Under low shear flow (300 s^{-1}) and stasis, $Mmrn1^{-/-}$ platelets visually showed reduced adhesion to collagen and formed smaller aggregates compared to wild-type platelets. These data suggest that Mmrn1 may enhance platelet adhesion to collagen through additional mechanisms than those that support Vwf adhesive functions. In regard to primary platelet adhesion to collagen, it is possible that Mmrn1 binding to collagen and to platelets may be sufficient to mediate platelet adhesion to collagen on its own under stasis and low shear flow. This could be tested using a model of combined Mmrn1 and Vwf deficiency compared to single-deficient $Vwf^{-/-}$ mice to eliminate the contribution of

ultra-large platelet Vwf to platelet adhesion to collagen, with and without anti-mouse $\alpha_2\beta_1$

and GpVI blocking antibodies to control the contribution of platelet collagen receptors.

4.2.2 Potential roles of Mmrn1 in supporting platelet aggregate formation onto
fibrillar collagen

An important observation from this thesis is that the presence of Mmrn1 enhanced

the size of platelet aggregates formed on Horm collagen surfaces under high and low

shear flow. This suggests that in addition to supporting initial platelet adhesion to

collagen, Mmrn1 has a role in enhancing the size or stability of platelet aggregates

formed under flow. Within the context of platelet adhesion to collagen, Mmrn1

interactions with collagen and platelets might also facilitate platelet activation, which is

suggested by the observation of enhanced stable primary platelet adhesion to collagen in

the presence compared to the absence of Mmrn1. Platelet activation is an important

modulator of platelet adhesion and aggregation onto collagen, given that it induces

conformational changes in platelet integrins, which increases the affinity of $\alpha_2\beta_1$ for

collagen and increases the affinity of $\alpha_{IIb}\beta_3$. This is supported by observations that GpVI-

or FcRγ-deficient mouse platelets (which do not express platelet GpVI) fail to form

aggregates on collagen under high shear flow,[182,415,464] and show significantly reduced

$\alpha_2\beta_1$-mediated stable adhesion onto collagen in real-time.[415] It is also supported by

observations that blockade of P2Y$_{12}$ and P2Y$_1$ significantly reduces platelet aggregation

onto collagen.[573] Mmrn1 may assist in primary adhesion onto collagen to facilitate

platelet activation by collagen, which would enhance the rate and amount of platelet aggregate formation. The transient on/off binding of VWF to GPIbα and the relatively weak interaction between collagen and GPVI means that additional ligands are required to facilitate stable adhesion and subsequent activation, partly evidenced by the delay in collagen-induced platelet aggregation observed in α_2-deficient mice.[574]

Notably, the ability of Mmrn1 to enhance platelet aggregate size under flow is not limited to immobilized collagen surfaces, which suggests that Mmrn1 may play a dual role in enhancing platelet adhesion and aggregate formation onto collagen by: 1) mediating platelet adhesion onto collagen through interactions with collagen and/or Vwf, and 2) enhancing platelet aggregate formation on collagen by supporting platelet-platelet interactions. The ability of MMRN1 to bind FG, fibrin, and FN indicates MMRN1/Mmrn1 could feasibly enhance platelet aggregate growth through multiple potential mechanisms. Similar to TSP-1, multimeric VN, and fibrillar FN, MMRN1 may be able to crosslink $\alpha_{IIb}\beta_3$ or $\alpha_{IIb}\beta_3$-bound FG on adjacent platelets. *Figure 32* outlines potential mechanisms through which MMRN1/Mmrn1 could support platelet-platelet interactions to enhance platelet aggregate formation. Large MMRN1/Mmrn1 multimers bound to the surface of activated platelets would decrease the distance required for platelet-platelet interactions to occur, which would facilitate greater platelet-platelet interactions. Increasing the number of platelet crosslinks would also enhance the avidity of platelet-platelet interactions. Lastly, MMRN1/Mmrn1 may bind to FG or FN to form larger complexes that are more effective at supporting platelet-platelet interactions.
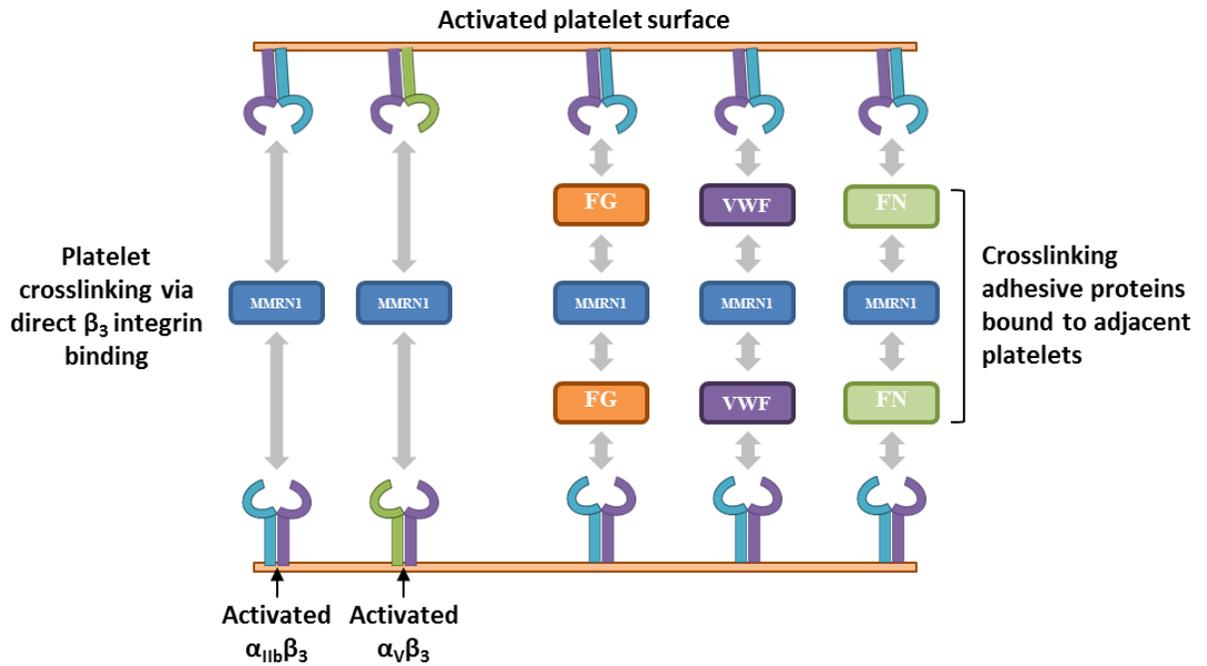
Figure 32: Potential mechanisms through which MMRN1/Mmrn1 enhances aggregate formation onto fibrillar collagens and other adhesive substrates.

Assays of static platelet adhesion using pre-activated mouse platelets to induce Mmrn1 release indicated that Mmrn1 was not required for stable adhesion to Fg, fibrin, or Fn, although the absence of Mmrn1 impaired platelet adhesion and aggregate formation onto Fg under low shear flow. This discrepancy could arise from the type of model and unit of measurement to measure adhesion in static adhesion and perfusion assays. Static adhesion assays commonly estimate platelet adhesion by quantifying conversation of p-Nitrophenyl phosphate by acid phosphatases released from lysed adherent platelets,[565] whereas perfusion assays directly visualize the quantity and extent of fluorescently-labelled platelets onto a protein surface. Another possible explanation is differences in the

binding kinetics of mouse platelet receptors to their respective ligands under stasis versus shear stress. Under conditions of low shear flow, the ability of Mmrn1 to enhance adhesion and aggregate size of activated but not resting platelets onto immobilized Fg is consistent with the requirement of platelet activation to release Mmrn1 from platelet α-granules. Additionally, it is possible that the active conformation of $\alpha_{IIb}\beta_3$ may be required for platelets to bind Mmrn1, similar to observations of human platelet adhesion to MMRN1.[1,2] These data indicate that Mmrn1 may have a supportive role in platelet adhesion to Fg, which would be immobilized onto the outer surface of a growing platelet aggregate. The binding site(s) of MMRN1 on FG are unknown, meaning it is presently unclear if MMRN1 bound to FG could prevent engagement of $\alpha_{IIb}\beta_3$ by the FG γ-chain. Future studies using recombinant FG domains, recombinant α_{IIb} and β_3, and rhMMRN1 could unravel this question.

Platelet adhesion to Fg, fibrin, and Fn were tested under low shear flow to recapitulate conditions that are favourable for $\alpha_{IIb}\beta_3$-mediated platelet adhesion, mostly independent of Vwf/GpIbα-mediated platelet tethering. FG-, fibrin-, or FN-coated surfaces do not support significantly platelet adhesion or aggregate formation under high shear rates,[174,265] which require VWF and GPIbα. Low shear perfusion assays are ideal for testing the effects of platelet Mmrn1 on platelet adhesion and aggregate formation through mechanisms involving FG and $\alpha_{IIb}\beta_3$. Although perfusion assays using Horm collagen or Vwf surfaces were performed at high shear, some comparisons can be made between high shear and low shear perfusion experiments. Following VWF/GPIbα-

mediated platelet tethering under high shear flow, stable platelet-collagen and platelet-platelet interactions mediate adhesion, which involve FG and VWF binding to $\alpha_{IIb}\beta_3$ on activated platelets either arrested onto collagen or slowly translocating along the collagen surface. In this way, stable platelet adhesion and platelet aggregate formation onto collagen under high shear flow also involves static or low-velocity interactions.

Analyses of *Mmrn1*$^{-/-}$ and wild-type platelet adhesion indicate that Mmrn1 contributes to platelet adhesion and aggregate formation onto immobilized Fg when platelets are pre-activated to release Mmrn1. TRAP activation prior to perfusion likely primes the platelet surface with large Mmrn1 multimers and releases some Mmrn1 into solution. Similar to previously discussed, Mmrn1 could decrease the distance required for platelets to contact surface-bound Fg, and also increase the frequency of platelet-platelet interactions to enhance the rate of aggregate formation. It is possible that Mmrn1 may also contribute to the stability of platelet aggregates formed on Fg surfaces. Additional experiments could be performed to answer this question, in which *Mmrn1*$^{-/-}$ and wild-type platelet adhesion to Fg under low shear is quantified by fluorescence microscopy and chambers are subsequently washed under elevated shear stress to quantify aggregate dissolution and platelet detachment. Lastly, Mmrn1 released from the platelet surface could even bind pFn to inhibit its ability to compete with FG for ligation of $\alpha_{IIb}\beta_3$,[211] thereby indirectly supporting FG-dependent platelet adhesion and aggregation.

Interestingly, $Mmrn1^{-/-}$ and wild-type platelet adhesion to fibrin was similar using resting or TRAP-activated platelets. Differences in the ultrastructure of surface-adsorbed Fg versus fibrin and/or differences in Fg- and fibrin-$\alpha_{IIb}\beta_3$ binding interactions could account for this divergence. Surface-adsorbed FG is shown to unfold into a highly adhesive conformation that supports platelet adhesion via RGD-independent mechanisms.[280] Conversely, fibrin forms large polymers that branch and crosslink into three-dimensional networks, and platelet adhesion to fibrin is predominantly mediated by $\alpha_{IIb}\beta_3$ binding to the RGD motifs in fibrin.

$Mmrn1^{-/-}$ and wild-type platelet adhesion to Fn is consistent with previous observations that FN does not support significant platelet adhesion and platelets do not aggregate on FN surfaces. This is hypothesized to be a result of the weak interaction between platelet receptors $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ with FN. Platelet adhesion to FN is mediated by its RGD sequence binding to β_3 integrins. Although $\alpha_5\beta_1$ on the platelet surface binds FN, it is not the key mediator of platelet adhesion to FN under stasis or flow. The molar ratio of FG to FN likely affects platelet adhesion to FN. Plasma FG concentrations range from 2 to 4 mg/ml in humans, whereas plasma FN concentrations range from 100 to 300 µg/ml. The affinity of FG for activated $\alpha_{IIb}\beta_3$ is so high that $\alpha_{IIb}\beta_3$ is likely immediately occupied by FG following platelet activation. As a result, in whole blood perfusion assays, plasma FG likely inhibits platelet adhesion onto FN, resulting in poor adhesion. Although MMRN1 binds to FN, it does not appear to affect the ability of platelets to adhere to or aggregate onto Fn under stasis or low shear flow. It could be that pFn

requires co-presentation with other proteins to modulate platelet adhesion. For example, pFn crosslinked to fibrin surfaces induces fibrillar matrix formation of pFn monomers that significantly enhances platelet adhesion,[575] and pFn requires fibrin in order to enhance platelet aggregation.[204] On its own, pFn is insufficient to induce fiber formation, and does not support significant adhesion. Similarly, VN incorporated into fibrin matrices enhances platelet adhesion, whereas it supports little adhesion on its own.[380] It is possible that Mmrn1 could modulate platelet adhesion to fibrillar Fn to synergistically enhance platelet adhesion and aggregate formation on surfaces with multiple adhesive proteins present, and a model of platelet adhesion to Fn alone is insufficient to detect an effect of Mmrn1 on Fn adhesive functions.

Although MMRN1 binds to $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_3$ on activated human platelets, it is interesting that static adhesion of *Mmrn1*[-/-] and wild-type platelets to rhMMRN1 is only partially inhibited by blockade of $\alpha_{IIb}\beta_3$. When presented with immobilized rhMMRN1, which contains the N-terminal integrin-binding RGD site, static adhesion was only inhibited by ≤45% on average for both wild-type and *Mmrn1*[-/-] platelets. This observation differs from previous studies of human platelets (*Table 5*). Differences in antibody epitopes or affinity could account for this discrepancy, but it is possible that additional mechanisms are involved in murine platelet adhesion to MMRN1/Mmrn1. One possibility is that, because MMRN1 is a ligand for FN, platelet Fn could potentially mediate platelet adhesion to MMRN1 by binding $\alpha_5\beta_1$ on platelets. Additionally, ULVWF secreted from activated platelets is able to bind platelets in the absence of shear stress,[576] and could

potentially bridge platelet-MMRN1 interactions, but stable platelet adhesion to VWF is mediated by $\alpha_{IIb}\beta_3$ binding to the RGD motif in the VWF C4 domain,[569] which may be inaccessible in the absence of high shear flow. Lastly, other platelet receptors, either known or uncharacterized, could bind MMRN1/Mmrn1. In analyses of human platelet adhesion to MMRN1, combined blockade of $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ on activated human platelets only inhibited adhesion by 77% on average,[1] which led the authors to suggest the presence of other receptors for MMRN1 on human platelets.

	ADP-activated human platelets		CRP-activated WT mouse platelets
Protein surface	Monoclonal anti-human $\alpha_{IIb}\beta_3$	Monoclonal anti-human $\alpha_{IIb}\beta_3/\alpha_v\beta_3$	Monoclonal anti-mouse $\alpha_{IIb}\beta_3$
MMRN1	75.5 ± 4.4	76.8 ± 4.9	45.1 ± 3.5
FG/Fg	82.6 ± 6.4	81.1 ± 7.2	67.4 ± 3.4
FN/Fn	67.2 ± 3.5	69.3 ± 4.3	63.6 ± 2.0

Table 5: Comparison of inhibition of static adhesion of ADP-activated human platelets to MMRN1, FG, and FN with inhibition of static adhesion of CRP-activated mouse platelets to MMRN1, murine Fg, and murine Fn. Methods, antibody sources, and data reported for ADP-activated human platelets are described in Adam *et al*.[1] In both sets of experiments, wells were coated overnight at 4°C using 100 µl of 110 µg/ml protein (1 µg/well) and platelets were incubated with 10 µg/ml of monoclonal antibodies before adding to microtiter wells. In experiments using CRP-activated mouse platelets, adhesion was tested using murine Fg or Fn.

Collagen-induced aggregation of $Mmrn1^{-/-}$ and wild-type platelets tested in whole blood was similar to studies of collagen-induced aggregation of $Mmrn1^{-/-}$ and wild-type platelets tested by LTA.[4] It was previously hypothesized that adhesive proteins in plasma, such as VWF and FG, mask the ability to detect a defect in platelet aggregation due to loss of platelet Mmrn1. Similarly, studies of human platelets show that adding plasma concentrations of purified FG or FN to washed platelets reduces platelet adhesion to MMRN1 by 50% or 23%, respectively, and adding plasma to washed platelets reduces platelet adhesion to MMRN1 by 88%.[1] Together, these data indicate that other proteins in plasma, such as FG and FN, likely compete with MMRN1 for ligation of the promiscuous $\alpha_{IIb}\beta_3$ receptor. This may account for why $Mmrn1^{-/-}$ platelets show normal aggregation responses in models wherein adhesive plasma proteins are present. In regard to how these findings relate to observations from *in vitro* perfusion experiments using whole blood, aggregometry testing measures either turbidity or electrical resistance, which measure the rate of aggregation and maximal aggregation, but these tests are not sensitive to detect changes in aggregate size or morphology. For example, anti-TSP-1 antibodies impair collagen- or thrombin-induced platelet aggregation measured by turbidity, but a striking feature is that anti-TSP-1 antibodies impair the size of aggregates formed when assessed by microscopy.[241,351] It is possible that Mmrn1 modulates aggregate size, but not the rate and amount of maximal aggregation in these assays. Analyses to measure the size of fixed platelet aggregates by fluorescence microscopy following LTA or whole blood aggregation tests could be used to answer this question.

It is surprising that Mmrn1 deficiency did not affect SIPA, considering that SIPA is initiated by VWF-mediated aggregation, followed by platelet activation, α-granule release, and $\alpha_{IIb}\beta_3$-mediated stabilization of platelet aggregates.[320,577] Under these conditions, it is anticipated that Mmrn1 would be released following platelet activation, which could contribute to platelet-platelet interactions and bind to plasma Vwf multimers to assist in further Vwf-mediated aggregation. The presence of adhesive platelet α-granule and plasma proteins such as Fg, Fn, Tsp-1, and Vn, could mask the ability of Mmrn1 to contribute to SIPA or even inhibit Mmrn1 binding to activated platelets via competition for ligation of $\alpha_{IIb}\beta_3$. However, whole blood was selected for these experiments because it is the most physiologic model to test SIPA. The data presented in this thesis suggests that platelet Mmrn1 does not significantly contribute to SIPA, but further experiments using washed platelets could determine if Mmrn1 modulates SIPA in the absence of adhesive plasma proteins. The time in which blood is exposed to shear affects the amount of SIPA, although SIPA was similar between *Mmrn1*$^{-/-}$ and wild-type samples when sheared for 30 s or 90 s (data not shown), in addition to the studies included herein that sheared samples for 60 s. It is possible that measuring SIPA at a greater number of time points could reveal a significant difference in SIPA between *Mmrn1*$^{-/-}$ and wild-type samples.

Part of the observed reduction in platelet number in response to high shear stress could be due to either receptor shedding or platelet fragmentation in addition to SIPA. Shedding of platelet GPIb/IX/V and $\alpha_{IIb}\beta_3$ occur following platelet activation, and

receptor shedding has also been reported under conditions of pathological shear stress.[578,579] In many experiments, smaller events were visible on scatterplots (*Figure 23*), which could reflect either platelet fragmentation or the release of CD42b-bearing platelet microparticles by activated platelets in response to elevated shear stress.[579] Notably, platelet count can also impact the degree of SIPA.[247,580] Platelet-platelet collisions are required for aggregation to occur, and 2-body collision theory asserts that collision frequency is proportional to the cell concentration at a given shear rate.[581,582] Variability in mouse platelet counts in whole blood could account for some variability in measurements of SIPA, which would make detection of less pronounced changes in SIPA due to Mmrn1 loss require extremely large sample sizes to reach statistical significance. Using platelet count-adjusted PRP for SIPA experiments could be useful for controlling variability in SIPA due to underlying differences in platelet count, but generating PRP requires sample manipulations that expose whole blood to shear stress (i.e. centrifugation), and eliminates the potential role of erythrocytes in SIPA, unless PRP is reconstituted with washed erythrocytes. Importantly, the upper limit of detection for particle size on the flow cytometer used to measure SIPA is may be insufficient to detect larger aggregates. Following exposure to a shear rate of $15{,}000$ s^{-1}, the aggregates detected by fluorescence microscopy in some experiments could reach up to $11{,}000$ μm^2 in size. This likely explains why samples show a significantly smaller number of CD42b+ events, but not a significant shift in the size distribution of collected events. Pilot experiments to detect P-selectin expression by flow cytometry as a measure of shear-induced platelet activation (SIPAct) failed to detect significant P-selectin expression on

the events collected by flow cytometry (data not shown), which suggests that most activated platelets expressing P-selectin are likely incorporated into large aggregates not detectable by flow cytometry. Detection of P-selectin expression on fixed platelet aggregates by fluorescence microscopy in future studies could answer this question.

4.3 UPDATED MODEL OF RECOGNITION MOTIFS IN FIBRILLAR COLLAGENS INVOLVED IN PLATELET ADHESION OR ACTIVATION

The GPAGPOGPX sequences in types I and III vessel wall fibrillar collagens are structurally and spatially distinct from other recognition motifs in collagen that support platelet adhesion or activation (*Figure 33*). Toolkit peptide III-38 containing GPAGPOGPQ does not bind VWF,[545] integrin $\alpha_2\beta_1$,[546] or GPVI.[547] Similarly, rhMMRN1 does not bind to peptides containing the VWF-binding GPRGQOGVMGF sequence, $\alpha_2\beta_1$-binding GXOGEX' sequences, or the GPVI-binding peptides CRP and III-30 (Subia Tasneem, unpublished data). The MMRN1-binding GPAGPOGPQ motif in Toolkit peptide III-38 is also distinct from other recognition motifs in collagen that have been identified using Collagen Toolkits.[583–590] The only other known ligand that binds to the region in collagen containing GPAGPOGPQ is leukocyte-associated Ig-like receptor-1 (LAIR-1), which binds to the discontinuous GPO repeats in Toolkit peptide III-38,[586] not specifically to the GPAGPOGPQ sequence.
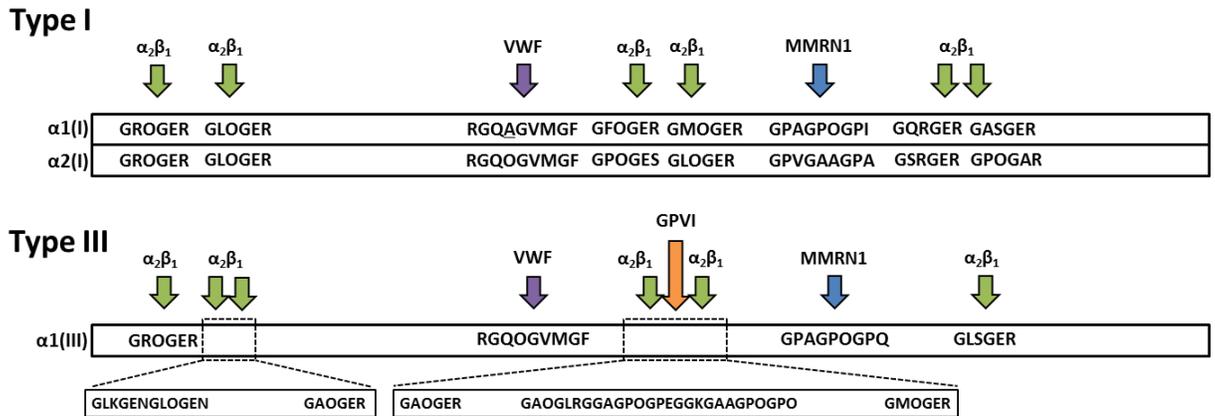
Figure 33: Updated model of recognition motifs on fibrillar collagens that support platelet adhesion.

The GPAGPOGPI (helix residues 667 to 675) sequence lies at the same locus as GPAGPOGPQ (helix residues 682 to 690) in D-period 3 of the collagen α_1(I) chain. At this locus, two copies of GPAGPOGPI would be present in a native heterotrimeric type I collagen molecule. The third α_2(I) chain of the heterotrimer has a corresponding GPVGAAGPA sequence at this position, but it is unclear if this sequence is involved in MMRN1 binding. Testing a triple-helical homotrimeric GPVGAAGPA peptide for MMRN1 binding would not be sufficient to answer this question, which is why it was not synthesized for testing. Specific residues from the COL α_2(I) chain GPVGAAGPA may enhance the ability of GPAGPOGPI to bind MMRN1, but not necessarily substitute for this sequence in MMRN1 binding. Interestingly, a homotrimeric isotype of type I collagen composed of three α_1(I) chains has been reported during wound healing, embryonically, and in certain tumour cell lines,[591] but its potential relevance to MMRN1 is unclear. In the absence of a Collagen Toolkit for the heterotrimeric type I collagen,

other techniques (e.g. cyanogen bromide fragmentation) have been used to map

recognition motifs in type I collagen.[540] In some cases, recognition motifs have been

inferred from data obtained using Collagen Toolkit III due to the high homology between

types I and III collagen.[436,545,546] Similar to the GPAGPOGPQ sequence in type III

collagen, GPAGPOGPI is spatially and structural distinct from $\alpha_2\beta_1$-binding GXOGEX'

sequences in collagen α_1(I)[436,541,546] and a postulated VWF-binding GPRGQAGVMGF

sequence in collagen α_1(I) that aligns with GPRGQOGVMGF in α_2(I) and provides the

necessary hydroxyproline residue at the 6th position.[545] Interaction "hot spots" have been

reported in type I collagen, and GPAGPOGPI is directly adjacent to the C-terminal

interaction hot spot (helix residues 680 to 830).[502,592] In general, more ligand-binding sites

have been identified on the C-terminal half of type I collagen. Fibrillar collagen

molecules trimerize from the C- to the N-terminus, and mutations within the COL domain

that disrupt helix assembly can affect downstream helix assembly toward the N-terminus.

As a result, it is hypothesized that mutations near the C-terminus that disrupt triple helix

assembly are less tolerated than N-terminal mutations because they have more significant

downstream effects of helix assembly.[593] Consequently, ligand-binding sites have

predominantly evolved closer to the C-terminus. Together, these data suggest

GPAGPOGPI may be the primary interaction site between type I collagen and MMRN1.

The ability of wild-type but not *Mmrn1*[-/-] platelets to adhere to GPAGPOGPX

peptides in static adhesion assays provides further evidence that these sequences in

fibrillar collagens are specific recognition motifs for MMRN1/Mmrn1. The overall poor

adhesion of wild-type platelets to GPAGPOGPI compared to GPAGPOGPQ may be a consequence of synthesizing the GPAGPOGPI sequence as a homotrimeric triple-helical peptide. As stated above, it is unclear if the corresponding GPVGAAGPA in the $\alpha_2(I)$ chain of type I collagen contributes key residues that are important for MMRN1 binding. Similarly, in solid-phase binding assays, MMRN1 binding to GPAGPOGPI was consistently lower compared to GPAGPOGPQ or GPAGPOGPV. This may be a result of using synthetic collagen mimetic peptides rather than full-length collagen fibrils. Toolkit and other triple-helical collagen mimetic peptides contain Cys residues at both the N- and C-termini, which allows them to crosslink to one another, but they do not aggregate into native fibrils. The dynamics of polyvalent binding of MMRN1 multimers to collagen fibrils may be important for the amount and stability of MMRN1 binding to GPAGPOGPX sequences, which could result in poor binding between MMRN1 and GPAGPOGPI peptides. The reduction in static adhesion of $Mmrn1^{-/-}$ to Horm collagen, which is predominantly composed of type I collagen (~95%), under stasis suggests that Mmrn1 is involved in platelet adhesion to type I collagen, likely through interactions with the GPAGPOGPI/GPVGAAGPV heterotrimer. Another possibility is that there may be MMRN1-binding motifs in type I collagen that are structurally distinct from GPAGPOGPX that support or enhance MMRN1 binding to collagen, which *in silico* searches for GPAGPOGPX-like sequences would not be sensitive to detect.

Fibrillar collagens are unlike many ligands because of their rod-like COL domain that encompasses almost its entire sequence and the propensity of collagens to form large

ultrastructures. This means that collagens have many possible interactions along the entire length of a collagen fiber, many of which would be low affinity, multi-valent interactions. As a result, GPAGPOGPI may be the single, physiologic ligand for MMRN1 in type I collagen, but polyvalent binding between numerous MMRN1 monomers in a larger polymer along the length of a collagen fiber may be required for GPAGPOGPI to support significant MMRN1 binding or platelet adhesion. Another consideration is that, as a consequence of ultrastructural organization of collagen molecules into D-periods, binding sites for platelet receptors and other molecules are not uniformly distributed across the fiber surface. It is hypothesized that binding sites may be presented as coherent bands around the circumference of the fiber,[594] allowing platelet receptors to be recruited in closely packed clusters, which may be required to induce signalling. Clustering of GPAGPOGPX motifs on a fiber surface may similarly be important for MMRN1 binding. Moreover, it is unclear what regions of the collagen monomer are accessible on the fiber surface. Collagen fibrils contain many inter-chain crosslinking sites, and additional molecules, such as type V collagen, are bound to the fibril surface to regulate its function. Some recognition motifs may be concealed by the architecture of a collagen fiber,[595] or, conversely, some motifs may only be present in a suitable conformation when collagen molecules are assembled into fibers.

Testing platelet adhesion to GPAGPOGPX peptides under high shear flow provided further evidence of a potential dual role of Mmrn1 in platelet adhesion to collagen, while also demonstrating specificity of GPAGPOGPQ for Mmrn1 under

laminar flow. Although VWF is considered to be required for significant platelet adhesion under high shear flow, the GPAGPOGPQ-containing peptide was able to enhance the formation of platelet aggregates in the absence of a surface ligand for Vwf when co-presented with GFOGER. It is possible that Mmrn1 released from activated platelets could bind to GPAGPOGPX to provide a surface for attachment of Vwf, which would allow for some degree of platelet tethering in the absence of a surface ligand for Vwf. Additionally, Mmrn1 multimers bound to the platelet surface or bound to GPAGPOGPQ may contribute to platelet tethering that is able to enhance $\alpha_2\beta_1$-mediated adhesion to GFOGER under high shear flow. However, it would be expected that if Mmrn1 released into solution binds to GPAGPOGPX to provide a surface for Vwf attachment and platelet tethering, presentation of the GPAGPOGPQ sequence alone would support some degree of platelet adhesion, with morphology similar to surfaces coated with either GPRQOGVMGF or immobilized Vwf. Following perfusion under high shear flow, strings of platelets can be observed on immobilized VWF, rVwf, and GPRQOGVMGF. Wild-type and $Mmrn1^{-/-}$ platelets failed to adhere to the GPAGPOGPQ-containing peptide under high shear flow, which suggests that Mmrn1 cannot substitute for Vwf in the absence of an additional adhesive ligand, such as GFOGER.

An important observation was that $Mmrn1^{-/-}$ platelets had impaired aggregate formation on GFOGER surfaces in the absence of a surface ligand for other proteins or receptors. These data provide further evidence that Mmrn1 plays a supporting role in platelet aggregate formation onto adhesive substrates. $Mmrn1^{-/-}$ platelets have similar

expression of β_1 integrin compared to wild-type platelets, [4] and static adhesion of activated $Mmrn1^{-/-}$ and wild-type platelets to GFOGER is also similar, which indicates the inability of $Mmrn1^{-/-}$ platelets to forms large aggregates GFOGER surfaces is likely not due to alterations in $\alpha_2\beta_1$ expression or the ability of $\alpha_2\beta_1$ to bind GFOGER.

In assays of static platelet adhesion using mouse platelets and triple-helical collagen peptides, GFOGER alone supported adhesion of CRP-activated mouse platelets as well as full-length Horm collagen, which hindered the ability to detect synergy between combinations of triple-helical peptides. In comparison, GFOGER supported roughly 50% of adhesion of CRP-activated human platelets compared to Horm collagen, which provided a sufficient model to test the synergistic effects of GFOGER, GPRQOGVMGF, and GPAGPOGPX on platelet adhesion. These differences between human and mouse platelets may result from the significantly higher copy number of $\alpha_2\beta_1$ on murine platelets (~16000 copies per platelet)[403] versus human platelets (~1000 to 3000 copies per platelet, *Figure 3*).[430] Even when the relative concentration of GFOGER compared to GPAGPOGPI/Q was titered down to 10% of GPAGPOGPX concentrations, it was not possible to detect synergy between GFOGER and GPAGPOGPX peptides using CRP-activated mouse platelets. Titration curves indicated that adhesion of activated mouse platelets to GFOGER was nearly all-or-nothing, in that adhesion jumped from no adhesion to maximal adhesion in a window of 0.9 µM. In assays using human platelets, GPAGPOGPX peptides were able to synergistically enhance platelet adhesion when presented with GFOGER or GFOGER and III-23. These data provide further evidence

that MMRN1 is able to additively enhance platelet adhesion to collagen without inhibiting VWF- or $\alpha_2\beta_1$-mediated adhesion to their respective recognition motifs in collagen. However, as these experiments were performed with triple-helical collagen peptides, it is unclear how the spatial arrangement of immobilized peptides versus native collagen fibrils impacts these findings.

The effect of platelet activation on static adhesion to combinations of triple-helical peptides is consistent with the requirement for platelet activation to release MMRN1. These data provide indirect evidence that GPAGPOGPX sequences may also have high specificity for MMRN1. Ligands with high specificity for MMRN1 would be expected to support minimal adhesion in the absence of MMRN1 on the surface of activated platelets unless the ligand can support adhesion through other mechanisms. Notably, a modest increase in the endpoint adhesion of washed, unstimulated human platelets to GPAGPOGPX peptides co-presented with GFOGER was observed, which may be the result of a small degree of platelet activation inevitably occurring during platelet preparation and less likely, other, unidentified adhesive molecules that can bind this motif to supporting platelet adhesion. The effect of platelet activation on platelet adhesion to GFOGER is also consistent with the observation that platelet activation induces conformational change in $\alpha_2\beta_1$ that increases the affinity of $\alpha_2\beta_1$ for GFOGER.[436]

With regard to the MMRN1-binding GPAGPOGPV sequence in type II collagen, it is unclear whether or not this sequence is physiologically relevant to platelet adhesion.

The GPAGPOGPV peptide was able to synergistically enhance platelet adhesion similar to either GPAGPOGPI or GPAGPOGPQ, but the tissue distribution of type II collagen is restricted to cartilage in mammals: fibrocartilage in cartilaginous joints of the pelvis and spine, hyaline cartilage on the articular surface of joints, in the trachea, or in the nose, and elastic cartilage in the epiglottis and outer ear. MMRN1 would likely only contact the GPAGPOGPV sequence in instances wherein flowing blood enters a type II collagen-rich joint capsule or when flowing blood is exposed to cartilaginous structures in the nose, ear, or trachea. Sequence conservation is not necessarily a good indicator of preservation of function for fibrillar collagens because of its repeating structure. Approximately 33% of the triple-helical domain of fibrillar collagens is composed of glycine.[485] Given the requirement of proline in each α-chain for the formation of a polyproline II-like helix, much of the COL domain is composed of highly conserved proline residues. As a result, fibrillar collagens tend to have highly conserved COL domains because of their structural requirements, and mutations in glycine and proline residues are not well-tolerated due to its ubiquitous distribution and essential developmental and structural role.

4.4 STRENGTHS AND LIMITATIONS OF THIS THESIS

The use of *in vitro* experimentation allows for the control and isolation of specific variables contributing to complex outcomes. MMRN1/Mmrn1 is a multipotent adhesive ligand that binds to adhesive proteins in platelets, plasma, and extracellular matrices, but also has potential roles in supporting thrombin generation and coagulation. It is difficult

to dissect the specific contributions of Mmrn1 in platelet adhesion using *in vivo* models that involve thrombin generation and coagulation, such as the $FeCl_3$-induced vessel injury model and the tail transection model. These models measure outcomes of thrombosis and hemostasis, respectively, which may be insensitive to detect specific alterations in platelet Mmrn1 adhesive functions, rather than measuring a holistic outcome. This is evidenced by studies of other supporting proteins in platelet adhesion, such as *Tsp-1$^{-/-}$*, *pFn$^{-/-}$*, and *Vn$^{-/-}$* mice, which have normal bleeding times and only somewhat altered thrombus formation (*Table 1*), despite numerous *in vitro* studies that demonstrate more complex supporting roles of these proteins in platelet adhesion and platelet aggregation.

The experiments performed in this thesis have the advantage of inhibiting or removing specific components of hemostatic or thrombotic responses (i.e. thrombin generation, platelet activation, presence or absence of plasma, or composition of extracellular matrices). These conditions enable the study of platelet adhesion at varying levels of complexity: whole blood vs. washed platelets, resting vs. pre-activated platelets, with or without antibody blockade of specific receptors, and control over the composition of immobilized adhesive substrates. *In vitro* experiments also enable strict control over rheological parameters (i.e. shear rate, tube diameter, and pulsatile vs. constant flow), which greatly affect platelet adhesive properties. Further, the use of triple-helical collagen mimetic peptides allows for the manipulation of individual recognition motifs in collagen independent of one another in order to define their specific and synergistic roles, and eliminates the variability observed between different fibrillar collagen preparations.[596]

Consequently, triple-helical collagen mimetic peptides have been instrumental in the current understanding of the type I, II, and III collagen interactomes as well as the functional properties of recognition motifs in collagens.[548] The use of purified proteins are useful for elucidating specific mechanisms, but it is equally important to consider that protein-coated surfaces fail to reflect the integrated function of all components present in the extracellular matrix of the vessel wall.

A major limitation in studying platelet adhesion and platelet-collagen interactions is establishing the relevance of *in vitro* findings using *in vivo* models. Fibrillar collagen exposure would be most evident in extravascular injuries, that is, when platelets in flowing blood are exposed to: 1) the collagen-rich structures of the tunica media and adventitia of blood vessels, 2) type I collagen-rich dense and loose fibrous tissues (i.e. skin, tendons, and ligaments), 3) type III collagen-rich reticular meshwork of adipose tissue, and 4) type II collagen-rich cartilage. The most commonly used *in vivo* models of arterial thrombosis include $FeCl_3$ treatment of the carotid artery[229,464,563,597] or mesenteric arterioles,[3,204,208,211,215] laser-induced injury of cremaster muscle arterioles,[33,203,204,598–600] or photochemical injury of the carotid artery following injection of Rose-Bengal dye.[440,601,602] Within these models, the degree and extent of collagen exposure is not well-understood and these models assess thrombosis, not hemostasis. Collagen-induced thrombosis can be measured directly by intravenous injection of collagen or collagen-like peptides,[310,440] but these models fail to recapitulate the conditions of exposed, insoluble collagen fibers in the vessel wall following injury. A major limitation of commonly used

murine models of thrombosis is that they induce platelet adhesion artificially within intact vessels. A model involving exposure of adventitial fibrillar collagen exposure to flowing blood has been reported,[603] which may be useful for studying Mmrn1 contributions to collagen-induced thrombosis in arteries and veins, but thrombosis also occurs within vessel lumina in this model.

An *in vivo* model has been developed to visualize hemostatic plug formation on the inner and outer surfaces of the vessel wall following puncture of the jugular vein in mice.[604] The findings of these studies highlight major differences in the architecture of hemostatic plugs versus thrombi formed within vessels, specifically that the collagen-rich outer surface of the vessel wall is the primary site of platelet activation, α-granule release, thrombin generation, and fibrin formation, whereas the luminal surface of the plug is composed predominantly of resting, discoid platelets that show minimal α-granule release and fibrin formation.[604]

The development of transgenic or knockout mouse models to study the importance of GPAGPOGPX sequences *in vivo* is particularly limited by the inherent properties of fibrillar collagens. Due to the ubiquitous distribution of fibrillar collagens, which make up approximately 30% of total protein mass in humans, mutations or deletions in collagen genes have systemic, often lethal consequences. Developing a mouse model in which GXOGEX', GPRQOGVMGF, and/or GPAGPOGPX sequences are mutated or deleted would likely have systemic, potentially lethal consequences.

Further, it would be difficult to interpret whether bleeding diatheses in collagen mutant mice are due to defects in platelet adhesion or because of vessel fragility as a consequence of compromised collagen triple-helices, which are pronounced clinical features of collagen-related diseases EDS and OI and in a mouse model of EDS.[516,593,605,606] Together, these considerations make it difficult to the effects of MMRN1-binding GPAGPOGPX motifs in fibrillar collagens through *in vivo* experimentation. As a result, this thesis relies on *in vitro* evidence to characterize the mechanisms through which Mmrn1 supports platelet adhesion to collagen via GPAGPOGPX sequences in fibrillar collagens.

4.5 CONCLUSIONS AND FUTURE DIRECTIONS

The questions addressed in this thesis contribute to the fundamental understanding of platelet adhesion, which is a critical first step in cessation of blood loss following injury or in the development of platelet-rich thrombi. Many of the mechanisms involved in hemostasis are conserved in thrombosis, making it crucially important to develop therapies for the prevention or treatment of thrombotic diseases that do not significantly compromise hemostasis. MMRN1 is an attractive potential therapeutic target because *in vivo* evidence suggests that Mmrn1 deficiency is anti-thrombotic without significantly impairing hemostasis. Future therapies that block the functions of "supporting proteins" such as MMRN1, TSP-1, multimeric platelet VN, or platelet FN could be useful in combination therapy to enhance the efficacy of anti-platelet drugs without significantly

impacting hemostasis. More specifically, given that MMRN1/Mmrn1 contributes to platelet adhesion to collagen, anti-MMRN1 therapy may be useful for exploring in the treatment of atherothrombosis, in which the type I collagen-rich fibrous cap formed over an atherosclerotic plaque ruptures and is exposed to flowing blood. Equally, it is important to consider that species differences may affect the phenotype of qualitative or quantitative defects in MMRN1 versus Mmrn1.

Although selective MMRN1 deficiency has not been described in human patients, the causes of many inherited platelet function disorders (PFDs) are uncharacterized.[607] It is possible that selective defects in MMRN1 could be the cause of some cases of PFD, but all of what is presently known about the phenotype of selective MMRN1 deficiency comes from studies of *Mmrn1* knockout mice. Based on studies of *Mmrn1[-/-]* mice, specific types of analyses (i.e. testing platelet adhesion to collagen under high shear flow or collagen-induced aggregation of gel-filtered platelets) would be required to suggest involvement of MMRN1. Typical laboratory tests of platelet function would not be sensitive to detect MMRN1 deficiency in human patients,[608] making it unlikely that a selective defect in MMRN1 would be identified through routine laboratory testing for platelet function disorders.

It has also been argued that mechanisms of platelet adhesion to fibrillar collagens are redundant in hemostasis,[531] based on the relatively mild phenotypes of transgenic and knockout mouse models and cases of human patients with deficiencies or defects in

platelet collagen receptors. What is not often considered is the process of wound healing, in which fibroblasts and smooth muscle cells infiltrate the area of injury to regenerate and remodel the damaged extracellular matrix structures. For example, in addition to the role of FN in platelet adhesion and aggregation, FN fibers are a normal part of extracellular matrices of connective tissues that appear to play an important role in wound healing.[275,609] Similarly, the role of MMRN1 may not be limited to the processes of platelet adhesion and aggregation in hemostasis, and may have important functions in the wound healing process during and following the dissolution of a hemostatic plug. Other proteins in the EMILIN/Multimerin family are normal constituents of extracellular matrices containing elastic fibers, including the outer layers of arteries and veins. Given the high homology between EMILINs and MMRN1, it is possible that platelet MMRN1 released at sites of injury may serve similar functions following hemostatic plug formation.

The results described in this thesis raised numerous questions regarding additional mechanisms of MMRN1 in platelet adhesion and identified a need for verifying *in vitro* data using models of collagen-mediated hemostasis or collagen-induced thrombosis *in vivo*. It is also interesting to consider how mutations in collagens might have effects on platelet adhesion and contribute to part of the bleeding phenotype exhibited by some patients with EDS or OI. The following sections will discuss additional studies that could be performed to answer some of these questions.

4.5.1 Possible effect of multimerin 1 on VWF multimer size

As suggested in *section 4.2.1*, Mmrn1 may contribute to platelet adhesion and

thrombus formation partly by binding plasma and platelet Vwf, which could: 1) generate

large, highly adhesive heteropolymers composed of Mmrn1 and Vwf, or 2) prevent

Adamts13-mediated cleavage of Vwf to facilitate the formation of and maintain the size

of ULVwf multimers at sites of injury. First, the ability of MMRN1 to form large

heteropolymers with VWF/Vwf could be tested *in vitro* using recombinant VWF or Vwf

and rhMMRN1. Purified VWF or VWF in normal or ADAMTS13-deficient plasma could

be exposed to ristocetin or shear to induce exposure of the cryptic MMRN1-binding sites

in VWF A1 and A3 in the presence or absence of varying concentrations of rhMMRN1.

Multimer size could then be resolved using non-reduced agarose-acrylamide gel

electrophoresis and Western blotting. There is also a possibility that Mmrn1 augments

Vwf multimer size within platelet and endothelial cell storage granules, although studies

of *Tsp-1*[-/-] mice generated on different background strains have reported conflicting

results.[225,363] *Tsp-1*[-/-] C57BL/6J mice show smaller Vwf multimers in platelets and

endothelial cells compared to the wild-type, but Vwf multimer size is similar between

Tsp-1[-/-] and wild-type Swiss mice. TSP-1 has been demonstrated to block VWF A2

cleavage by ADAMTS13 *in vitro,*[362] which has been demonstrated *in vivo* using *Tsp-1*[-/-]

mice.[225] These assays could be adapted to test the ability of MMRN1 to competitively

bind VWF to prevent cleavage by ADAMTS13 in a dose-dependent manner *in vitro* and

measure ultra-large Vwf degradation *in vivo* using *Mmrn1*[-/-] mice. These experiments

would clarify part of the mechanism through which MMRN1 supports VWF adhesive functions.

4.5.2 Generation and testing of additional mouse models

The ability of Mmrn1 to augment platelet adhesion and thrombus formation following exposure to fibrillar collagens *in vivo* could be investigated using methods described in *section 4.4*. The jugular vein puncture model is most appropriate for studying the effects of Mmrn1 on platelet adhesion to collagens because it best recapitulates conditions of extravascular injury involving collagen-rich matrices. It may also be possible to modify this model to assess hemostatic plug formation in arteries or differently-sized vessels. Additionally, there is a collagen-induced thrombosis model in which a section of the epigastric artery is dissected from donor mice and inserted into the carotid artery or femoral vein of anesthetized recipient mice to induce thrombus formation by exposure of the fibrillar collagen-rich adventitia of the epigastric artery to flowing blood.[603] However, this model involves intravascular thrombus formation, which might be useful as a model for studying collagen-induced thrombosis, but not for studies of hemostatic plug formation. The collagen-dependent thrombosis model that measures pulmonary embolism following collagen or peptide injection is less appealing because it would not directly test platelet adhesion to immobilized fibrillar collagen surfaces at a site of injury.

The presence of Mmrn1 in both platelet and endothelial cell granules combined with its proposed contributions to Fv storage and thrombin generation complicates interpretations of *in vivo* observations. Using global *Mmrn1* knockout animals, it is difficult to ascertain whether defects in platelet adhesion are due to platelet Mmrn1 loss or if the endothelial cell pool of Mmrn1 plays an important, or potentially greater, role in platelet adhesion and thrombus formation. The specific contributions of platelet Mmrn1 in fibrillar collagen-dependent thrombus formation could be studied using bone marrow transplantation, which has already been used to identify the relative contributions of platelet vs. endothelial cell Vwf in thrombosis.[332] These studies would untangle the relative importance of platelet vs. endothelial cell Mmrn1 in experimental models of thrombosis, and help orient the findings of this thesis in an *in vivo* setting, which centre on platelet Mmrn1. The jugular vein puncture model, coupled with the generation of cell type-specific *Mmrn1* knockout mice, could be used to validate some of the results in this thesis *in vivo* and address some ambiguities of the results obtained using the $FeCl_3$-induced mesenteric vessel injury model and global *Mmrn1*[-/-] mice.

4.5.3 Impact of triple-helical conformation and mutations in MMRN1 binding to GPAGPOGPX sequences

Triple-helical conformation is required for GPVI binding to GPO repeats or $\alpha_2\beta_1$ binding to GFOGER.[540,543] It may be similarly required for MMRN1 binding to GPAGPOGPX motifs. As previously reported, synthesizing GPAGPOGPX peptides

within (GAP)$_5$ host sequences, which do not spontaneously assemble into triple-helices, could be used to determine if triple-helical conformation of GPAGPOGPX peptides is required for MMRN1 binding. Although the GPAGPOGPI and GPAGPOGPQ sequences are unique to collagen, there may be non-triple-helical GPAGPOGPX-like sequences in other proteins. The substitution of the hydrophobic residue V to another hydrophobic residue I is a conservative mutation,[566] and both the GPAGPOGPV and GPAGPOGPI peptides support MMRN1 binding. It could be that other proteins contain GPAGPOGPX sequences where X is occupied by a different hydrophobic residue, which could consequently support MMRN1 binding. An *in silico* search for sequences containing GPAGPOGPX sequences where X is occupied by any of the amino acids with a hydrophobic side chain could reveal additional MMRN1-binding motifs in other proteins. Interestingly, substitution of V to Q is not a conservative mutation,[566] but both GPAGPOGPV and GPAGPOGPQ support MMRN1 binding very well, meaning that residues in the X position may not be limited to hydrophobic residues alone. Further, without performing studies of site-directed mutagenesis of the GPAGPOGPX motif, it is unclear which residues in this sequence are critical for MMRN1 binding. This means that while GPAGPOGP(I/V/Q) is unique to collagens, there could be other variants of this sequence that possess the key residues for MMRN1 binding, but differ dramatically in others, which would make detection of these types of potential MMRN1-binding sequences by *in silico* analyses difficult. Firstly, determining whether or not linear (non-triple-helical) GPAGPOGPX peptides bind MMRN1 would provide evidence for whether

or not linear GPAGPOGPX-like sequences in other proteins could potentially support MMRN1 binding.

As mentioned in *section 4.3*, mutations in the MMRN1-binding GPAGPOGPX motifs could affect MMRN1 binding and platelet adhesion, similar to reports of Gly mutations in the GFOGER sequence.[610,611] Disease-causing mutations have been reported in the GPAGPOGPI and GPAGPOGPQ sequences. Mutations in the GPAGPOGPI motif (G845R and G848R) are associated with type II (perinatal lethal) OI[517] and a mutation in GPAGPOGPQ (G852C) is associated with type IV (vascular type) EDS. The effects of missense Gly mutations within and surrounding the GFOGER sequence on integrin binding have been tested using recombinant bacteria that express a triple-helical collagen-like protein, and this model could be applied to study the effects of Gly mutations in GPAGPOGPX motifs on MMRN1 binding. In these studies, mutation of either Gly residue in GFOGER abolishes $\alpha_2\beta_1$ binding and results in disruption of inter-chain hydrogen bonds within the affected portion of the triple helix.[610] Additionally, substitution of Gly residues to the N-terminal side of the inserted GFOGER sequence is able to impair $\alpha_2\beta_1$ binding.[611] It has been previously postulated that mutations in fibrillar collagens that locally disrupt triple helix assembly could have systemic consequences on ligand binding at other areas within the COL domain.[593] It is therefore difficult to conclude how specific mutations might affect other ligand binding sites in collagen, and consequently, different aspects of platelet adhesion to collagen. Other studies have used cultured fibroblasts from patients with fibrillar collagen mutations to test their effects on

ligand binding and cell adhesion. Cell databases could be accessed to obtain fibroblasts from patients with COL domain mutations in recognition motifs in collagens that support platelet adhesion or activation in order to study the systemic effects of various collagen mutations on platelet adhesion and MMRN1 binding to collagen.

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