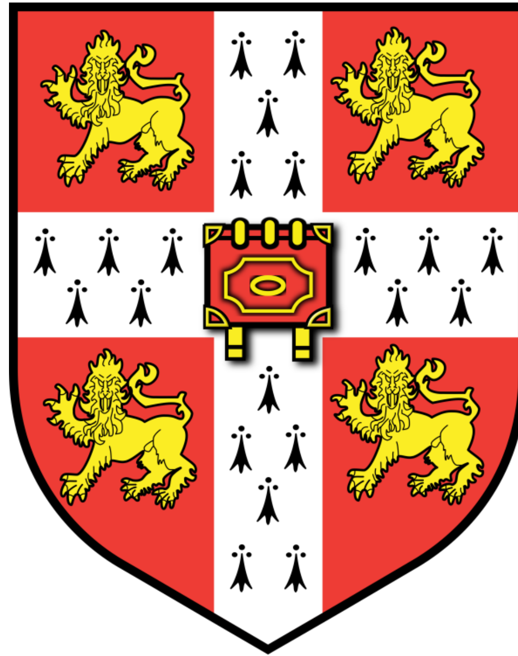

PLATELET RECEPTOR GLYCOPROTEIN VI IN ISCHAEMIC STROKE

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This dissertation is submitted for the degree of Doctor of Philosophy

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Declaration

This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Preface and specified in the text. It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. It does not exceed the prescribed word limit for the Clinical Medicine and Clinical Veterinary Medicine Degree Committee (60,000 words).

Summary

Platelet Receptor Glycoprotein VI in Ischaemic Stroke

Isuru Pasanna Induruwa

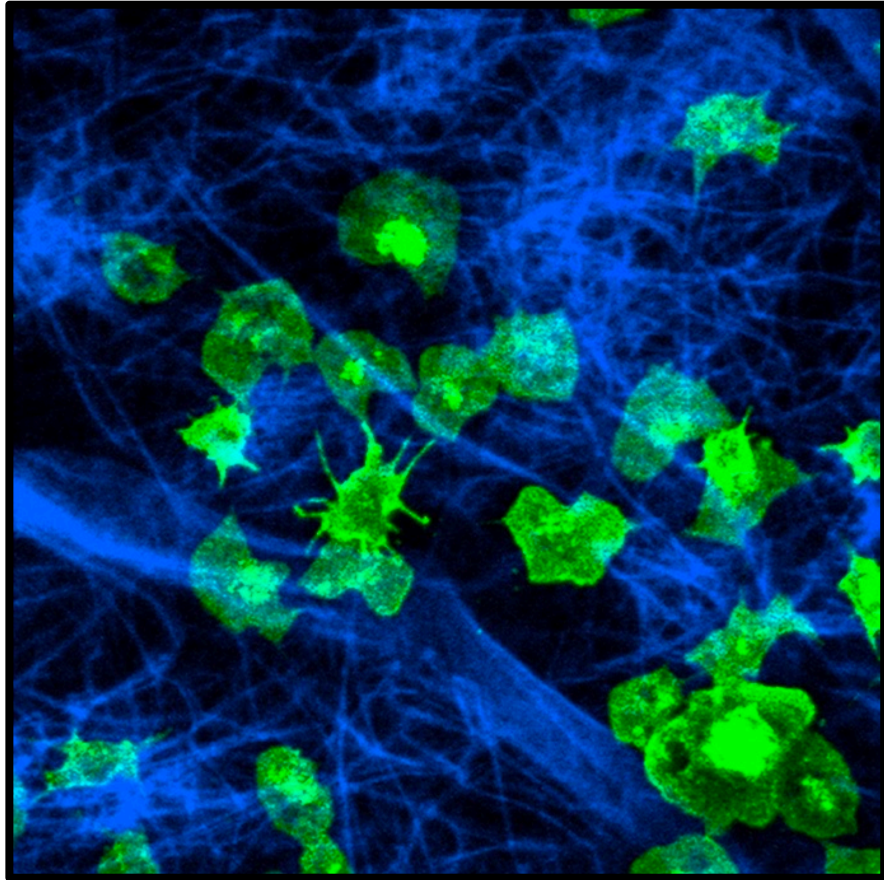
Platelet activation, thrombus growth and subsequent thromboembolism underpins the pathophysiology of ischaemic stroke. At sites of atherothrombotic plaque rupture, it is established that platelet surface receptor glycoprotein (GP) VI-dimer binds to exposed sub-endothelial collagen, initiating the signalling required to cause platelet activation. Then, separate platelet-fibrin interactions cause the thrombus to grow, culminating in distal tissue ischaemia; crucial in thrombotic diseases such as ischaemic stroke.

The work in this thesis demonstrates that GPVI-dimer binds to fibrin also, causing platelet activation through a mechanism independent of collagen. In ischaemic stroke this is important as it not only implicates GPVI-dimer in the large-artery atherosclerotic stroke subtype, it suggests a key role in cardioembolic stroke, where fibrin, rather than collagen, is the key platelet ligand.

We compared the platelet surface expression of GPVI-dimer in a control, pre-stroke atrial fibrillation (AF), and a stroke population admitted to hospital using flow cytometry. We also measured platelet activation by platelet P-selectin exposure in all the cohorts.

The results demonstrate that both AF and stroke patients have more 'active' circulating platelets compared to the controls. Furthermore, both AF and stroke patients – irrespective of AF type or stroke aetiology, express more GPVI-dimer on their platelets compared to controls. In the stroke cohort, GPVI-dimer expression was significantly higher at day-90 post-stroke than at admission.

The ability of GPVI to interact with the two main ligands that drive thrombosis, collagen and fibrin, cements its role as a key platelet receptor in human thromboembolic disease. These results intimate an important role for GPVI-dimer in driving thrombotic risk pre-stroke, as well as after having a stroke, suggesting that the direct inhibition of GPVI-dimer could be a promising future antithrombotic target.



Arachnoid platelets trapped in an intricate fibrin web

Image courtesy of Dr S Jung

Research Training

The work described in this thesis has been conducted primarily in the Departments of Clinical Neurosciences (under the supervision of Dr Elizabeth Warburton) and Biochemistry (Farndale Laboratory; under the supervision of Dr Stephanie Jung) at the University of Cambridge.

The work was conducted with the support of the following individuals:
Professor Richard Farndale, Professor of Matrix Biochemistry, Department of Biochemistry, University of Cambridge. Professor Willem Ouwehand, Professor of Experimental Haematology, Department of Biochemistry, University of Cambridge. Dr Kate Downes, senior research associate within the Ouwehand laboratory.

The basic sciences experiments detailed in chapters IV and V were carried out under the guidance of Dr M Moroi, Dr S Jung and Professor Farndale. This included training in human platelet preparation, ELISA, flow cytometry, flow adhesion, aggregation and confocal microscopy.

All patients for both clinical studies were recruited from Addenbrooke's Hospital, Cambridge University Hospitals NHS Foundation Trust. I created the study protocol for the Glycoprotein VI in Atrial Fibrillation and ThromboEmbolism *(GRAFITE)* study (chapter VI). As chief investigator, I led the application through Research and Development and Research Ethics Committee approvals. I screened and recruited all participants into the study, performed data collection and analysis including statistical analysis.

For the Glycoprotein Six in StrokE (*GYPSIE)* study (chapter VII), I analysed all the data and carried out statistical analysis. I also subclassified all the ischaemic strokes and wrote the manuscript, which is currently under review.

The methods for quantification of GPVI expression and platelet function were developed by Dr M Moroi and Dr S Jung. These measurements, as part of the two clinical studies, as well as data analysis were carried out by laboratory technicians (Harriet McKinney, Carly

Kempster and Patrick Thomas) at the Ouwehand Laboratory, supervised by Dr Kate Downes.

Assistance and review of statistical methodology was provided by Dr Ali Amin, Department of Clinical Neurosciences, University of Cambridge who is an epidemiological statistician with experience in cerebrovascular disease and risk factor analysis and Dr Kate Downes, Department of Haematology who is a clinical geneticist with experience in application of epidemiological and haematology-specific statistical methods.

Dedication

I have enormously enjoyed creating this piece of work over the last three years. Dare I say it, I do not recognise the person that first started this PhD as I have grown on both a personal and professional level. It must be said that I could not have achieved this without the relationships forged with the fantastic people that surround me.

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I would like to acknowledge our collaborators in the Ouwehand lab. Kate Downes for the time spent doing statistics with me and for keeping me on my 'scientific' toes. Thank you to Harriet, Carly, Patrick, and Joana – the clinical studies would not be a success without your hard work.

I would like to end by thanking my family for their love and support. To my parents and sister, being in a position to do this work is only a snapshot of how you have guided me through the years. You laid the foundations for everything I achieve and are a constant source of motivation to achieve more. Words cannot express my gratitude. To my father-in-law, thank you for always being there and always being enthusiastic about my work.

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This is dedicated to you.

Glossary of terms

ACS	Acute coronary syndrome
ADAM	A disintegrin and metalloproteinase
ADP	Adenosine diphosphate
AF	Atrial fibrillation
aPTT	Activated partial thromboplastin time
ATP	Adenosine triphosphate
BMI	Body mass index
BSA	Bovine serum albumin
BTG	Beta-thromboglobulin
CAA	Cerebral amyloid angiography
CCF	Congestive cardiac failure
CES	Cardioembolic stroke
CI	Confidence interval
CKD	Chronic kidney disease
CLEC-2	C-type lectin-like receptor 2
CRP	Collagen related peptide
CRP-XL	Crosslinked collagen related peptide
C-RP	C-reactive protein
CT	Computerised tomography
CUH	Cambridge University Hospitals NHS Foundation Trust
DAPT	Dual antiplatelet therapy
DNA	Deoxyribonucleic acid
DVT	Deep vein thrombosis
ECM	Extracellular matrix
ED	Emergency department
EGRESS	Emboli and Gene RESearch in Stroke Study
ELISA	Enzyme-linked immunosorbent assay
ESUS	Embolic stroke of undetermined source
Fab	Antigen binding fragment of antibody
FBC	Full blood count
FCγR	FC receptor γ-chain
GP	Glycoprotein
GPCR	G-protein coupled receptor
$GPVI_{ex}$	Recombinant GPVI monomer
GPVI-Fc_2	Recombinant GPVI-dimer
GT	Glanzmann's thrombasthenia
GWAS	Genome-wide association studies
hsCRP	High sensitivity C-reactive protein

Hb	Haemoglobin
HBS	Hepes buffered saline
HT	Hepes-Tyrodes
Ig	Immunoglobulin
IL	Interleukin
INR	International normalised ratio
IV	Intravenous
K_d	Dissociation constant
LAA	Left atrial appendage
LAS	Large artery atherosclerotic stroke
mAb	Monoclonal antibody
MACE	Major adverse cardiovascular events
mFibrin	Laboratory prepared monomeric fibrin
MI	Myocardial infarction
MK	Megakaryocytes
MMP	Matrix metalloproteinase
MPV	Mean platelet volume
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
mRS	Modified Rankin Scale
MTH	Mean thrombus height
NEWS	National Early Warning Score 2
NICE	National Institute for Health and Care Excellence
NHS	National Health Service
NNT	Number needed to treat
OCS	Open canalicular system
OR	Odds ratio
pAF	Paroxysmal atrial fibrillation
PAF	Platelet activating factor
PAR	Protease-activated receptor
PCI	Percutaneous coronary intervention
PDGF	Platelet derived growth factor
pFibrin	Laboratory prepared polymerised fibrin
PF4	Platelet factor 4
PKC	Protein kinase C
PLC	Phospholipase C
Plt	Platelet
PPP	Platelet poor plasma
pP-selecin	Platelet P-selectin
PRP	Platelet rich plasma
PS	Phosphatidylserine

RNA	Ribonucleic acid
ROS	Reactive oxygen species
rtPA	Recombinant tissue plasminogen activator
SA	Surface area coverage
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sGPVI	Shed or soluble GPVI
SNP	Single nucleotide polymorphisms
sP-selectin	Plasma/soluble P-selectin
SVD	Small vessel disease
SVO	Small vessel occlusion
TF	Tissue factor
TIA	Transient ischaemic attack
TOAST	Trial of Org 10172 in Acute Stroke Treatment
TP	Thromboxane receptor
TXA_2	Thromboxane A_2
vWF	von Willebrand factor

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CHAPTER I. PLATELET ACTIVATION IN ISCHAEMIC STROKE

1.01 Introduction

Cardiovascular diseases are responsible for one-third of worldwide mortality, and haemorrhagic and ischemic strokes were the second- and third-largest cardiovascular causes of disability in 2015 (Roth, 2017).

In both ischaemic heart disease (IHD) and large-artery ischaemic stroke, atherosclerotic plaque rupture is followed by thrombus formation at the site of injury. Subsequently the unstable thrombus can become detached, resulting in sudden distal arterial occlusion. Specific to ischaemic stroke, thrombi can also originate from a cardiac source, driven by a thrombogenic cardiac arrhythmia called atrial fibrillation (AF).

The common pathway in both these types of events is the dysfunction of the vascular endothelial layer, causing activation of platelets as well as the coagulation system – the end product of which is polymerised fibrin. Many studies have shown that patients with thrombotic diseases and vascular risk factors have 'primed', hyperactive platelets that can readily form thrombi (Davì, 2007).

The currently available anti-platelet drugs are effective in secondary prevention of acute coronary or cerebrovascular events (Rothlisberger, 2015), but many patients still suffer from thrombotic disease, despite antiplatelet therapy (Burn, 1994; Yamamoto, 1998). One of the problems with drugs such as aspirin and clopidogrel is that their mechanism of action targets the products of platelets that are already activated. Alongside this, the risks of haemorrhage increase with antiplatelet use, and in some cohorts like the older population, this may outweigh the benefit of reduction in thrombotic events. This is also the same for anticoagulant medication used in stroke prevention, where due to the perceived risk of increased haemorrhage from these drugs, many patients who are at high-risk of thrombotic events do not receive it (Denoël, 2014).

Therefore, there is a need for anti-thrombotic drugs that strongly inhibit platelet function but do not induce bleeding; a major challenge which lies ahead of those doing translational research. Ideally it would be through a mechanism that decreases platelet activity by

targeting a pathway different from those addressed by current antiplatelets or anticoagulants.

A promising candidate 3here is the platelet receptor glycoprotein (GP) VI which represents a logical and unexploited target for anti-thrombotic medications. GPVI inhibition has gained much traction as a possible novel antithrombotic target over the last few years due to its specific mechanism of action on inhibiting platelet activation and expected lack of bleeding complications as seen in both pre-clinical trials and animal models (Induruwa, 2016). Its role in thrombus formation with respect to ischaemic stroke will form the basis of the work described in this thesis.

1.02 Ischaemic stroke

Stroke is a considerable cause of death and disability worldwide, with nearly two-thirds of patients left with a drastically reduced quality of life after their stroke (Langhorne, 2009; Katan, 2018). Stroke not only affects the patients, but also their family, carers as well as the wider society. Ischaemic stroke accounts for around 85% of all strokes and a wealth of research has explored the various genetic, molecular, clinical and epidemiological factors that increases an individual’s risk of stroke.

Broadly, ischaemic stroke can be divided into its subtypes according to aetiology: large artery atherosclerotic stroke (LAS), cardioembolic stroke (CES) and small-vessel stroke (SVO) (Adams, 1993). Recently, large genome-wide association studies (GWAS) have shown that specific genetic loci are attributed to individual subtypes of stroke (Malik, 2018), although some loci bestride two or more stroke subtypes (Lindgren, 2014; Malik, 2016), highlighting that ischaemic stroke is a complex syndrome with a heterogenous aetiology.

Hyperacute treatment for ischaemic stroke is based upon achieving recanalization of the occluded artery through recombinant tissue plasminogen activator (rtPA) and/or endovascular thrombectomy followed by appropriate anti-thrombotic therapy (NICE NG128 May 2019). However, only a small proportion of stroke patients are eligible for

hyperacute treatment and even still, the rates of recanalization can vary between individuals.

Frustratingly, approximately 30-40% of all ischaemic stroke cases cannot be sub-classified either and are labelled as embolic stroke of undetermined source (ESUS), which has enhanced the previous Trial of Org 10172 in Acute Stroke Treatment (TOAST) definition (Adams, 1993) of cryptogenic stroke (Hart, 2017). The difficulty in these cases is knowing which medication would be best to prevent their future risk of stroke. Even if the aetiology of the stroke can be determined, based on the degree of carotid stenosis (LAS), presence of AF (CES) or a diagnosis of lacunar stroke (SVD), individuals with risk factors common to cerebrovascular disease remain at risk of stroke from all subtypes.

For example, a commonly encountered scenario in transient ischaemic attack (TIA) clinic is a patient with AF, who also has some carotid disease, presenting with symptoms of amaurosis fugax. In this situation, the culprit is likely to be the inflamed carotid lesion and thromboembolism to the retinal vasculature. In the absence of AF, this patient would clearly benefit from antiplatelet therapy. However, given the patient has AF, and that neither single or dual antiplatelet therapy would not sufficiently alleviate their risk of CES (ACTIVE Investigators, 2006; You, 2012), they would still continue their anticoagulation for this and not receive antiplatelets – leaving them at risk of further ischaemic events from a large artery source, especially in the immediate period following the TIA (Rothwell, 2005).

Therefore, there is a need for novel anti-thrombotic targets that can be used for treating multiple aetiologies of stroke. Platelets are central to the thrombus that is formed in ischaemic stroke. The process of platelet 'priming' for thrombus formation, called platelet activation, is a key process leading to stroke, driven by vascular risk factors and is discussed later in this chapter. Understanding platelet activation and platelet function with respect to increasing thrombotic risk and thrombus formation in ischaemic stroke, therefore, is a crucial component of the pathway to developing new anti-thrombotic agents for ischaemic stroke.

1.03 Platelets

(a) Platelet Overview

Platelets are derived from megakaryocytes (MK) that play a major haemostatic role in the body. They are also vital components in other processes within the body including inflammation, immunity and angiogenesis. The majority of platelets circulate around the body in a resting state until they are cleared at the end of their lifespan of 7-10 days (Michelson, 2019).

Platelets stem from the MK cytoplasm; their much larger (30-100 μm), highly specialised, precursor cells present in the bone marrow (Michelson, 2019). After entering the circulation, around one-third of platelets migrate to the spleen for storage, awaiting release when required. The average adult must produce $1x10^{11}$ platelets each day to maintain a steady count (Kaushansky, 2005). However, at times of increased demand or thrombocytopaenia, negative feedback causes thrombopoietin to increase megakaryocytopoiesis and platelet production as needed (Sato, 1998).

(b) Platelet anatomy and platelet activation

Platelets are small (1-3 μm diameter, 0.5 μm thickness), anucleate cells, that are discoid in shape when inactive within the circulation. Despite their lack of a nucleus, there is evidence that platelets are able to translate pre-mRNA received from MKs into proteins (Weyrich, 2009). This means that platelets do contain post-translational mechanisms which allow alteration of their phenotype and function in response to cellular activation induced by various platelet-ligands (Zimmerman, 2008), although this maybe time-limited (Angénieux, 2016).

Much of the platelet's anatomy is geared towards its primary function of activation and subsequent thrombus formation. Multiple regions work in unison, firstly, to ensure that platelets remain quiescent in the circulation, but then also allow rapid activation at sites of vessel injury. For example, the spectrin-based membrane skeleton, microtubule system and rigid actin-filament network which form the platelet cytoskeleton help maintain the

platelet plasma membrane in a discoid shape when inactive, but also facilitate shape change and spreading when platelets become activated (Cerecedo, 2013).

Internally, the platelets contain three major types of secretory organelles namely α-granules, dense bodies (δ granules) and lysosomes; as well as mitochondria, glycogen-storing lysosomes, electron-dense chains and clusters and a dense tubular system, which is the site of prostaglandin and thromboxane A_2 (TXA_2) synthesis, and calcium sequestration (Michelson, 2019).

The most important for haemostasis and thrombosis are the α-granules and dense bodies. There are around 40-80 α-granules per platelet, each containing adhesion proteins such as von Willebrand factor (vWF) and fibrinogen, as well as chemokines and growth factors such as platelet factor 4 (PF4), Beta thromboglobulin (BTG), coagulation factor V, P-selectin, platelet derived growth factor (PDGF) and thrombospondin. Dense bodies are lower in number and size compared to α-granules and are rich in adenosine triphosphate (ATP), adenosine diphosphate (ADP), calcium, magnesium and serotonin. The α-granules and dense bodies are able to release their contents upon platelet activation, by fusing with the open canalicular system (OCS). The OCS is a network of invaginations of the platelet membrane that has two primary functions. Firstly, to transport stored products from the interior of the platelet plasma membrane upon activation and secondly to increase the platelet surface area to absorb, release and store coagulation factors from the plasma (White, 1991).

Interiorly, the platelet plasma membrane primarily contains phospholipids which are distributed so that the surface of a resting platelet does not support coagulation until platelets undergo activation. However, once platelets become activated, the assembly of the membrane changes, bringing a phospholipid called phosphatidylserine and cholesterol-rich lipid islands, which contain tissue factor (TF) to the outer platelet membrane. This reorganisation generates a pro-coagulant platelet surface driven by TF and thrombin formation, allowing platelets to interact with the coagulation system.

The exterior surface of the platelet membrane, the glycocalyx, is rather different containing a thicker, diverse layer of glycoproteins, glycolipids and absorbed plasma proteins. This layer is negatively charged, which provides a repulsive force against other platelets, preventing spontaneous aggregation and retaining plasma proteins on the platelet surface (Lewandrowski, 2009; Nurden, 2014). Probably the most important attribute of this glycoprotein layer is that it is densely populated with platelet receptors. These receptors allow the platelet to recognise and respond to vascular damage and are, therefore, crucial in facilitating haemostasis and thrombosis.

1.04 The role of platelet activation in ischaemic stroke sub-types

Thrombosis and its resultant symptoms can present in many ways. Slow progression of atherosclerotic arterial disease can present as angina or peripheral arterial disease; acute plaque-rupture as myocardial infarction (MI), TIA or ischaemic stroke; deep vein thrombosis (DVT) and pulmonary embolism which constitute venous thromboembolism and finally, intra-cardiac thrombosis from AF, as CES.

Because the pathophysiology behind developing an arterial or venous thrombus is largely distinct, these conditions have often been considered different. However, the similarities of risk factor profiles in patients who develop both arterial and venous thromboses suggest an inherent common link between patients. Several epidemiological studies have shown that those with the conventional risk factors for arterial thrombosis, namely, hypertension, diabetes, dyslipidaemia, obesity and smoking, are at similar risk of venous thrombosis, (Ageno, 2008; Previtali, 2011) as well as developing atrial fibrillation (Violi, 2016; Lutsey, 2018) and therefore CES. Understanding how platelets are involved in the development of these conditions and how their activity and function subsequently change as they progress to the development of thrombosis and disease has to be considered a vital part of platelet research in the field of stroke medicine.

(a) Platelet activation in atherosclerosis and large artery ischaemic stroke

Atherosclerosis is a chronic disease characterised by lipid deposition and inflammation of the arterial intima. The initial accumulation of lipoproteins (mainly LDL cholesterol) within the intima is called a "fatty streak", but as the condition develops, this starts off a myriad of inflammatory reactions driven by monocytes, neutrophils, T- and B- cells, and platelets. As well as these inflammatory cells, lipid deposits are now joined by necrotic cell debris and extracellular matrix proteins leading to the development of a lipid-rich atheroma or "plaque". The molecular and cellular mechanisms that drive the development of a plaque to its rupture are largely governed by disordered collagen metabolism, superficial erosion of the intimal layer, death of arterial smooth muscle cells, thrombin generation and inflammation (Libby, 2009). With respect to stroke, the development of an atherosclerotic plaque and its subsequent rupture specifically leads to LAS, causing between 20-25% of all ischaemic strokes (Ekker, 2018).

Whilst atherosclerosis is undoubtedly a lipid-driven disease, there are other components of the atherosclerotic microenvironment that may perpetuate the disease further (Finney, 2017). For example, atherosclerotic plaques begin to develop in arterial regions exposed to turbulent patterns of high shear blood flow. Studies have shown that arterial branchpoints or curvatures exhibit the highest likelihood of developing a lesion, whereas unidirectional, laminar flow show protection from plaque formation (Hahn, 2009). This is because with laminar flow, endothelial cells which line blood vessels, are aligned in the direction of flow and remain quiescent. However, in turbulent areas or areas with disrupted shear, the endothelial cells have an activated, pro-inflammatory phenotype with high rates of cell turnover and high expression of leukocyte adhesion receptors such as E-selectin, P-selectin, vascular cell adhesion-molecule-1 (VCAM-1) and intracellular adhesion-molecule-1 (ICAM-1) and cytokines such as monocyte chemotactic protein 1 (MCP-1). As a result, increased numbers of leukocytes bind to the endothelium and migrate into the vessel wall early on in the development of a atherosclerotic lesion (Hahn, 2009).

Platelets in the development of an atherosclerotic lesion

Platelets also play an important role in the development of atherosclerosis by acting as a bridge between both endothelial cells and circulating or activated leukocytes. It is established that the classical risk factors that lead to atherosclerosis can cause underlying activation of circulating platelets (Finney, 2017) and this can be measured experimentally using a variety of platelet activation markers.

P-selectin (CD62P), the cell adhesion molecule found in platelets and endothelial cells, is only expressed on the platelet surface upon their activation (pP-selectin). Therefore, it has been widely used as a parameter to measure system-wide platelet activation (Bath, 2018). There are approximately 10,000 P-selectin molecules on the surface of an activated platelet and the ability to detect it easily and reproducibly using flow cytometry has put this molecule to the foreground of platelet research (Blann, 2003). However, what we do not know is the variability of P-selectin response in a population and how this is affected by genetic variance, pharmacology and existing vascular risk factors. Furthermore, even though P-selectin is the most commonly used platelet activation marker, other markers such as plasma/soluble P-selectin (sP-selectin; reflecting largely endothelial activation), fibrinogen, vWF, BTG, PF4 are also commonly used to measure systemic, underlying platelet activation.

Specific to atherosclerotic disease, immunohistochemical analysis of atherosclerotic plaques has shown strong expression of P-selectin by the endothelium overlying active atherosclerotic plaques and fatty streaks, whereas P-selectin was not detected in normal arterial endothelium or in endothelium overlying inactive fibrous plaques (Johnson-Tidey, 1994). Koyama et al showed that the percentage P-selectin-positive platelets were higher in patients with carotid plaques and that pP-selectin expression was significantly and positively correlated with carotid wall thickness and stiffness (Koyama, 2003) suggesting an intricate yet important relationship between atherosclerotic lesions and platelet activation. Blann et al demonstrated that sP-selectin, vWF, and BTG are all raised in patients with atherosclerotic peripheral vascular disease compared to healthy controls (Blann, 1997) and it maybe that endothelial release of P-selectin accelerates atherosclerotic plaque progression (Woollard, 2014).

P-selectin expression is not, however, specific to atherosclerosis and rather represents a common marker of platelet and endothelial activation in response to the presence of vascular risk factors. For example, increased platelet activation in adolescents with diabetes is observed as measured by increased pP-selectin and PF4 exposure (Israels, 2014). Similarly, raised pP-selectin is observed in patients who solely have hypertension as a vascular risk factor (Preston, 2007), or CCF (O'Connor, 1999) or AF (Goette, 2000). This highlights that vascular risk-factors resulting in ischaemic stroke likely drive platelet activation and in turn, activated platelets help promote atherosclerosis and increase an individual's risk of ischaemic stroke.

How activated platelets assist in the progression of atherosclerotic disease is largely established. Circulating activated platelets are able to tether to the dysfunctional endothelial cells, forming a monolayer around the site of atherosclerotic plaque development using pP-selectin. Early on, these platelets are able to activate endothelial cells through the release of cytokines such as interleukin (IL) -1β (Gawaz, 2000). Furthermore, these activated platelets are able to capture circulating and rolling monocytes and neutrophils on the endothelium and then help them adhere to activated endothelial cells (Kuijper, 1998; Ed Rainger, 2015). This is achieved through a coordinated response of P-selectin exposure to the platelet surface, release of pro-inflammatory microparticles and cytokines such as platelet activating factor (PAF) from platelet vesicles (Wang, 2016), and the expression of fibrinogen bound to activated integrin αIIbβ3 (Weber, 1997). The ability of platelets to capture leukocytes is further enhanced if the they are already activated with thrombin or ADP (Reininger, 1998; Stone, 1999).

These recruited leukocytes are then extravested into the growing arterial intima where they mature into macrophages (from monocytes) and endocytose LDL, transforming into foam cells which accumulate to form atherosclerotic plaques (Lievens, 2011). As this process continues, small areas of endothelial desquamation occurs, characterised by the presence of focal microthrombi adherent to foam cells (Moore, 2013). Inflammatory activation then promotes the evolution of these early atherosclerotic lesions into more fibrous ones, containing a core of lipids, smooth muscle cells, collagen and leucocytes.

Activated platelets also have an important role here, through promoting the migration of smooth muscle cells into the intima through the release of the chemo-attractants such as PDGF (Weber, 1999).

As this lipid-rich core expands the vessel lumen becomes stenosed and limits blood flow and perfusion of distal tissue. A collagenous fibrous cap also develops over the core. However, the recognition that this itself does not determine the development of ischaemic stroke and rather, the change from a stable to a vulnerable plaque (Libby, 2009) and how platelets may have a role to play here and in the subsequent thrombosis is paramount to comprehending the role of platelets in LAS.

From atherosclerosis to atherothrombosis

The concepts of arterial thrombus formation including the specific platelet receptors involved at each stage will be discussed in detail in chapter II. Broadly speaking, the heralding event in arterial thrombus formation is the rupture of the fibrous-cap of a vulnerable atherosclerotic plaque, causing disruption of the endothelial cell barrier. As each vascular layer is revealed through endothelial disruption, both sub-endothelial collagen and TF are exposed to the circulatory system and its contents. Platelets adhere and activate on collagen, driving arterial thrombosis, whilst TF causes the generation of thrombin which serves to both activate platelets and generate fibrin, driving thrombosis and triggering thrombus stability and growth through platelet–platelet and platelet–fibrin interactions (Furie, 2008). Distal tissue ischaemia occurs through embolism of the whole, or part of, the thrombus from the carotid or vertebral arteries.

(b) Platelet activation in atrial fibrillation and cardioembolic stroke

Atrial Fibrillation: a growing health and economic problem

AF is the most common and sustained cardiac arrhythmia and is increasing in prevalence, making it a worldwide burden on healthcare resources (Reiffel, 2014). AF leads to a five-fold increased risk of CES (Kannel, 1982) ranging from 0.5% to over 15% per year depending on an individual's risk-factor profile (Lip, 2010).

Thrombus formation and CES can be by the arrhythmia itself, causing low velocity blood flow within the cardiac left atrial appendage (LAA) leading to local endothelial dysfunction through relative hypoxia of endothelial cells and the development of a pro-thrombotic state. Previous studies have shown that over 90% of all thrombi in AF patients, if detected, are located within the LAA (Ammash, 2011; Mahajan, 2012).

However, evidence of a systemic prothrombotic state can also be detected outside the LAA, from measurements of inflammation and thrombosis, suggesting that AF leads to a global prothrombotic state leading to an increased risk of CES (Lip, 1995; Llombart, 2013). This partly explains why strokes in patients with AF can also originate from alternate sources including the left ventricle, aortic arch and extracranial arteries (Miller, 1993).

Outside the classic vascular risk factors for AF: hypertension, CCF, diabetes, hypercholesterolaemia, both common and rare genetic variants identified by GWAS, have shown to increase susceptibility to AF in an individual (Feghaly, 2018).

The growing concern with AF is that its prevalence is higher in the already expanding elderly population and that cardioembolic thrombi tend to be larger compared to other subtypes such as LAS, causing the subsequent strokes to be more disabling, as well as carrying an increased likelihood of haemorrhagic transformation (Hannon, 2014; Tu, 2015). Whether these thrombi are also more resistant to thrombolysis, is yet to be fully determined and is discussed later in chapter VIII.

Currently, we have settled on anticoagulation with warfarin or direct oral anticoagulants (DOACs) because this is currently the only pharmacological treatment to prevent CES (NICE CG180 May 2019). However, the risk of adverse effects such as systemic bleeding with these agents, especially in the older population, leads to under prescription, leaving those with the highest risk of stroke most vulnerable (Denoël, 2014).

It is well established that platelets are central to the development of both arterial and venous thrombosis and also link thrombosis and inflammation through their interactions with the vascular endothelium and circulating leukocytes. Likewise, in AF, inflammation

driving a prothrombotic state and subsequently unwanted platelet activation plays a crucial role in the development of the thrombus, embolism and eventual stroke.

Platelet activation and function in atrial fibrillation

As discussed, AF is recognised as a condition of increased thrombotic tendency. Patients with AF have been shown to have elevated levels of coagulation markers such as fibrinogen, D-dimer, factor VIIIa; indicators of endothelial dysfunction such as vWF and thrombomodulin; and evidence of platelet activation through higher expression of PF4, BTG and P-selectin compared with individuals with sinus rhythm (Gosk-Bierska, 2016). Not only have studies observed increased pP-selectin (Goette, 2000) and sP-selectin (Fu, 2011) in AF patients, raised sP-selectin levels have been found to be predictive of adverse clinical outcomes in AF (Heeringa, 2006).

What is not clear, however, is whether these measurements that represent a pro-thrombotic state in AF are primarily due to AF itself or the confounding effect of the other vascular comorbidities known to influence these biomarkers found in AF patients.

This is because similar platelet activation profiles are observed in patients with cardiovascular disease without AF (Blann, 2003) and it has been shown that AF patients, compared to disease-matched patients without AF, can have similar pP-selectin and sP-selectin levels, further suggesting that platelet activation in AF could be the combination of related vascular risk factors themselves rather than AF alone (Choudhury, 2007). Results from the SPAF-III study of 1321 AF patients revealed that diabetes, smoking and peripheral vascular disease were independently associated with increased sP-selectin levels (Conway, 2002). Therefore, we can hypothesise that the presence of relevant risk factors such as hypertension, diabetes and CCF, all of which can in fact increase an individual's risk of AF development in the first place and also contribute to low-level systemic inflammation, can lead to unwanted platelet activation and increased risk of CES (O'Connor, 1999; Nomura, 2002).

Nevertheless, studies have also demonstrated that BTG and PF4 levels are higher in pAF patients without cardiovascular risk factors, and seemed to be related to the duration of

pAF (Sohara, 1997). Other studies have also shown that acute AF induction caused platelet activation within 15 minutes, suggesting that an acute episode of AF could enhance the pro-coagulable state in AF through platelet activation (Akar, 2008; Hayashi, 2011).

Finally, it is difficult to fully ascertain whether these peripheral measurements of platelet activation mimic what is occurring in the LAA. There is limited evidence here, however, Li-Saw-Hee and colleagues found no statistical difference in D-dimer, sP-selectin, BTG and vWF levels when comparing blood sampled from the atria or from the femoral vessels in valvular AF patients, suggesting systemic platelet activation (Li-Saw-Hee, 1999).

Inflammation and platelet activation in AF

Increasing evidence supports a role for inflammation in AF. A common link is probably the low-level inflammatory nature of all the vascular risk factors that contribute to AF, as well as AF itself. This is highlighted further as even outside of cardiovascular risk-factors, sepsis-related inflammation can drive the development of AF, and AF is also observed in the context of cardiac inflammatory conditions (Kuipers, 2014). Histological analysis of atrial biopsies from AF patients have shown increased recruitment of immune cells compared to those in sinus rhythm (Yamashita, 2010), and inflammatory changes comparable to myocarditis in AF but not control patients has been previously observed (Frustaci, 1997).

Furthermore, high levels of circulating high sensitivity C-reactive protein (hsCRP), tumour necrosis factor-α and IL-2, -6 and -8 are also seen in AF, compared to those in sinus rhythm (Marcus, 2008; Patel, 2010; Yo, 2014). Platelet 'hyper' activation is observed by electron microscopy in the presence of IL-1β, -6 and -8 even in healthy control blood (Bester, 2016), suggestive of an active role for pro-inflammatory cytokines in the pathophysiology of platelet activation in AF and also explains why we see platelet activation systemically, as part of a body-wide inflammatory process.

The systemic inflammatory process may also be reflected in platelet size, through measurement of mean platelet volume (MPV). Increasing platelet volume is observed as a sign of underlying platelet activation and patients with chronic AF have higher MPV

values compared to controls (Tekin, 2013) and restoration of sinus rhythm from AF appears to significantly reduce mean MPV as well as pP-selectin expression (Makowski, 2017).

Further work is necessary to completely determine whether inflammation is causative or merely associated with atrial fibrillation and what factors within an inflamed vascular system leads to thrombus formation and subsequent embolism from the LAA.

From arrhythmia to embolism

Having established that platelet activation and inflammation drive a systemic pro-thrombotic state in AF and are likely protagonists to the development of a LAA thrombus, it is important to understand the local factors within the LAA that precedes thromboembolism in some patients.

In venous thrombosis, it is a combination of low blood flow and stasis which causes the local release of pro-coagulant materials such as TF, P-selectin and vWF from the endothelium, which drives thrombosis (Wakefield, 2008). Collagen plays much less of a role here as the endothelium remains largely intact, although dysfunctional. This is most prevalent in valve pockets of lower limb veins and can be a site of thrombus initiation due to disturbed blood flow and local endothelial hypoxia (van Hinsbergh, 2012).

The thrombus development in CES is believed to be through a similar process to venous thrombosis, where endothelial dysfunction through low velocity blood flow within the left atrium of AF patients is observed, due to the fibrillating movement of the cardiac muscle (Li, 1994). These abnormal flow dynamics are associated with endocardial cell damage with the LAA (Goldsmith, 2000). Endocardial remodelling can then occur with thrombin, factor V, VII and X release, activating platelets through PAR and GPCRs followed by fibrin deposition attaching along the endocardium to platelets through integrin αIIbβ3, resulting in a thrombogenic surface on which a thrombus can form (Masawa, 1993).

The low velocity flow within the LAA can be due to the length (1.2-4.5cm) and shape of the LAA itself, which can vary within a population. Individuals who have an LAA with a

small orifice, larger neck dimension and extensive LAA trabeculation are associated with a higher stroke risk by potentiating low flow and blood stasis (De Backer, 2014). Studies have shown that at the level of atrial tissue, AF leads to increased release of Ca^{2+} and reactive oxygen species (ROS), to cause increased release of inflammatory, prothrombotic material within the LAA. AF and Low flow rates within the LAA were also associated with higher concentrations of D-dimer, BTG and PF4 and a greater incidence of spontaneous echo contrast (a radiological sign of low velocity flow and a risk factor for thromboembolism in AF) (Shinohara, 1998). Increased pro-inflammatory cytokines such as IL-6 have also been associated with increased left atrial size, further supporting a link between inflammation and atrial remodelling (Guo, 2012).This is likely to be driven by chronic endothelial scarring within the LAA and new endothelial cell migration as well as inflammatory cell infiltration (Goldsmith, 2000; Luo, 2014) leading to a nidus for local platelet activation and thrombus development within the LAA.

The risk of thromboembolism in AF has been studied extensively and varies widely depending on the presence of several risk-factors. Clinicians now have scoring systems such as the CHA_2DS_2-VASc (see Appendix 2) to estimate the annual risk of CES (Lip, 2010), with guidelines suggesting that the those with a CHA_2DS_2-VASc score ≥ 1 should be started on anticoagulation to reduce their risk of ischaemic stroke (NICE CG180 May 2019, no date). Large studies have shown that anticoagulants reduce the risk of CES by nearly two-thirds (ACTIVE Investigators, 2006) and in groups not on anticoagulation it has been observed that the chances of a discovering an LAA thrombus are higher (Mahajan, 2012). This efficacy in reducing the risk of thromboembolism in AF patients clearly indicates that the mechanism of thrombosis is at least partially dependent on the coagulation cascade (Blustin, 2014).

What is most likely to be occurring in CES is a mixture of all of the above. Systemic inflammation leading to platelet activation and release of procoagulant factors from platelets and endothelial cells, as well as LAA endothelial dysfunction, resulting in a local pro-thrombotic state, release of factors such as thrombin and fibrin, leading to platelet activation, thrombus growth and eventual embolism.

(c) Platelet activation in small vessel disease and lacunar stroke

Lacunar strokes are small, deep infarcts resulting from the occlusion of deep branch arteries that occur as a result of progressive leukoaraiosis and accounts for around 25% of all ischaemic strokes (Pantoni, 2010). Common risk factors such as age, hypertension and diabetes lead to the development and progression of SVD and its consequences can not only be seen as SVO, but also as areas of atrophy and white matter damage using conventional imaging such as computerised tomography (CT) or magnetic resonance imaging (MRI). Furthermore, SVD can manifest as cerebral microbleeds, small intraparenchymal bleeds in perivascular spaces and represent the leakage of blood constituents through the affected vessel wall, part of the constellation of pathologies that make up cerebral amyloid angiopathy (CAA). This in turn likely carries a greater risk for large cerebral intraparenchymal bleeds (Zupan, 2016).

Anatomically, SVD preferentially affects the vessels of the basal ganglia, peripheral white matter, leptomeningeal arteries, thalamic and cerebellar white matter vessels and vessels of the brainstem (Wardlaw, 2013; Rincon, 2014). We now know that the progression of SVD not only leads to stroke but also to vascular dementia and vascular parkinsonism and that these different features of SVD, although appearing to be distinct, are in fact more related and dynamic than previously thought (Gorelick, 2011; van Norden, 2011).

The difficulty with understanding the pathophysiology of SVD is the lack of in vivo models, meaning that most of the information we have learnt so far has stemmed from histological analysis of autopsies. However, we now know that SVD is a result of several contributing factors including cerebral hypoperfusion, arteriosclerosis – a collection of changes within the small vessels such as concentric wall thickening due to hyalinosis, disorganised fibrosis and luminal narrowing - and increased blood-brain-barrier (BBB) permeability (Pantoni, 2010).

There is certainly a role for both systemic and local endothelial dysfunction in SVD, which in turn affects both cerebral hypoperfusion and BBB dysfunction, which is at least partly governed by an individual’s genetic susceptibility to leukoaraiosis as well (Markus, 2008).

Endothelial dysfunction also leads to prothrombotic changes, as it does in other stroke aetiologies and this is likely to be driven by inflammation. This is evidenced in the blood of SVD patients where markers of inflammation and thrombosis such as thrombomodulin, TF, vWF, P-selectin, hs-CRP, and IL-6 are raised, even when adjusted for the presence of vascular risk factors (Hassan, 2003; Knottnerus, 2009). Cherian and colleagues showed that both E-selectin and sP-selectin levels were higher in the blood of SVO patients, compared to controls (Cherian, 2003).

BBB permeability may also be increased through the effects of these pro-inflammatory molecules, as well as matrix metalloproteinases (MMP). MMP 9 in particular may be directly involved in BBB disruption through its ability to breakdown collagen (especially type IV) and laminin (Lakhan, 2013). There is evidence of increased BBB failure in individuals with SVD, but also in normal appearing white matter, with the highest permeability seen in those with the most extensive white matter disease (Topakian, 2010; Wardlaw, 2017).

The failure of antiplatelets to halt the progression of SVD suggests a more limited role for platelets in this stroke subtype compared to LAS and CES (Bath, 2015; Mok, 2015). Furthermore, many of the robust studies investigating platelet activation in SVD have yielded mixed results. Lavallée and colleagues calculated platelet activation by measuring activated αIIbβ3, pP-selectin and platelet microparticles, and measured endothelial activation/inflammatory markers (vWF and hs-CRP) in patients with recent SVO compared to controls. They found that only the endothelial activation or inflammatory markers were increased in patients in comparison to controls and concluded that chronic platelet activation does not seem to play a role in the pathogenesis of SVD (Lavallée, 2013). Similarly, Oberheiden et al measured pP-selectin, TF and serum IL-6 and -7 levels in patients with SVD. They observed that SVD patients express significantly higher pP-selectin, significantly elevated amounts of platelet-monocyte aggregates and significantly enhanced TF exposition compared to controls, although this platelet and monocyte activation did not correlate with the severity of SVD in these individuals (Oberheiden, 2010).

1.05 Stroke treatment: antiplatelets and anticoagulation

(a) Antiplatelets

Antiplatelets remain the cornerstone in treatment of thrombotic diseases such acute coronary syndrome (ACS), ischaemic stroke and peripheral vascular disease. They are most commonly used as secondary prophylaxis, reducing unwanted platelet activation at the sites of endothelial injury, and thereby the risk of further vascular events.

Aspirin is the most commonly used antiplatelet agent. For the secondary prevention of thrombotic disease, it is effective at doses between 75-150mg daily in ischaemic stroke, and data on the use of higher doses fail to show added net benefit when taking into account adverse events (Antithrombotic Trialists' Collaboration, 2002). At these doses aspirin exerts its effects through acetylating the serine529 residue of cyclo-oxygenase (COX) -1 (at higher doses COX-2 too) and therefore, inhibiting >95 % of TXA_2 production (Wood, 1994). As platelets are non-nucleated cells, the inhibition of TXA_2 is permanent for the platelet lifecycle (Meek, 2010). Furthermore, aspirin achieves peak plasma levels within 30-40 minutes of oral intake and maximal platelet inhibition by one hour, making it a potent anti-thrombotic agent (Marder, 2012).

Clopidogrel, prasugrel and ticagrelor represent other drugs that exhibit antiplatelet activity through inhibiting ADP-dependent platelet aggregation by irreversibly inhibiting the P2Y12 subtype of ADP receptor (Savi, 1998). Unlike clopidogrel and prasugrel, ticagrelor is not a pro-drug and does not need liver cytochrome (CYP) 450 conversion into their active metabolites (Johnston, 2016).

Despite antiplatelet agents being the mainstay of treatment in ischaemic stroke, the evidence stems from a few older and heterogenous trials. A Cochrane review concluded that the odds-ratio (OR) for reduction of death and dependence after stroke with antiplatelets was 0.95 (95% CI 0.91-0.99, P=0.008) and the number needed to treat (NNT) was 79 and the OR for all-cause mortality at 6-months was 0.93 (95% CI 0.87-0.99, P=0.01), NNT = 108 (Sandercock, 2003). Antiplatelets do confer long term benefits in reduction of major adverse cardiovascular events (Hackam, 2019). However, it could be argued that

the greatest evidence for the use of antiplatelets is within the first month (Antithrombotic Trialists' Collaboration, 2002); the Cochrane review concluding that the OR for recurrent stroke in the first 30-days as 0.77 (95% CI 0.68 – 0.86, *P*< 0.0001), NNT = 140. This is backed-up the results of the meta-analysis by Rothwell et al, who describe the greatest effect of aspirin within the first two weeks of either TIA or minor stroke, with a reduction in the six week risk of recurrent ischaemic stroke by about 60% (Rothwell, 2016).

There is some evidence for the use of dual antiplatelet therapy (DAPT), aspirin and clopidogrel, to reduce the risk of recurrence after minor stroke or high-risk TIA in the first 30-days (Kheiri, 2019). The evidence as to whether this lasts to 90-days is contentious, as a higher risk of major haemorrhage has been observed in some studies (Wang, 2013; Johnston, 2018). There is no evidence for triple antiplatelet therapy (DAPT + dipyridamole) in ischaemic stroke (Bath, 2018). However, even as a single agent their general mechanism of action can lead to unwanted systemic effects of haemorrhage; for example in the case of aspirin, COX-1 inhibition impairs prostaglandin-dependent defences in the gastric mucosa, leading to ulceration and bleeding (Silverstein, 1995) and withdrawal of the drug causes a disproportionately swift recovery in platelet function (Marder, 2012). The use of clopidogrel, which arguably has better evidence than aspirin in thrombotic disease (CAPRIE Steering Committee, 1996), is also marred as its effectiveness depends on its conversion to an active metabolite by CYP2C19 and individuals who carry 2 non-functional copies of the CYP2C19 gene have a higher risk of cardiovascular events whilst taking clopidogrel, compared to those who do not (Simon, 2009).

(b) Anticoagulation

In ischaemic stroke, starting anticoagulation is reserved for those with AF, ideally prophylactically or as secondary prevention after CES. Rarer initiation of anticoagulation in ischaemic stroke are for patients with a left ventricular intracardiac thrombus or pro-thrombotic conditions such as antiphospholipid syndrome. For many years vitamin K antagonist therapy, through the initiation of warfarin, had been used for CES prevention. This was because its efficacy over aspirin for the prevention of CES in patients with AF had been well established in meta-analyses which demonstrated that warfarin reduced stroke

by 64% compared to only 22% by aspirin; although with a modest increase in intracranial haemorrhage (Hart, 2007). The results from the BAFTA study then went on to show that this benefit in stroke reduction is also evidenced in patients over 75 years of age (Mant, 2007).

The advent of the DOAC era were heralded by the three landmark trials comparing dabigatran (a direct thrombin inhibitor), rivaroxaban and apixaban (both factor Xa inhibitors) compared to warfarin (RELY, ROCKET-AF and ARISTOTLE, respectively) (Connolly, 2009; Granger, 2011; Patel, 2011). This was followed by the results of the ENGAGE-TIMI AF 48 trial comparing another factor Xa inhibitor, edoxaban, to warfarin (Giugliano, 2013). High-dose dabigatran and apixaban demonstrated superiority to warfarin in terms of ischaemic stroke and rivaroxaban and edoxaban demonstrated non-inferiority. All 4 direct oral anticoagulants demonstrated lower rates of intracranial haemorrhage compared to warfarin.

Some of the shortcomings of warfarin, for example, its narrow therapeutic window and the need for lifelong coagulation monitoring owing to a marked variation in its effect may have been overcome by DOACs, however, many limitations still remain with anticoagulation in AF.

These issues revolve around the increased risk, or perceived increased risk, of adverse effects such as bleeding from anticoagulation (Pugh, 2011; O'Brien, 2014). This means that many patients who should be receiving anticoagulation, especially the elderly, who are at the highest-risk of ischaemic stroke, do not receive it (Denoël, 2014; Han, 2018). Results from the Sentinel Stroke National Audit Programme show us that in the East of England, only 47% of those with known AF presenting with stroke were on anticoagulation (Sentinal Stroke National Audit Programme (SSNAP), 2017).

Another unresolved challenge is deciding when to initiate oral anticoagulation in patients after CES. Although the risk of early recurrent ischaemic stroke is high, early oral anticoagulation is also suspected to increase the risk of potentially harmful intracranial haemorrhage, including haemorrhagic transformation of the infarct. This means that

many patients are not receiving appropriate anticoagulation therapy until up to two weeks after their stroke (Seiffge, 2019).

1.06 Limitations of current medication

Despite the evidence that antiplatelets significantly reduces the odds of death or dependency, as well as recurrent stroke and chance of a complete recovery post stroke, the use of antiplatelet agents do increase an individual's risk of haemorrhage. This is particularly important to consider, as symptomatic ischaemic haemorrhages post antiplatelet therapy is more likely to cause death and disability than ischaemic stroke recurrence (Sandercock, 2003). However, robust evidence shows that with aspirin, the absolute risk reductions in serious vascular events and ischemic strokes in secondary prevention outnumber the absolute risk increases in haemorrhage, with a favourable net risk-benefit ratio (Hackam, 2019). This is also true with clopidogrel, when compared with aspirin in the CAPRIE trial, there was a relative risk reduction in recurrent ischaemic stroke, myocardial infarction or death in stroke patients with similar rates of intracranial and gastrointestinal haemorrhage rates compared to aspirin (CAPRIE Steering Committee, 1996).

Another limitation of current medication, and one that is most pertinent to this thesis, is that there is not one agent that is efficacious in all ischaemic stroke subtypes. Antiplatelets are effective at reducing LAS and SVO risk but not CES, and conversely, anticoagulation is effective in CES but recommended routinely for LAS and SVO in the absence of AF. However, patients are heterogenous and have complex and over-lapping risk factors for multiple stroke aetiologies.

The dilemma is further complicated by the 25% or so strokes which are ESUS, that are currently treated with antiplatelet therapy. The question remains as to whether some of these patients should receive anticoagulation. However, the results of the recent Navigate ESUS trial (rivaroxaban vs aspirin in ESUS) and RE-SPECT ESUS trial (dabigatran vs aspirin in ESUS) demonstrated no net benefit in anticoagulation compared to aspirin in recurrent stroke prevention, with rivaroxaban demonstrating higher risks of bleeding (Hart, 2018;

Diener, 2019). However, both trials were marred by heterogenous patient inclusion. We await the results of the ATTICUS trial (apixaban vs aspirin in ESUS).

Investigators have also trialled whether anticoagulation plus antiplatelets could be safely used to prevent ischaemic stroke. Results from the COMPASS study demonstrated a significant reduction in stroke with low-dose rivaroxaban combined with aspirin, compared to aspirin or higher dose rivaroxaban alone in a group of patients with stable coronary or peripheral artery disease (Eikelboom, 2017). However, those taking rivaroxaban and aspirin had significantly more episodes of major bleeding compared to aspirin or rivaroxaban alone, suggesting that although beneficial, anticoagulation and antiplatelet treatment have significant risk of adverse effects.

1.07 Chapter summary

Ischaemic stroke is a worldwide cause of disability and death (Katan, 2018). The currently employed anti-thrombotic medication for acute stroke has its limitations in that they only adequately reduce stroke-risk in specific aetiologies of ischaemic stroke, and also carry a risk of haemorrhagic complications.

Platelets are cells central to the pathology behind different stroke subtypes including LAS, CES and SVO. When they become activated, they are not only involved in the formation of the thrombus that leads to ischaemic stroke, they are also involved in the progression of atherosclerosis, atherothrombosis, cardioembolism, and are linked to various inflammatory processes that increase thrombotic-risk prior to having a stroke. Therefore, it is conceivable that controlling and downregulating platelet activation and its thrombogenic response could be a viable solution in the search for new anti-thrombotic targets.

Chapter II will discuss the vascular endothelium, platelets and platelet activation in more detail. Specifically, how endothelial dysfunction or disruption can transform platelets from their quiescent circulating state to becoming activated in response to various platelet ligands, and how this results in the development of a thrombus which eventually culminates in thrombotic disorders like ischaemic stroke.

CHAPTER II. PLATELET ACTIVATION IN THROMBUS FORMATION

2.01 Platelets in haemostasis and thrombosis

Platelets have a circulating concentration of 150 – 400 x10^9/L in humans, and normal laminar blood flow causes platelets to circulate nearer to the vessel wall, ideally positioned to detect and respond to vascular damage (Tangelder, 1985). Primarily, platelets are associated with haemostasis, but uncontrolled and overwhelming platelet activation in the circulation leads to pathological thrombosis. Where haemostasis can limit blood *loss* from a damaged vessel in a coordinated and organised fashion, thrombosis blocks blood *flow* in an otherwise intact vessel, leading to distal tissue ischaemia and embolism of less organised thrombi (Colman, 2006). Therefore, it is essential that platelet function is tightly governed and that platelets remain quiescent in the circulation until they become activated at sites of blood vessel damage and endothelial disruption.

Under normal haemostatic conditions vascular endothelial cells form a monolayer lining the entire circulatory system and act as a protective barrier against thrombosis by preventing platelets from coming into contact with the highly pro-thrombotic subendothelial matrix. A multitude of mechanisms coordinate together to preserve the non-thrombogenic behaviour of the endothelial lining, including enzymatic buffering of ROS, release of substances such as endothelial nitric oxide, prostacyclin (PGI_2), CD39/ENTPD1 and promotion of numerous anticoagulation pathways (Marcus, 1997; Rajendran, 2013; Gimbrone, 2016). Furthermore, endothelial cells are able to help limit clot formation to the areas where haemostasis is needed to restore or maintain vascular integrity (Rajendran, 2013).

However, damage or dysfunction of the endothelium bypasses these protective steps and exposes circulating platelets to the sub-endothelial matrix. The sub-endothelial matrix is composed of layers of collagen beneath the endothelium, and TF, exposed from medial and adventitial layers of the vessel wall (Furie, 2008). Platelets are able to rapidly recognise and respond to vessel damage and the sub-endothelial matrix provides a surface to which platelets can adhere to. Platelets subsequently spread on the damaged surface and become activated, causing shape change as well as release stored pro-thrombotic contents from their α-granules and dense bodies. The contents of these granules support

the activation of nearby platelets catalysing the formation of a thrombus. This initiates the thrombosis cascade which culminates with platelets adhering to fibrin, the end-product of the coagulation cascade and forming a haemostatic plug or pathological thrombus (Furie, 2008). The role of platelets in thrombus formation can therefore be split into several stages, from initial adhesion within the prothrombotic collagenous layer to aggregation and finally, stable clot formation.

(a) Platelet tethering, rolling and adhesion

The ability of platelets to adhere to exposed subendothelial collagen is highly influenced by the rate at which blood flows within a vessel and disturbances to this blood flow cause important changes that lead to the development of cardiovascular diseases. The velocity of blood layers is highest at the middle of the vessel, usually where erythrocytes flow, and gradually decreases when approaching the vessel wall. However, vessel shear is defined by both shear rate (s^{-1}); the velocity at which fluid layers pass each other and shear stress (*dyn*/cm^2); the force per unit area exerted in the direction of flow (can be thought of as the frictional force of the blood on the endothelial layer). Opposite to flow velocity, both shear rate and shear stress are maximal at the vessel wall, where the flow velocity is close to zero (Michelson, 2019). Under normal flow conditions, the shear rate increases as the vessel diameter narrows e.g. from veins to arteries and also from non-stenosed to stenosed vessels. Low (venous) shear rates are estimated at between 10-500 s^{-1} and high (arterial) shear at 250-5000 s^{-1} (Jackson, 2007). The corresponding shear stress therefore also increases as vessels become narrowed (Sakariassen, 2015).

For the purposes of this chapter, I will discuss organised, haemostatic thrombus formation at high shear rates. In vessels with high shear, such as arteries, platelets cannot directly bind to exposed sub-endothelial collagen at sites of atherosclerotic plaque rupture or vessel damage. Instead, the first-step is dependent on the large multimeric glycoprotein vWF, released from endothelial cells into the plasma, that binds to subendothelial collagen through its A3 domain (Ruggeri, 2007). The higher the shear, the greater the reliance on vWF. Now, platelet GPIbα, as part of the GP-Ib-V-IX complex, can bind to the A1 domain of collagen-bound vWF and later thrombin as platelets become more active. This

interaction initially has a fast off-rate of disassociation, meaning that platelets are unable to stably adhere to collagen and instead translocate or "roll" on the sub-endothelial surface in the direction of blood flow.

The slowly rolling platelets are able to interact with collagen through their two main platelet surface receptors, GPVI and integrin $\alpha2\beta1$ (Varga-Szabo, 2008). These receptors bind to different peptide motifs on the collagen fibril; GPVI to glycine-proline-hydroxyproline (GPO) repeats, and the A-domain of $\alpha2\beta1$ to glycine-phenylalanine-hydroxyproline-glycine-glutamate-arginine (GFOGER) (Knight, 1999, 2000).

The functional form of GPVI is a dimer, constitutively present in resting platelets as a proportion of total GPVI (monomers and dimers) (Jung, 2012). First, GPVI-dimers bind to collagen in a relatively weak interaction, but one that is sufficient to initiate signalling pathways leading to platelet activation. This includes the shift of platelet integrins, especially $\alpha2\beta1$ [but also αIIb$\beta3$ (discussed later)] from a low-affinity to a high-affinity state via inside-out signalling, which binds strongly to collagen, allowing firm adhesion of platelets. Other integrin receptors such as $\alpha5\beta1$ and $\alpha6\beta1$ also bind to fibronectin and laminin, respectively, in the extracellular matrix (ECM), contributing to platelet adhesion and activation (Moroi, 2004; Varga-Szabo, 2008).

GPVI and more specifically GPVI-dimer is the receptor of interest in this piece of work. The binding of GPVI-dimer to collagen and subsequent signal induction and thrombosis is further discussed in section 3.02.

(b) Platelet spreading and activation

Platelets are now firmly adhered to collagen using GPVI-dimer, supported by integrin $\alpha2\beta1$, as well as vWF on collagen, supported by GPIb-V-IX. The process of platelet activation now starts, initially through platelet shape change and spreading. Morphological changes to the platelet occur through calcium mediated cytoskeletal remodelling. Platelets lose their discoid shape and flatten onto the sub-endothelial matrix forming a monolayer. Long finger-like projections called filopodia extend from the platelet

cytoplasm ensuring sustained attachment to the sub-endothelial matrix and to other platelets.

Coinciding with their shape change, activating platelets release the contents of their α-granules and dense bodies, mainly TxA_2 and ADP. TxA_2 (though the thromboxane receptor) and ADP (which signals through $P2Y_1$ and $P2Y_{12}$ receptors) in turn attract and activate nearby platelets to the establishing monolayer. Importantly, inside-out signalling converts integrin αIIbβ3 to its active fibrinogen-binding form. Because fibrinogen is bivalent, it acts as a bridge between platelets and platelet aggregates are formed, eventually forming a thrombus.

(c) Platelet aggregation, stable clot formation and clot retraction

Parallel to platelet activation, the coagulation system generates thrombin (Factor IIa). Activated platelets also facilitate the generation of thrombin on their surface, accelerated by exposure of phosphatidylserine (PS) on the platelet surface and its interaction with coagulation factors from the coagulation system (Furie, 2008). Thrombin can subsequently activate nearby platelets through the protease activated receptors (PAR) 1 and 4 on the platelet surface (Brass, 2003).

Local circulating soluble fibrinogen and platelet released fibrinogen are converted to fibrin through the action of thrombin. This results in the development of a polymerised, insoluble fibrin mesh, which together with platelets, through integrin αIIbβ3, strengthens the thrombus.

In the final stages, platelets are able to generate contractile forces through interlocking platelet cytoskeletons to cause clot retraction, and the thrombus becomes stabilised through fibrin-bound αIIbβ3. The final result is a haemostatic plug comprised of activated platelets, embedded within a mesh of polymerised fibrin. When properly organised, it is a structure that is able to withstand the forces generated by the high shear flow within the arterial circulation (Michelson, 2019). The stages of the platelet cascade are summarised in Figure 2.1 and the main platelet receptors involved are discussed in Table 2.1.

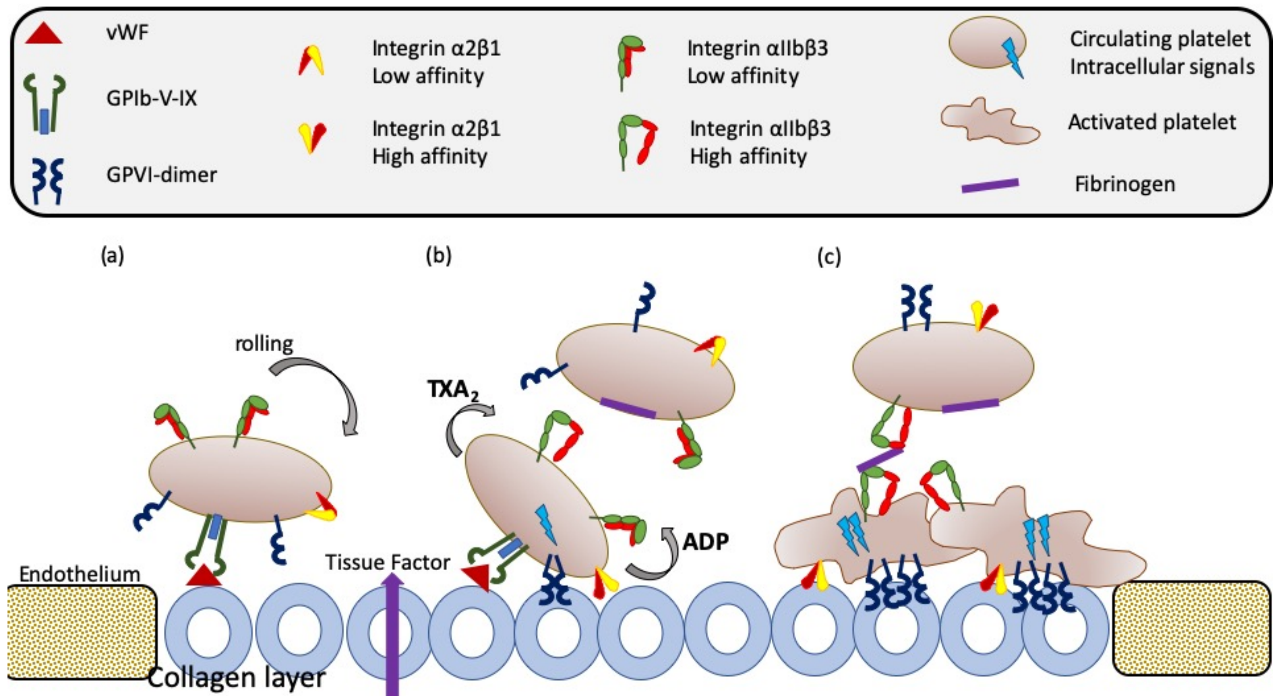


Figure 2.1 Stages of haemostatic plug formation and thrombosis. (a) At sites of vessel injury, the endothelium is disrupted, and sub-endothelial collagen is exposed, allowing plasma vWF to bind to it. Through GPIb-V-IX, platelets bind to vWF in a low affinity interaction, in which they bind and detach from the collagen surface (tethering), causing them to roll across the collagen surface; (b) Platelet GPVI-dimer, constitutively present on platelets, interact with collagen, initiating a signalling cascade to activate platelets, including inside-out signalling to convert integrins α2β1 and αIIbβ3 to their high affinity forms. Activated α2β1 binds with high affinity to collagen, arresting the platelets on the collagen surface. Activated platelets release fibrinogen, ADP, and TxA_2 and other thrombogenic substances. (c) Activated αIIbβ3 on binds to bivalent fibrinogen, bridging platelets to form aggregates. Locally released ADP and $TXA_{2,}$ induces shape change and platelet spreading on the collagen surface. As a further level of regulation, GPVI-dimers can cluster, amplifying their intracellular signalling.

Receptor	Ligand	Function	Receptor Inhibition	Risks of haemorrhage
GPIb-V-IX	vWF, Ristocetin snake toxin, Thrombin, P-selectin	Platelet adhesion to vWF adhered to collagen	Antibodies do exist, but none have reached clinical trials	Not known
GPVI	Collagen Convulxin snake toxin Laminin Adiponectin *Fibrin(ogen)*	Initial adhesion to collagen and activation of $\beta 1$ and $\beta 3$ receptors during thrombosis	Revacept – chimeric Fc fusion protein ACT017 – anti-GPVI antibody	Phase I (both agents) - no changes in coagulation parameters or platelet count in healthy volunteers. Phase II data awaited.
$\alpha 2\beta 1$	Collagen	Firm adhesion to collagen during thrombosis	No specific data available	Not known
$\alpha IIb\beta 3$	Fibrin(ogen) vWF Vitronectin Fibronectin	Platelet aggregation Clot stabilisation and retraction during thrombosis	Abciximab Eptifibatide Tirofiban	AbESTT-II trial stopped early due to haemorrhage See section 2.02 (f)
CLEC-2	Podoplanin	Platelet signalling Thrombus stabilisation	No specific data available	Not known
$P2Y_1$ and $P2Y_{12}$	ADP	Ca^{2+} mobilisation Platelet shape change and activation	See section 1.05	See section 1.06
TP	TXA_2		Terutroban – did not meet non-inferiority criteria to aspirin	Increase in minor bleeds compared to aspirin
PAR 1 and 4	Thrombin		Vorapaxar and Atopaxar (PAR1) and several early clinical molecules for PAR4 awaiting data.	TRA2P-TIMI50 trial (Vorapaxar). Significantly higher moderate-severe and intracranial haemorrhage. Metanalysis; Atopaxar > Vorapaxar in recurrent MACE but increased bleeding compared to placebo

Table 2.1 Major human platelet receptors involved in thrombosis, their important ligands, function, effect of inhibition in stroke and risks of haemorrhage. CLEC-2= C-type lectin-like receptor 2. TP = thromboxane receptor.

2.02 Platelets and the coagulation system

Haemostasis or thrombosis requires concurrent activation of the coagulation system, as well as platelets, to form a stable blood clot. Platelets mainly interact with what was previously known as the *extrinsic* coagulation pathway, which starts with the release of TF from the smooth muscle and adventitial layers of the vessel wall upon endothelial disruption or dysfunction (Furie, 2008). From this initial step, coagulation proceeds through a series of proteolytic steps that act as a biochemical amplifier, culminating in the generation of thrombin, which then cleaves fibrinogen to fibrin (Gailani, 2007). Activated platelets also expose PS which promotes thrombin accumulation, fibrin generation and crosslinking at the platelet surface, further capturing circulating platelets to the growing thrombus. This facilitates thrombus stability through integrin αIIbβ3-fibrin binding; linking the platelet and coagulation pathways together (Swieringa, 2018). The exact contribution of each pathway to thrombosis is heterogenous, largely depending on whether the endothelium is disrupted and collagen is exposed at high shear, often in the case in arterial thrombosis, or merely dysfunctional at low shear, in the case of venous or LAA thrombosis (Colace, 2012; Casa, 2015).

(a) The coagulation cascade

There are two main pathways within the coagulation cascade (Figure 2.2). The most relevant in thrombosis and haemostasis is the 'extrinsic' cascade. In this pathway flowing FVII is exposed to TF following vascular injury. TF forms a complex with factor VII, which activates it. The TF-FVIIa complex then activates both FIX and FX, causing the generation of a small amount of trace thrombin. This trace amount of thrombin then activates FVIII and FV, which act as co-factors for together with FIXa and FXa, respectively, to convert prothrombin to thrombin (Schenone, 2004). The accumulated enzyme complexes on the platelet surface support further thrombin generation and platelet activation in the propagation phase, as well as the conversion of fibrinogen to fibrin, supported by FXIIIa (Palta, 2014).

An alternative method of supporting the clotting cascade is though the contact or 'intrinsic' pathway. This pathway is important in working to amplify the signals of the

extrinsic pathway, as well as in inflammation. To start, FXII becomes activated when in contact with negatively charged substances. FXIIa then converts the serine protease plasma prekallikrein to kallikrein which then converts FXII to more FXIIa. Eventually, this cascade propagates clotting through FIX activation.

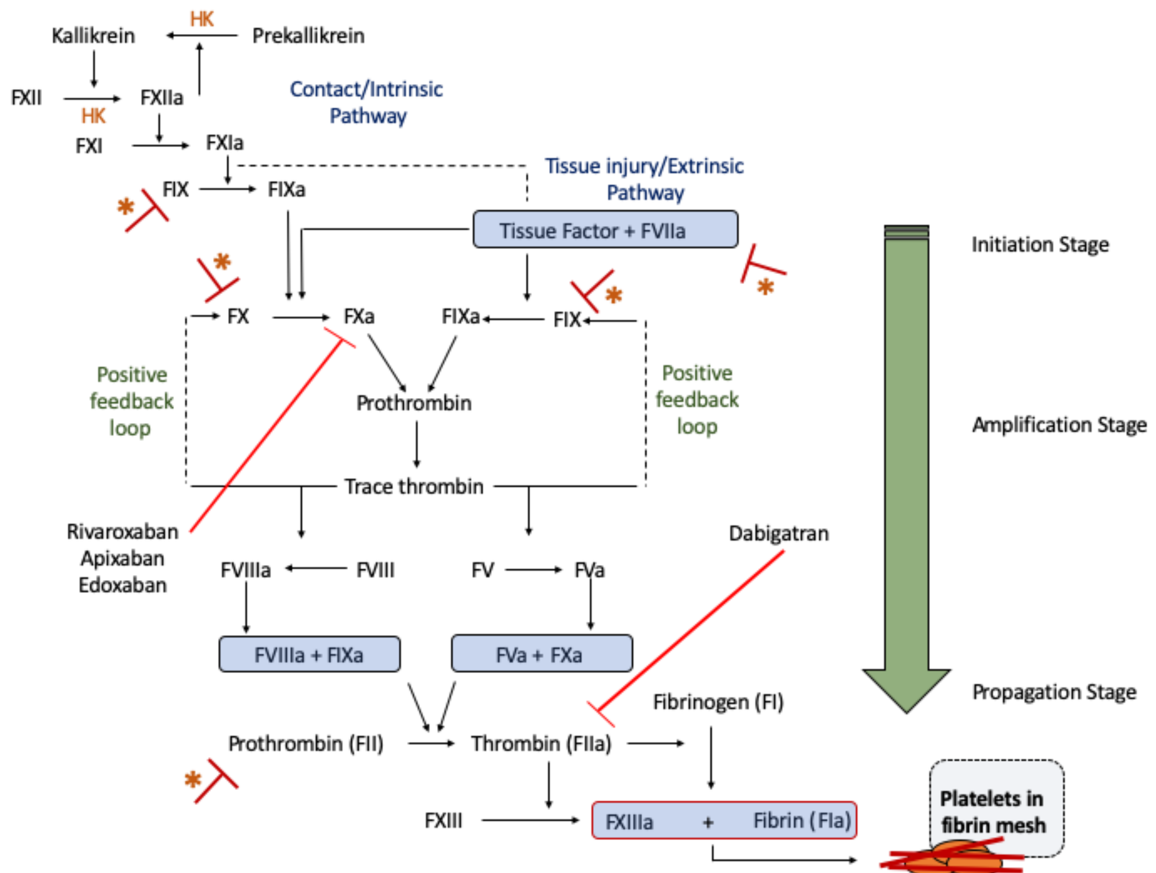


Figure 2.2 The coagulation cascade is a series of proteolytic steps that lead to the formation of an insoluble fibrin mesh that helps to stabilise the growing thrombus. The red lines denote the action of DOACs and the orange star (*) denotes the mechanism of action of warfarin. HK= high molecular weight kininogen.

(b) Thrombin

Thrombin (factor IIa) is the most potent platelet activator in the human circulation system and can activate platelets at concentrations as low as 0.1 nM (Davey, 1967; Andersen, 1999). It is a serine protease formed from pro-thrombin (factor II) in the last stages of blood coagulation. Prothrombin cleaves two internal peptide bonds, catalysed by FXa and assisted by FVa and FVIIIa (Crawley, 2007).

The primary function of thrombin is to convert fibrinogen to fibrin, through cleaving fibrinopeptides from the central fibrinogen N-terminal domain, exposing a binding site that then allows fibrin polymerisation in the presence of FXIIIa and Ca^{2+}.

The majority of thrombin generation occurs on the surface of activated platelets, and thrombin can directly interact with platelets through cleaving their GPCR PAR1 and PAR4 receptors, (Kahn, 1998). It is likely that PAR1 is the predominant signalling receptor when local thrombin concentrations are low, and PAR4 requires between 10- to 100- fold higher concentrations of thrombin (Andersen, 1999). However, PAR4 signals for longer than PAR1 (Coughlin, 1999; Holinstat, 2006). A secondary mechanism of platelet activation by thrombin involves proteolysis of glycoprotein GPV, as part of the GPIb-V-IX complex, in an ADP dependent manner (Ramakrishnan, 2001). GPVI also has a role with platelet interactions with thrombin, probably supporting maintenance of platelet activation (Hughan, 2007).

(c) Factor XIII

Factor XIII was originally identified as a serum protein that made fibrin clots insoluble, hence its initial recognition and naming as fibrin-stabilizing factor (Lorand, 1981), until later rebranding as a clotting factor. FXIII circulates in the plasma as a 325 kDa hetero-tetramer (FXIII-A_2B_2), where the two A subunits have the catalytic active site and the two B units have an inhibitory function. FXIII-A_2 in dimeric form, is found in the cytoplasm of macrophages and platelets as well. Essentially all FXIII-A_2B_2 circulates in a complex with fibrinogen (Greenberg, 1982), likely associated with regions in the fibrinogen αC and γ chains. Platelets contain a 150-fold higher concentration of FXIII-A compared to the

plasma (Katona E, 2001; Muszbek, 2011) and can also uptake a fraction of fibrinogen-bound FXIII-A_2B_2 to be stored in their α-granules (Byrnes, 2016).

Factor XIII is activated by thrombin into FXIIIa through a series of proteolytic reactions. Thrombin first catalyses the cleavage of activation peptide(s) from the N-termini of the FXIII-A subunits to produce FXIII-$A_2'B_2$ and then Ca^{2+} causes release of the two FXIII-B subunits, creating activated FXIII-A_2* (FXIIIa) (Muszbek, 2011). FXIIIa catalyses formation of covalent bonds between adjacent fibrin molecules to polymerise and stabilise the fibrin mesh within a growing thrombus and also crosslinks α2-antiplasmin inhibitor to fibrin, making the fibres more resistant to fibrinolysis through plasmin (Sakata, 1980; Hethershaw, 2014). Therefore, studies reveal that factor FXIII plays a central role in both arterial and venous thrombus generation (Byrnes, 2016), reduces risk of embolization through increasing thrombus stability (Gosk-Bierska, 2011) and could make clots more resistant to rtPA (Leidy, 1990).

(d) Fibrinogen and fibrin

Fibrin formation from fibrinogen, is a central process of both arterial and venous thrombosis, and inhibiting its formation or causing its breakdown is the target of thrombolytic medications used to treat both of these clinical presentations (Byrnes, 2016).

Fibrinogen (Factor I) is the most abundant circulating clotting factor in the blood and is also released from platelet α-granules upon activation (Lopaciuk, 1976). It circulates as a 340 kDa dimer consisting of three pairs of heterogenous, disulphide linked, polypeptide chains $(A\alpha,B\beta,\gamma)_2$ arranged in a central E-domain containing their N-termini, and an outer 'globular' D-domain containing their C-termini (Mosesson, 2005) (Figure 1.5). The two D-domains, or when broken down by plasmin, called D-fragments (80 kDa each), account for about 55% of the molecular weight of fibrinogen compared to only 15% from the E-fragment and are oriented approximately 130 degrees to each other (Spraggon, 1997). The fibrinogen α-chains are important in binding to platelet integrins using their Arg-Gly-Asp (RGD) peptide motif as well as fibrin crosslinking. The main integrin αIIbβ3 binding motif is in the fibrinogen γ-chains, which are also paramount in fibrin crosslinking.

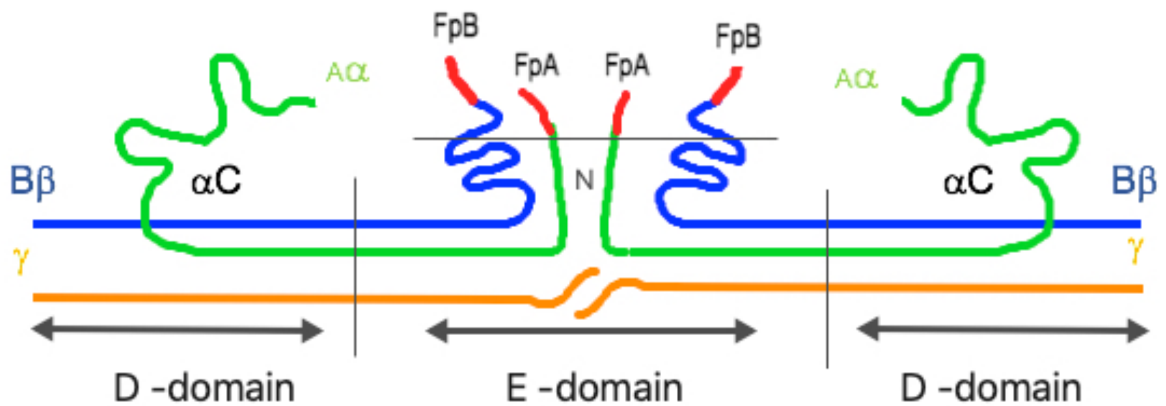


Figure 2.3 The structure of human fibrinogen. Fibrinogen molecules are made of six polypeptide chains $(A\alpha, B\beta, \gamma)_2$ comprising two outer D-domains, connected to a central E-domain. Thrombin cleaves fibrinopeptides (FP) A and B, exposing N-terminal binding sites between fibrinogen chains (horizontal black line). The γ-chains within the D-domain undergo reciprocal crosslinking mediated by FXIIIa and Ca^{2+}, leading to a strengthened fibrin mesh. Plasmin can then act on fibrinogen to release D- or E- domains (vertical black line), or on fibrin to release D-dimer, which typically consists of two crosslinked D-domains from two original fibrinogen molecules. Adapted from (Medved, 2009).

The first stage of converting soluble fibrinogen into an insoluble fibrin (factor Ia) clot occurs through the removal of fibrinopeptides A and B from the centrally located α-chain N termini via thrombin, resulting in the formation of a fibrin monomer. At this stage, new N-terminal E-domain sequences known as 'knobs' are exposed which rapidly and spontaneously interact with 'holes' on the distal D-domains of other fibrinogen molecules. Specifically, it is the newly exposed Gly-Pro-Arg (GPR) sequence within the E-domain α-chain (Pratt, 1997) which binds to the D-domain γ-chain (Shimizu, 1992). This results in the transient creation of a two-molecule thick protofibril with a half-molecule staggered overlap at a D- and E- domains.

A peptide analogue to the E- to D-domain binding site GPR, called Gly-Pro-Arg-Pro (GPRP), inhibits the continuation of polymerisation, arresting the fibrinogen to fibrin conversion at a the stage of fibrin monomers (Pratt, 1997).

Otherwise, the fibrin oligomers are then reinforced by FXIIIa, which introduces γ-glutamyl-ε-amino-lysine isopeptide crosslinks between the carboxyl-segments of abutting γ-chains. The γ-γ dimers join two D-domains in an end-to-end conformation (Chen, 1971; Spraggon, 1997) although different investigators have reported different γ-γ dimer configurations (Everse, 1998). The proto-fibrils subsequently associate laterally to form additional crosslinks between the carboxyl regions of a small fraction of α-chains, strengthening the fibrin structure further into a polymerised mesh. Even though the D-domain in fibrinogen and fibrin differ from each other due to the presence of the γ-γ crosslinks, it has been shown that the E-domain in fibrinogen and fibrin are identical, except for the loss of the fibrinopeptides (Pizzo, 1973).

The proteolysis of polymerised fibrin through plasmin is called fibrinolysis and is tightly controlled, just like the coagulation cascade. Plasminogen activator inhibitor-1, plasminogen activator inhibitor-2, α2-macroglobulin and α2-antiplasmin usually protect fibrin from plasmin degradation. However, conversion of plasminogen to plasmin and local increases in plasmin concentrations allows cleavage of fibrin. Plasmin initially cleaves the C-terminal region of the α- and β-chains within the D-domain of fibrin (Cesarman-Maus,

2005). Then plasmin cleaves the three polypeptide chains that connect the D- and E-domains, releasing fibrin degradation products; an E-fragment, an unstable complex called D_2E and the widely measured biomarker in thrombotic disease, D-dimer (Cesarman-Maus & Hajjar 2005; Chapin & Hajjar 2015)

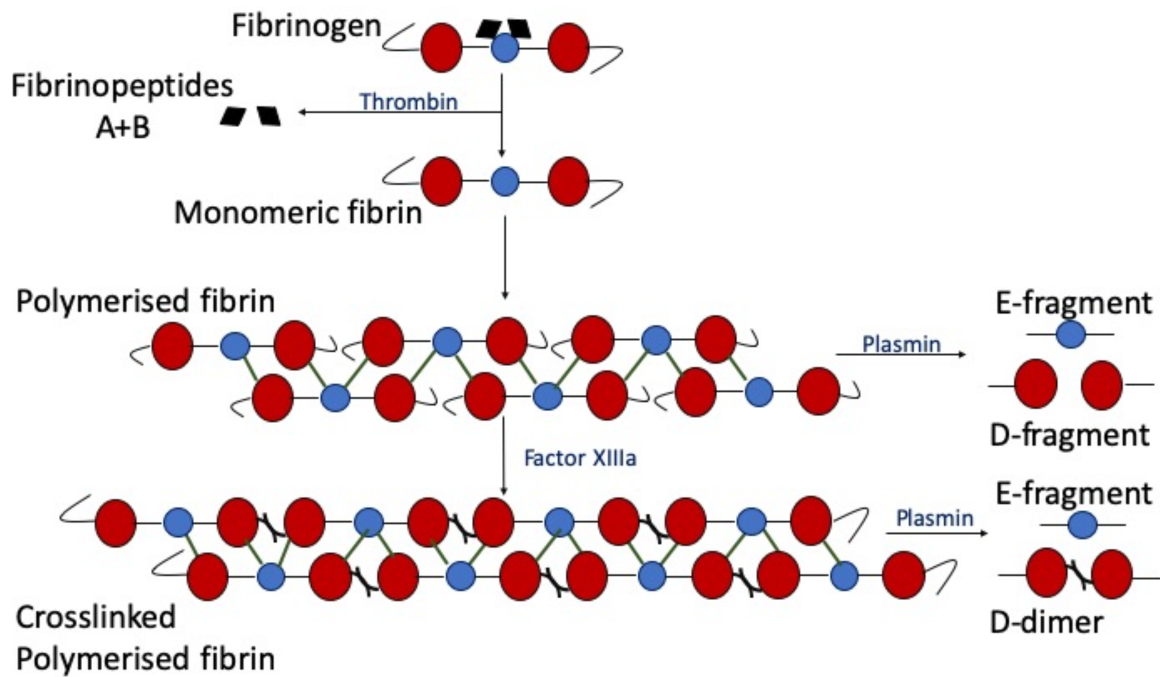


Figure 2.4 Soluble fibrinogen is converted to insoluble crosslinked fibrin and the action of plasmin creates fibrin degradation products (D-fragment, E-fragment and D-dimer).

(e) Fibrin(ogen) receptor integrin αIIbβ3

Integrin αIIbβ3 is the most abundant platelet receptor, with approximately 80,000 copies per platelet membrane (Andrews, 2014), which can increase by up to a further 50% through the transport of αIIbβ3 stored in platelet α-granules to the platelet surface upon platelet activation (Niiya, 1987; Joo, 2012). The main ligands for αIIbβ3 are fibrinogen, vWF, fibronectin and vitronectin, which cause signalling through this receptor. Although platelets can bind fibrin using αIIbβ3, it is not felt that this causes any platelet signalling (Bennett, 2001).

The αIIb subunit consists of 1008 amino acids, which is composed of a heavy chain and a light chain, whereby the light chain consists of a 20 amino acid cytoplasmic tail and a transmembrane helix. The entire heavy chain of αIIb is extracellular and is linked to the light chain by disulphide bonds. The β3 subunit is a single polypeptide chain of 762 amino acids and the αIIb and β3 subunits assemble into a heterodimer during its synthesis (Joo, 2012). Both subunits have their N-terminus residing extracellularly, with the C-terminus in the platelet cytoplasm. The extracellular portion of αIIb can be divided in to a β-propeller domain and the thigh, calf-1 and calf-2 domains arranged as a β-sheet extending down towards the platelet membrane. The extracellular portion of β3 is notable for containing the metal ion-dependent adhesion site (MIDAS) within its N-terminal, the β3 A domain, important as integrin activity is based on cation (Mn^{2+}, Mg^{2+}) availability and binding at MIDAS and Ca^{2+} binding at a site known as ADMIDAS (adjacent to MIDAS site).

In resting platelets αIIbβ3 remains in a low affinity, quiescent state. This is a protective mechanism to inhibit unwanted platelet activation in the circulation which contains a high concentration of fibrinogen. Platelets are therefore capable of ignoring soluble circulating fibrinogen whilst avidly binding to immobilised fibrinogen at sites of vessel injury (Zhang, 2018). Activation of αIIbβ3 requires the generation of inside-out signals throughout the process of platelet adhesion and activation. Synergistic signalling from receptors such as GPIb-V-IX, GPVI, CLEC-2 and GPCRs (PAR and ADP) ultimately result in phospholipase C (PLC) activation, elevation of cytosolic Ca^{2+}, protein kinase C (PKC) activation and subsequent protein phosphorylation to activate αIIbβ3 (Michelson, 2019). The activation

process involves an unclasping of the cytoplasmic tail between the α and β subunits and a disruption of the αIIb and β3 transmembrane helix associations, causing a conformational change of αIIbβ3 from a bent/coiled to an extended configuration (Du, 1993; Ma, 2007). This creates a new ligand binding site between the N-termini of the αIIb and β3 subunits, which has high affinity for its ligands.

Once activated, integrin αIIbβ3 is able to recognise its main ligands vWF and fibrinogen through their Arg-Gly-Asp (RGD) domains (Varon, 1993). Whereas these RGD motifs in the Aα-chains are not unique to integrin αIIbβ3 binding, fibrinogen also contains a unique Lys-Gln-Ala-Gly-Asp-Val (KQAGDV) sequence at the C-terminal of its γ-chain which binds only to αIIbβ3 assisted by Mg^{2+} and Ca^{2+} binding (Springer, 2008). This frees up the fibrinogen RGD domain to bind other platelet receptors such as αvβ3 during haemostasis or thrombosis.

Inside-out activation now causes αIIbβ3 clustering and generation of outside-in signalling. The early phase of this outside-in signalling happens through the β3 cytoplasmic tail and activation of Src family kinases and then phosphorylation of the tyrosine kinase Syk. Syk can weakly associate with FC receptor γ-chain (FCγR) IIa following tyrosine phosphorylation of its immunoreceptor tyrosine-based activation motif (ITAM) residues due to the low-level expression of FCγRIIa. Platelet Src family kinaes mainly function to activate PLCγ2, focal adhesion kinase (FAK), and PKC, further propagating outside-in signalling required for αIIbβ3 to assist in platelet spreading, thrombus stability and clot retraction (Durrant, 2017).

(f) Inhibition of integrin αIIbβ3

The integrin αIIbβ3 receptor has been a target for anti-thrombotic therapy for many years. Currently there are three intravenously administered αIIbβ3 antagonists available to use clinically – Abciximab, Eptifibatide (Integrillin) and Tirofiban, which primarily work on nullifying platelet aggregation.

Abciximab is a chimeric human-murine monoclonal antibody (mAb) which predominantly inhibits αIIbβ3, with weak effects on αMβ2 and αVβ3 (Coller, 1999). It inhibits αIIbβ3 by binding to its β-chain and causing steric hindrance to ligand access. In vitro studies in patients with ACS demonstrated that it prevented platelet aggregation as well as fibrin formation (Dangas, 1998). The use of abciximab is currently limited as an adjunct to percutaneous coronary intervention (PCI) or during procedures such as intra-arterial thrombolysis due to its high risk of bleeding. Specific to patients with ischaemic stroke, the AbESTT-II trial had to be stopped early due to high rates of intracranial haemorrhage within the first 5 days after abciximab administration (Adams, 2008).

Both eptifibatide and tirofiban are small molecule inhibitors of αIIbβ3 activity. Eptifibatide competitively inhibits αIIbβ3 and in comparison to abciximab, has higher binding specificity but lower binding affinity (Stangl, 2010). Unfortunately, the clinical use of eptifibatide is narrow due to its short half-life of 2 hours. It demonstrated a reasonable improvement in outcomes compared to placebo in clinical trials (Tcheng, 1997; O'Shea, 2001), as well as a similar efficacy and safety profile to abciximab (Singh, 2012). Tirofiban is currently used as an adjunct to PCI in patients with ACS.

(g) Platelets and the coagulation system

As discussed, platelets play a key role in the response to vessel injury, alongside the coagulation system; not just during thrombus formation but also during specific areas within a formed thrombus (Swieringa, 2018). We now know that platelets are not only activated by collagen, but also through thrombin formed from TF through the coagulation system (Dubois, 2006). These collagen and TF mediated pathways run parallel to each other and formulate a highly coordinated thrombotic response which can contribute to thrombosis in both arteries and veins (Rosenblum, 1997; Furie, 2008).

Platelets interact with the coagulation system in multiple ways. In high-shear dependent arterial thrombus formation such as in LAS, platelets become adhered and activated on collagen beds and the release of TF from endothelial cells means that traces of thrombin are also available locally (Nieswandt, 2011). Platelets regulate these reactions leading to

the amplification of thrombin generation in multiple ways. Firstly by PS exposure, secondly by binding coagulation factors via GPIb-V-IX, GPVI or integrin αIIbβ3, and finally via thrombin-induced direct platelet activation through PARs (Swieringa, 2018). Thrombin can in fact directly activate platelets, without prior collagen exposure, as seen in low-shear venous thrombosis, which would be the mechanism of thrombus formation in CES (Esmon, 2009).

The exposure of PS on the platelet surface is an important and key step in haemostasis and thrombosis and occurs due to increased cytosolic Ca^{2+} generated through signalling throughout platelet activation (Martín-Molina, 2012). GPVI-signalling is key here, as well as that through CLEC-2, to formulate prolonged calcium elevation via their downstream signalling pathways. PS exposing platelets are now able to form complexes with coagulation factors V, VII, IX and X, significantly enhancing the activities of these coagulation factors (Heemskerk, 2002). PS exposure on platelets is also essential for FXIIIa mediated crosslinking of fibrin on the platelet surface. Now fibrin is also able to accelerate the interaction between platelets whilst also allowing αIIbβ3 to facilitate platelet-fibrin interactions and also further enhancing procoagulant PS exposure, maintaining thrombus growth and stability under flow (Swieringa, 2018).

Thrombin acts on platelets at both the early and late stages of thrombus development. During the early stages, thrombin activates platelets through their PAR receptors, supporting the signalling processes by potentiating Ca^{2+} increases, activation of integrins α2β1 and αIIbβ3, and exposure of PS (Heemskerk, 2002). Once the thrombus starts to grow, PS exposed platelets around the growing fibrin network serve as a catalyst to amplify this process, helped by fibrin capturing more thrombin and increasing its local concentration around a thrombus (Monroe, 2002).

2.03 Chapter summary

Platelets are essential for both haemostasis and thrombosis. In both LAS and CES, platelets become activated at the site of thrombus formation and assist in thrombus growth and stability. When platelets bind to collagen, initially through vWF and GP1b-V-IX and then

firmly through GPVI-dimer and integrin α2β1, platelets become activated. Activated platelets then release locally acting platelet mediators such as thrombin, P-selectin, TXA_2, ADP and PS to recruit and activate nearby platelets, developing a thrombus (Figure 2.5). Platelets then interact with fibrinogen and fibrin through integrin αIIbβ3, their main link to the coagulation system, to stabilise the growing thrombus and cause clot retraction. In ischaemic stroke, platelets, therefore, play a role in arterial thrombus formation through platelet-collagen adhesion and activation through GPVI-dimer and in CES through platelet-fibrin interactions via integrin receptors. However, a novel role for GPVI in linking platelets with the coagulation system was reported in 2015 which suggested that it is far more than just a receptor for collagen. GPVI, its structure and function within thrombosis related to ischaemic stroke is discussed in chapter III.

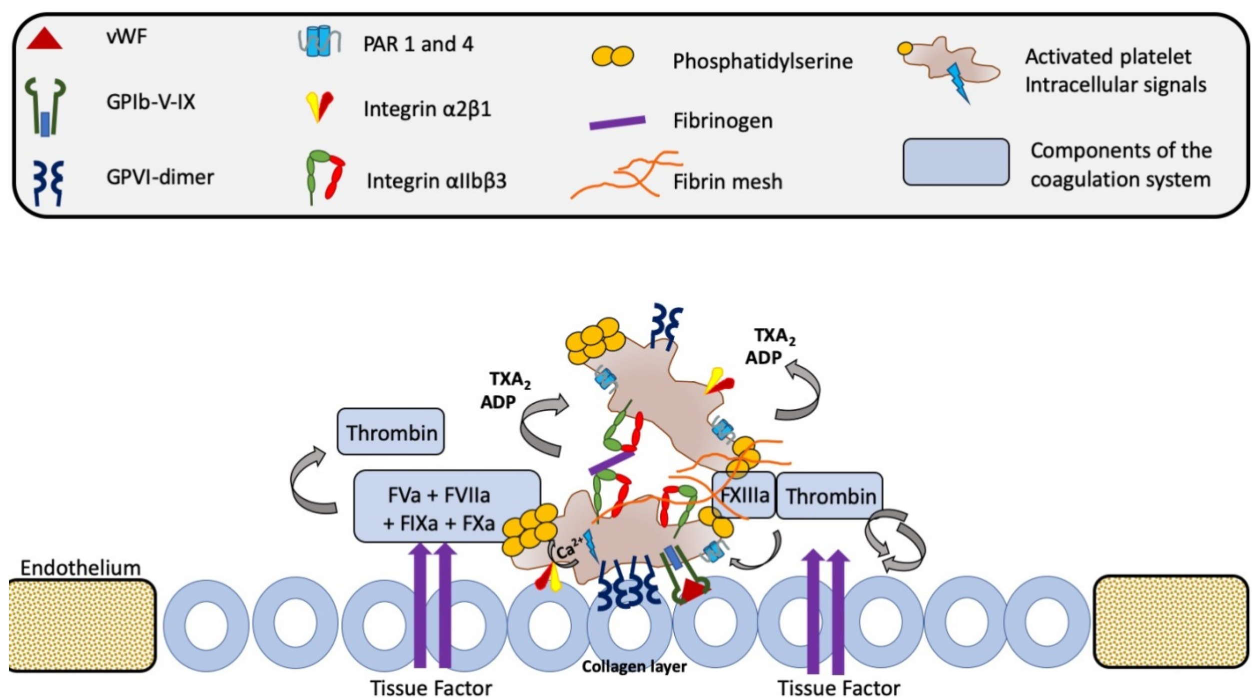


Figure 2.5 The integration of the coagulation cascade and activated platelets. Activated platelets express phosphatidylserine which can form complexes with coagulation factors as well as promote the conversion of fibrinogen to fibrin on the platelet surface. Local thrombin generation coordinated through the coagulation system is integral to fibrin mesh formation. Thrombin can also directly activate platelets through their PAR receptors.

CHAPTER III. PLATELET RECEPTOR GLYCOPROTEIN VI

3.01 Structure of Glycoprotein VI

Sugiyama and colleagues (Sugiyama, 1987) first reported a patient with idiopathic thrombocytopenic purpura whose platelets did not respond to collagen and lacked a 62 kDa platelet protein. Masaaki Moroi and Stephanie Jung then reported another patient who was genetically deficient in a 62 kDa glycoprotein that was requisite for the interaction of platelets with collagen; they named this protein glycoprotein VI, in accord with the Phillips nomenclature for platelet glycoprotein receptors (M Moroi, 1989). Over the last 30 years, research has highlighted the important role GPVI plays in human thrombosis, and has been targeted in the design of novel antiplatelet agents (Dütting, 2012).

GPVI is a transmembrane glycoprotein receptor which belongs to the immunoglobulin superfamily and is expressed in both platelets and megakaryocytes (Clemetson, 1999; Jandrot-Perrus, 2000). GPVI contains two IgG-like extracellular domains (D1 and D2). D1 contains an N-glycosylation site. D2 is connected to the membrane by a rigid mucin-like stalk segment that is rich in O-glycosylation sites, followed by a 19 amino acid long transmembrane portion and a short cytoplasmic domain (Moroi, 2004). The transmembrane portion contains an Arg273 that forms a salt bridge with Asp11 of FcRγ, a subunit that is essential GPVI expression and signal transmission through GPVI; FcRγ itself is a disulphide bonded dimer (Tsuji, 1997; Nieswandt, 2000). The cytoplasmic domain contains a basic domain that serves as a calmodulin binding site and a proline-rich domain before the C-terminal that binds to the Src family kinases Fyn and Lyn (Moroi, 2004).

There are between 6000-10000 copies of GPVI on the platelet surface (Andrews, 2014). GPVI exists as single-chain monomers of GPVI or GPVI-dimers, comprising two monomers. GPVI-dimers are constitutively present on resting platelets (20–29 % of total GPVI) and have high affinity for collagen, thus being the functional form of this receptor (Jung, 2009, 2012). Upon platelet activation, the number of dimers on the platelet surface increases (Jung, 2012) and as a further level of regulation, activation induces clustering of the GPVI-dimers, which may bring GPVI-associated signalling molecules in closer proximity, facilitating signalling (Poulter, 2017).

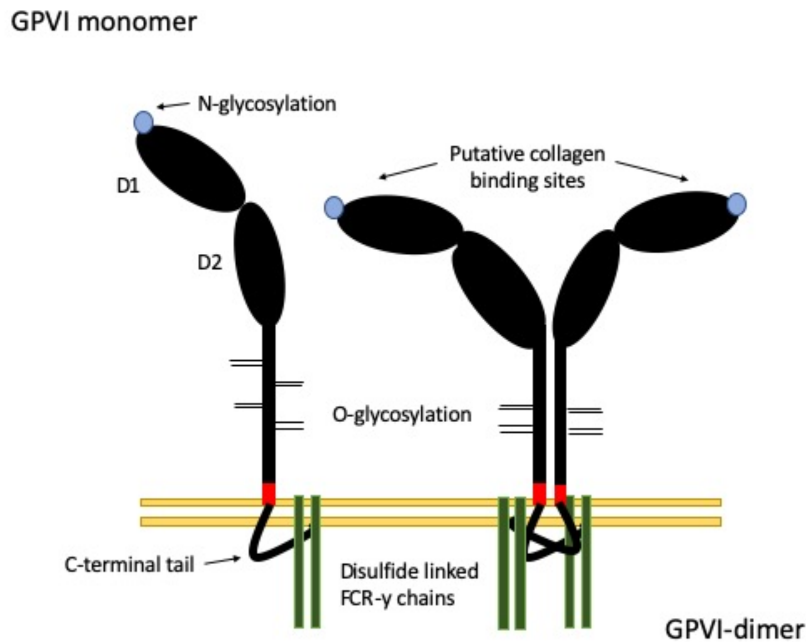


Figure 3.1 The structure of GPVI monomer and dimer. The extracellular domains contain two IgG domains D1 and D2, where the collagen binding sites are found in D1. Each monomer is non-covalently associated through a salt bridge with FCRγ. Both GPVI-monomers and -dimers recognize the GPO triplets in the primary sequence of collagen. However, GPVI-dimers also recognise a structure specifically found in collagen fibres, which may contribute to its high affinity. Adapted from (Induruwa, 2016).

3.02 Glycoprotein VI ligands: collagen and collagen related peptide

Collagen is the most abundant protein in humans, comprising one-third of the total protein, and is the most prevalent component of the sub-endothelial ECM (Shoulders, 2009). It is made of three parallel polypeptide chains arranged in a triple-helix. Each chain can be homo- or hetero trimeric and within the chains, every third amino-acid residue is glycine, with GPO being the most common triplet (Ramshaw, 1998) and also the most abundant platelet stimulus, through its interaction with GPVI (Morton, 1995). These collagen triple helices are arranged as highly ordered fibrils which then stack together to form collagen fibres.

Other than collagen, the ECM is comprised of elastin, laminin and fibronectin and the extracellular interstitial spaces are filled with a proteoglycan gel within which the fibrous proteins above are embedded (Järveläinen, 2009). Numerous types of collagens exist in the ECM, but the most abundant and important in platelet interactions and thrombosis are types I and III (Varga-Szabo, 2008). The way collagens are arranged in a fibrillar network forms a strong structure that can withstand high-tensile forces.

Bovine collagen, horse collagen as well as collagen 'toolkits' have been successfully used in vitro to further elucidate receptor-ligand properties between platelets and collagen (Raynal, 2006; Hamaia, 2012). The toolkits (TK) are comprised of overlapping triple-helical peptides which encompass the entire collagen domains of human collagens II and III. Advances in peptide synthesis have enabled the synthesis of collagen-like peptides (collagen related peptide; CRP), a powerful GPVI agonist comprised of 10 GPO motifs which can spontaneously form stable triple-helices (crosslinked CRP; CRP-XL) under physiological conditions (Morton, 1995). We now know that platelet adhesion to CRP-like peptides varies with their GPO triplet content and that two GPO triplets is the smallest motif within collagen that recognises and activates platelet GPVI (Smethurst, 2007).

(a) Glycoprotein VI adhesion to collagen and resultant signalling

Fibrillar collagen type I is the major physiological collagen type that binds to GPVI-dimer, and together with collagen type III, predominate the collagen found in the ECM (Kehrel, 1998). It was suspected that the collagen binding region within GPVI was located in D1D2 and that binding required the dimeric conformation of GPVI because a dimeric recombinant Fc-fusion protein of the GPVI-extracellular domain (GPVI-Fc_2) bound to collagen fibres with high affinity whereas the monomeric GPVI-extracellular domain, $GPVI_{ex}$ did not (Miura, 2002).

Horii and colleagues obtained a crystal structure of D1D2, which was consistent with a dimer formed by a “back-to-back” arrangement of D1D2 monomers (Horii, 2006). They observed a shallow groove on the surface of D1 when GPVI is in this dimeric configuration, which matched the orientation and dimensions of collagen triple helices. This suggests that the dimeric configuration is vital for GPVI adhesion to collagen, explaining the nearly

200-fold difference between the affinities of monomeric and dimeric GPVI to collagen (Jung, 2012) (Figure 1.3). Table 1.2 summarises the affinity of GPVI monomer and dimer with collagen type I, III and CRP and TK III-30.

Furthermore, the proportion of pre-existing GPVI-dimers on the platelet surface can increase upon collagen or thrombin induced platelet activation, by up to 35% (Jung, 2012). Rapid assembly of dimers on the platelet surface causes further platelet reactivity to collagen (Loyau, 2012). As discussed, GPVI proteins can oligomerise upon ligand binding, into higher-order clusters (Horii, 2006) which in turn enhances the strengths of the signal induced from GPVI and thereby platelet activity (Jiang, 2015; Poulter, 2017). This distribution and assembly of GPVI across the platelet membrane is regulated by platelet cytoskeletal proteins (Haining, 2017; Poulter, 2017). Thus far only crosslinked CRP (Barnes, 1998) and fibrous collagen (Jung, 1998) had been established in being able to induce a platelet aggregation response through GPVI.

	GPVI-dimer (K_d, nM)	**GPVI-monomer (K_d, μM)**
CRP-XL	22.0 ± 2.1	2.7 ± 0.3
Collagen type I	41.7 ± 0.4	8.1 ± 1.2
Collagen type III	58.3 ± 9.4	13.8 ± 2.5
TK III-30	11.5 ± 0.6	10.2 ± 1.9

Table 3.1 The dissociation constants (binding affinity between a molecule e.g. protein and a ligand; K_d) ± SD for GPVI dimer and monomer binding to various collagen substrates. GPVI-dimer has much higher affinity for the collagenous substrates than the monomer, indicating that it is the functional form of GPVI on platelets. TK III-30: peptide from collagen toolkit III has the highest affinity for GPVI. As reported in (Jung, 2012). Similarly, others have reported it to be within the 10^{-7} to 10^{-9} M range (Miura, 2002; Massberg, 2004).

The final outcome of GPVI signalling is to activate integrins α2β1 and αIIbβ3 to further propel platelet activation and aggregation, leading to thrombus formation. Even though GPVI is the major signalling receptor for collagen on peptides, once activated, integrin α2β1 has higher for collagen compared to GPVI and enhances the activity of GPVI by fixing platelets on the collagen surface under flow conditions, although α2β1 cannot activate platelets by itself (Jarvis, 2002). The cytoplasmic region of FcRγ contains an ITAM and once adhered to collagen, the GPVI/FcRγ complex induces sequential activation of the Src kinases Lyn, and Fyn which are bound to the cytosolic tail of GPVI (Watson, 2005). In particular Lyn is important for catalysing phosphorylation of ITAM and activation of Syk (Gibbins, 1996). Downstream of this lies a series of proteins, including linker for activation of T-cells (LAT) and adaptor proteins SLP76 and Gad, which lead to the activation of PLCγ2, generation of intracellular calcium signals, protein kinase C activation, finally leading to the activation of β1 and β3 integrins (Nieswandt, 2003). Clustering is likely to be caused by the dimerisation of the intra- and extracellular regions of GPVI/FcRγ, bringing the ITAMs within the cytoplasmic tail in closer proximity to each other (Poulter, 2017) with the cell membrane lipid rafts also aiding in GPVI signalling and cluster formation after FcRγ-chain phosphorylation (Locke, 2002).

The components of the major GPVI signalling pathways are also shared with other platelet receptors that bind ligands other than collagen. For example, the signalling induced through GPIb-V-IX binding to vWF is similar to that of GPVI, in that both involve activation of PLCγ2 via FcRγ-chain, Src kinases and Syk (Liu, 2005). Another example is CLEC-2, which is activated through podoplanin but produces powerful platelet activation signals in conjunction with Src, Syk and PLCγ2, similar to the GPVI/FcRγ-chain complex (SuzukiI-Inoue, 2011), as well as other integrin receptors such as αIIbβ3 (Watson, 2005).

3.03 Genetic variability of GPVI expression and function

The gene coding for GPVI is located on chromosome 19q13.4 and contains different single nucleotide polymorphisms (SNPs). Variation at the *GP6* locus accounts for a large part of the population vitiation of GPVI platelet surface expression, as well as the response to collagen or CRP-XL. Early reported variations in platelet GPVI content amongst healthy

individuals were identified by their responses to the snake venom protein convulxin which activates platelets like collagen (Furihata, 2001) and since mapping the *GP6* DNA sequence, numerous heterozygous mutations in the *GP6* gene have been reported, which can cause a lack of collagen-induced platelet activation (Dumont, 2009; Hermans, 2009). Alternative spliced variants have also been observed to lack responses to collagen (Arthur, 2007b) suggesting that GPVI is responsible for collagen based platelet activity and function.

The alleles of the common haplotype of 5 coding *GP6* non-synonymous single nucleotide variations accounts for over 15% of the population variation of GPVI surface expression and studies have concluded that the *GP6* locus accounted for 35% of the variation in responsiveness to CRP-XL (Joutsi-Korhonen, 2003; Jones, 2007). This highlights the direct effect that the variants have on the ability of a platelet to signal through GPVI.

The minor *GP6* haplotype occurs in homozygosity in 24 of 6000 (0.4%) individuals genotyped so far through recent work within the Ouwehand group, who were our collaborators for parts of this work. Previous studies have shown that this haplotype could protect against recurrent MI in patients treated with antiplatelets, indicating an underlying role of GPVI in thrombosis (Snoep, 2010) and intracellular signalling (Trifiro, 2009).

Even at the time of writing this thesis, less than 20 patients with GPVI-deficiency have been widely reported, with the cause of GPVI deficiency ranging from an auto-antibody effect, congenital defect or an acquired deficiency. The common themes of presentation in GPVI deficient patients are purpura, petechiae and ecchymosis, resembling an ITP which responds to steroids, with a careful history revealing other signs of bleeding time prolongation such as menorrhagia and epistaxis (Arthur, 2007). Thrombocytopaenia is common also, and platelets demonstrate severe impairment of aggregation on collagen.

Matus et al. recently reported a novel homozygous *GP6* mutation in five subjects from four unrelated Chilean families who presented with ecchymoses and minor bleeding (Matus, 2013). Interestingly, all the patients demonstrated normal PT, APTT, platelet and

vWF counts, similar to their heterozygous relatives. By contrast, their platelets completely failed to aggregate in response to collagen and convulxin. This data is pertinent as they indicate the benefits and risks of possible future antibody-mediated GPVI-inhibition, if we were to consider it as a pharmacological target in human thrombosis. Furthermore, the work suggests a rather benign nature of symptoms associated with GPVI deficiency, but the cases themselves are heterogenous. We require additional data to understand how GPVI deficiency relates to an individual's arterial or venous thrombotic risk in the setting of vascular risk factors.

3.04 Glycoprotein VI in thrombotic disease

This section will focus on GPVI-mediated thrombosis through its main ligand, collagen. How GPVI contributes to platelet activation in the setting of haemostasis and thrombosis has been discussed in section 2.01 and 3.02.

Much of the work investigating the role of GPVI in thrombosis has been in knockout mice models, particularly as GPVI deficiency in humans appears to be a very rare platelet disorder (Arthur, 2007b). Therefore it is difficult to elucidate the functional role of GPVI from these patients alone, apart from that these individuals only exhibit a mild bleeding tendency, which in turn has driven the research on GPVI as an potential anti-thrombotic target (Sugiyama, 1987; M. Moroi, 1989; Arthur, 2007; Matus, 2013).

Much of the work from mouse models has helped to shift the focus of GPVI's activity from an platelet adhesion receptor, to an activatory one (Misra, 2014). In both arterial and venous thrombosis development, the lack of GPVI appears to be protective (Nieswandt, 2001; Lockyer, 2006), suggesting that the major function of GPVI may not be limited to the denuded endothelium at sites of plaque rupture; an important fact to be discussed in further detail in this thesis.

The majority of the GPVI studies in humans have focussed around the quantification of platelet surface GPVI via flow cytometry and the plasma detection of soluble GPVI (sGPVI), a shed ectodomain of GPVI via enzyme-linked immunosorbent assay (ELISA) (Al-Tamimi,

2009). Many of the studies looking into platelet detection of GPVI have quantified the presence of total (monomeric and dimeric) GPVI, compared to controls. Interestingly, patients with thrombotic diseases such as acute coronary syndromes (ACS) (Bigalke, 2010, 2011), TIA and ischaemic stroke (Bigalke, 2010) demonstrate higher platelet total GPVI expression and poorer clinical outcomes. Similarly, sGPVI has been shown to be increased in ACS (Bigalke, 2009) and ischaemic stroke (Al-Tamimi, 2011; Wurster, 2013) suggesting that it could be used as a potential biomarker in human thrombotic disease.

3.05 Glycoprotein VI in roles outside thrombosis

Outside of the classical role of haemostasis, platelets contribute to many other physiological conditions, for instance, inflammation, immunity, vascular integrity and repair (Moroi, 2004). A contribution to these roles from GPVI is increasingly recognised, although its mechanism is not as well established as it is in thrombosis and appears to be pathology and organ dependent (Rayes, 2018).

In inflammatory conditions such as glomerulonephritis, GPVI has been shown to potentiate neutrophil recruitment to inflamed glomeruli (Devi, 2010). GPVI has also shown to promote cutaneous macrophage expression of inflammatory mediators such as prostaglandin E_2 and IL-6 (Pierre, 2017). As observed in thrombotic disease, plasma sGPVI levels are shown to be higher in patients with inflammatory conditions such as rheumatoid arthritis (Stack, 2017), liver cirrhosis (Egan, 2017) and thermal injury patients with sepsis (Montague, 2018) but lower in patients with Alzheimer's disease (Laske, 2008).

Conversely, GPVI has also been shown to protect vascular integrity during local inflammation by sealing the sites of damage at the inflamed vessel wall and preventing neutrophil-induced bleeding (Gros, 2015), possibly in conjunction with CLEC-2 (Boulaftali, 2018). However, unlike in haemostasis, single platelets tend to adhere to sites of vascular inflammation, preventing blood leakage into the oedematous tissue rather than forming a haemostatic plug (Gros, 2015).

Within the immune system, GPVI also appears to have multiple roles. GPVI has been shown to facilitate platelet-leucocyte interactions, helping recovery in mice with pneumonia (Claushuis, 2017). However, GPVI has also shown to bind and assist Hepatitis C in spreading (Zahn, 2006). The role of GPVI in cancer is less well understood also with conflicting research based mainly on mouse models suggesting a role in facilitating lung metastasis (Jain, 2009) and conversely increasing efficacy of chemotherapy (Volz, 2019).

3.06 Glycoprotein VI shedding and downregulation

After ligand binding and platelet activation, the GPVI extracellular domain is rapidly and irreversibly shed from the platelet surface at the juxtamembrane cleavage site (Bergmeier, 2004; Gardiner, 2004). This GPVI shedding is mediated by the A disintegrin and metalloproteinase (ADAM) family of MMPs, and in humans, it is largely attributed to ADAM10 where as both ADAM 10 and 17 have a role on GPVI shedding in murine platelets (Gardiner, 2007). They are located on the membrane of resting platelets and results in a roughly 55 kDa GPVI-fragment being released from platelets the platelet surface (sGPVI) (Bigalke, 2009).

Other regulators of GPVI shedding also exist. Platelet granules also contain MMPs which are released from storage into extracellular and interstitial spaces (Gardiner, 2018). Specifically, for GPVI, MMP-9 and 13 may have a role in downregulating GPVI function in thrombosis, although it is not clear whether this is through receptor cleavage (Claushuis, 2017; Howes, 2018). Other studies have shown that GPVI can be shed in the absence of collagen in a factor Xa-dependent manner, where drugs such as factor Xa-inhibitor rivaroxaban inhibited the coagulation induced GPVI shedding in vitro which is of extreme importance since the availability and use of DOACs (Al-Tamimi, 2011). Finally, GPVI deficiencies have been described in patients with immune thrombocytopenic purpura, where auto-antibodies are felt to induce GPVI shedding (Boylan, 2004; Rabie, 2007).

Although downregulating the platelet response through receptor shedding after thrombosis is likely to be a protective mechanism to reduce platelet responsiveness and govern cellular functions, the exact role of platelet shedding is yet to be fully elucidated.

Elevated plasma sGPVI levels have been detected after myocardial infarction and stroke, suggesting a potential role in diagnostic, prognostic and therapeutic strategies in thrombotic diseases (Bigalke, 2010; Wurster, 2013). Therefore, the clinical significance of GPVI downregulation on platelets is the subject of much interest and research within the field (Chatterjee, 2017).

3.07 Glycoprotein VI inhibition

Much of the early work on GPVI-inhibition were focussed on mouse models or in vitro studies, progressing through work performed by Nieswandt and colleagues using the rat anti-GPVI mAb, JAQ1, on mouse platelets (Nieswandt, 2001). They showed that JAQ1 caused a sustained loss of GPVI from the mouse platelet surface, with a corresponding downregulation of platelet responsiveness and a near-complete loss of aggregation to collagen (Massberg, 2003). Interestingly, JAQ1 did cause a transient thrombocytopaenia which lasted less than 2 weeks on average, with normalisation of the murine platelet count. Kleinschnitz and colleagues showed that GPVI inhibition leads to significantly reduced infarct size after transient middle cerebral artery occlusion (tMCAO) in mice, without a change in platelet count and increase in bleeding complications, although a non-significant increase in tail bleeding times were observed (Kleinschnitz, 2007) and Pachel et al have recently shown that anti-GPVI protects against myocardial reperfusion injury in mice (Pachel, 2016). GPVI-inhibition also appears to translate well when used in elderly and comorbid mice with induced tMCAO ischaemic stroke, demonstrating reduced infarct size and improved functional outcome (Kraft, 2015).

Since JAQ1, numerous other antibodies have been raised against human GPVI. The fragment antigen-binding (Fab) fragments of such antibodies – notably 5C4 (Massberg, 2004), OM2 (Matsumoto, 2007), OM4 (Li, 2007), 9012 (Lecut, 2003), and mFAB-F (GPVI-dimer specific) (Jung, 2009) are potent inhibitors of GPVI-mediated platelet activation. Single domain antibody clones (Walker, 2009) as well as single-chain variable fragments (scFv) have been developed against GPVI (Smethurst, 2004; Muzard, 2009). Researchers are also investigating the feasibility of blocking kinases involved in GPVI-signalling pathways as a method of inhibiting GPVI-mediated platelet activation (Spalton, 2009),

although their success maybe limited due to the shared signalling pathways used in platelets.

Human studies investigating GPVI-inhibition have pushed the field forward in terms of developing GPVI as a thrombotic target. The most successful so far has been Revacept, a recombinant fusion protein formed between the collagen binding extracellular domain of GPVI and the Fc part of human IgG, creating a soluble GPVI-dimer analogue (GPVI-Fc) (Massberg, 2004). It specifically binds to collagen with high affinity and competes with native platelet GPVI to the exposed GPVI-binding site on collagen at sites of atherothrombosis in vivo (Bültmann, 2006).

Revacept was used in an open-label Phase 1 dose-escalation study in 30 healthy men to better understand safety and tolerability of the drug after a single intravenous dose (5 doses with n=6 in each group). Revacept was shown not to affect international normalised ratio (INR), activated partial thromboplastin time (aPTT) or platelet count, whilst dose-dependently inhibiting collagen induced platelet aggregation for up to a week in higher doses with almost full inhibition seen at 24 hours (Ungerer, 2011). Specifically for ischaemic stroke, Revacept has shown to significantly reduce infarct size and improve functional recovery after tMCAO occlusion in mice (Goebel, 2013), showing potential as a single agent, or an adjunct to low dose rtPA administration (Reimann, 2016).

In vitro models have shown that crosslinking GPVI-Fc onto collagen fibres can be a site-selective method of inhibiting plaque induced platelet activation (Jamasbi, 2016). Furthermore, the addition of GPVI-Fc to in vitro atherosclerotic plaques appears to be able to compound the reduction in platelet activation by aspirin and ticagrelor individually or both together (Mojica Muñoz, 2017). The advantage of GPVI-Fc based pharmacological therapy is it is highly selective to collagen exposed sites of plaque-rupture only. This may convey additional benefits in those prone to bleeding by not effecting the haematological system, as it only competes with the binding site of GPVI-dimer on collagen, not on the platelets themselves. However, where in stroke treatment Revacept could be utilised and its efficacy and side-effects at higher doses in larger numbers of patients with cardiovascular disease is yet to be determined. We await the results of the Phase 2 study

investigating its use in symptomatic carotid artery stenosis, TIA, amaurosis fugax or stroke where patients receive either Revacept (single dose) plus antiplatelet monotherapy or monotherapy alone, which has just completed recruitment in October 2018.

The other anti-GPVI therapeutic agent of note is ACT017, a humanised Fab fragment has also concluded a randomised, placebo-controlled, double blind, phase 1 trial in 6 cohorts of 48 healthy volunteers. ACOT017 demonstrated a modest reduction in collagen induced platelet aggregation lasting up to 2 days alongside a non-significant increase in bleeding times and no significant variation in platelet count (Voors-Pette, 2019). Similar to the Revacept data, we will await further results on the efficacy of ACT017 in phase II trials to come. The main antibodies or fusion proteins developed against GPVI are listed in Table 3.2.

Although not through an antibody mediated effect, Losartan has recently been proposed as a novel GPVI-receptor antagonist, unique within the *sartan* family (Taylor, 2014). Active metabolites of Losartan have been identified as a specific GPVI inhibitor by reducing GPVI-dependent human platelet adhesion to collagen under shear in vitro and aggregation of platelets in vivo (Grothusen, 2007), possibly through inhibition of collagen induced Ca^{2+} release (Taylor, 2014) or inhibition of collagen-induced clustering (Jiang, 2015). Although it must be highlighted that administration of a therapeutic dose of losartan to normotensive patients revealed no measurable effect on platelet reactivity, suggesting that it is unlikely to exert any conventional antiplatelet effects at routine dosing (Jiang, 2015).

	Type	Effects
JAQ1	Rat anti-mouse mAb	Reduction of platelet adhesion to SEM Inhibition of collagen-induced platelet aggregation in mice
9012	Mouse anti-human GPVI Fab	Inhibition of platelet adhesion to collagen and CRP Inhibition of thrombus formation under in vitro flow studies
5C4	Rat anti-human GPVI Fab	Inhibition of platelet adhesion to fibronectin
OM2	Mouse anti-human GPVI Fab	Inhibition of collagen-induced platelet aggregation ex vivo in monkeys
OM4	Mouse anti-human GPVI Fab	Inhibition of collagen induced platelet aggregation in vitro and ex vivo Reduction of rat carotid artery thrombus formation in vivo under flow
10B12	Human scFv against human GPVI	Inhibition of collagen- and CRP-XL-induced platelet aggregation
mFAB-F	Human anti-GPVI-dimer Fab	Inhibition of recombinant GPVI binding to collagen and CRP Inhibition of thrombus formation under in vitro flow studies
ACT017	Human anti-GPVI Fab	Reduction in collagen-induced platelet aggregation in healthy human volunteers
Revacept	Dimeric recombinant GPVI-Fc fusion protein	Inhibiting collagen-induced platelet aggregation in healthy men Reduce cerebral infarct size and improve functional recovery in mice

Table 3.2 Antibodies and their inhibitory effect on GPVI-mediated platelet activity. SEM = subendothelial matrix.

3.08 Glycoprotein VI ligands: GPVI binding to fibrinogen and fibrin

It has long been felt that GPVI has a role in thrombosis outside of exposure to collagen. For example, it has been demonstrated that GPVI can support thrombin induced platelet activation, outside of its normal collagen-induced signalling pathway (Hughan, 2007) Furthermore, laminin was shown to stimulate platelet spreading through GPVI activation (Inoue, 2006) and GPVI inhibition was observed to significantly reduce platelet adhesion to fibronectin (Bültmann, 2010) and vitronectin (Schönberger, 2012), suggesting multiple GPVI-ligands outside the classic GPVI-collagen binding pathway.

At the time of starting this PhD, two groups had also shown that GPVI could be a receptor for fibrin, but not much was known about this interaction at the time (Alshehri, 2015; Mammadova-Bach, 2015). Mammadova-Bach and colleagues demonstrated that thrombin generation in fibrinogen-deficient patients was reliant on functional GPVI, that 9012 Fab (anti-GPVI) causes a reduction in both static adhesion of recombinant GPVI to fibrin, and human platelet adhesion to fibrin at different shear rates, and that recruitment of circulating platelets to fibrin-rich clots was GPVI-dependent. Alshehri et al. showed that the pattern of tyrosine phosphorylation through thrombin in human and mouse platelets, in the absence of integrin αIIbβ3 and PAR4, was similar to what is seen when induced by GPVI, and that the GPVI ectodomain (sGPVI) bound to immobilised fibrin. Both groups concluded that fibrin is a novel ligand for the platelet collagen receptor GPVI; however, from these studies it was difficult conclusively decide whether it was the monomeric, dimeric or both forms of GPVI that was stipulated to bind to fibrin.

What made the GPVI-fibrin interaction tantalising in terms of ischaemic stroke was that it suggested that the main collagen binding, platelet activating receptor, GPVI-dimer, could have an important role in the thrombus formation in both large artery and cardio embolic stroke. If indeed GPVI did bind fibrin, this suggested a key role in stabilising thrombus growth through GPVI-fibrin interactions during atherosclerotic plaque rupture in large artery stroke, after platelets had been activated through GPVI-collagen binding. This was previously suggested from observations that GPVI plays a role in the latter stages of

thrombus formation where platelet-fibrin interactions predominate, although at the time it was not known whether this was due to an relation with fibrin or not (Bender, 2011).

What is also interesting is that Mammadova-Bach et al report a marked impairment in thrombin generation at both low and high sear rates, along with reduced platelet adhesion to fibrin in GPVI- deficient patients (Mammadova-Bach, 2015). This suggested that GPVI may also play a role in propagation of thrombus formation at low shear, and also during the early stages of thrombus formation too. Therefore, GPVI-dimer could also have an instrumental part to play in the development of non-arterial thrombi, independent of plaque rupture, ECM exposure and GPVI-collagen binding, and instead through GPVI-fibrin interactions alone. This is especially important in thrombotic diseases such as venous thromboembolism or cardioembolic stroke, where low shear, hypoxia-driven endothelial dysfunction causes activation of the coagulation system, fibrin deposition and platelet activation.

3.09 Objectives of the thesis

This thesis aims to address the following research questions

1) Does GPVI-dimer bind to fibrin(ogen) and activate platelets? (Chapter IV and V)
2) Does it play a primary, or supportive role in these interactions? (Chapter IV and V)
3) Does inhibition of GPVI-dimer lead to reduced thrombus formation on both collagen and fibrin? (Chapter V)
4) Do patients with atrial fibrillation, at risk of cardioembolic stroke, have more active platelets compared to healthy controls, and is part of this activity related to GPVI expression on platelets? (Chapter VI)
5) Similarly, do patients who present with stroke have more active platelets compared to controls, and is part of this activity related to GPVI expression on platelets? (Chapter VII)

6) What does this tell us about the role of GPVI-dimer in thrombotic disease with respect to thrombus formation and structure in ischaemic stroke and could it represent a viable anti-thrombotic target? (Chapter VIII)

3.10 Thesis Hypotheses

Primary hypothesis

It is the dimeric, collagen binding form of GPVI that also binds to fibrin and therefore plays a key role in thrombus formation in ischaemic stroke patients.

Secondary hypotheses

1. Inhibition of GPVI-dimer will reduce thrombus formation on collagen and fibrin surfaces in vitro
2. GPVI-dimer is over-expressed on the platelet surface of patients at *risk* of cardioembolic stroke from atrial fibrillation
3. GPVI-dimer is over-expressed on the platelet surface of patients with ischaemic stroke
4. GPVI-dimer represents a viable anti-thrombotic target

CHAPTER IV. METHODS: INVESTIGATING THE BINDING OF GLYCOPROTEIN VI-DIMER TO FIBRINOGEN AND FIBRIN

4.01 Materials

(a) Buffers

Phosphate buffered saline

Phosphate buffered saline (PBS) was made from dissolving a phosphate-saline tablet with 200 mL of distilled water to obtain a 137 mM NaCl, 2.7 mM KCl and 10 mM phosphate buffer solution (pH 7.4 at room temperature; Sigma- Aldrich, USA), stored at 4 °C.

Hepes-Tyrodes buffer

Hepes-Tyrodes (HT) buffer contains the following; 134 mM NaCl, 2.9 mM KCl, 0.34 mM NaH_2PO_4, 5.5 mM glucose, and 5mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES) and was filtered prior to storage at 4 °C. The buffer is used at room temperature and a pH of 7.3 was measured and adjusted as needed prior to experimental use.

Flow buffer

Flow (Fc) buffer (136mM NaCl, 2.7mM KCl, 5mM HEPES, 10 mM Glucose, 2mM $MgCl_2$, 2mM $CaCl_2$, 0.5% bovine serum albumin (BSA)) was used in flow adhesion experiments. The buffer was filtered prior to storage at 4 °C and a pH of 7.4 was verified prior to experimental use.

(b) Collagen, Collagen-Related Peptide and fibrinogen

Collagen

Pepsin-treated bovine collagen type-III (col-III; (non-fibrous; Koken, Japan and horse collagen (HORM; fibrous; Takeda, Austria) were mainly used instead of synthetic peptides where described.

The synthetic crosslinked collagen related peptide CRP-XL; [GCO–$(GPO)_{10}$–GCOG-NH_2] was used as a GPVI-specific agonist (Morton, 1995). GPP10-XL [GCP-$(GPP)_{10}$G–NH_2] is an inactive analogue where the third hydroxyproline is substituted for a proline; therefore it does not bind GPVI, justifying its use as a negative control (Smethurst, 2007). Both

peptides were synthesised and crosslinked in-house, by Drs A Bonna and J-D Malcor at the Farndale laboratory.

Fibrinogens and their substrates

Different human fibrinogen preparations were used in the initial experiments, prior to settling on Fibrinogen 3 (Fib-3; Enzyme Research Laboratories, UK) because it was plasminogen-, vWF- and fibronectin-depleted, but not factor XIII-depleted.

Human fibrinogen D-fragment and E-fragment was commercially obtained (Thermo Fisher Scientific, USA) as well as fibrin D-dimer (Abcam, UK). Table 4.1 summarises the commercial preparations of human fibrinogen and derivatives used. The manufacturer's stated purity was confirmed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) purity (Supplementary Figure 4.1).

Name	Abbreviation	Characteristics	Commercial Source
Fibrinogen	Fib-Sigma	340 kDa. Other characteristics not stated by manufacturer	Sigma Aldrich, UK
Fibrinogen 1	Fib-1	330 kDa. Plasminogen-depleted	Enzyme Research Laboratories, UK
Fibrinogen 3	Fib-3	330 kDa. Plasminogen, vWF, fibronectin-depleted	Enzyme Research Laboratories, UK
Fibrinogen Peak 1	Fib Peak 1	340 kDa. Plasminogen, fibronectin, factor XIII-depleted	Enzyme Research Laboratories, UK
Fibrinogen D-fragment	D-fragment	Approx. 83 kDa, plasmin-leaved	Thermo Fisher Scientific, USA
Fibrinogen E-fragment	E-fragment	Approx.50 kDa, plasmin-cleaved	Thermo Fisher Scientific, USA
Fibrin D-dimer	D-dimer	200 kDa, plasmin-cleaved	Abcam, UK

Table 4.1 The commercial preparations of human fibrinogen, D-, E- fragments and D-dimer used as well as the manufacturer stated structure and composition.

(c) Recombinant glycoprotein VI monomer and dimer

$GPVI_{ex}$ is a 41 kDa recombinant protein comprising the extracellular domain of GPVI, including the collagen binding portions D1 and D2 (amino acids 1-214), alongside most of the highly glycosylated extracellular stem portion.

$GPVI\text{-}Fc_2$ is a 150 kDa fusion protein of the GPVI extracellular domain (same amino acid sequence as $GPVI_{ex}$) and the Fc domain of IgG, which spontaneously dimerizes [$(GPVI\text{-}Fc)_2$, abbreviated as $GPVI\text{-}Fc_2$]. Both proteins were expressed, designed and characterized by Dr M Moroi and colleagues (Miura, 2002).

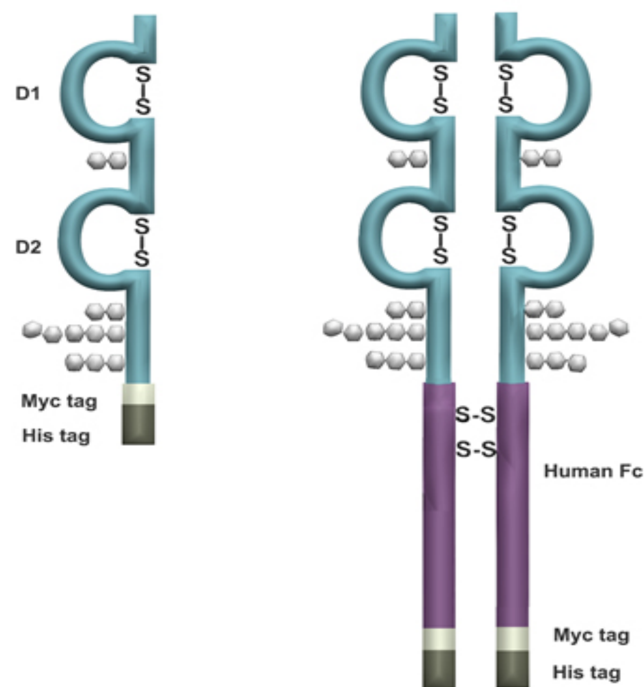


Figure 4.1 Recombinant proteins of GPVI-extracellular domain containing the collagen binding D1 and D2 domains and the heavily glycosylated stem portions. S-S denotes disulphide bonds. Figure courtesy of Dr Stephanie Jung.

(d) Antibodies used in GPVI detection

mFAB-F is a GPVI-dimer–specific, inhibitory, human Fab that recognises the dimers on resting and activated platelets as well as inhibiting collagen induced platelet aggregation. 204-11 mAb was first developed as a mouse antibody against GPVI and was found to detect both forms of GPVI on resting or activated platelets, without exerting any inhibitory of activatory properties. The Fab portion from this mAb, 204-11 Fab, was developed later as a GPVI-dimer–specific, non-inhibitory Fab. All antibodies were developed by Drs M Moroi and S Jung.

1G5 (BioCytex, France) is an mouse mAb raised against recombinant $GPVI_{ex}$ (Inoue, 2006; Al-Tamimi, 2009), but binds to both monomers and dimers with similar affinity and therefore used to detect total GPVI. GPVI-specific antibodies their affinities for GPVI monomer and dimer are summarised in Table 4.2 (Jung, 2009, 2012).

Antibody	K_d (dimer), nM	K_d (monomer), nM	Form	Specificity
mFAB-F	10.83 ± 0.60	Undetectable	Fab	Dimer
204-11 Fab	1.05 ± 0.10	Undetectable	Fab	Dimer
204-11 mAb	0.16 ± 0.01	4.09 ± 0.44	IgG	Both
1G5	0.37 ± 0.03	0.20 ± 0.02	IgG	Both

Table 4.2 The dissociation constants (K_d) for binding of recombinant GPVI-dimer and monomer to antibodies against the extracellular domain of GPVI. Both mFAB-F and 204-11 Fab are specific to GPVI-dimer, therefore their binding to GPVI-monomer is too low to calculate K_d values. 1G5 and 204-11 can be used to detect both forms of GPVI. Data reported by (Jung, 2012).

4.02 Methods

(a) Preparation of fibrin from fibrinogen

To prepare polymerised fibrin (pFibrin) for static adhesion, aggregometry and flow adhesion studies, thrombin (2 U/mL; Sigma-Aldrich, UK) was added to fibrinogen (10 µg/mL, unless otherwise stated), incubated for 30 min at room temperature before coating onto the required surface and left overnight at 4°C. The coated surface was blocked with 0.5% BSA, treated with hirudin or protease inhibitor cocktail for 10 min to inhibit active thrombin, and then washed with PBS. Presence of fibrin strands was confirmed by Dr S Jung (Supplementary Figure 4.2) by staining with anti-fibrin (UC45)/Alexa-Fluor-Plus 555 anti-mouse IgG (ThermoFisher Scientific).

Immobilized monomeric fibrin (mFibrin) was prepared by adding GPRP (2 mM) to fibrinogen (10 µg/mL, unless otherwise stated), and incubated for 30 min at room temperature before adding thrombin as above; other procedures are the same as for pFibrin.

(b) Glycoprotein VI-fibrin binding ELISA assay

96-well black optical polymer-bottom ELISA plates (Thermo Fisher Scientific, USA) or Maxisorp ELISA plates (Thermo Fisher Scientific, USA) were incubated with fibrinogen or collagenous substrates in PBS as required. The coated plates were left overnight at 4°C and washed with PBS (250 µL x 3) the following morning, blocked with 0.5% BSA and left for 1-hour. The required concentrations of $GPVI_{ex}$ or GPVI-Fc_2 (usually 0–50 µg/mL unless otherwise stated) diluted in 0.5% BSA was then added before a further 1-hour incubation.

Bound GPVI was detected using the pan-GPVI antibody 1G5 as the primary antibody (4 µg/mL in 0.5% BSA), followed by (1:10000 in 0.5% BSA) IRDye®800CW goat anti-mouse IgG (Li-cor Biosciences, USA) or 1:4000 Alexa-fluor 647–conjugated anti–human Fc (BioLegend, USA). The wells were thoroughly washed with 250 µL PBS x3 after each incubation stage.

GPVI binding was quantified using fluorescence (AU) through the immunofluorescence or near infra-red detection capabilities of the Li-cor Odyssey CLx system.

(c) Human washed platelet preparation

Blood was taken by venepuncture from healthy volunteers with informed consent under the ethics obtained (Human Biology Research Ethics Committee, HB REC.215.18). The universal 9 mL Sarstedt blood tubes were pre-prepared with the addition of 900 μL of citrate prior to venepuncture (1.62 g of trisodium citrate/50 mL of distilled water) to make a final ratio of 1:9.

To prepare washed platelets, whole blood was centrifuged at 1200 RPM for 12 minutes to retain platelet rich plasma (PRP). 4.5 μL of 1 mg/mL prostaglandin E_2 (PGE_2) was added per 9 mL of PRP to inhibit unwanted platelet activation. The PRP was removed and then centrifuged at 2200 RPM for 10 minutes, creating platelet poor plasma (PPP) and a platelet pellet at the bottom of the tube. The PPP is removed and can be used in aggregometry if needed. 1 mL of HT per 9 mL of original blood volume is then added to the platelet pellet and mixed to re-suspend.

A Beckman Coulter Counter was used to establish the platelet count by using 5 μL of re-suspended platelets in 10 mL of isotonic counter solution. The platelets were allowed to rest for 20 minutes prior to experimentation.

(d) Platelet aggregometry

A PAP-8E light transmission aggregometer (Bio/Data corporation, USA) was used for all aggregation experiments. 300 μL of washed platelets at a concentration of 2.5×10^8 were added to siliconized glass tubes, either untreated or pre-incubated with 100 μg/mL of mFAB-F to inhibit GPVI-dimer, or 45 μM of Eptifibatide to inhibit integrin αIIbβ3. The platelets ± inhibitors were left for 10 minutes prior to starting aggregometry. Platelets are then warmed to 37°C for 1 minute and the stimulations are allowed to proceed under stirring. At 3 minutes, D-fragment, D-dimer, fibrinogen or pFibrin (5- 20 μg/mL) was added to the platelet mixture. If no aggregation occurred by 8 min, HORM collagen was added to induce platelet aggregation, verifying that the platelets were active.

(e) Whole blood flow adhesion

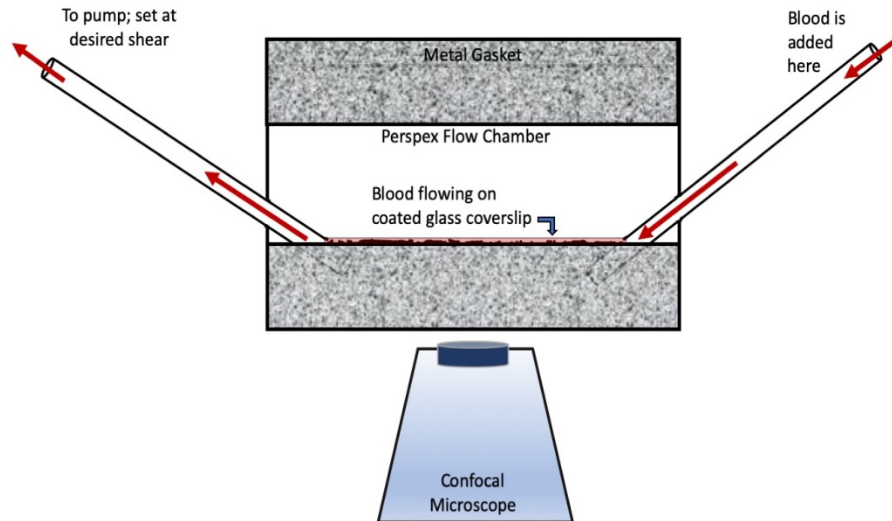


Figure 4.2 Flow adhesion set up. Whole blood is perfused through a Perspex flow chamber placed within a metal gasket. This is then mounted onto the confocal microscope dock for image acquisition.

The flow adhesion set-up within the Farndale lab is illustrated in Figure 4.2. Glass coverslips were coated with fibrinogen, fibrin, their relevant substrates and collagen III at 10 µg/mL in PBS and left overnight at 4°C in a black humid chamber. The following day, 36 µL of the anticoagulant D-phenylalanyl-prolyl-arginyl chloromethyl ketone (PPACK) was added to a 9 mL universal blood and healthy volunteer blood or blood from patients with Glanzmann's thrombasthenia (GT) which was collected via venepuncture, with informed consent under the ethics obtained as above. Glass coverslips with the immobilised substrate are then attached to a 125 µm deep flow chamber within a metal gasket and then mounted to an Olympus FV300 laser-scanning confocal microscope.

Platelets in whole blood were fluorescently labelled with 1:4500 DiOC6 (3,3'-dihexyloxacarbocyanine iodide) and left for 5 minutes. After this stage, any required platelet receptor inhibitors are added (human Fab 100 µg/mL as a control Fab; mFAB-F

100 μg/mL for GPVI-dimer; Eptifibatide 45 μM for αIIbβ3 and Gi9 10 μg/mL for α2β1) and left for a further 15 minutes. After perfusion of Fc buffer (treated with protease inhibitor cocktail for preparations that required thrombin) for 1-minute, whole blood was perfused over the coverslips at a either 350 s^{-1} or 1000 s^{-1}. Whole blood preparations are topped up with 1:1000 PPACK every hour.

Shear rate $\gamma_{(cs)}$, s^{-1}	100	300	1000	3000
Flow rate ml/sec	0.000391	0.001174	0.003915	0.011744
Flow rate (Q) ml/min	0.023	0.070	0.235	0.705

Table 4.3 Pump settings for flow adhesion experiments. The flow rate (Q) in mL/min that corresponds to 300 s^{-1} and 1000 s^{-1} were used to mimic venous and arterial flow, respectively.

Analysis

After 5 min, Z-stacks (ΔZ = 0.69 μm increments from the coverslip plane) were collected and analysed by ImageJ 1.35 (National Institutes of Health). The coverslip plane was defined as the Z-plane with the largest fluorescent platelet area and used to calculate platelet surface area coverage (SA; μm^2). Thrombus volume (μm^3) was calculated as the sum of the detected surface areas of all images in the Z-stack, multiplied by ΔZ. Mean thrombus height (MTH) was calculated as thrombus volume/field area (Figure 4.3).

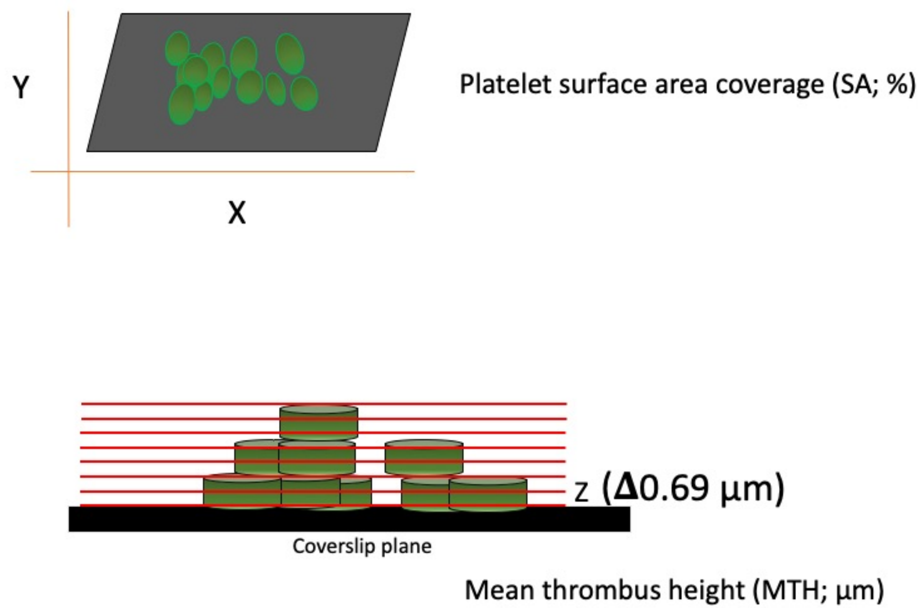
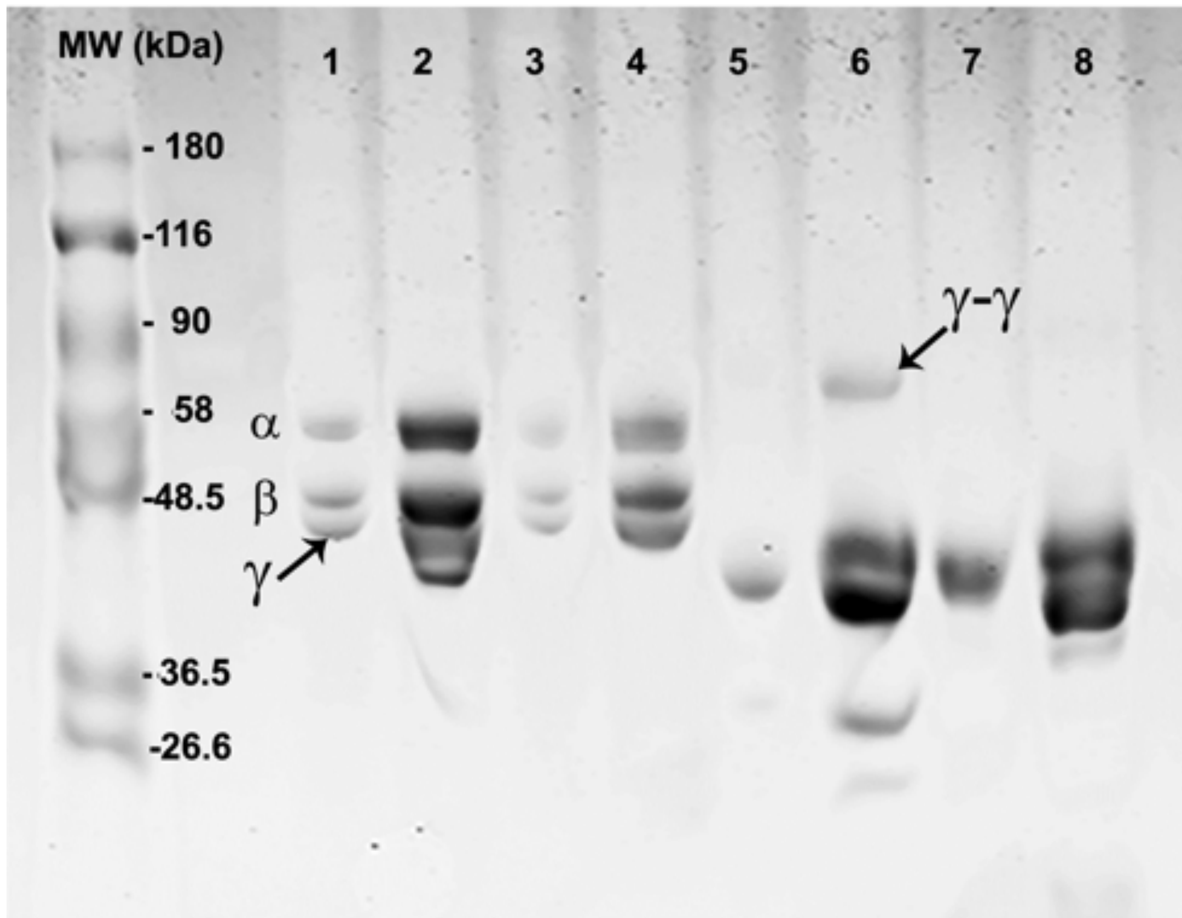


Figure 4.3 Calculation of platelet surface area coverage (SA) and mean thrombus height (MTH).

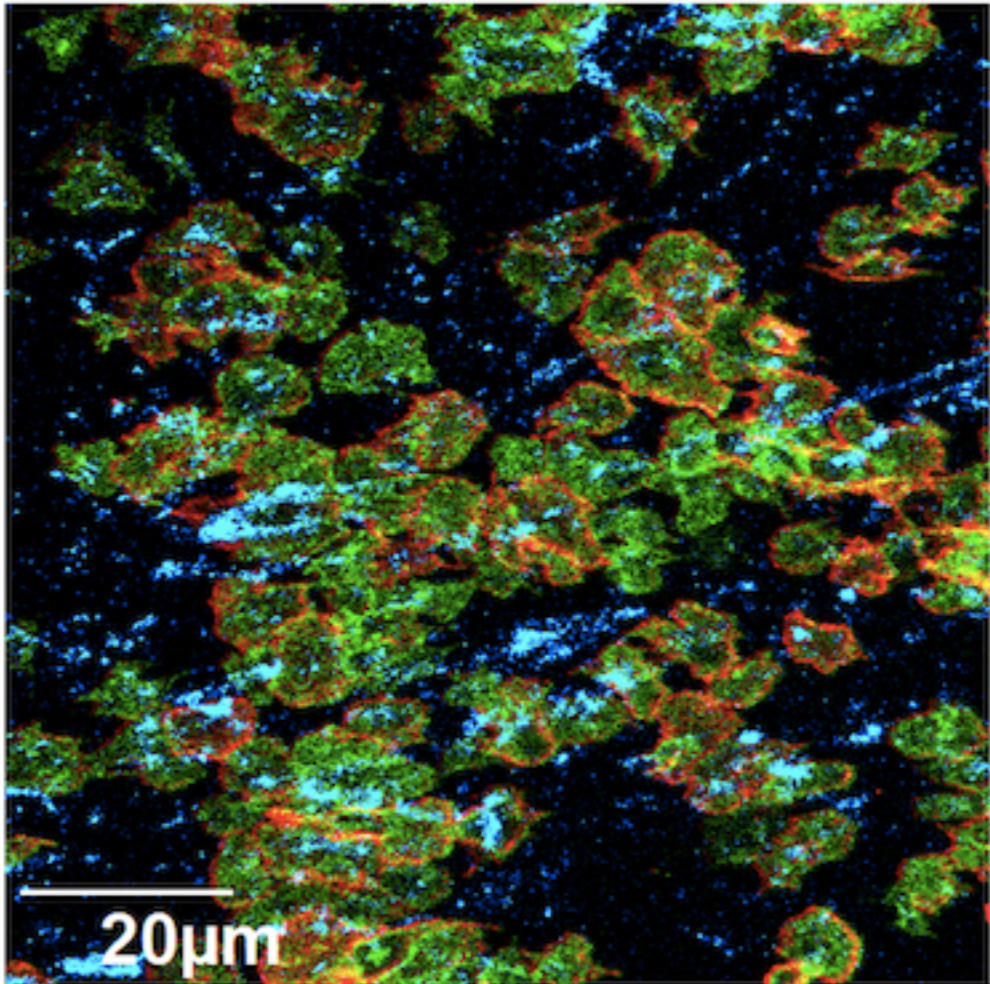
4.03 Statistical analysis

K_d were recalculated using non-linear regression (one-site model). For means testing for parametric and non-parametric data, student's t-test and Mann-Whitney U test were performed after normality testing. Prism version 7.2 (GraphPad, CA) was used for statistical analysis and Image J version 1.22 (NIH) was used for analysis and quantification of flow adhesion images and data.

4.04 Supplementary Figures



Supplementary Figure 4.1 SDS-PAGE confirming the manufacturer's stated purity of fibrinogen and its derivatives. Fib-3, Fib-S, D-fragment, and D-dimer were applied at protein concentrations of 1- and 5-µg/protein per lane, Western-blotted, stained by Coomassie blue, and then scanned by the Licor Odyssey. Fib-3 (lanes 1, 2) and Fib-S (lanes 3, 4) show similar banding patterns, with the expected α, β, and γ subunits (as indicated in lane 1 only for simplicity). With D-dimer, lanes 5 and 6) the approximately 70 kDa band visible corresponds to the covalently crosslinked gamma chain, indicated in lane 6 as γ–γ. D-fragment only shows the γ and β chains, and no γ–γ as expected. The D-fragment α-chain is in a degraded form with a lower molecular weight. This experiment was done by Dr M Moroi.



Supplementary Figure 4.2 pFibrin was created as per the methods. pFibrin was immobilised on MatTek glass dishes and stained with anti-fibrin/Alexa-fluor 547–anti-mouse IgG (blue) after the surface was blocked with 1% BSA. The dishes were then thoroughly washed. pFibrin (blue) is clearly evident. P-selectin (red) expression (platelet activation marker) and aggregate formation is evident on the surface of the adhered platelets. Images were obtained with a 60x oil immersion objective on an Olympus FV500 confocal microscope. Experiment done by Dr S Jung.

CHAPTER V. RESULTS: GLYCOPROTEIN VI-DIMER CONTRIBUTES TO THROMBUS FORMATION THROUGH BINDING TO FIBRINOGEN AND FIBRIN

Original Publication:

Platelet collagen receptor Glycoprotein VI-dimer recognizes fibrinogen and fibrin through their D-domains, contributing to platelet adhesion and activation during thrombus formation. **Induruwa I,** Moroi M, Bonna A, Malcor JD, Howes JM, Warburton EA, Farndale RW, Jung SM. *J Thromb Haemost*. 2018; 16(2): 389-404.

5.01 Results

(a) GPVI-Fc_2 binds to immobilised fibrinogen D-domain and fibrin D-dimer

Integrin αIIbβ3 has been established as the main receptor for fibrinogen on platelets, where it exists in its inactive, low-affinity form until inside out signalling which opens up a fibrinogen binding site. This conformational change is pivotal for platelet aggregation and thrombus build up, which occurs through the initiation of outside-in signalling causing thrombin generation and platelet PS exposure (Ma, 2007). Integrin αIIbβ3 can now also capture locally available fibrin to engage it in the growing thrombus, vital for thrombus growth and clot retraction.

GPVI was identified as a receptor for fibrin potentially through fibrin-mediated GPVI-signalling which supported continued thrombus growth through thrombin generation and increased PS exposure (Alshehri, 2015; Mammadova-Bach, 2015). Both studies also concluded that the polymerised structure of fibrin was essential for GPVI binding and that there was no interaction between fibrinogen and mFibrin with GPVI.

As GPVI-dimer is the functional, collagen binding, form of GPVI, we wanted to investigate whether it was the GPVI-dimer that also bound to fibrin and whether it could also bind to fibrinogen.

First an ELISA-based adhesion assay was used to investigate whether either of the recombinant GPVI constructs ($GPVI_{ex}$ monomer and GPVI-Fc_2 dimer) bound to immobilised fibrinogen or fibrin. The initial experiments were done using GPVI-Fc_2 due to the limited supply of $GPVI_{ex.}$ Fibrinogen (Fib-3) as well as the pFibrin and mFibrin created from each of the original concentrations of Fib-3 (10 – 200 μg/mL) were coated on plates as described in the methods section. The following day, GPVI-Fc_2 was added at increasing concentrations between 0-50 μg/mL. At all coating concentrations of fibrinogen, mFibrin, or pFibrin, binding was too low and scattered to determine and accurate K_d. Therefore, it was determined that GPVI-Fc_2 did not bind fibrinogen or fibrin under these conditions (Figure 5.1).

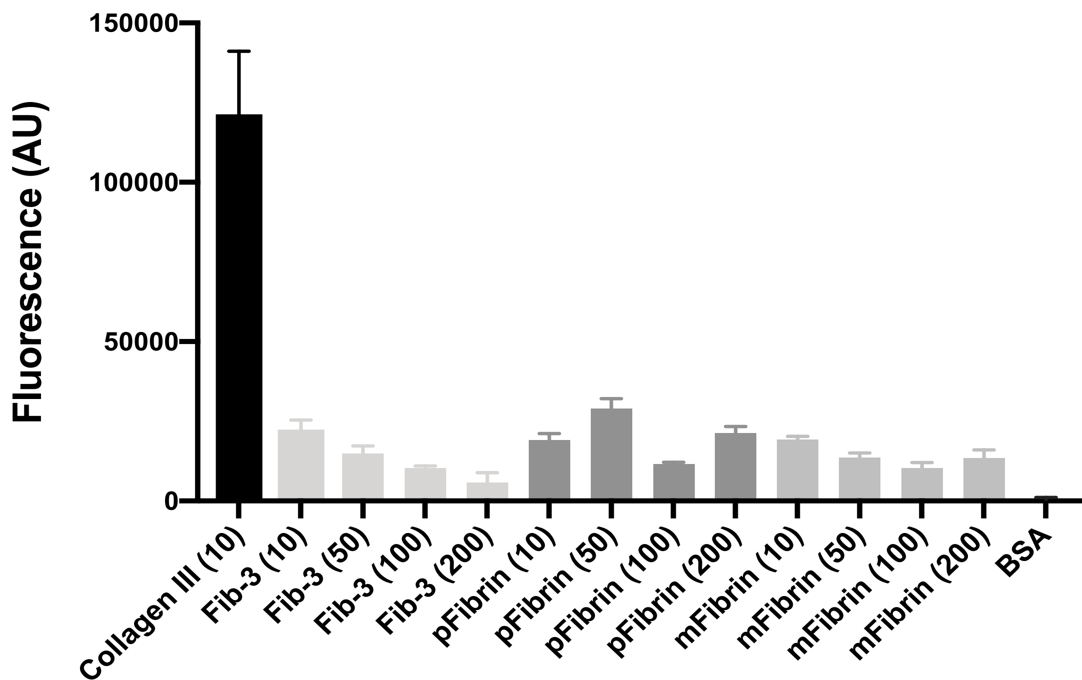
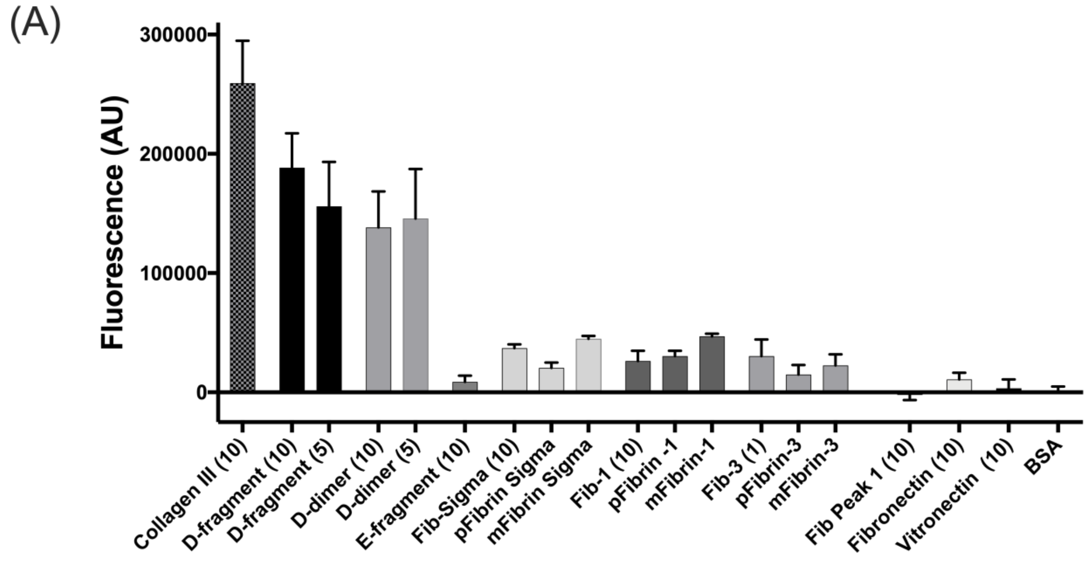


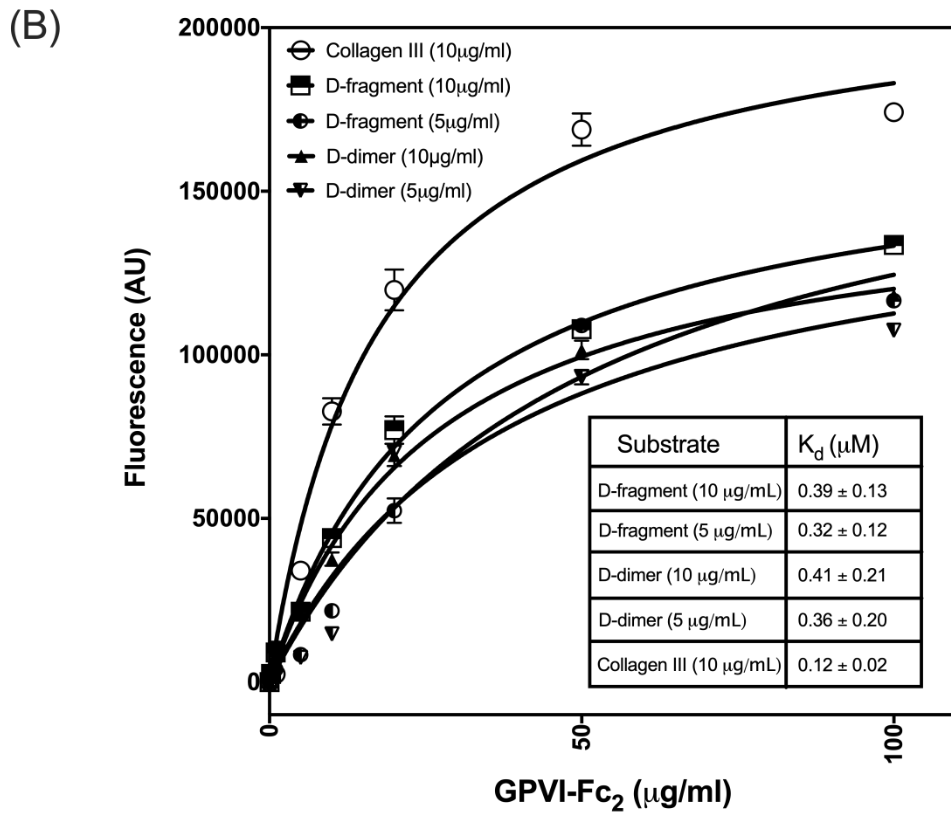
Figure 5.1 GPVI-Fc$_2$ does not bind fibrinogen, pFibrin or mFibrin up to 200 μg/mL. The numbers in parentheses indicate the concentrations of fibrinogen used to coat the ELISA plate wells (μg/mL in PBS). GPVI-Fc$_2$ (50 μg/mL shown) does not bind immobilised fibrinogen or their constituent pFibrin or mFibrin in this assay, up to 200 μg/mL. GPVI-Fc$_2$ binds well to collagen (10 μg/mL). Fluorescence values shown are the mean ± SEM, from 3 different experiments, with duplicate repeats.

Since the full-length fibrinogen or fibrin did not bind GPVI-Fc$_2$ in this assay, we decided to use plasmin cleaved fibrinogen (D-, E-) and fibrin subdomains (D-dimer) alongside different commercially available fibrinogens as described in Table 2.1 and fibronectin and vitronectin. Collagen III was used as a positive control and 0.5% BSA as a negative control. GPVI-Fc$_2$ (0-50 μg/mL tested) demonstrated specific and saturable binding to D-fragment and D-dimer demonstrating classic receptor-ligand properties but did not bind E-fragment (Figure 5.2).

(A)



(B)



Substrate	K_d (μM)
D-fragment (10 μg/mL)	0.39 ± 0.13
D-fragment (5 μg/mL)	0.32 ± 0.12
D-dimer (10 μg/mL)	0.41 ± 0.21
D-dimer (5 μg/mL)	0.36 ± 0.20
Collagen III (10 μg/mL)	0.12 ± 0.02

Figure 5.2 (previous page) GPVI-Fc_2 binds to D-fragment, D-dimer and collagen III. (A) GPVI-Fc_2 (50 μg/mL shown) binds to D-fragment and D-dimer as well as collagen. In contrast, there is only very low binding to Fib-Sigma, Fib-1, Fib-3, and the corresponding pFibrin and mFibrin produced from thrombin +/- GPRP addition respectively. GPVI-Fc_2 demonstrates no binding to E-fragment, Fib Peak 1, fibronectin or vitronectin. Fluorescence values shown are the mean ± SEM, from 5 different experiments, with duplicate repeats. (B) Kinetic analyses of the binding of GPVI-Fc_2 to D-fragment and D-dimer to determine K_d values. GPVI-Fc_2 (0-100μg/mL) exhibits saturable binding to both D-fragment and D-dimer (coated at 5- and 10μg/mL). The K_d values for D-fragment and D-dimer are comparable to the K_d value for collagen III (B-inset).

The recombinant GPVI monomer, $GPVI_{ex}$ (0–50 µg/mL), did not bind D-fragment, D-dimer or fibrinogen and exhibited poor collagen binding compared to GPVI-Fc_2, as previously reported (Jung, 2012) (Figure 5.3). We were not able to test pFibrin binding due to the limited availability of $GPVI_{ex}$ but since it did not bind to D-dimer, it was felt binding to pFibrin was unlikely. These results suggest that it is the dimeric form of GPVI, rather than GPVI-monomer is involved in binding to fibrinogen and fibrin, through their D-domains and D-dimer regions respectively.

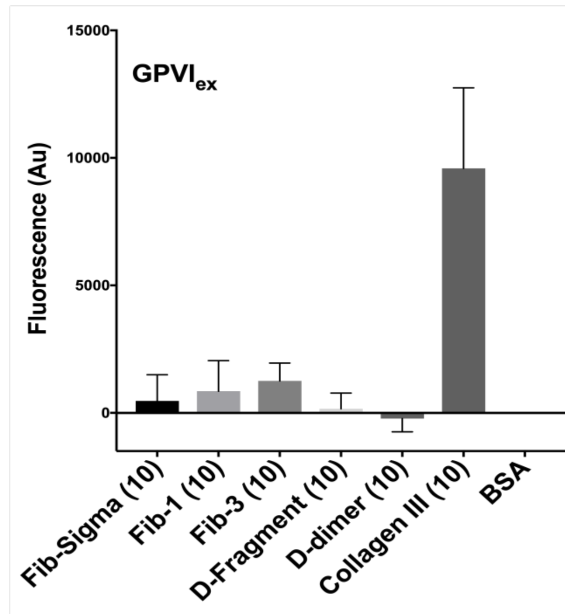


Figure 5.3 $GPVI_{ex}$ does not bind D-domain or D-dimer. $GPVI_{ex}$ (50 µg/mL shown) demonstrates no binding to immobilized Fib-Sigma, Fib-1, Fib-3, D-fragment or D-dimer with some binding to collagen III (all coated at 10µg/mL). Fluorescence values shown are the mean ± SEM, from 3 different experiments, with duplicate repeats.

To ensure that it was the extracellular D1D2 portion of GPVI-Fc_2 and not the IgG or Fc portions of GPVI-Fc_2 that bound to D-fragment and D-dimer, human Fc and DDR2-Fc_2 (recombinant Fc-fusion protein of discoidin domain–containing receptor 2, another collagen-binding protein, provided by Dr B Leitinger (Xu, 2011)) (0-20 μg/mL) and human IgG (0-25 μg/mL) were used in a similar binding assay with the fibrinogen substrates and collagen III as a positive control. As expected, DDR2-Fc_2 bound to collagen, but, neither DDR2-Fc_2, Human Fc or Human IgG were able to bind to fibrinogen derivatives. GPVI-Fc_2 bound well to fibrinogen derivatives and collagen (Figure 5.4).

Furthermore, human integrin α2- and α1-domains (kindly provided by Dr S Hamaia) were tested to see if they bound to D-fragment or D-dimer (all coated at 10 μg/mL). These α-domains bind their respective peptide sequences GFOGER and GLOGEN in the presence of Mg^{2+}, whereas EDTA abolishes α-domain binding (Hamaia, 2012). As expected, α2 bound to GFOGER in the presence of Mg^{2+} and not EDTA and α1 bound to GLOGEN in the presence of Mg^{2+} and not EDTA. Neither of the α-domains were able to bind D-fragment or D-dimer irrespective of the availability of Mg^{2+} further suggesting that the GPVI-dimer interaction with the D-domain and D-dimer is specific to GPVI (Figure 5.5)

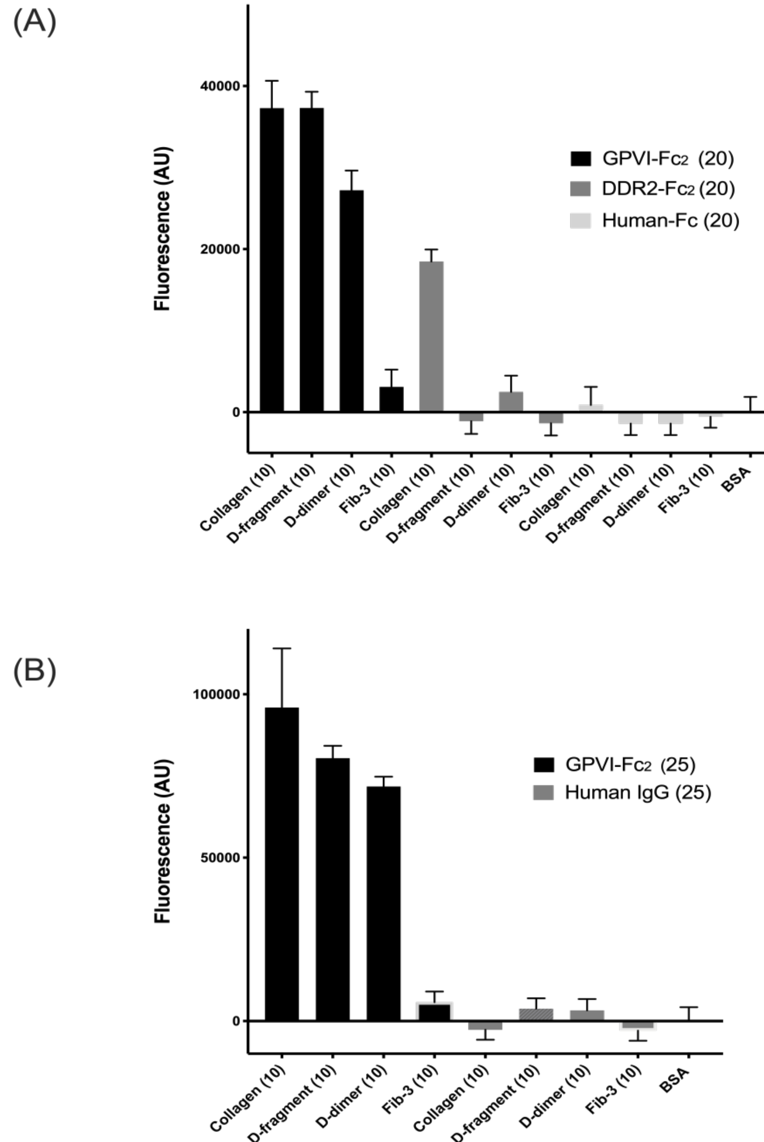


Figure 5.4 GPVI-Fc$_2$ binds D-domain and D-dimer through its D1D2 collagen binding extracellular domain. (A) Fib-3, D-fragment, D-dimer and collagen III were coated at 10 μg/mL and reacted with 20 μg/mL of GPVI-Fc$_2$ (black), DDR2-Fc$_2$ (dark grey) and Human - Fc (light grey). Only GPVI-Fc$_2$ is able to bind D-domain, D-dimer and collagen. DDR2-Fc$_2$ bound to collagen as expected. (B) Fib-3, D-fragment, D-dimer and collagen III were coated at 10 μg/mL and reacted with 25 μg/mL of GPVI-Fc$_2$ (black), Human IgG (grey). Human IgG did not bind fibrinogen, its derivatives or collagen III. Fluorescence values shown are the mean ± SEM, from 2 different experiments, with duplicate repeats.

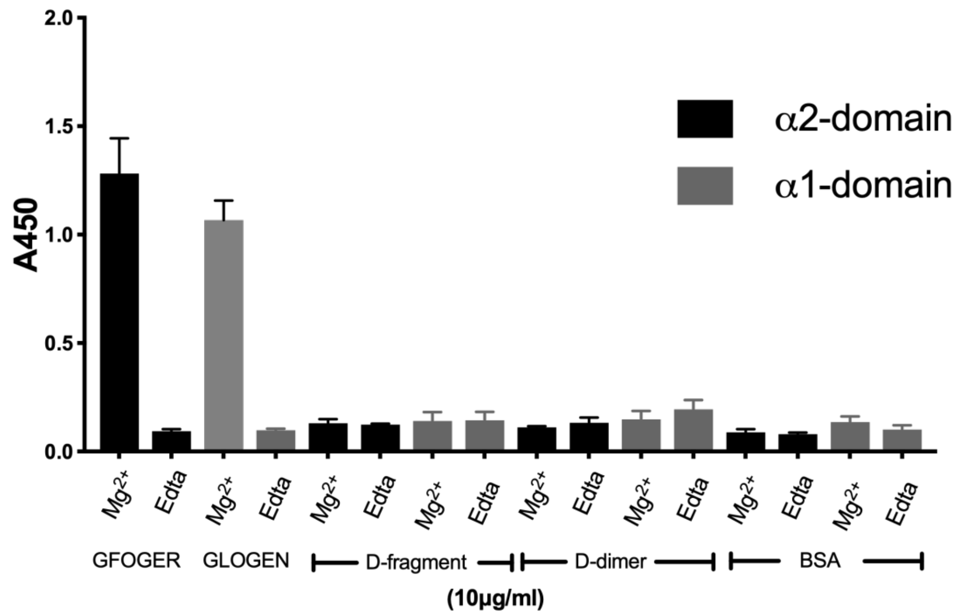


Figure 5.5 Integrin α2- and α1-domains do not bind D-domain or D-dimer. GFOGER, GLOGEN, D-fragment and D-dimer were coated at 10 μg/mL and left for 2 hours on Maxisorp 96-well clear plates. At each incubation step, 250 μL of PBS was used to wash x3. The GST-conjugated α2- and α1-domains (10 μg/mL) were then added to the coated wells. After 1 hour, a buffer containing 1:10000 HRP-conjugated anti-GST antibody was added. Colour was developed using a TMB substrate kit (Pierce) as per manufacturer's instructions. Absorbance was measured using a 96-well plate reader at A_{450}. Absorbance values shown are the mean ± SEM, from 1 experiment, with triplicate repeats. The α-domains bind their respective peptide sequences in the presence of Mg^{2+}, but not D-fragment or D-dimer.

(b) mFAB-F inhibits GPVI-Fc_2 binding to immobilised D-fragment, D-dimer and collagen

The theory that GPVI-dimer binds to D-fragment and D-dimer in a specific manner is further supported by the ability of GPVI-dimer specific inhibitory antibody mFAB-F to reduce its binding to D-fragment and D-dimer (and collagen). Compared to the non-specific human Fab, mFAB-F significantly reduced GPVI-Fc_2 binding to collagen (P=0.01), D-fragment (P=0.001) and D-dimer (P=0.004).

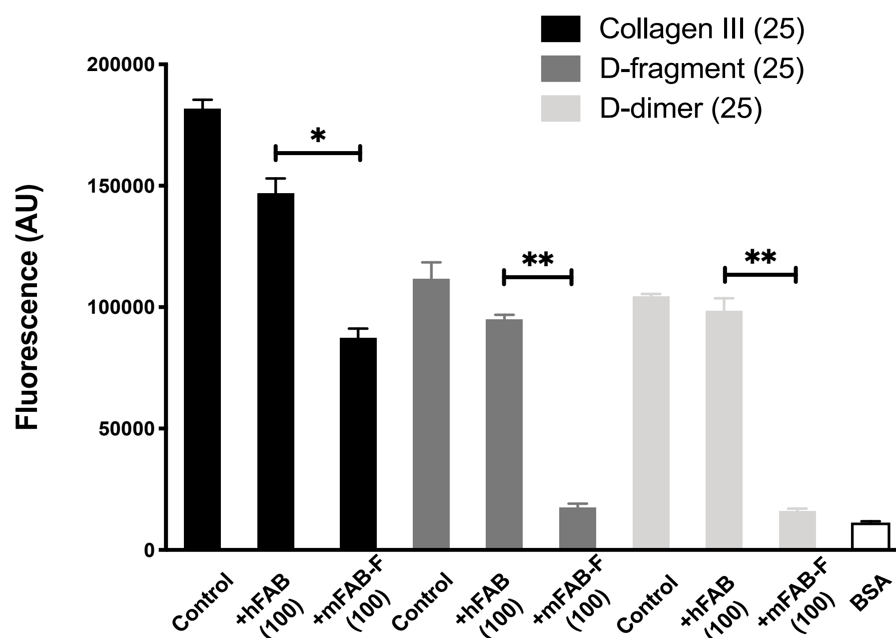


Figure 5.6 mFAB-F significantly inhibits GPVI-Fc_2 binding D-domain, D-dimer and collagen. Collagen III, D-fragment and D-dimer were coated at 25 μg/mL and left overnight. GPVI-Fc_2 (50 μg/mL) was incubated for 15 minutes in the presence of either human Fab (hFab; 100 μg/mL), mFAB-F (100 μg/mL) or no inhibitor (control). mFAB-F is able to significantly reduce the binding of GPVI-Fc_2 to D-fragment, D-dimer and collagen III. Fluorescence values shown are the mean ± SEM, from 2 different experiments, with duplicate repeats.

(c) The GPVI-dimer binding site on fibrin(ogen) is proximate to the collagen binding site

The evidence so far suggested that the GPVI-dimer binds the D-domain of immobilised fibrinogen and the D-dimer region of fibrin. However, it had not yet been determined where the D-domain or D-dimer would interact with GPVI-dimer. Therefore, another ELISA assay was setup to investigate whether the binding site on GPVI-dimer to fibrin(ogen) was proximate to that of collagen, based on the current understanding that the collagen binding site on platelet GPVI-dimer was in its extracellular D1 domain (Horii, 2006).

The results demonstrate that if GPVI-Fc_2 is incubated in solution with collagenous substrates (collagen III, HORM, CRP-XL), its ability to bind to both immobilised D-fragment and D-dimer is reduced in a concentration-dependent manner. CRP-XL showed the most effective inhibition of GPVI-Fc_2 binding to D-fragment and D-dimer at concentrations $\geq$0.05 μg/mL. Collagen III and HORM were able to inhibit GPVI-Fc_2 binding at concentrations of $\geq$0.5 μg/mL. GPP10-XL was used as a negative control and did not inhibit the binding of GPVI-Fc_2 to D-fragment or D-dimer (Figure 5.7).

The converse experiment, where immobilised collagen substrates were reacted with GPVI-Fc_2 incubated with fibrinogen, fibrin and their respective subdomain fragments was also performed. None of these substrates were able to inhibit GPVI-Fc_2 binding to collagen. This may because their affinity to GPVI-dimer is too low, compared with that between GPVI-Fc_2 and collagen when they are in suspension, or possibly that D-fragment and D-dimer may adopt a different conformation in solution so that they may not be able to interact with GPVI-dimer (Figure 5.8).

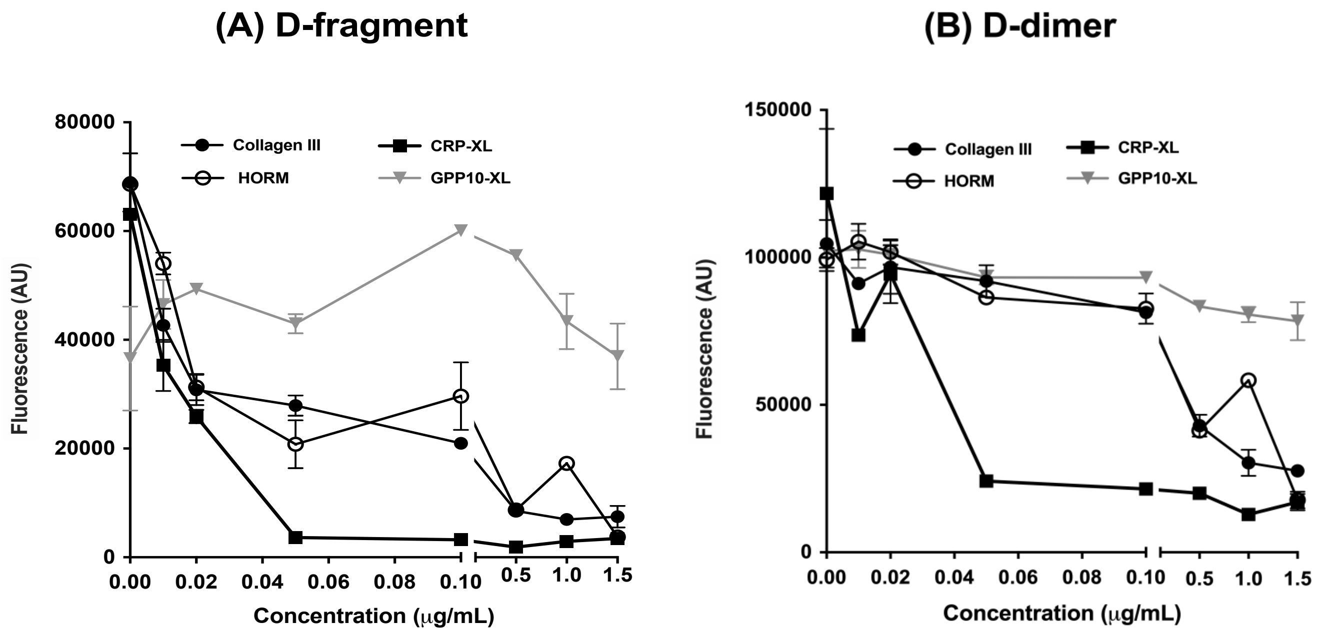


Figure 5.7 Collagenous substrates compete with GPVI-dimer in binding to immobilised D-fragment and D-dimer. ELISA plates were coated with D-fragment and D-dimer (10 µg/mL) and left overnight. GPVI-Fc_2 (20 µg/mL) was incubated with increasing concentrations of 0–20 µg/mL of collagen III, HORM, CRP-XL (0-1.5 µg/mL shown), or the negative control GPP10-XL in solution. (A) Collagen III and HORM showed similar concentration-dependent abilities to inhibit binding of GPVI-Fc_2 to D-fragment, with complete inhibition at ≥0.5 µg/mL. CRP-XL was effective at lower concentrations, producing complete inhibition at ≥0.05 µg/mL. (B) The inhibition of GPVI-Fc_2 to D-dimer showed a similar inhibition profile to D-fragment. As expected, GPP10-XL had little effect against GPVI-Fc_2 binding. Data is presented as mean ± SEM obtained from 3 separate experiments with each data point determined in duplicate.

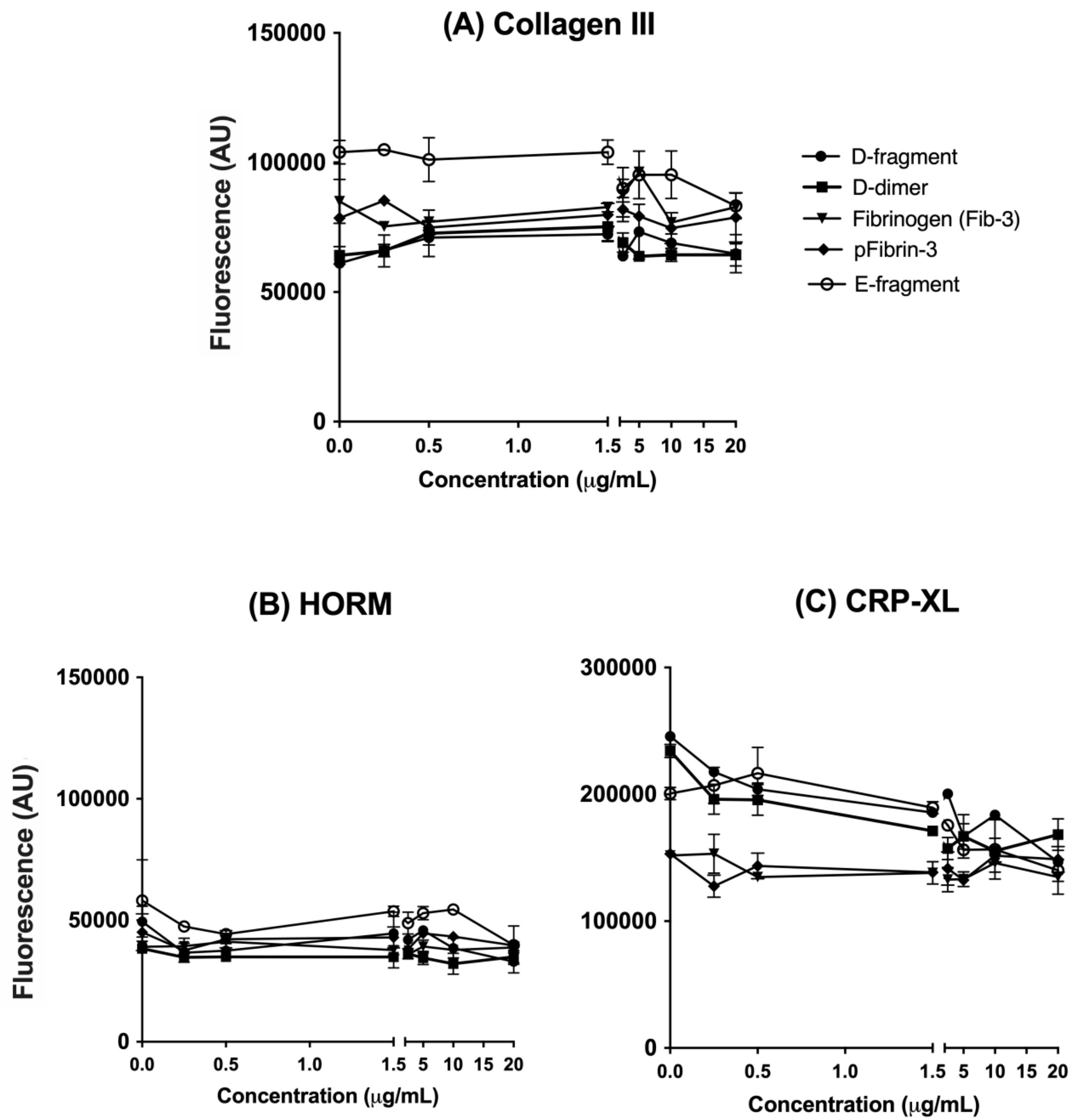


Figure 5.8 GPVI-Fc$_2$ is not able to interact with fibrinogen, fibrin or their subdomains in solution. ELISA plates were coated with collagen III, HORM and CRP-XL (10 μg/mL) and left overnight. GPVI-Fc$_2$ (20 μg/mL) was incubated with 0–20 μg/mL of D-fragment, D-dimer, Fib-3 or pFibrin-3. E-fragment was used as a negative control. D-fragment, D-dimer, Fib-3 pFibrin-3 or E-fragment could not inhibit binding of GPVI-Fc$_2$ to immobilized collagenous substrates, suggesting that GPVI-Fc$_2$ cannot bind to them in solution. Data is presented as mean ± SEM obtained from 3 separate experiments with each data point determined in duplicate.

The work so far helped to establish that recombinant GPVI-dimer and not the monomer bound to fibrinogen and fibrin, through their D-domain and D-dimer, and that GPVI-dimer specific inhibition by mFAB-F was able to significantly reduce binding. Furthermore, a competition assay using collagenous substrates in solution with recombinant GPVI-dimer inhibited its binding to D-fragment and D-dimer. However, it was curious as to why fibrinogen substrates incubated with recombinant GPVI-dimer were not able to inhibit GPVI-dimer binding to collagen.

The ELISA assays described above are purified systems and serve to give us an indication of receptor-ligand interactions. However, these reactions in platelets are far more complex and can also be heterogenous. Various factors, including the activity of other receptors such as integrin αIIbβ3, availability of GPVI-dimer on the platelet surface, and signalling mechanisms via GPVI-dimer that may affect the affinity of GPVI-dimers to fibrinogen and fibrin. The next logical step, therefore, was to investigate how platelet GPVI interacted with fibrinogen and fibrin.

(d) D-dimer inhibits collagen induced platelet aggregation in solution

Aggregometry was used to investigate if fibrinogen or fibrin interacted with platelet GPVI in the same way as collagen. Washed platelets were stimulated by fibrinogen and its derivatives or by HORM and light transmission was recorded at 37°C under stirring, as described in the methods. The addition of fibrinogen (Fib-3; 5-20 μg/mL), D-fragment (5-10 μg/mL) or D-dimer (5-20 μg/mL), did not cause platelets to aggregate, but upon the addition of HORN collagen, platelet aggregation occurred (Figure 5.9A), consistent with the findings earlier that fibrinogen and its subdomains did not inhibit GPVI-Fc_2 binding to collagen. pFibrin was able to cause spontaneous platelet aggregation (Figure 5.9B). The pFibrin induced aggregation was not inhibited by mFAB-F (100 μg/mL), however, Eptifibatide (45 μM) completely abrogated it. This suggests that the platelets are likely to become slightly activated during the washing process, especially integrin αIIbβ3, but more importantly that platelet aggregation through pFibrin in suspension doesn't require GPVI-dimer.

An interesting finding was that the addition of D-dimer to the platelet suspension inhibited HORM–induced aggregation of washed platelets in a concentration dependent manner (Figure 5.9C; raw values displayed in Supplementary Table 5.1). In platelets pre-incubated with 100 µg/mL of mFAB-F, thereby inhibiting GPVI-dimer activity, the addition of 5- or 10 μg/mL of D-dimer concentrations to the platelet + mFAB-F suspension did not affect mFAB-F inhibition of HORM-induced platelet aggregation. Higher D-dimer concentration (20 μg/mL) however, relieves the inhibition (Figure 5.9D). This suggests that D-dimer may bind at a separate, but nearby site, perhaps producing a conformational change that may reduce the affinity of mFAB-F.

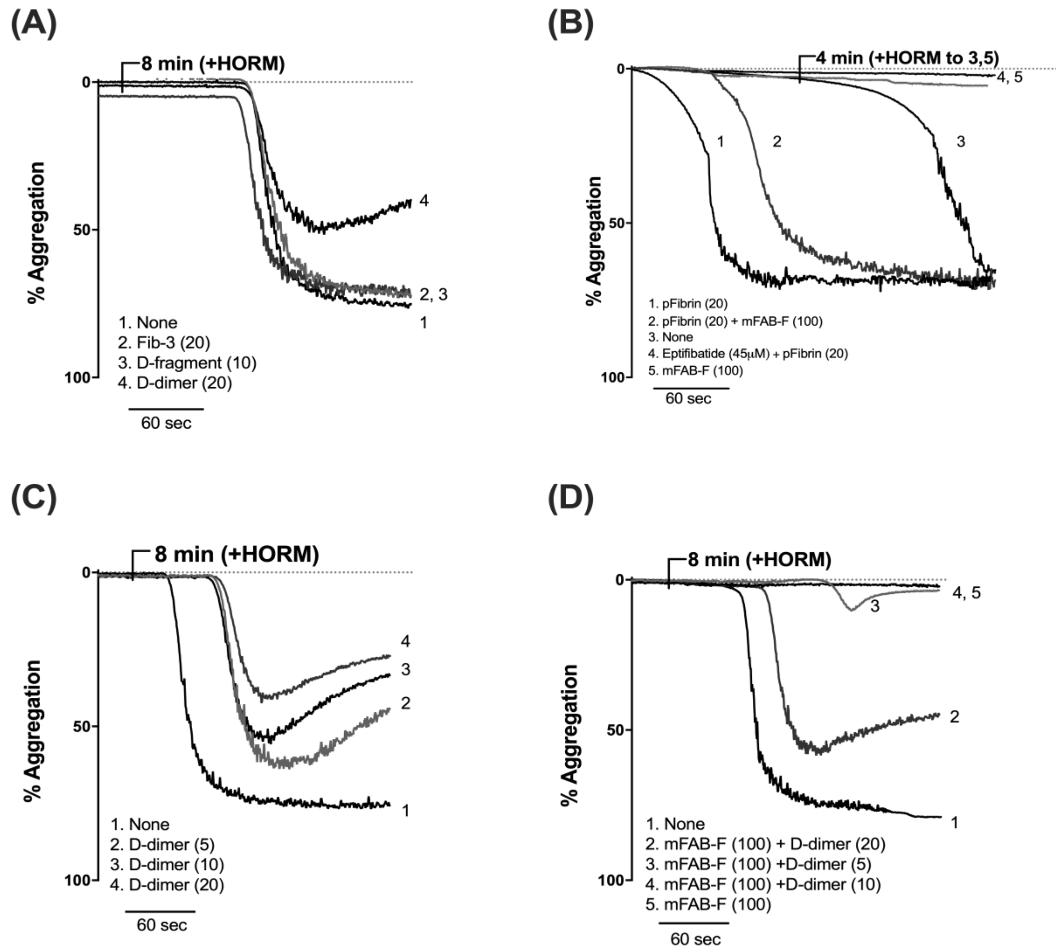


Figure 5.9 D-dimer is able to inhibit collagen induced platelet aggregation in a concentration dependent manner. Aggregometry of washed platelets ($2.5x10^8$ platelets/mL) either untreated (A-C) or incubated with mFAB-F (D; 100 μg/mL) was performed. At 3 minutes, D-fragment, D-dimer, Fib-3 or pFibrin was added to the platelets as indicated. If no aggregation occurred by 8 minutes, HORM (3 μg/mL) was added to induce aggregation and confirm platelet activity. (A) Fib-3, D-fragment and D-dimer (10–20 μg/mL) did not cause platelet aggregation. (B) pFibrin (20 μg/mL) was able to cause spontaneous platelet aggregation, prior to the addition of HORM. This was unaffected by mFAB-F (100 μg/mL) and abolished by Eptifibatide (45 μM). (C) D-dimer concentration-dependently inhibited HORM–induced aggregation of platelets. (D) Pre-incubation of platelets in mFAB-F prior to addition of D-dimer caused an inhibition of platelet aggregation at 20μg/mL of D-dimer only. The above are representative figures as each experiment (A-D) was repeated 3 times, using different donor blood each time.

(e) Platelet glycoprotein VI-dimer inhibition reduces thrombus formation on fibrin(ogen) under flow

To mimic the formation of a thrombus under venous or arterial shear, a flow chamber set-up was used as described in the methods. Glass coverslips were coated with the substrates (below) and left overnight.

- D-fragment 10 μg/mL
- D-dimer 10 μg/mL
- Fib-3 10 μg/mL
- pFibrin 10 μg/mL
- mFibrin 10 μg/mL
- Collagen III 10 μg/mL

For the initial experiments, DiOC6 fluorescently labelled, PPACK anticoagulated healthy volunteer blood was used, with either:

- Control No antibodies added
- Human Fab 100 μg/mL (control Fab)
- mFAB-F 100 μg/mL (GPVI-dimer inhibition)
- Eptifibatide 45μM (integrin αIIbβ3 inhibition; Tocris Biosciences, UK)
- Gi9 10 μg/mL (integrin α2β1 inhibition; GeneTex, UK)

Shear rates of 350s^{-1} to mimic venous shear or 1000 s^{-1} to mimic arterial shear were employed. Higher shear rates were not used due to the increased volume of blood, and also antibodies required to perform these experiments. Further information specific to the experiment can be found in the corresponding figure legends. Statistical significance between control/human-Fab vs. antibody inhibition was calculated using paired t-tests.

In general, platelets adhered to and formed thrombi on collagen, fibrinogen and mFibrin the best at 350 s^{-1}, with poorer platelet adhesion to both D-fragment and D-domain being observed. This may suggest that under shear, the full-length fibrinogen maybe required to capture platelets efficiently. Platelets also did not adhere to pFibrin very well; however,

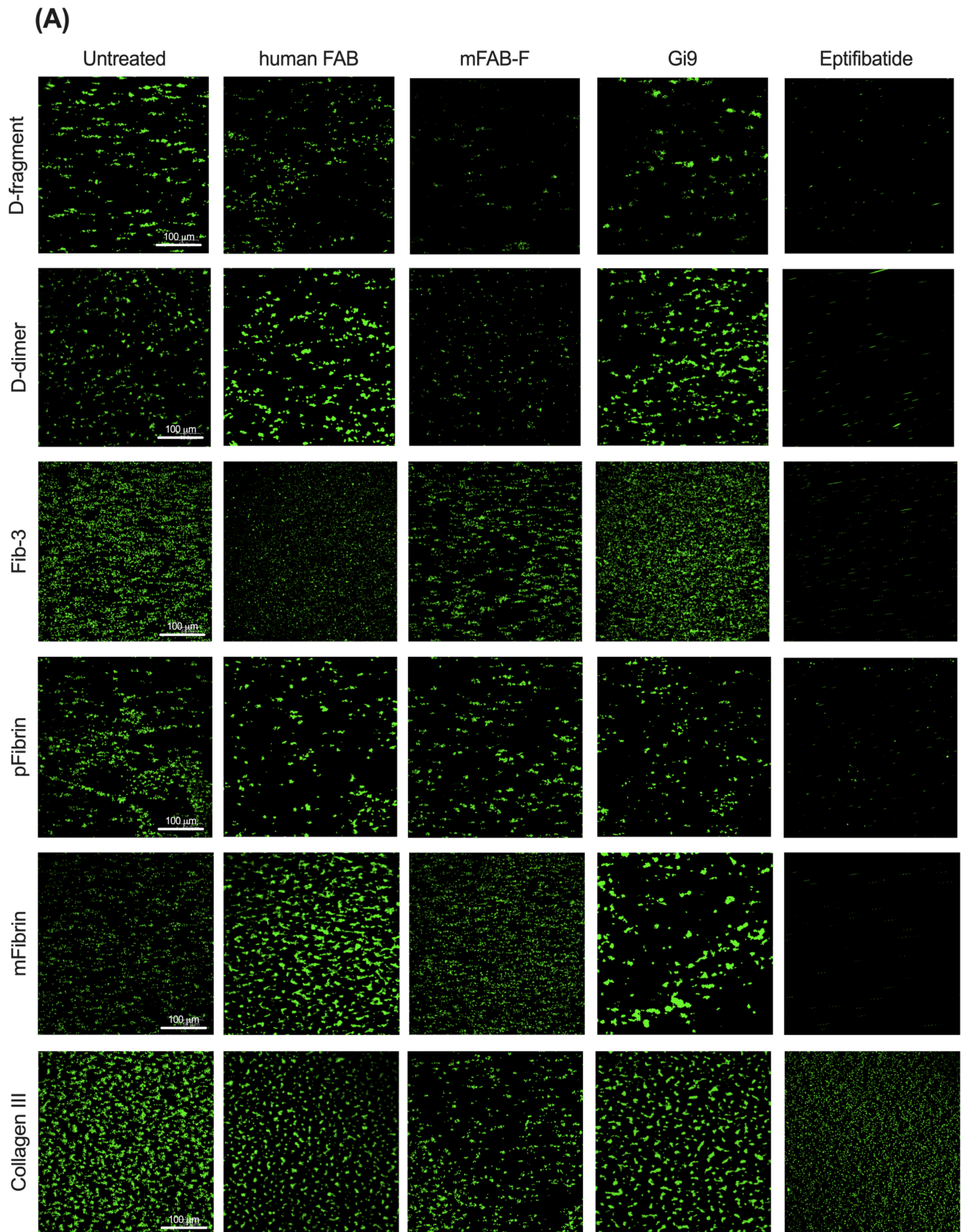
when they did adhere, they tended to make thrombi better than on D-fragment and D-dimer alone. As expected, the platelets formed the largest thrombi on collagen, their MTH were often twice as large any of the other fibrinogen substrates (Figure 5.10)

GPVI-dimer inhibition through mFAB-F significantly reduces SA of all fibrinogen substrates (D-fragment (*P*=0.0045), D-dimer (*P*=0.046), Fib-3 (*P* = 0.025), pFibrin (*P*=0.027), mFibrin (*P*=0.046)) and collagen III (*P* = 0.01) compared to the control. Although only significantly reduced on D-fragment (*P*=0.0018) and mFibrin (*P*=0.0029), MTH was non-significantly reduced on all coated substrates with GPVI-dimer inhibition (Figure 5.10: B&C).

The poor reduction in MTH maybe explained due to the fact that platelets poorly adhered to fibrinogen substrates with GPVI-dimer inhibition in the first place, and that this may precludes them from forming thrombi. This suggests that the primary role of GPVI-dimer with respect to a GPVI-fibrin interaction is to assist platelets in adhering to fibrin(ogen) surfaces. As expected, the greatest inhibition of MTH with mFAB-F was seen on collagen III (*P*= 0.0091) (Figure 5.10 B&C).

Inhibition of αIIbβ3 using Eptifibatide nullified platelet adhesion and thrombus formation on all fibrinogen surfaces (Figure 5.10). On collagen, platelets adhered well, using their functional GPVI-dimer and $\alpha 2\beta 1$, but were unable to form more than a platelet monolayer under shear (A, bottom right).

The inhibition of $\alpha 2\beta 1$ using Gi9 made no significant difference to SA or MTH on fibrinogen substrates or collagen (Figure 5.10: D&E). To ensure specificity, mFAB-F was tested against a concentration-matched human FAB preparation in healthy volunteer blood. mFAB-F demonstrated a reduction in SA and MTH on all tested substrates, with significant SA inhibition on fibrinogen (*P*=0.0061), pFibrin (*P*=0.0069), mFibrin (*P*=0.015), and collagen III (*P*=0.035). MTH was reduced on D-fragment (*P*=0.048), D-dimer (*P*=0.042), Fib-3 (*P*=0.031), pFibrin (*P*=0.030), mFibrin (*P*=0.020) and collagen III (*P*=0.0058) (Figure 5.10: D&E).


(A)
Untreated
human FAB
mFAB-F
Gi9
Eptifibatide
D-fragment
D-dimer
Fib-3
pFibrin
mFibrin
Collagen III
100 µm
100 µm
100 µm
100 µm
100 µm
100 µm

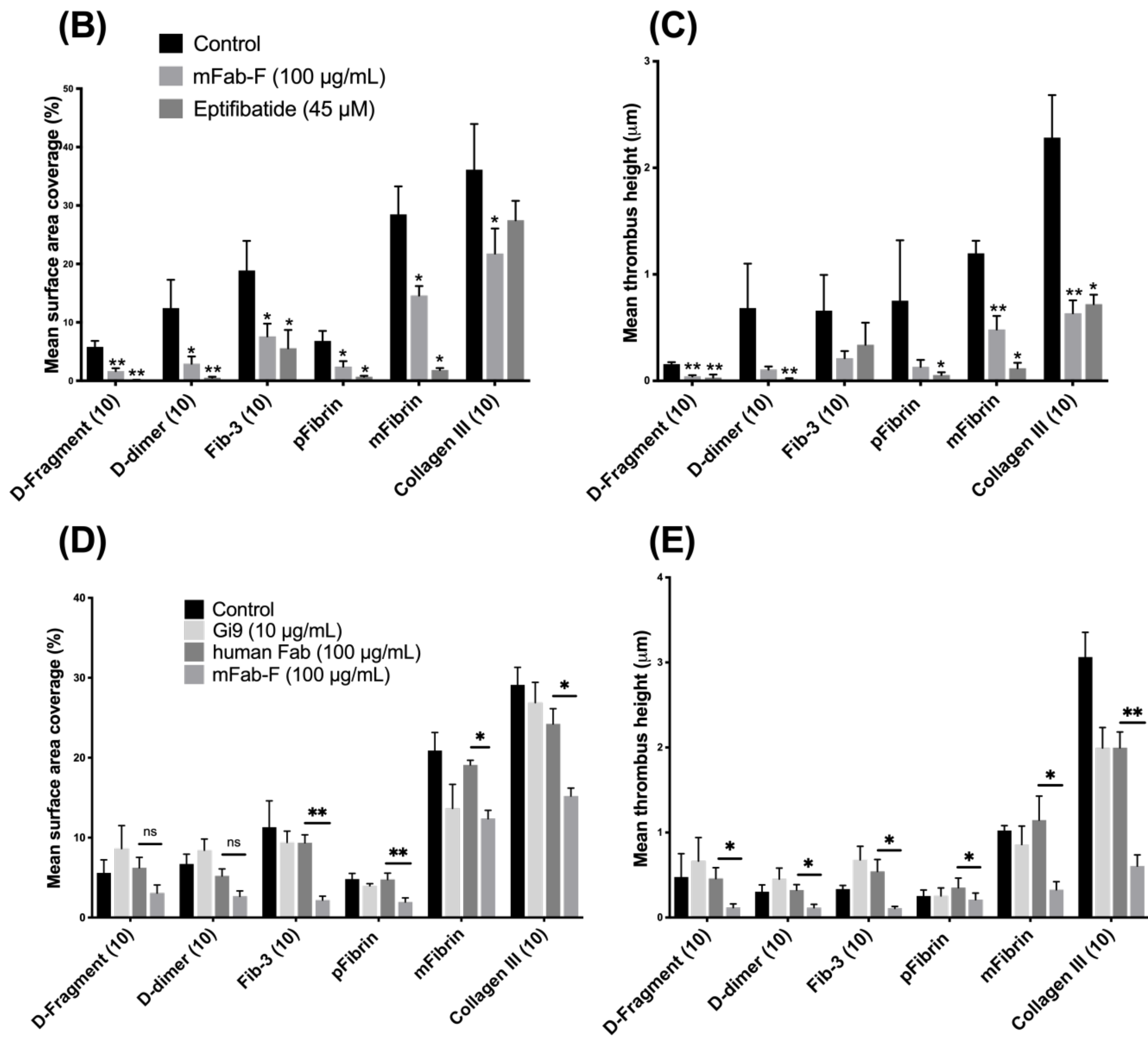
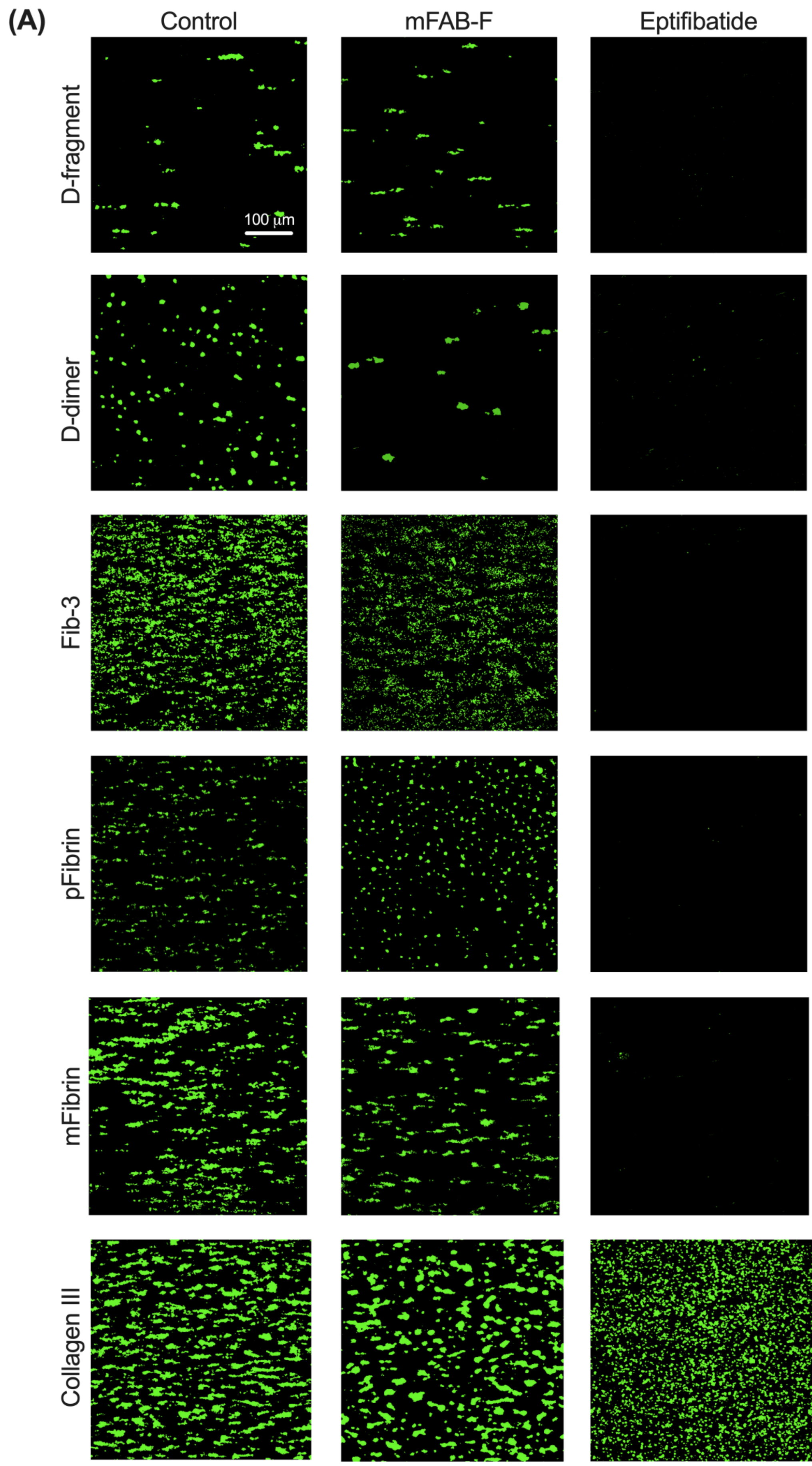


Figure 5.10 Quantification of platelet SA and MTH at 350 s^{-1}. mFAB-F significantly reduces thrombus formation on fibrinogen substrates. (A) The images in are representative of 6 flow experiments performed with blood from different healthy donors. (B +C) Inhibition of αIIbβ3 abrogated SA and MTH to all fibrinogen derivatives. mFAB-F reduced platelet adhesion (SA) to all 5 fibrinogen derivatives and significantly suppressed MTH on D-fragment, mFibrin and collagen compared to the control. (D+E) The inhibition of GPVI-dimer with mFAB-F compared to the effect of human Fab and Gi9 (anti-α2β1) under the same conditions. Compared to human Fab, mFAB-F significantly reduced SA and MTH on all tested substrates, except SA on D-fragment and D-dimer, which nevertheless tended towards significance. *P<0.05, **P<0.01 ***P<0.001).

In contrast to collagen-bound vWF binding to GPIbα at high-shear, the fibrinogen binding through platelet integrin αIIbβ3 occurs at low shear rates (Savage, 1996). This is crucial when considering what role GPVI-dimer may play in its interaction with fibrin. Having established that at shear rates of 350 s^{-1} GPVI-dimer played an important role in platelet attachment to fibrinogen substrates, it was important to establish whether this would translate to shear rates of 1000 s^{-1}.

The same coating conditions, as well as antibody concentrations were used. Fluorescently labelled, PPACK anticoagulated whole blood was perfused over fibrinogen substrates and collagen. Platelets tended to adhere less to all substrates, including collagen at this shear rate and even though thrombus formation is seen on fibrinogen, pFibrin, mFibrin and col III, platelets are less able to form large thrombi. The inhibition of αIIbβ3 again abrogates SA and MTH at this shear rate, with platelets not adhering to the coverslip plane at all. mFAB-F has a significant effect in reducing SA to D-dimer ($P = 0.053$), fibrinogen ($P=0.021$), pFibrin ($P=0.040$), and collagen ($P=0.020$), as well as reducing MTH on fibrinogen ($P=0.026$) and collagen ($P=0.0028$) (Figure 5.11 B&C). This suggests that GPVI-dimer forms less stable interactions with fibrinogen derivatives at higher shear rates and that αIIbβ3 plays the predominant role here.

When comparing the inhibitory effects of mFAB-F on SA and MTH between the two shear rates used (Table 5.1), it seems clear that GPVI-dimer plays its predominant role in platelet adhesion to fibrinogen and fibrin at low shear. At the higher shear rate, the effect of GPVI-dimer inhibition was most effective in reducing MTH, but this was more variable and harder to explain, as it was only significantly effective on fibrinogen.


(A)
Control
mFAB-F
Eptifibatide
D-fragment
100 µm
D-dimer
Fib-3
pFibrin
mFibrin
Collagen III

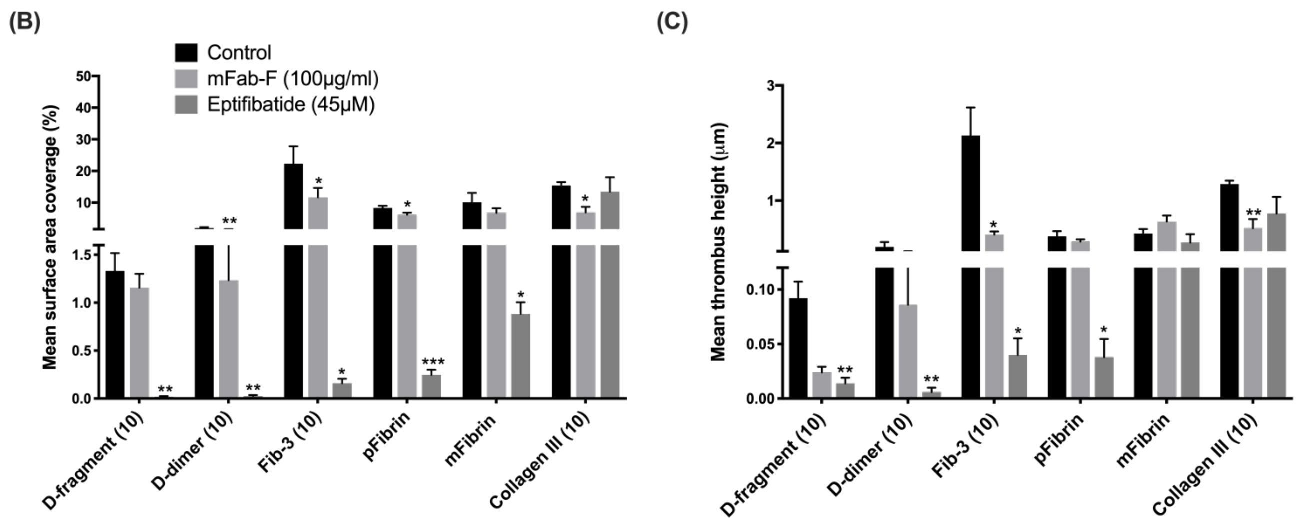


Figure 5.11 Quantification of platelet SA and MTH at 1000 s^{-1}. (A) The images are representative of 5 flow experiments performed with blood from different healthy donors. (B + C) At high shear, platelets struggle to attach to, and form thrombi on D-fragment and D-dimer (SA and MTH around 3-fold less compared to at 350 s^{-1}). Thrombus formation is seen on fibrinogen, pFibrin, mFibrin and col III. Eptifibatide abrogates SA and MTH, except on mFibrin and collagen even at 1000 s^{-1}. The effect of mFAB-F is more variable, with the most consistent inhibitory effect seen on fibrinogen and collagen. *$P<0.05$, **$P<0.01$ ***$P<0.001$

% reduction in SA or MTH with GPVI-dimer inhibition (mFAB-F; 100 μg/mL) compared to control (no inhibition)				
	SA 350 s^{-1}	SA 1000 s^{-1}	MTH 350 s^{-1}	MTH 1000 s^{-1}
D-fragment	76.2**	13.2	26.3**	60.9
D-dimer	86.5*	36.5**	15.6	56.6
Fib-3	59.8*	47.7*	68.8	80.7*
pFibrin	35.7*	26.0*	17.4	24.2
mFibrin	43.4*	33.2	50.8**	Increased
Collagen III	39.8**	55.5*	32.2**	59.5**

Table 5.12 Comparing surface area coverage (%) and mean thrombus height (μm) at low (350 s^{-1}) and high shear (1000 s^{-1}). The table shows the % reduction in surface area coverage (SA) or mean thrombus height (MTH) on all tested substrates with mFAB-F compared to the control. The higher reduction in SA or MTH achieved when comparing 350 s^{-1} and 1000 s^{-1} is highlighted in light grey. Statistical significance was calculated using paired t-tests and significance of inhibition compared to the control is denoted where applicable *P<0.05, **P<0.01 ***P<0.001.

Glanzmann's thrombasthenia (GT) is a bleeding disorder where patients have either no αIIbβ3 or non-functional αIIbβ3 on their platelet surface. GT platelets can adhere to exposed SEM and undergo shape change but the subsequent platelet spreading and thrombus formation is defective (Nurden, 2006).

Blood was obtained from 2 individuals with GT (patients A +B). The absence of integrin αIIbβ3 but not GPVI on the platelet surface was confirmed by flow cytometry using blood from patient A (Supplementary Figure 5.1)

Labelled whole blood was then perfused over surfaces of D-fragment, D-dimer, fibrinogen, pFibrin and mFibrin (100 μg/mL) and collagen (10 μg/mL). Whole blood was obtained from a healthy volunteer for control measurements. Thrombasthenic blood was either perfused with or without the addition of mFAB-F (100 μg/mL) at 350 s^{-1}.

GT platelets retain some ability to adhere on all fibrinogen substrates, albeit at dramatically reduced levels compared to normal platelets. Despite this, no thrombus formation is seen on any of the fibrinogen substrates. Adhesion to collagen, through functioning GPVI and α2β1, is within normal values, although only a platelet monolayer is seen, rather than large thrombi. This is once again due to lack of αIIbβ3 and the inability of platelets to properly aggregate. Therefore, the effect of adding mFAB-F to GT blood in terms of MTH cannot be assessed. However, SA appears to be further reduced when mFAB-F is added to GT blood. It is difficult to say with these results whether GPVI-dimer has an independent role in platelet adherence to fibrinogen and fibrin, but these results suggest that in the presence of functioning αIIbβ3, GPVI-dimer has a supportive and faciliatory role in GPVI-fibrin interactions (Figure 5.12).

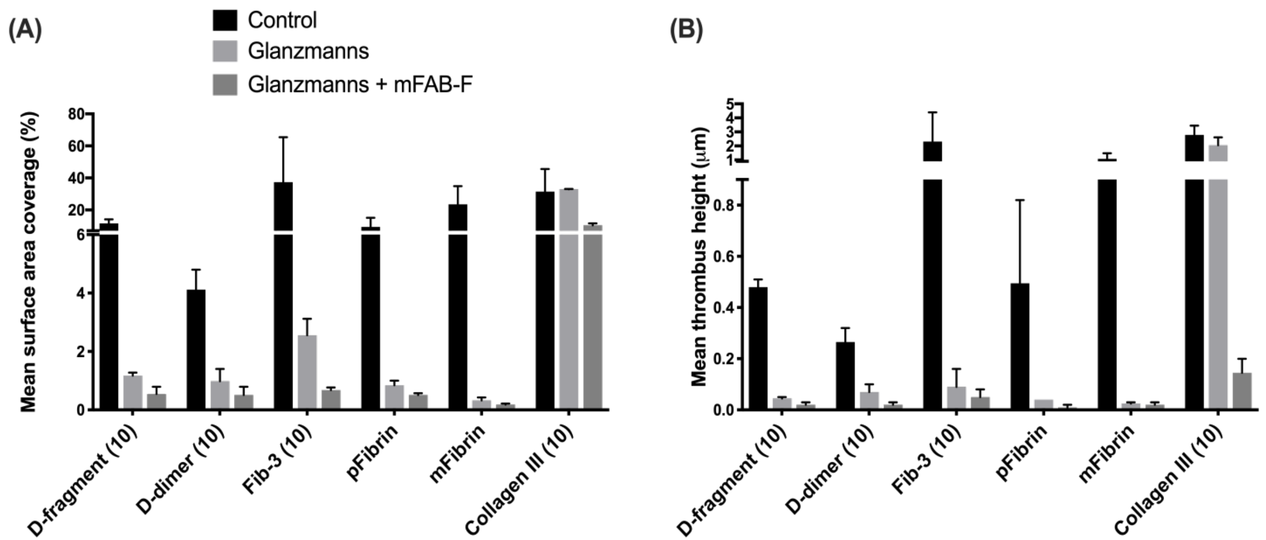


Figure 5.12 Analysis of Glanzmann's thrombasthenia platelets (n=2) under flow conditions. (A) GT platelets retain some ability to adhere on all fibrinogen substrates but only adhesion to collagen is within normal values. (B) There is no aggregate formation except a monolayer of platelets on collagen. mFAB-F appears to cause a reduction in platelet adherence to fibrinogen and collagen the most, with also a reduction of thrombus formation on collagen, as expected.

5.02 Discussion

The two seminal papers by Mammadova-Bach et al. and Alshehri et al. in 2015 opened up a new field of research on GPVI as a possible receptor for fibrin (Alshehri, 2015; Mammadova-Bach, 2015). At the time of carrying out the experiments detailed in chapters IV and V, very little was known about the GPVI-fibrin interaction. In fact, many in the field of platelet research did not believe the initial discoveries to be correct. This newly identified role for GPVI still remains a little contentious, but there is now a growing evidence base that recognises GPVI as a receptor for fibrin.

Mammadova-Bach's paper from the Jandrot-Perrus group in France was the first to describe that GPVI is a functional receptor for fibrin. They used thrombin assays on PRP from control and GPVI-deficient patients to demonstrate that tissue-factor-triggered thrombin generation was defective in the GPVI-deficient patients. Similarly, this effect was reduced by the anti-GPVI Fab 9012. In a static binding assay using recombinant GPVI-Fc, 9012 inhibited the interaction between GPVI-Fc and fibrin. Under flow, 9012 reduced platelet ability to adhere to fibrin at 300 s^{-1} and 1500 s^{-1} and finally, 9012 was able to decrease the recruitment of platelets to fibrin-rich clots by up to 85%.

Three months later in August 2015, Alshehri and colleagues at the Watson group in Birmingham published their work, recognising that fibrin can activate GPVI-signalling in human and mouse platelets. They first showed that human and mouse platelets could undergo tyrosine phosphorylation induced by fibrin in the absence of $\alpha IIb\beta 3$ (in mice) or presence of Eptifibatide (in mice and humans). The authors reported that the pattern of tyrosine phosphorylation was similar to that induced by the activation of GPVI, including the FcRγ-chain. Furthermore, they reported that the GPVI shed from the surface of platelets, which is presumed to be monomeric GPVI, binds to immobilised fibrin but not to fibrinogen. The spreading of platelets on fibrin was abolished in the absence of GPVI in humans and mice. Fibrin was also able to induce a threefold increase in the number of PS-exposing platelets which as inhibited in GPVI-deficient patients. Possibly of most interest was the theory that GPVI plays an important role in thrombus stability. Using an $FeCl_3$ injury model they showed a 6.6-fold increase in the rate of embolisation from formed

thrombi in GPVI-deficient mice compared to wild type mice. Taking the papers together, the authors had described that GPVI was able to bind to fibrin, induce signalling causing tyrosine phosphorylation, thrombin generation together with platelet PS exposure and support thrombus formation under flow.

The main results from this chapter are as follows:

1) Recombinant GPVI-dimer (GPVI-Fc_2), but not the monomer ($GPVI_{ex}$) binds to immobilised D-domain of fibrinogen and the D-dimer of fibrin.
2) Collagen substrates competitively inhibit the binding of GPVI-Fc_2 to immobilised D-fragment and D-dimer in ELISA, suggesting proximity of the binding sites.
3) GPVI-Fc_2 does not bind to, and platelets are not activated by, fibrinogen substrates in solution. However, D-dimer in a platelet suspension can inhibit HORM induced platelet aggregation in a concentration dependent manner.
4) GPVI-dimer specific inhibitory antibody mFAB-F is able to significantly reduce GPVI-Fc_2 binding to D-domain and D-dimer under static conditions, as well as platelet adhesion and thrombus formation on fibrinogen substrates under shear (350^{s-1} and 1000^{s-1}).

This work was the first to conclusively report that the active, collagen binding form of GPVI, the GPVI-dimer, also binds to fibrinogen and fibrin through the D-domain region. However, in our static adhesion assays, GPVI-dimer was not able to bind to coated fibrinogen and fibrin directly. To investigate this further we looked into whether this was due to poor binding of the larger proteins (fibrinogen and fibrin) when coated in wells (Supplementary Figure 5.2) As suspected, fibrinogen and fibrin adhered poorly to the wells. However, since they each have two D-domains per molecule, the availability of fmoles D-domains/mm^2 of well surface was similar. Therefore, we hypothesise that the reason we observed no binding of GPVI-dimer to fibrinogen and fibrin under our ELISA conditions could be due to the conformation the large fibrinogen and fibrin chains take up when immobilised in a well, where the possible D-domain binding sites are not available for recognition for GPVI-dimer (Zhang, 2017).

Mammadova-Bach and colleagues also did not note any binding to fibrinogen in a similar assay, although they noted binding to fibrin. To overcome this, when the plasmin cleaved fibrinogen and fibrin subdomains were coated, we observed specific and saturable binding of GPVI-dimer to D-fragment and D-dimer. GPVI-dimer did not bind to the central fibrinogen E-domain.

At the time of carrying out this work, one of the main questions we had was whether it was the dimeric, monomeric or both versions of GPVI that bound to fibrin – the answer to which would have had huge implications on future translational work. Our results show a clear preference of GPVI-dimer in binding to fibrinogen and fibrin, where the monomer showed no binding to fibrinogen substrates at all (we were not able to test $GPVI_{ex}$ against polymerised fibrin, however, it did not bind to D-dimer). Furthermore, mFAB-F, which is able to recognise constitutive dimers on the platelet surface inhibited binding of GPVI-Fc_2 and platelets to fibrinogen and fibrin, suggesting a key role for GPVI-dimer here.

As mentioned earlier, it remains controversial as to which form of GPVI, the monomer or the dimer binds to fibrin. Certainly, the GPVI-Fc construct used by Mammadova-Bach closely resembles our dimeric GPVI-Fc_2, with the exception of an amino acid linker sequence (Slater, 2019). The antibody they used, 9012 Fab, can however inhibit both monomeric and dimeric GPVI on platelets. Comparisons between the GPVI-ectodomain used by the Watson group and our $GPVI_{ex}$ are harder to make, especially as they have not characterised its binding to collagen. The physiological consequence of GPVI-monomer binding to fibrin is also unclear. It is likely that *if* the GPVI monomer is able to bind to fibrin as suggested by the Watson group, it would have to quickly dimerise/oligomerise in order to initiate the signalling required for platelet activation (Alshehri, 2015).

The ability of collagens in solution with GPVI-Fc_2 to inhibit its binding to coated D-fragment and D-dimer suggests that the collagen and D-domain binding sites on GPVI-dimer are proximate. This is further confirmed by the observation that soluble D-dimer is able to inhibit collagen-induced platelet aggregation in suspension.

A key finding from our work is that the immobilised nature of the fibrinogen substrates is important for platelet interactions through GPVI-dimer. Firstly, fibrinogen sub-domains in solution with GPVI-dimer cannot inhibit its binding to collagen. Secondly, aggregometry confirms that D-fragment, D-dimer and fibrinogen in solution do not cause platelet aggregation either and together these results suggest that GPVI-dimer cannot bind to fibrinogen, fibrin or their subdomains unless they are immobilised. Fibrin is able to induce platelet aggregation in suspension, but through a pathway that is independent of GPVI-dimer, presumably through αIIbβ3. This is crucial as platelet aggregation through binding to soluble fibrinogen does not occur in the circulation under normal conditions either, unless initial platelet activation has occurred, converting αIIbβ3 to its high-affinity state (Li, 2010).

Measuring the effect of GPVI on platelet adhesion and thrombus formation on fibrinogen substrates under flow is more complex than measuring direct binding using ELISA alone, especially as it involves a role for αIIbβ3 at shear. GPVI-dimer inhibition using mFAB-F demonstrated that at low shear, GPVI-dimer inhibition significantly reduced platelet adhesion to all immobilised fibrinogen substrates. At higher shear, this effect was more varied, suggesting that GPVI-dimer exerts most of its effects on binding to fibrinogen and fibrin at low shear. It is clear that integrin αIIbβ3 remains the most important receptor in platelet interactions with fibrinogen and fibrin. Its inhibition lead to completer abrogation of platelet adhesion and thrombus formation at both shear rates, particularly at high shear. Integrin α2β1 does not play an important role in platelet-fibrin adhesion, as it does with collagen.

Integrin αIIbβ3 has always been recognised as the main receptor involved in platelet aggregation and thrombus growth. However, an interesting observation was that even with GPVI-dimer inhibition alone, the ability of platelets to form large thrombi was compromised, despite the presence of αIIbβ3. A question that is then raised is whether GPVI-dimer inhibition leads to the reduction of signalling that could lead to platelet activation and whether GPVI-dimer alone, or in conjunction with αIIbβ3 is necessary for this.

To explore this further, firstly we quantified thrombus formation under flow using GT platelets, with or without GPVI-dimer inhibition. Under flow, GT platelets were able to support binding to fibrinogen and fibrin, but these adhered platelets did not appear spread nor activated, suggesting that signalling may not occur in the absence of αIIbβ3. GT platelets with additional GPVI-dimer inhibition adhered to fibrinogen substrates less than the GT platelets alone, suggesting that GPVI-dimer has a weak but independent role in platelet adhesion to fibrinogen substrates but cannot activate them without the presence of functioning αIIbβ3.

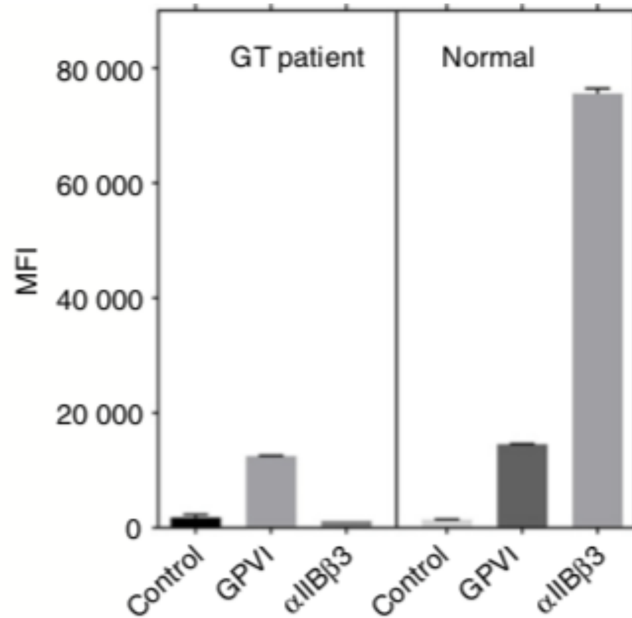
Previous studies have indicated that there are convergent tyrosine kinase signalling pathways downstream from GPVI and αIIbβ3. For example, the cross-linking of GPVI induces Src-dependent tyrosine phosphorylation of FcRγ (Gibbins, 1997). Similarly, signalling mediated by αIIbβ3 also includes tyrosine kinases such as Syk and Src (Clark, 1994; Gao, 1997), with specific blockade reducing the tyrosine phosphorylation of signalling proteins downstream from Syk in both the GPVI- and αIIbβ3-dependent signalling pathways (Spalton, 2009). Our results demonstrate that the Src kinase inhibitor, Dasatinib, and mFAB-F each exert a similar inhibitory effect on platelet adhesion and spreading on fibrinogen substrates, indicating platelet-adhesion-induced signalling through GPVI-dimer is an important step in platelet spreading and activation on fibrinogen and fibrin (Supplementary Figure 5.3)

To summarise, these results suggest that it is the GPVI-dimer that recognises immobilised fibrinogen and fibrin through their D-domain and D-dimer, respectively. Integrin αIIbβ3 remains the most important platelet receptor with respect to fibrin(ogen) interactions. GPVI-dimer exerts its main effects on platelet adhesion to fibrinogen and fibrin and may have a weak but independent role here. It is also likely that αIIbβ3 and GPVI-dimer have collaborative roles in platelet activation when binding to fibrinogen and fibrin, demonstrated by platelets treated with both mFAB-F and Hip8 showing a further decrease in adhesion to fibrinogen substrates compared with Hip8 (integrin αIIbβ3 inhibitor) alone (Supplementary Figure 5.3). The exact nature of this collaboration is yet to be determined,

but GPVI-dimer is likely to assist $\alpha IIb\beta 3$ in platelets binding to immobilised fibrinogen on the surface of other platelets, as well as increasing the stability of platelets already adhered to fibrin within a clot. Whether GPVI therefore has an effect in making clots less permeable and more resistant to lysis, which would have an impact on the effectiveness of acute stroke treatments such as rtPA needs to be established.

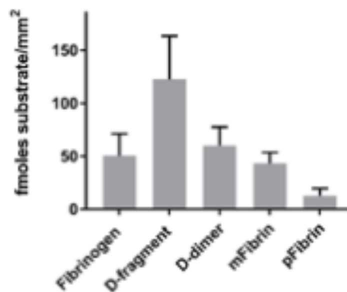
GPVI-dimer clearly plays a key part in both initiation of thrombosis at sites of atherosclerotic plaque rupture through collagen and then the propagation and stability of these thrombi through binding to fibrin. However, what is tantalising is that our findings also intimate a role for GPVI-dimer in conditions where blood clots form without prior collagen exposure, including cardioembolic thrombi in AF, and other thromboembolic conditions such as DVT. Since this work was completed, the active research and evidence base on the GPVI-fibrin interaction has increased. A further discussion on this topic as well as how it relates to thrombus formation in ischaemic stroke can be found in chapter VIII.

5.03 Supplementary files



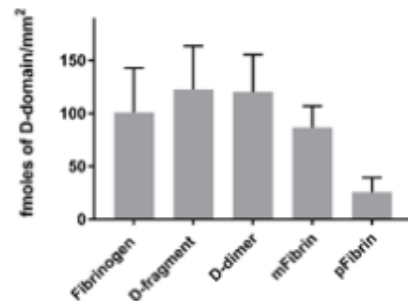
Supplementary Figure 5.1 Flow cytometry analysis verified that the GT platelets from patient A contain no integrin αIIbβ3 but had normal levels of GPVI. Samples were measured in an Accuri C6 flow cytometer. The normal platelets had appropriate levels of αIIbβ3 and GPVI. The GT platelets show have no αIIbβ3 but normal levels of GPVI. This work was done by Dr M Moroi. MFI = Median fluorescent intensity.

fmoles immobilized protein per mm^2 of well surface (bottom)



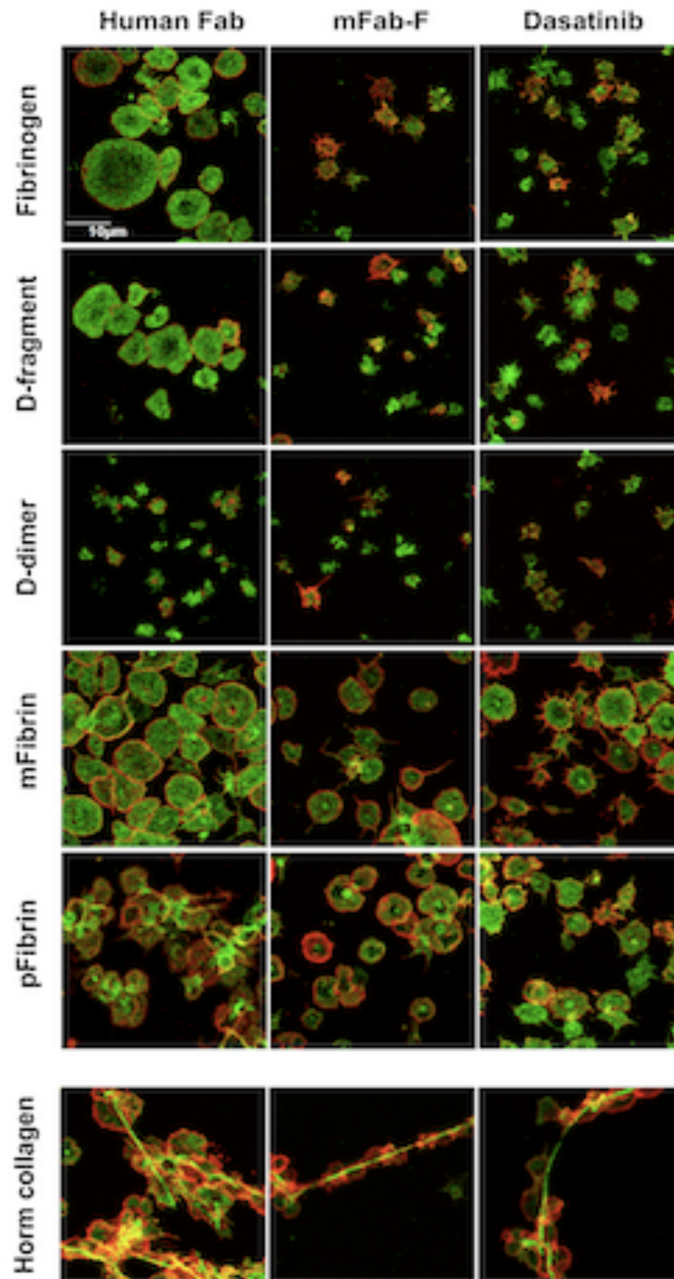
Well diameter = 6.5 mm, area of bottom = 33.18 mm^2

fmoles of D-domain per mm^2 of well surface (bottom)



Fibrinogen, D-dimer, mFibrin, and pFibrin contain 2 D-domains

Supplementary Figure 5.2 Analysis of fibrinogen, D-fragment, D-dimer and fibrin adherence to plates. Nunc fluorescence black 96-well glass-bottomed plates were coated with a fibrinogen substrate (5, 50 µL/well of 10, 50, 100 µg/mL), 3 wells each, and left overnight at 4ºC. The substrate was discarded, and the wells washed 4 times (200 µL PBS, 5 min, each wash). A 25 µL aliquot of 1%/8M urea was added to each well, and after 30 min, protein was assayed by using the Pierce™ BCA Protein Assay Kit. Experiment performed by Dr S Jung.



Supplementary Figure 5.3 Confocal images of washed platelets adhered to fibrinogen derivatives and HORM collagen and the effect of inhibitors. Washed platelets suspended in HT buffer were pre-treated with human Fab (control, 200 mg/mL), inhibitory anti-GPVI-dimer mFAB-F (200 mg/mL) or the Src inhibitor Dasatinib (50 nM) for 10 min and then were allowed to adhere to fibrinogen, fibrinogen derivative or HORM collagen immobilized on MatTek glass dishes, followed by fixation and staining with fluorescently labelled antibody. Images show platelets stained with Alexa-fluor 488–1G5 (green; pan

GPVI) and Alexa-fluor 647–anti-P-selectin (red) to measure platelet activation. Images were obtained with a 60x oil immersion objective on an Olympus confocal microscope. As shown in the left-most column of images, platelets spread well on fibrinogen, D-fragment, mFibrin and HORM collagen, with evident surface expression of P-selectin. Platelet aggregate formation was seen on pFibrin, whereas platelets spread but did not appear aggregated on mFibrin. HORM collagen supported aggregate formation, with the characteristic binding of GPVI along the HORM fibres. In contrast, platelets did not spread well on D-dimer, with only some platelets showing P-selectin expression. Treatment with mFAB-F (middle column of images) prevented platelet spreading on fibrinogen, D-fragment and HORM collagen, and decreased the extent of spreading, with evident filopodia observed, on mFibrin and pFibrin, whereas there was little change in the less-spread platelets on D-dimer. mFAB-F prevented aggregate formation on both pFibrin and HORM collagen, consistent with inhibition of activation through GPVI. Dasatinib (A, right-most column of images) shows effects similar to those of mFAB-F. Experiments carried out by Dr S Jung.

Seconds	None	D-dimer (5)	D-dimer (10)	D-dimer (20)
500	1	1.3	1.5	1.3
515	14.8	1	1.5	1.4
530	44.3	0.9	1.2	1.5
545	59.8	0.9	1.5	1.5
560	67.8	0.9	1.6	1
575	70.2	3.8	7.4	2
590	70.8	22.8	28	9.9
605	69.9	42.8	43.3	24.7
620	74.3	52.6	51.2	35
635	74.2	57.8	52.4	39.3
650	73.5	61.5	55.2	41
665	74.7	62.7	52.2	39.7
680	74.8	60.3	49.9	38.5
695	76	60.2	47.6	36
710	75.2	60.4	45.4	34.5
725	75.1	56.7	43	33.2
740	76.3	54.2	40.7	31.9
755	75.4	53.9	38.8	30.4
770	74.9	50.6	37.5	30.1

Supplementary Table 5.1 Platelets were either pre-incubated with D-dimer (dose indicated in parentheses) or not (none). HORM collagen was added at 8 minutes (480 seconds) to each of the wells. This table displays the raw values of % aggregation for each of the parameters. The values are shown from 500 seconds onwards, at 15 second intervals as there was no aggregation prior to the addition of HORM.

CHAPTER VI. FIBRIN BINDING PLATELET RECEPTOR GLYCOPROTEIN VI-DIMER IS SIGNIFICANTLY OVEREXPRESSED IN PATIENTS WITH ATRIAL FIBRILLATION

The **G**lycoprotein VI **R**eceptor in **A**trial **FI**brillation and **T**hrombo**E**mbolism *(GRAFITE)* Study

Chapter Summary

Patients with AF express significantly lower total GPVI but significantly higher levels of GPVI-dimer on their platelet surface compared to controls without AF. They also have more active circulating platelets as measured by P-selectin exposure compared to controls. pAF patients express significantly lower total GPVI expression, but similar GPVI-dimer expression compared to those in permanent AF. Those taking anticoagulation express more total GPVI and GPVI-dimer, which could be due to factor Xa inhibitors downregulating GPVI shedding from the platelet surface. Serum fibrinogen, BNP and hs-CRP are all correlated with higher GPVI expression, and the presence of congestive cardiac failure was associated with total GPVI expression. In the whole population, higher GPVI-dimer expression was independently associated with the presence of AF.

6.01 Introduction

From in vitro models and animal studies, it can be established that GPVI-dimer is likely to play a key role in the pathogenesis of ischaemic stroke, through its interactions with collagen and fibrin. However, there are only a small number of clinical studies investigating GPVI expression in thrombotic disease, and even fewer looking specifically at ischaemic stroke. These studies are further limited as they have either quantified platelet surface expression of total GPVI (monomer and dimer) or soluble GPVI (sGPVI; the extracellular portion of GPVI that is shed after platelet activation).

Since GPVI-dimer, the active form of GPVI, is constitutively present on circulating platelets and its expression increases with platelet activation and platelet-ligand interactions, it is imperative to establish how GPVI-dimer levels vary in response to stroke risk factors or thrombotic disease.

Individuals with AF represent a cohort that are at high-risk of stroke and previous studies have shown that the whole coagulation system demonstrates a hypercoagulable state, with platelet priming, ready to be involved in thrombosis (Hayashi, 2011; Danese, 2014). What is not established is whether this priming also means that they express more GPVI, especially more GPVI-dimer, and how this in turn may relate to their platelet function with respect to thrombus formation and CES.

The aim of the *Glycoprotein VI Receptor in Atrial FIbrillation and ThromboEmbolism (GRAFITE) study* was to investigate platelet GPVI-dimer expression and platelet activity in patients with AF who have not had a stroke using flow cytometry analysis of venous whole blood and compare it to healthy controls who do not have AF, who had their platelets analysed for the same parameters.

(a) Rationale for flow cytometry assessment of AF patients' platelets

Whole blood flow cytometry is now a commonly used method to measure different modalities of platelet activation and receptor expression. It has several advantages:

1. Physiological platelet activation can be studied in presence of the necessary clotting factors present in whole blood
2. There is minimal manipulation of the samples, preventing in vitro platelet activation
3. Only small volumes of blood are required for analysis, but the method has a high sensitivity for the detection of platelet sub-populations

Dr Jung and Dr Moroi have developed a flow cytometry method with appropriate antibodies to separately assess the levels of GPVI-dimer and total GPVI in platelet populations. Indeed, their 204-11 Fab is the only currently available antibody that binds to GPVI-dimers in resting and activated platelets; it's characteristics have been thoroughly tested in previous in vitro work (Jung, 2012). We compared dimer levels with those of total GPVI measured by the antibody HY101, which recognises both GPVI monomers and dimers (Chen, 2002).

P-selectin exposure on the platelet surface and fibrinogen binding represent two commonly used methods of quantifying platelet activation. Fibrinogen binding to platelets necessitates the conversion of integrin αIIbβ3 to its high-affinity state and requires lower concentrations of activating stimuli compared to platelet P-selectin secretion, representing an early phase of platelet activation. P-selectin exposure requires repeated stimuli and platelet degranulation (α-granules), reflecting a later-phase of platelet activation (Ruf, 1995).

In this study, to determine if patients with AF have more active platelets, both fibrinogen binding and P-selectin exposure in non-activated "resting platelets" or after exogenous stimulation by ADP or the CRP-XL was quantified. These agonists activate platelets through distinct and separate activation pathways. CRP-XL binds specifically to GPVI to initiate an

activation cascade (Smethurst, 2007) and ADP activates platelets through purinergic receptors P2γ1 and P2γ12 (Li, 2010).

Flow cytometry compatible antibodies to detect platelet P-selectin exposure and fibrinogen binding are commercially available. Although there are a lack of studies measuring platelet-fibrinogen binding in AF patients, elevated serum fibrinogen levels have been observed in patients with both paroxysmal (pAF) and persistent AF (Lip, 1996; Mukamal, 2006; Fu, 2011). Increased platelet exposure of P-selectin has been previously observed in both AF and ischaemic stroke (Goette, 2000; Marquardt, 2002), representing a validated marker to ensure the robustness of our results.

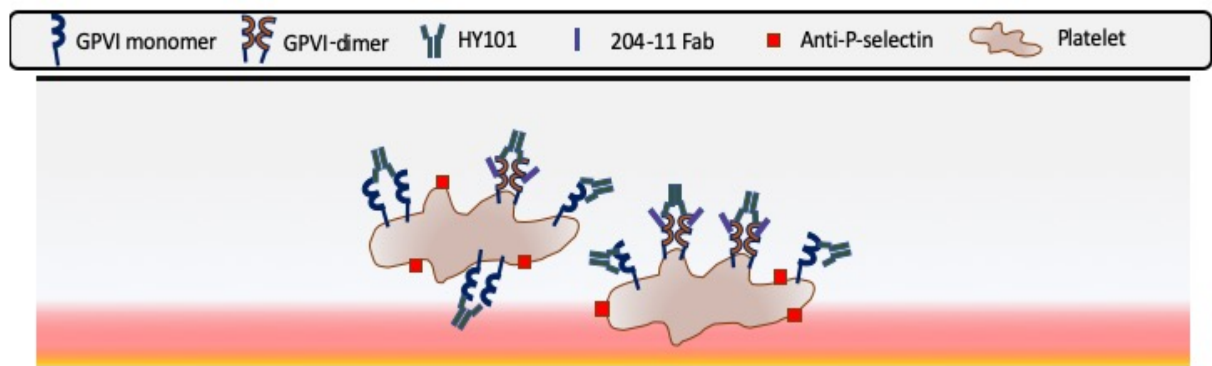


Figure 6.1 Schematic demonstrating the binding of HY101, 204-11 Fab and anti-P-selectin (CD62P) on platelets. HY101 as a whole antibody would bind to GPVI monomer and dimer through its two Fab binding regions. Each dimer would bind two molecules of 204-11 Fab. The exact binding sites of HY101 and 204-11 Fab are not known.

(b) Rationale for serum analysis of AF and stroke biomarkers

BNP, D-dimer and hs-CRP represent commonly measured AF and stroke biomarkers. BNP is a validated biomarker in cardiac failure and myocardial infarction. Because it is released from a pressure loaded left atrium, it is a measure of atrial stretch and atrial dysfunction during AF as well (Wożakowska-Kapłon, 2004). Studies have shown an association between AF and BNP with persistently higher plasma levels than in healthy matched controls and a reduction to that of control subjects following successful restoration of sinus rhythm (Wozakowska-Kapłon, 2010).

Similarly, published data on D-dimer levels in AF demonstrate that elevated D-dimer values may be associated with the presence of atrial thrombosis, correlated with infarct volume and may also represent a useful parameter for assessing the degree of hypercoagulability of AF patients (Danese, 2014).

Elevated serum levels of C-RP and hs-CRP has been shown to predict the presence of a prothrombotic state in AF (Lip, 2007), the recurrence of AF after cardioversion (Yo, 2014) and risk of CES (Dawood, 2016) and represents a close link between AF and a pro-inflammatory state.

Whether there is a link between GPVI levels, either total or specifically GPVI-dimer, and these serum biomarkers in AF has never been established.

6.02 *GRAFITE* study aims

1) Identify whether patients with AF who have not had a stroke express higher levels of GPVI (total or dimer) on their platelet surface compared to healthy controls without AF
2) Investigate whether these patients have more active platelets as demonstrated by increased fibrinogen binding and P-selectin exposure compared to healthy controls
3) Identify if there are any differences in platelet GPVI expression and platelet activity considering:
    a) Type of AF: Paroxysmal vs Persistent AF
    b) Whether on anticoagulation treatment or not
    c) Duration of AF
4) Identify correlations between GPVI expression and levels of serum AF biomarkers

6.03 *GRAFITE* study approvals and support

The study was conducted at Cambridge University Hospitals NHS Foundation Trust (CUH) between December 2016 and March 2019. The study protocol was approved by the Cambridge East Research Ethics Committee (16/EE/0436) and was registered in the ISRCTN registry (ISRCTN33370579).

6.04 Participant eligibility to recruitment

(a) Inclusion and exclusion criteria

CUH admits around 1300 patients a month under general medicine via the emergency department. **Patients with atrial fibrillation who are admitted under a medical team with AF as a main diagnosis or a comorbidity were eligible to participate in the study.**

Patients with a new onset of AF would be diagnosed by 12-lead ECG which is routinely carried out for every patient whilst they are in the emergency department. Paroxysmal AF (pAF) carries the same risk of stroke as persistent AF and therefore, those patients were eligible for participation in the study if the patient was documented to be in AF at the time of recruitment. Patients with a confirmed TIA or stroke in the last 10 years were excluded

from the study. The recruitment to the study included a number of other strict inclusion and exclusion criteria indicated in Table 6.1.

Inclusion	Exclusion
Able to give informed consent	No clear confirmation of the presence of atrial fibrillation in medical notes or ECG
Presence of AF on ECG either as a new or existing diagnosis	Not admitted under a medical team to CUH
Paroxysmal or persistent AF (as ascertained by medical history)	Age less than 18 years
On anticoagulation (warfarin or DOACs) or not on anticoagulation	Pregnancy
	Active or previous history of malignancy
	Known platelet disorder
	Haemoglobin <90g/L at the time of blood sampling
	Known HIV/AIDS
	Known hepatitis B or hepatitis C infection
	TIA or stroke – ischaemic or haemorrhagic, within the last 10 years and no active myocardial infarction.

Table 6.1 Inclusion and exclusion criteria for the *GRAFITE* study population

(b) Sample size

From the existing data we had at the time of *GRAFITE* study design, the median fluorescence intensity (MFI) of the GPVI dimer in patients presenting with stroke was (MFI= 0.6±0.15) compared to healthy control (MFI =0.5±0.14). Power calculations using the following study parameters: α 0.05, 1-β0.8, suggested that we can resolve differences in GPVI dimer MFI between two donor populations (P<0.05) using a sample size of less than 20.

(c) Patient recruitment

The details of patients admitted from the previous day's medical take are discussed at morning report, a meeting that is routinely attended by members of the research team, as well as all medical teams within CUH. Patient comorbidities including the presence of AF, are discussed at this meeting and was the main source of identification of patients during the study. The list of medical patients admitted was also available on the CUH electronic patient medical record database.

The direct care team approached the patient on behalf of the research team and ask their consent for the research team to see if they fit the inclusion criteria, and if the individual would be willing to consider participation. The research team then approached the individual in order to provide more information about the study and allow time to consider participation. Patients were typically recruited from medical inpatient wards, which included a mixture of acute medical, cardiology and geriatric inpatients. Participants were consented to provide a venous blood sample to carry out study investigations (Figure 6.2).

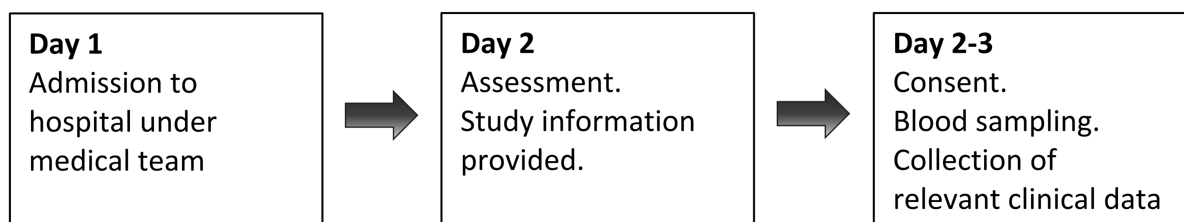


Figure 6.2 Flow diagram of patient approach to recruitment. Assessment by the research team typically took place on day 2 of admission. Consenting and blood sampling was

carried out after the study information was provided to the patient, – typically 24h later, when they had had a chance to consider participation.

(d) Healthy control recruitment

Healthy donors from the National Institute for Health Research Cambridge BioResource were invited to participate in the study alongside study participants. The NIHR Cambridge BioResource is a pool of over 15000 volunteers, both with and without health conditions who are willing to be approached for research. For the purposes of these studies, only those without previous thrombotic disease (IHD or stroke) and those not on antiplatelets were approached. No age limits were set. Potential donors attended an appointment where written consent was obtained for participation. The study protocol was given ethics approval by the Cambridge East Ethics Committee (ref 10/H0304/65).

Blood samples were drawn from the antecubital fossa vein using a 21-gauge butterfly needle. The first tube drawn was a K2 EDTA Vacutainer® (BD, NJ) with tourniquet applied that was solely used for a full blood count measurement. Blood was then drawn into a 0.109mol/L sodium citrate Vacuette® (Greiner, Kremsmünster, Austria) with the tourniquet removed to prevent artefactual platelet activation for functional assays. Samples were transferred to the laboratory on-site for immediate analysis.

Controls with a history of pAF, permanent AF or stroke were excluded from the analysis for the purposes of this study.

The following data were collected from the control cohort.

1. Basic demographic variables
2. Medication history: anti-hypertensive, statin, antiplatelet and anticoagulation
3. Clinical details: relevant vascular risk factors: hypertension, CCF, diabetes, hypercholesterolaemia, IHD, any previous stroke history.

6.05 Study investigations

(a) Platelet GPVI expression, function & haematological investigations

Blood was obtained via venepuncture from patients who were willing to participate in the study as well as from the healthy control population.

Typically, between 25-30 mL of blood was collected into S-monovette® tubes (Sarstedt, Germany) for the investigations outlined below. All blood samples were taken on a general medical ward at CUH and taken to either the Cell Function Laboratory, Ouwehand group at the Department of Haematology, University of Cambridge for GPVI quantification and platelet function testing, or the Department of Clinical Biochemistry, CUH for serum biomarker analysis within 20 minutes of venepuncture.

S-monovette® 3 mL, 0.106 mol/L citrate tube

1. Platelet total GPVI (monomer and dimer) expression and GPVI-dimer expression
2. Platelet function:
   a) P-selectin exposurewithout agonist or with of ADP or CRP-XL agonism
   b) Fibrinogen binding in platelets without added agonist or in platelets induced with ADP or CRP-XL.

S-monovette® 1.6 mL, 1.6 mg/mL potassium-EDTA tube

1. Full blood count

S-monovette® 3 mL, 0.106 mol/L citrate tube

2 x S-monovette® 4 mL, 1.6 mg/mL Serum Gel with Clotting Activator

1. International Normalised Ratio (INR)
2. D-dimer
3. Fibrinogen
4. Brain Natriuretic Peptide (BNP)
5. Hs-CRP

Serum biomarker analysis carried out by Department of Clinical Biochemistry, CUH.

S-monovette® 3.4 mL, 1.6 mg/mL potassium-EDTA tube

S-monovette® 4.9 mL, 1.6 mg/mL potassium-EDTA tube

S-monovette® 4 mL, 1.6 mg/mL Serum Gel with Clotting Activator

1. Genotyping, gene expression
2. Metabolomic analysis

Done in collaboration with the Ouwehand Laboratory, Department of Haematology, University of Cambridge. Results not yet available, therefore, will not form part of this thesis.

(b) Clinical data collection

After consent was obtained from the patient, further details were gained from the patient's electronic medical notes including:

1. Basic demographic variables
2. Medication history: anti-hypertensive, statin, antiplatelet and anticoagulation
3. Clinical details: admission diagnosis, relevant vascular risk factors: hypertension, CCF, diabetes, hypercholesterolaemia, ischemic heart disease, any previous stroke history and AF type and duration if known.
4. CHA_2DS_2-VASc score
5. Modified Rankin Scale (mRS) at admission

6.06 Methods

(a) Total GPVI and GPVI-dimer quantification using flow cytometry

Citrated whole blood was diluted at a 1:5 ratio with HEPES-Buffered Saline (HBS: 0.15 M NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM HEPES, pH 7.4) (Sigma-Aldrich Ltd, UK).

The relevant primary antibody was added:

1. Either anti-total GPVI HY101, 12.5 µg/mL (IBGRL, UK)
2. Anti-GPVI-dimer 204-11 Fab, 5 µg/mL (developed by Dr M Moroi and Dr S Jung, in collaboration with Kaketsu-ken, Japan);
3. IgG2a negative control, 12.5 µg/mL (BioLegend, UK) or

4. Mouse Fab negative dimer control, 5 µg/mL (BioLegend)

and incubated at room temperature for 10 min.

The fluorescent secondary antibody FITC-F(ab')$_2$ (Jackson Laboratory, USA) was added at 40 µg/mL and incubated in the dark for 10 min. The sample was subsequently diluted in 0.5 mL HBS prior to flow cytometric analysis using a FC500 flow cytometer (Beckman-Coulter Ltd., High Wycombe, UK). Platelets were identified using light scatter and results recorded as Median Fluorescent Intensity (MFI).

(b) Platelet function analysis using flow cytometry

To assess platelet activation, 5 µl of aspirinated (100 µM) and hirudinized (4 U/ml) citrated whole blood was added to 45 µl of HBS (after 5 minutes incubation) to make up final volume of 50 µL containing PE-conjugated anti-CD62P (Clone Thromb6, Bristol Institute for Transfusion Science, UK) at a final dilution of 1:50 and with FITC-conjugated anti-fibrinogen (F0111; Dako Ltd, UK) and either
no agonist

1. HBS

or addition of exogenous agonists

1. ADP (final concentration of 0.5 µM, Sigma-Aldrich) or
2. CRP-XL (final concentration of 4 µg/ml; Farndale Laboratory, University of Cambridge).

For the CRP-XL–induced platelets, apyrase (4 U/mL, final concentration) was added to inhibit activation through the ADP-induced pathway (Sigma-Aldrich) before addition of CRP-XL.

All activation reactions were stopped after 20 minutes by adding 100-fold volume of saline containing 0.2% formyl (37% formaldehyde, 0.85% NaCl; Sigma-Aldrich) (further 10-minute incubation) prior to flow cytometry analysis. Negative controls for the anti-P-selectin were set using a 9E10 isotype control (Bristol Institute for Transfusion Science)

and for anti-fibrinogen, the antibody was incubated in the presence of 10 mM EDTA (Sigma-Aldrich Ltd, Gillingham, UK).

Platelets were identified by light scatter and results were recorded as the percentage of platelets positive (%PP) for the relevant activation marker, calculated as the percentage of platelets expressing the relevant activation marker with MFI greater than 98% of the isotype control.

(c) Serum AF and stroke biomarker analysis

Serum samples from AF patients was sent to the on-site Department of Clinical Biochemistry for immediate analysis. Results were anonymised and electronically delivered after analysis. Control patients did not undergo biomarker analysis.

6.07 Statistical analysis

A *P*-value of <0.05 was taken as statistically significant. Linear regression revealed that age and mean platelet volume (MPV) were associated with GPVI expression in the patients. Therefore, GPVI expression and P-selectin exposure results from all cohorts were adjusted for age and MPV and predicted values calculated from adjusted unstandardized residuals were subsequently used when comparing mean values between cohorts. Specific statistical tests employed are included in the figure legends.

Age- and MPV-adjusted values were also entered into a simple linear regression analysis to determine association between total GPVI or GPVI-dimer expression with other single predictor variables. Significantly associated predictor variables ($P<0.05$) were then entered into a multiple regression model to determine independent predictors for GPVI-dimer expression. Unstandardized coefficient (B), standard error (SE) and significance (*P*) are reported for each of the significantly associated variables. Multivariable logistic regression analysis included the following predictors: total GPVI, GPVI-dimer, age and MPV to determine which variables were independently associated with AF. Data was analysed using Prism v8.0 (GraphPad, CA) and SPSS v.26 (IBM, CA).

6.08 Results

(a) Recruitment and population details

Over the duration of the study, a total of 79 patients with AF were recruited. However, 4 patients were excluded from analysis as detailed in Table 6.2.

Date	Study ID	Age (Yrs)	Anticoagulated	Reason for exclusion
10/01/2017	GRA0001	59.1	YES	On heparin infusion, recent MVR
30/03/2017	GRA0030	58.0	NO	Error in GPVI quantification
14/12/2018	GRA0071	79.9	YES	Error in GPVI quantification
05/03/2019	GRA0099	66.3	NO	Incomplete platelet function data

Table 6.2 Patients with AF excluded from the study analysis. MVR = mitral valve replacement

The presence of any platelet disorders, active or previous history of malignancy or any other reason that met exclusion criteria were thoroughly checked at admission and then at 4 months after inclusion into the study.

The remaining 75 patients were included in subsequent analysis. Their demographic details, blood parameters, comorbidities, admission medication of the control and AF populations are supplied in Table 6.3.

Compared to the control population, the AF population were significantly older (median age (Q_1-Q_3): controls, 60 years (47–68); AF, 74 years (66–79), $P<0.0001$) and included a smaller proportion of female (control, 65.8%; AF, 38.7%, $P<0.0001$). Full blood count analysis revealed that haemoglobin (Hb) and platelet counts were not different between the control and AF groups, however, the AF population had significantly larger platelets as measured by mean platelet volume (MPV (fL)$\pm$SD: control 10.4$\pm$0.9; AF 10.6$\pm$1.0, $P=0.04$).

As expected, the AF population had a greater burden of vascular risk factors compared to healthy controls (P<0.0001), none of the patients had had a stroke in the control group. Two patients with AF had had small ischaemic infarcts around 20 years ago, with no residual neurological deficit and this was felt to be of unlikely significance now and therefore included in the analysis. 2 patients in the AF group had chronic kidney disease (CKD) stage 2-3, whereas none of the patients in the control group had CKD.

A significantly higher proportion of AF patients were taking an antiplatelet, anticoagulation, anti-hypertensives and a statin at admission compared to the control group. The median (Q_1-Q_3) CHA_2DS_2-VASc score of the AF group was 3. There was no association between BMI or NEWS scores and GPVI expression or platelet function. None of the AF patients had died within six months of data collection.

	Control	**AF**	**Significance**
n	299	75	-
Median age (Q_1-Q_3)	60 (47-68)	74 (66-79)	**<0.0001**
Female (%)	65.8	38.7	**<0.0001**
Mean Haemoglobin (g\L) ± SD	136.5±11.4	134.0±20.2	0.15
Mean Plt Count ($10^x 9$/L) ± SD	259.6±57.3	247.0±80.1	0.11
Mean Plt Volume (fL) ± SD	10.4±0.9	10.6±1.0	**0.04**
Risk factors for thrombotic disease, n (%)			
Congestive Cardiac Failure	0 (0)	22 (29.3)	**<0.0001**
Hypertension	26 (8.7)	51 (68.0)	**<0.0001**
Diabetes	1 (0.33)	19 (25.3)	**<0.0001**
Ischaemic Heart Disease	0 (0)	14 (18.6)	**<0.0001**
Cholesterol	23 (7.7)	31 (41.3)	**<0.0001**
Stroke in last 5 years*	0 (0)	0 (0)	**-**
Admission medication n (%)			
ACE inhibitor or ARB	20 (6.7)	28 (37.3)	**<0.0001**
Aspirin	0 (0)	14 (18.6)	**<0.0001**
Clopidogrel	0 (0)	3 (4.0)	**0.02**
Apixaban	0 (0)	9 (12.0)	**<0.0001**
Dabigatran	0 (0)	1 (1.3)	**0.20**
Rivaroxaban	0 (0)	25 (33.3)	**<0.0001**
Warfarin	0 (0)	12 (16.0)	**<0.0001**
Statin	22 (7.4)	34 (45.3)	**<0.0001**

Table 6.3 The demographic details, blood parameters, comorbidities, admission medication of the control and AF populations. Controls were younger, had fewer risk factors and were on fewer anti-thrombotic medication. Individuals were classified as having a risk factor if there was a documented history of it, were on treatment for it, or were diagnosed with it during the admission.

*2 patients within the AF group had had strokes previously in 1997 and 1998 but were included in the analysis.

(b) General platelet characteristics of AF patients

Patients with AF had larger platelets as measured by MPV, compared to the controls (*P*=0.04), although neither their platelet count nor their haemoglobin level was significantly different.

As expected, the total GPVI and GPVI-dimer levels are strongly associated with each other (B 0.32, SE 0.01, *P*<0.0001). However, GPVI expression appears to be negatively associated with P-selectin exposure post ADP and CRP-XL as well as fibrinogen binding post CRP-XL addition (Table 6.4).

	Total GPVI		**GPVI-dimer**	
	Coefficient B	Significance (*P*)	Coefficient B	Significance (*P*)
P-Selectin Resting	**0.03**	**<0.0001**	0.001	0.26
P-Selectin exposure (+ ADP)	**-0.03**	**0.02**	**-0.004**	**<0.0001**
P-Selectin exposure (+ CRP-XL)	**-0.08**	**<0.0001**	**-0.007**	**<0.0001**
Fibrinogen Binding Resting	0.23	0.16	-0.19	0.15
Fibrinogen Binding (+ ADP)	**0.12**	**<0.0001**	**0.01**	**<0.0001**
Fibrinogen Binding (+ CRP-XL)	**-0.04**	**<0.0001**	**-0.005**	**<0.0001**

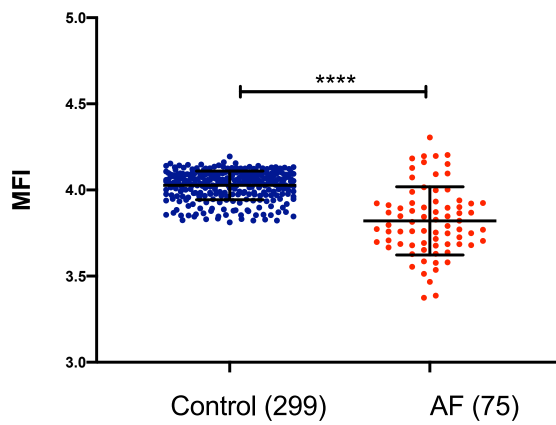
Table 6.4 Bivariate comparisons between either total GPVI or GPVI dimer and measured platelet function.

(c) Platelet GPVI expression in individuals with AF compared to controls

Compared to controls, AF patients' platelets expressed significantly lower total GPVI (MFI ± SD; control 4.03±0.08, AF 3.82±0.20, *P*<0.0001). Conversely, GPVI-dimer levels were significantly higher in the AF cohort compared to controls (MFI ± SD; control 0.57± 0.01, stroke 0.60±0.02, *P*<0.0001) (Table 6.5, Figure 6.3)

	Control (n=299)	**AF (n=75)**	***P***
Platelet GPVI expression (MFI) ± SD			
Total	**4.03±0.08**	3.82±0.20	**<0.0001**
Dimer	0.57± 0.01	**0.60±0.02**	**<0.0001**

Table 6.5 Total GPVI and GPVI-dimer expression as measured by median fluorescence intensity (MFI) in the control cohort compared to individuals with AF. The higher value is indicated in bold. Significance (*P*) was calculated using unpaired t-test.



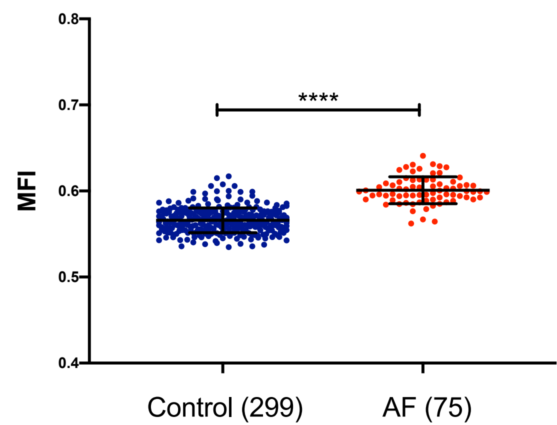


Figure 6.3 Expression of (A) total GPVI and **(B) GPVI-dimer between control and AF patients.** Significant differences between means of each cohort were calculated using an unpaired t-test and the error bars represent the mean MFI ± SD.

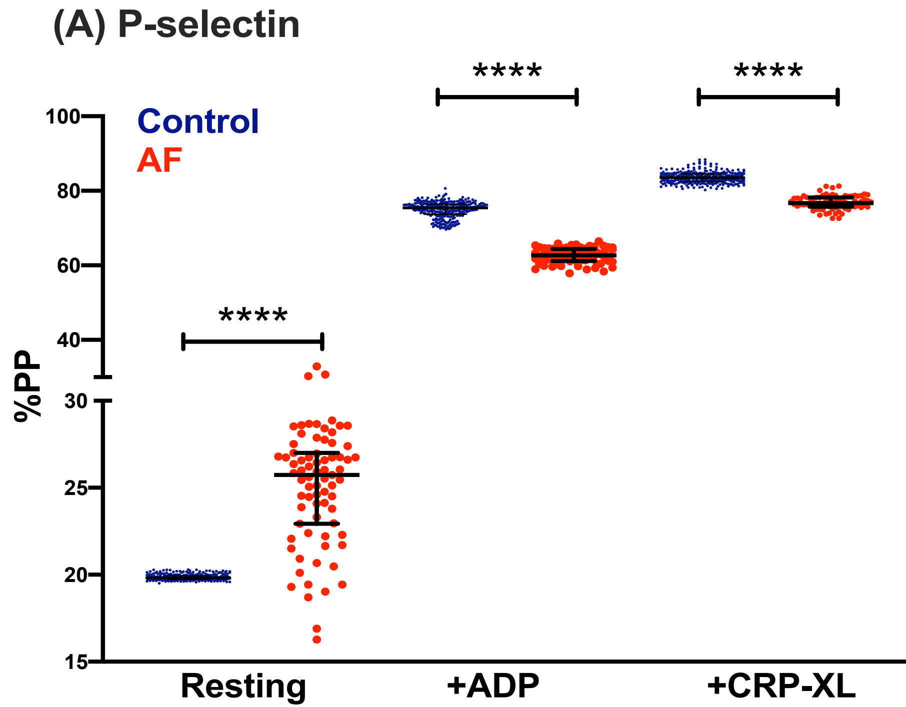
(d) Platelet P-selectin exposure and fibrinogen binding in AF cohort

Compared to controls, AF patient's resting platelets expose more P-selectin on their surface (*P*<0.0001). However, after the addition of ADP or CRP-XL, they expose significantly less P-selectin compared to controls (P<0.0001).

Conversely, AF platelets have significantly less bound fibrinogen on their surface compared to controls prior to addition of exogenous agonists (P<0.0001). After ADP or CRP-XL are added, they are able to bind more fibrinogen, but this remains significantly less compared to the control cohort (*P*<0.0001) (Table 6.6, Fig 6.4).

	Control (n=299)	**AF (n=75)**	***P***
Median (Q_1-Q_3) P-Selectin Exposure (%PP)			
Resting	19.8 (19.7-20.0)	25.7 (22.3-27.0)	**<0.0001**
+ADP	75.4 (73.7-76.4)	62.6 (61.1-64.4)	**<0.0001**
+CRP-XL	83.5 (82.5-84.6)	76.7 (75.7-78.3)	**<0.0001**
Median (Q_1-Q_3) Fibrinogen Binding (%PP)			
Resting	1.02 (0.94-1.08)	0.68 (0.57-0.7)	**<0.0001**
+ADP	49.2 (47.1-50.7)	30.2 (29.3-30.8)	**<0.0001**
+CRP-XL	33.4 (32.4-34.5)	21.7 (20.5-23.8)	**<0.0001**

Table 6.6 % Platelet positive (%PP) for P-selectin exposure and fibrinogen binding in control vs AF patients. Resting refers to circulating platelets where in vitro ADP or CRP-XL had not been added to activate the platelets.



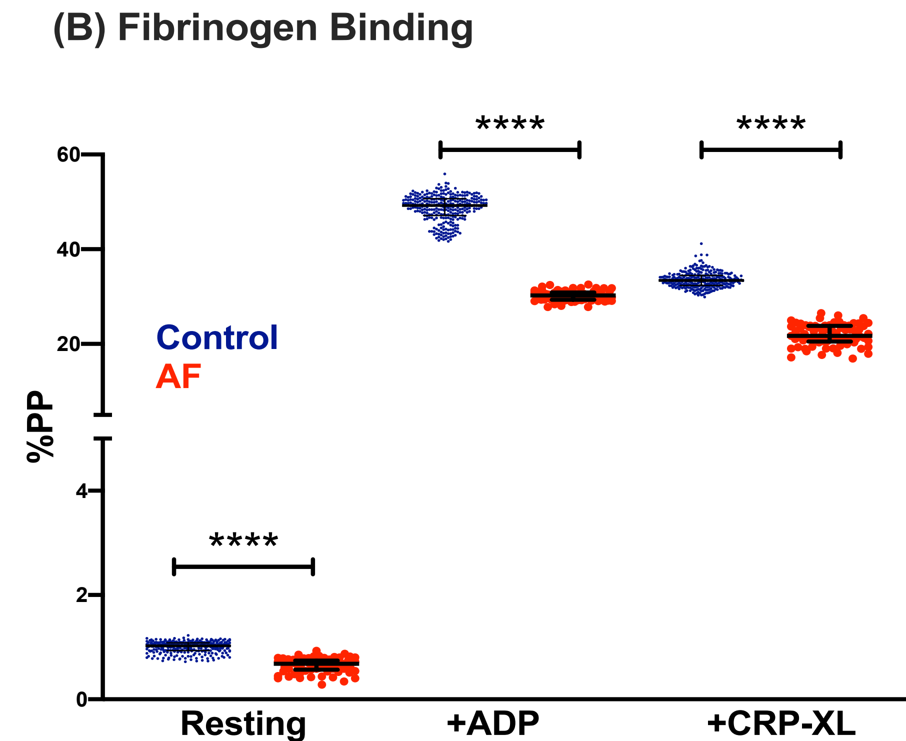


Figure 6.4 (A) P-selectin exposure and (B) fibrinogen binding in resting platelets or after addition of ADP or CRP-XL in the control and AF groups. Significant differences between means of each cohort were calculated using a Mann Whitney-U test and the error bars represent the median and interquartile range.

(e) GPVI expression between individuals with paroxysmal or permanent AF

There are mixed reports estimating the risk of stroke with pAF, but current evidence suggests that permanent AF and pAF carry the same risk of CES; therefore, guidelines tell us that pAF patients should be treated with anticoagulation too (Hohnloser et al. 2007; NICE NG128 May 2019). Whether GPVI expression varies within permanent AF and pAF and how this could relate to stroke risk is not established. Out of the 75 AF patients, there were 33 patients with pAF, meaning that the groups were reasonably evenly matched in terms of numbers. It was no surprise that the pAF patients were significantly younger (P=0.0004). They also had a significantly lower INR, but this is because many patients take a mixture of warfarin and DOACs, making the INR value difficult to interpret. A similar proportion of patients with pAF were on anticoagulation compared to those in permanent AF and their median CHA_2DS_2-VASc scores were not significantly different (P=0.26).

What was striking was that pAF patients had significantly lower serum fibrinogen (P=0.004), hs-CRP (P=0.004) and BNP (P=0.002) levels despite being in AF at the time of blood collection. The MPV of permanent AF patients was also significantly higher (mean$\pm$SD, permanent AF 10.85$\pm$0.94, pAF 10.33$\pm$1.04, P=0.03) but there were no differences in haemoglobin level or platelet count between the two groups (Table 6.7).

There was also no difference in the admission NEWS score or BMI between the two cohorts (not shown).

	Permanent AF	**Paroxysmal AF**	**P**
Mean age (years) ± SD	75.3±8.2	67.5±10.1	**0.0004**
Female (%)	21 (50.0)	8 (25.8)	**0.03**
On anticoagulation (%)	31 (73.8)	19 (57.6)	0.14
Mean Haemoglobin (g\L) ± SD	130.4.4±19.7	138.5±20.2	0.09
Mean Plt Count ($10^x 9$/L) ± SD	243.4±82.0	251.7±78.6	0.66
Mean Plt Volume (fL) ± SD	10.85±0.94	10.33±1.04	**0.03**
Risk factors for thrombotic disease, n (%)			
Congestive Cardiac Failure	15 (35.7)	7 (21.2)	0.21
Hypertension	29 (69.0)	22 (66.7)	1.0
Diabetes	7 (16.7)	12 (36.4)	0.06
Ischaemic Heart Disease	10 (23.8)	4 (12.2)	0.24
Cholesterol	14 (33.3)	17 (51.5)	0.16
Biomarkers and risk-scores			
Admission CHA_2DS_2-VASc	3 (2-5)	3 (2-4)	0.26
INR	1.63 (1.2-2.1)	1.21 (1.04-1.71)	**0.008**
Serum Fibrinogen	4.2 (3.1-6.6)	3.04 (2.8-4.3)	**0.004**
D-dimer	223 (96-405)	141 (75-367)	0.37
Serum hs-CRP	8.05 (3.9-39.3)	3.39 (1.26-12.7)	**0.004**
BNP	1645 (861-3231)	580 (124-1704)	**0.002**
Serum Creatinine	79 (64-93)	83 (71-89)	0.84

Table 6.7 The demographic details, comorbidities, blood parameters and biomarkers of the permanent AF (n=42) and pAF (n=33) populations. Median values are presented with IQR (Q_1-Q_3).

Patients with pAF demonstrated significantly lower total GPVI expression (MFI$\pm$SD; permanent AF 3.87$\pm$0.18; pAF 3.75$\pm$0.20, P =0.006) but their dimer levels were not different (MFI $\pm$ SD; permanent AF 0.60$\pm$0.01; pAF 0.60$\pm$0.01, P =0.11) (Table 5.7, Figure 5.4). The lower total GPVI in the pAF cohort is maybe an explanation as to why the whole AF cohort (which included permanent and pAF) patients exhibited lower total GPVI expression compared to the controls (Table 6.8, Figure 6.5).

pAF patients also demonstrated significantly less P-selectin exposure (P<0.0001) and fibrinogen binding compared to those in permanent AF (P=0.002), as well as more P-selectin exposure after ADP addition (P=0.03). However, there were no significant differences in P-selectin exposure or fibrinogen binding between the two groups after the addition of CRP-XL which may reflect the similar dimer levels between the two groups (Table 6.8).

	Control (n=299)	**Permanent AF (n=42)**	**Paroxysmal AF (n=33)**	***P^1***
Platelet GPVI expression (MFI)±SD				
Total	4.03±0.08	3.87±0.18	3.75±0.20	**0.007**
Dimer	0.57± 0.01	0.60±0.01	0.60±0.01	0.11
Median (Q_1-Q_3) P-Selectin Exposure (%PP)				
Resting	19.8 (19.7-20.0)	26.7 (25.1-28.0)	24.1 (20.6-26.0)	**<0.0001**
+ADP	75.4 (73.7-76.4)	63.2 (62.0-64.5)	61.8 (60.0-64.2)	**0.03**
+CRP-XL	83.5 (82.5-84.6)	76.9 (75.9-78.1)	76.4 (75.3-78.4)	0.83
Median (Q_1-Q_3) Fibrinogen Binding (%PP)				
Resting	1.02 (0.94-1.08)	0.71 (0.64-0.79)	0.59 (0.46-0.72)	**0.002**
+ADP	49.2 (47.1-50.7)	30.1 (29.4-30.8)	30.5(29.3-31.1)	0.58
+CRP-XL	33.4 (32.4-34.5)	21.0 (22.4-23.9)	21.1 (19.2-23.8)	0.10

Table 6.8 Differences in platelet GPVI expression, P-selectin exposure and fibrinogen binding between those in permanent AF and paroxysmal AF. Significant differences (P^1) between means of the permanent and paroxysmal cohorts only were calculated using an unpaired t-test for parametric or Mann Whitney-U test for non-parametric data.

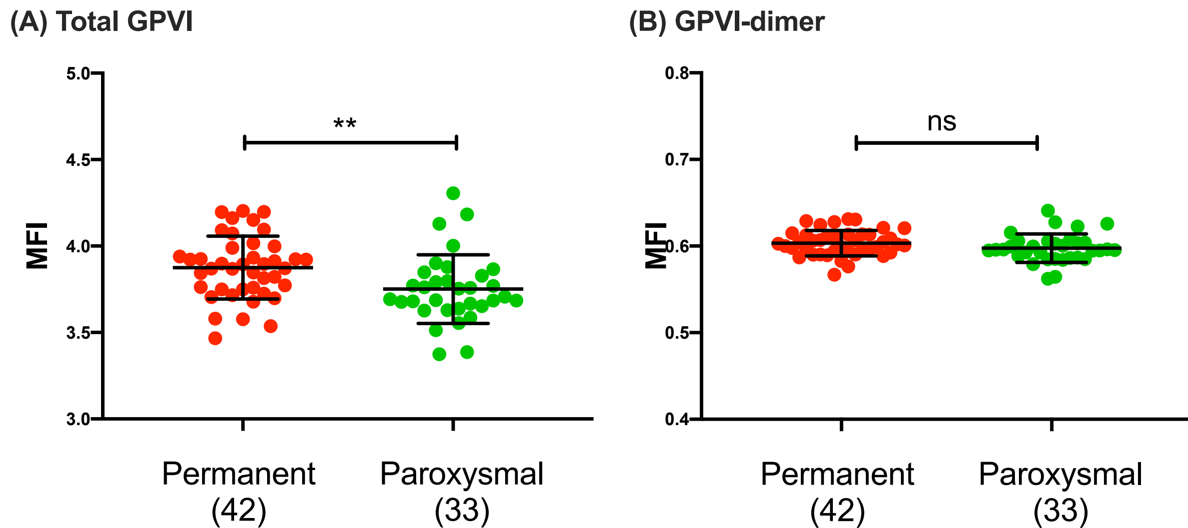


Figure 6.5 Expression of (A) total GPVI and **(B) GPVI-dimer between permanent AF (AF) and paroxysmal AF (pAF) patients.** Significant differences between means of each cohort were calculated using an unpaired t-test and the error bars represent the mean MFI ± SD.

(f) Differences in GPVI expression according to anticoagulation status

There were 50 patients (75%) with AF taking a variety of anticoagulation (+OAC) as summarised in Table 6.9. Rivaroxaban was the most popular choice of the DOACs, and 12 patients were prescribed warfarin.

Anticoagulation	n (%)
Apixaban	10 (20.0)
Dabigatran	1 (2.0)
Edoxaban	0
Rivaroxaban	25 (50.0)
Warfarin	12 (24.0)
Therapeutic dose dalteparin*	2 (4.0)

Table 6.9 Different anticoagulation medications taken by the AF patients.
*The patients on therapeutic dalteparin had been taking it for over 72 hours and therefore were included in the analysis as (+OAC).

As expected, a higher proportion of patients not on anticoagulation (-OAC) took antiplatelets. There were no statistical differences in antihypertensive or statin prescription. Patients on anticoagulation had a significantly higher MPV (P=0.02) and median creatinine (P=0.03) but age, haematological parameters and serum biomarkers were not significantly different as shown in Table 6.10. Also, a similar proportion of patients had permanent AF between the two groups. There was also no difference in the admission NEWS score or BMI between the two cohorts (not shown).

	AF (+OAC) (n=50)	**AF (-OAC) (n=25)**	**P**
Median age	74.7 (67–80)	73.1 (63-78)	0.37
Female n, (%)	20 (40.0)	9 (36.0)	1.0
Permanent AF (%)	31 (62.0)	11 (44.0)	0.15
Mean Haemoglobin (g\L) ± SD	133.4±20.4	135.0±20.1	0.75
Mean Plt Count ($10^x 9$/L)± SD	249.1±89.6	242.8±58.0	0.75
Mean Plt Volume (fL) ± SD	10.80±1.1	10.23±0.82	**0.02**
Median CHA_2DS_2-VASc Score	3 (2-4)	2 (1-4)	0.12
Median INR	1.75 (1.3-2.2)	1.07 (1.01-1.20)	**<0.0001**
Median Fibrinogen	4.2 (3.0-5.3)	3.16 (2.8-4.2)	0.11
Median D-dimer	161 (75-385)	153 (89-451)	0.61
Median Hs-CRP	7.6 (3.2-26.3)	3.58 (1.32-15.5)	0.13
Median BNP	2364 (514-2857)	863 (258-3147)	0.31
Median Creatinine	88 (66-95)	78 (67-82)	**0.03**
Admission medication n (%)			
ACE inhibitor or ARB	21 (42.0)	7 (28.0)	0.31
Aspirin	6 (12.0)	8 (32.0)	0.06
Clopidogrel	2 (4.0)	1 (4.0)	1.0
Statin	23 (46.0)	11 (44.0)	1.0

Table 6.10 The demographic details, blood parameters, biomarkers and other admission medication of AF patients taking anticoagulation (+OAC; n=50) and not (-OAC; n=33). The two cohorts were evenly matched in terms of measured parameters, except mean platelet volume and creatinine. Median values are presented with interquartile range (Q_1-Q_3).

There were no significant differences between P-selectin exposure or fibrinogen binding between the two cohorts overall. AF patients taking anticoagulation expressed significantly more total GPVI and GPVI-dimer compared to those not taking anticoagulation (Table 6.11).

	Control (n=299)	AF + OAC (n=50)	AF - OAC (n=25)	P^1
Platelet GPVI expression (MFI)±SD				
Total	4.03±0.08	3.86±0.20	3.74±0.16	**0.01**
Dimer	0.57± 0.01	0.60±0.02	0.60±0.01	**0.03**
Median (Q_1-Q_3) P-Selectin Expression (%PP)				
Resting	19.8 (19.7-20.0)	26.1 (23.9-27.6)	24.7 (22.0-26.6)	0.13
+ADP	75.4 (73.7-76.4)	62.8 (60.9-64.5)	62.5 (61.2-64.3)	0.92
+CRP-XL	83.5 (82.5-84.6)	76.7 (75.2-78.0)	77.1 (76.1-78.6)	0.14
Median (Q_1-Q_3) Fibrinogen Binding (%PP)				
Resting	1.02 (0.94-1.08)	0.69 (0.59-0.77)	0.67 (0.53-0.74)	0.44
+ADP	49.2 (47.1-50.7)	30.3 (29.5-31.1)	30.1 (29.1-30.6)	0.21
+CRP-XL	33.4 (32.4-34.5)	21.9 (19.6-23.7)	21.5 (20.7-23.9)	0.62

Table 6.11 Differences in platelet GPVI expression, P-selectin exposure and fibrinogen binding between AF patients taking anticoagulation (+OAC) and those who were not (-OAC). Significant differences (P^1) between means of the AF anticoagulated and not anticoagulated groups only were calculated using an unpaired t-test for parametric or Mann Whitney-U test for non-parametric data.

(g) Differences in GPVI expression according to anticoagulation type

35 patients out of 50 were on factor Xa (+FXa) inhibitors (Table 6.9; rivaroxaban 25, apixaban 10). There were only low numbers; 15 patients who were on non-FXa inhibitors (other OAC, warfarin 12, dabigatran 1, dalteparin 2), therefore, the results are difficult to interpret with certainty.

However, those taking FXa inhibitors appeared to have higher total GPVI expression those who were not on anticoagulation all together (-OAC, n=25) (MFI ±SD; +FXa 3.86±0.21, -OAC 3.74±0.16, P=0.02) but not compared to those taking other anticoagulation (other OAC, n=15) (MFI ±SD 3.84±0.21, P=0.74). Similarly, GPVI-dimer expression was also significantly raised in those on FXa inhibitors compared to those not on anticoagulation (MFI ± SD; +FXa 0.60±0.01, -OAC 0.59±0.01, P=0.04) but not compared to those taking other anticoagulation (MFI ± SD; 0.60±0.02, P=0.92) (Figure 6.6). There were no differences in resting P-selectin exposure or fibrinogen binding between any of the above tested groups (not shown).

Similar results are also demonstrated when comparing total GPVI (P=0.002) and GPVI-dimer expression (P=0.006) between those just on rivaroxaban (n=25) compared to those on no anticoagulation (n=25) (Figure 6.7).

The fact that patients on FXa inhibitors may express more GPVI-dimer on their platelet surface is an interesting finding, supported by existing literature which will be discussed in section 6.09. It may explain some of the differences observed between the anticoagulated vs non-anticoagulated patients seen in the previous section.

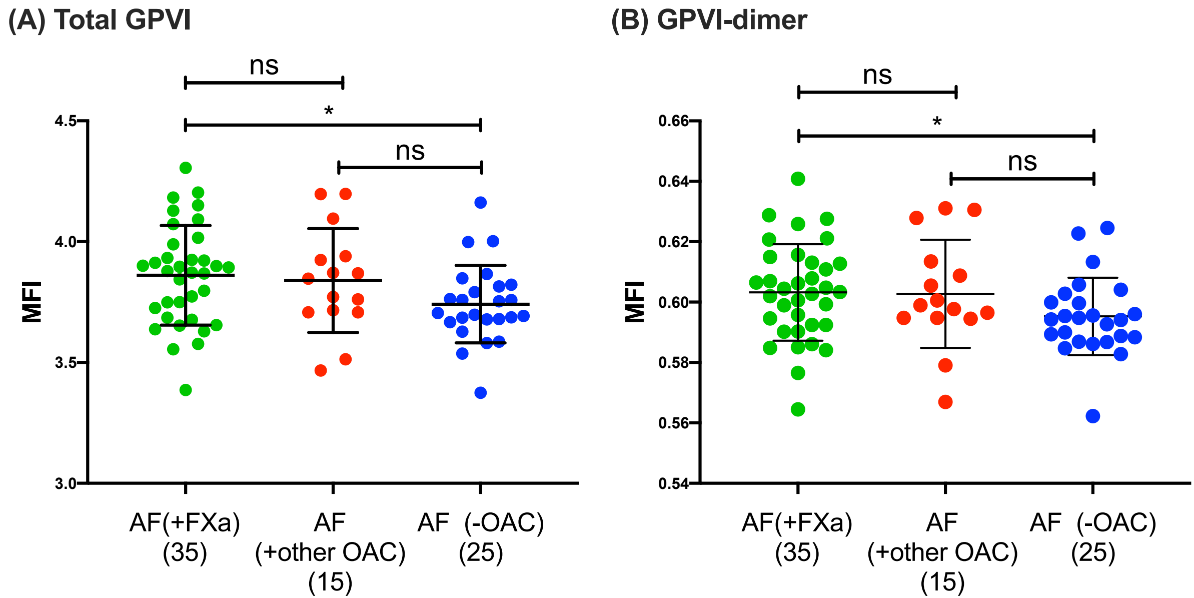


Figure 6.6 Expression of (A) Total GPVI and (B) GPVI-dimer between patients on all factor Xa inhibitors (+FXa), non-factor Xa inhibitors (other OAC) and AF patients not on anticoagulation (-OAC). Significant differences between means of each cohort were calculated using an unpaired t-test and the error bars represent the mean MFI ± SD

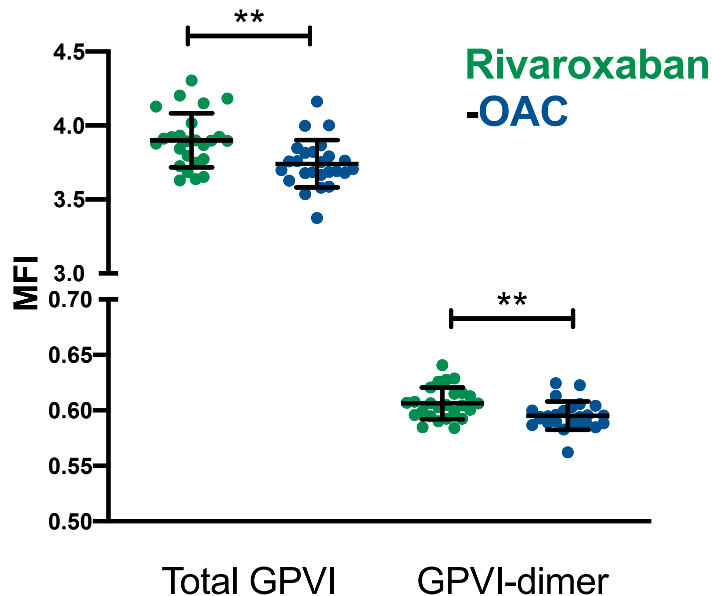


Figure 6.7 Total GPVI and GPVI-dimer expression in patients taking Rivaroxaban compared to those not on anticoagulation. Significant differences between means of each cohort were calculated using an unpaired t-test and the error bars represent the mean MFI ± SD

Simple linear regression analysis with total GPVI or GPVI-dimer expression as dependent variables demonstrates that being on rivaroxaban is a predictor of significantly higher total GPVI expression (*P*=0.047) and GPVI-dimer expression (*P*=0.03) (Table 6.12).

	Total GPVI		**GPVI-dimer**	
	Coefficient B	Significance (*P*)	Coefficient B	Significance (*P*)
All anticoagulants	**0.12**	**0.01**	**0.008**	**0.03**
Factor Xa inhibitors	0.09	0.06	0.005	0.14
Rivaroxaban	**0.12**	**0.047**	**0.008**	**0.03**

Table 6.12 Predictors of total GPVI and GPVI-dimer according to anticoagulation type

(h) GPVI expression and AF duration

The subclinical nature of AF means it is difficult to truly assess duration of the disease. However, for the 75 AF patients there were 61 with a documented year of AF diagnosis in the medical notes. The data therefore firstly divided into whether this diagnosis was made in the last 2 years, or longer. 30 patients had an AF diagnosis made within the last 2 years and 31 over 2 years ago. 7 patients in fact had their AF diagnosed within the month prior to recruitment.

There were also no differences between P-selectin exposure or fibrinogen binding between the two cohorts but both total GPVI and GPVI-dimer expression were higher in patients diagnosed with AF over 2 years ago (Table 6.13, Figure 6.8).

	Control (n=299)	AF < 2 years (n=30)	AF > 2years (n-31)	P[1]
Platelet GPVI expression (MFI)±SD				
Total	4.03±0.08	3.76±0.19	3.89±0.17	**0.01**
Dimer	0.57± 0.01	0.60±0.02	0.60±0.01	**0.01**
Median (Q_1-Q_3) P-Selectin Expression (%PP)				
Resting	19.8 (19.7-20.0)	25.3 (22.5-26.8)	26.0 (23.6-27.4)	0.82
+ADP	75.4 (73.7-76.4)	63.0 (61.2-64.8)	62.4 (60.9-63.8)	0.19
+CRP-XL	83.5 (82.5-84.6)	77.3 (76.0-78.7)	76.4 (75.0-77.9)	0.06
Median (Q_1-Q_3) Fibrinogen Binding (%PP)				
Resting	1.02 (0.94-1.08)	0.68 (0.52-0.76)	0.68 (0.56-0.74)	0.92
+ADP	49.2 (47.1-50.7)	30.2 (29.2-30.8)	30.5 (29.6-31.1)	0.82
+CRP-XL	33.4 (32.4-34.5)	21.8 (20.4-24.4)	21.3 (19.8-23.2)	0.25

Table 6.13 Differences in platelet GPVI expression, P-selectin exposure and fibrinogen binding between patients diagnosed with AF in the last 2 years or earlier. Significant differences (P^1) between means of the AF <2 and >2 years groups only were calculated using an unpaired t-test for parametric or Mann Whitney-U test for non-parametric data.

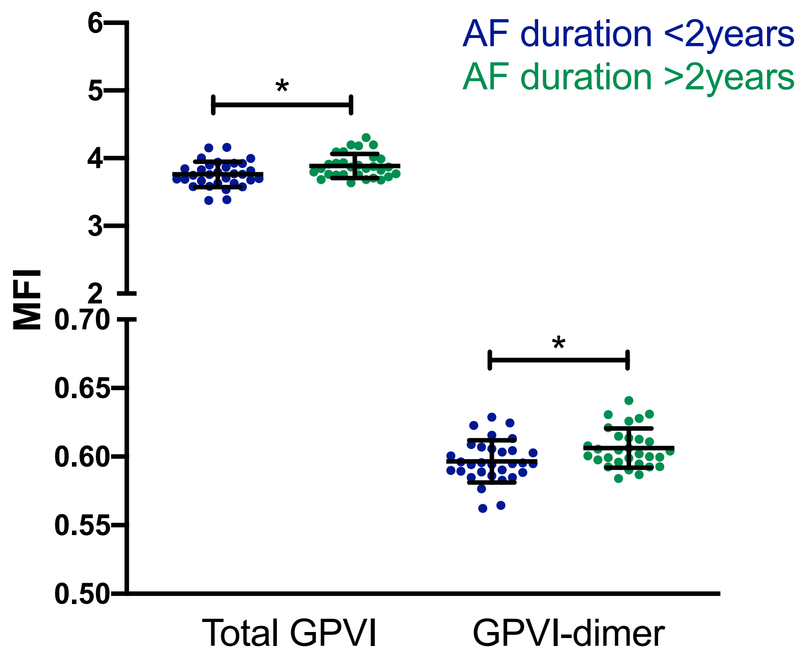


Figure 6.8 Total GPVI and GPVI-dimer expression in AF with a diagnosis made within 2 years compared to over 2 years ago. Significant differences between means of each cohort were calculated using an unpaired t-test and the error bars represent the mean MFI $\pm$ SD.

(i) Correlation between GPVI expression with AF and stroke biomarkers

The serum biomarkers included in analysis were D-dimer, fibrinogen, BNP and hs-CRP, collected at the same time as the blood sample for GPVI quantification. After adjusting for age and MPV, Spearman's rho correlation coefficient testing was used to assess for any correlation between these biomarkers and total GPVI or GPVI-dimer expression.

Fibrinogen was only positively correlated with total GPVI expression whereas, BNP and hs-CRP demonstrated significant positive correlation with total and GPVI-dimer expression (Table 6.14). D-dimer and GPVI-dimer were significantly negatively correlated (Figure 6.9).

	D-dimer (ng/mL)	Fibrinogen (g/L)	BNP (pg/mL)	hs-CRP (mg/L)
Spearman's r, Significance (*P*)				
Total GPVI	0.02, 0.89	**0.38, 0.001**	**0.78, <0.0001**	**0.93, <0.0001**
GPVI-dimer	**-0.34, 0.003**	0.02, 0.88	**0.52, <0.0001**	**0.72, <0.0001**

Table 6.14 Correlation between total GPVI or GPVI-dimer expression and serum biomarkers in stroke patients (n=127) .

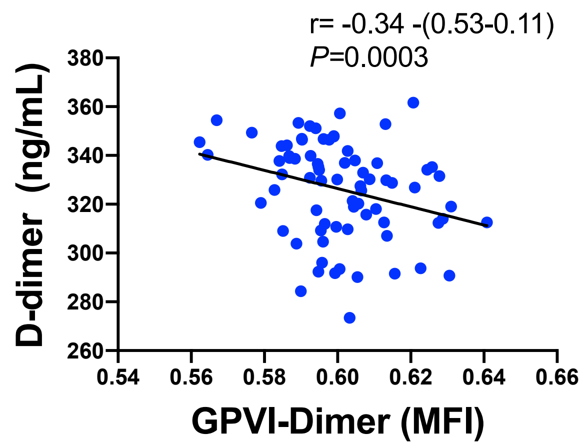


Figure 6.9 Correlation between GPVI-dimer expression and serum d-dimer levels. Calculated using Spearman's rho correlation coefficient. Regression line from simple linear regression is presented in black.

(j) Variables associated with GPVI expression in a population with AF

Associations between single predictor variables and total GPVI or GPVI-dimer which were adjusted for age and MPV, were carried out using simple linear regression (Table 6.15). Supplementary table 6.01 shows the associations between age and MPV with GPVI expression.

As expected from the results demonstrated in section 6.1.5, pAF was associated with significantly lower total GPVI expression (B -0.124, SE 0.04, *P*=0.006), whereas the presence of CCF and being on anticoagulation at admission (as suggested in section 6.1.6) were associated with higher total GPVI expression (CCF; B 0.11, SE 0.05, *P*=0.02, +OAC; B 0.12 SE 0.05, *P*=0.01). Fibrinogen, BNP and hs-CRP were associated with higher total GPVI expression and D-dimer, BNP and hs-CRP with higher GPVI-dimer expression.

Neither gender or any of the illness acuity measurements taken on admission within the AF cohort (hsCRP and NEWS) were associated with total GPVI or GPVI-dimer expression.

	Total GPVI		GPVI-dimer	
	Coefficient B	Significance (*P*)	Coefficient B	Significance (*P*)
Female Sex	0.002	0.97	-0.001	0.78
Paroxysmal AF	**-0.12**	**0.006**	-0.006	0.11
Comorbidities				
CCF	**0.11**	**0.02**	0.008	0.054
Hypertension	0.06	0.22	0.004	0.33
Diabetes	0.04	0.40	0.005	0.22
Ischaemic Heart Disease	0.02	0.70	-0.001	0.88
Cholesterol	0.01	0.72	0.001	0.78
BMI	-0.001	0.77	0.00	0.21
Serum Biomarkers				
D-dimer (ng/mL)	0.00	0.77	**0.01**	**0.01**
Fibrinogen (g/L)	**0.31**	**0.001**	0.00	0.62
BNP (pg/mL)	**0.02**	**<0.0001**	**0.01**	**<0.0001**
hs-CRP (mg/L)	**0.02**	**<0.0001**	**0.01**	**<0.0001**
Creatinine (μmol/L)	0.001	0.20	0.00	0.55
Admission Parameters				
Anticoagulation at admission	**0.12**	**0.01**	0.02	0.15
CHA_2DS_2-VASc score	0.02	0.11	0.00	0.92
NEWS score	0.00	0.00	0.00	0.78

Table 6.15 Simple linear regression to determine associations between single predictor variables and total GPVI or GPVI-dimer expression in patients with AF.

(k) Associations between the presence of AF in all participants (n=374) with GPVI-dimer expression

Associations between single predictor variables and GPVI-dimer expression for the whole population (control n=299 and AF n=75), were carried out using simple linear regression (Table 6.16). Higher GPVI-dimer levels were associated with the presence of AF.

	GPVI-dimer	
	Coefficient B	Significance (*P*)
AF	**0.69**	**<0.0001**
Permanent AF	0.16	0.15
pAF	-0.018	0.11

Table 6.16 Simple linear regression to determine associations between single predictor variables; the presence of AF, permanent AF or paroxysmal AF (pAF) and GPVI-dimer expression in patients all participants, n= 374.

(l) Presence of AF and relationship to GPVI-dimer

The whole population of study participants (control, and AF; n=374) were dichotomised into whether they had AF or not, which was then used as the dependent variable in a multivariable binary logistic regression analysis. Unadjusted values of total GPVI, GPVI-dimer, age and MPV were tested as independent variables.

Parameters	**Coefficient B**	**Odds Ratio**	**95% CI**	**Significance (*P*)**
Age (years)	0.126	1.13	1.10 – 1.18	<0.0001
GPVI dimer	**2.798**	**16.4**	**1.5– 75.5**	**0.02**
MPV (fL)	0.263	1.3	0.95 – 1.71	0.09
Total GPVI	**-0.694**	**0.5**	**0.32 – 0.78**	**0.002**

Table 6.17 Binary logistic regression analysis of parameters: total GPVI, GPVI-dimer, age and MPV, to assess for predictors of stroke.

The results suggest GPVI-dimer expression is an independent predictor of AF (Odds Ratio 16.4 (1.5-75.5), *P*=0.02), when modelled together with total GPVI, age and MPV. Lower total GPVI expression is independently predictive of the presence of AF in this model (Odds Ratio 0.5 (0.32-0.78), *P*=0.002).

6.09 Discussion

The aim of the *GRAFITE* study was to further the knowledge on a key platelet receptor, GPVI by studying its expression on the platelet surface in a cohort of individuals with AF who are at high risk of having an ischaemic stroke.

Previous work investigating GPVI expression in AF is limited to just one study. As part of a sub-group analysis of GPVI expression in patients with ACS or stable angina, Bigalke and colleagues measured platelet surface expression of total GPVI and P-selectin exposure using flow cytometry in patients who were either in sinus rhythm or AF at the time of recruitment (Bigalke, 2009). They also determined the plasma concentration of sGPVI in these patients. They demonstrated that total GPVI expression was significantly decreased in patients with ACS who were in AF, compared to those in sinus rhythm (P=0.023). Furthermore, the corresponding sGPVI levels were significantly higher in the AF group (P=0.038). What was interesting was that these differences in GPVI expression between AF and sinus rhythm was not seen in patients with stable angina and that P-selectin measurements were also not significantly different between any of the groups. It is difficult to interpret these results as the AF cohort had concurrent ACS, because ACS has been previously shown to increase total GPVI expression (Bigalke, 2010, 2011). The fact that AF patients had lower GPVI expression and higher sGPVI only with ACS and not stable angina suggests that there could be increased GPVI shedding in AF patients in the ACS setting. The authors imply that this is due to a significantly higher platelet activation in the ACS and AF cohort in the first place leading to more shedding compared to the others.

Recently Barrachina and colleagues used 1G5 and 204-11 Fab (supplied by Dr S Jung) to quantify total GPVI and GPVI-dimer expression respectively, as well as pP-selectin in 12 severely obese individuals (BMI >40) and 11 grade 1 obese individuals (BMI 26-35), compared to 12 lean controls (Barrachina, 2019). What they observed was higher expression of total GPVI and GPVI-dimer in both the obese cohorts compared to lean individuals (severe obese vs control total GPVI; P=0.0029 and GPVI-dimer; P=0.016), with increased platelet aggregation in response to CRP-XL or collagen in the obese cohorts. They observed no significant differences in P-selectin expression between the groups.

GPVI expression was also significantly correlated with BMI in the severely obese patients (total GPVI r=0.70, *P*=0.0001, GPVI-dimer r=0.49*, P*=0.01*)*.

These studies illustrate that there is higher GPVI expression in individuals at-risk of thrombotic disease. The fact that P-selectin exposure was not different between obese compared to lean individuals, whereas both total GPVI and GPVI-dimer were serves to highlight this point further. Similarly in the work by Bigalke, both stable angina and ACS patients with AF showed no significant differences in P-selectin, whereas total GPVI expression was higher in the ACS group (Bigalke, 2010).

<u>The main results from this chapter are as follows</u>

1) Patients with AF express less total GPVI (*P*<0.0001) compared to controls but have more GPVI-dimer on their platelet surface (*P*<0.0001).
2) Patients with paroxysmal AF express significantly less total GPVI compared to those in permanent AF (*P*=0.007), although their GPVI-dimer levels were not different (*P*=0.11). The presence of paroxysmal AF is an independent predictor of lower total GPVI expression (*P*=0.006).
3) AF patients taking anticoagulation expressed more total GPVI (*P*=0.01) and GPVI-dimer (*P*=0.03) compared to those not on anticoagulation. This was specifically seen in those taking Factor Xa inhibitors (total GPVI, *P*=0.02; GPVI-dimer, *P*=0.04).
4) Higher GPVI-dimer expression was an independent predictor of the presence of AF within our population (*P*=0.02*)*.

This is the first study to look at GPVI expression and platelet function in a cohort of AF patients, who are at high risk of ischaemic stroke. Apart from AF, they also had other vascular risk factors described in Table 6.3 which in turn would contribute to their stroke risk; as demonstrated by a median CHA_2DS_2-VASc score of 3. The presence of AF as well as these other risk factors create a systemic pro-thrombotic state, driven by inflammation endothelial dysfunction and platelet activation as discussed in Chapter I (Lip, 1995; Llombart, 2013) and how platelet levels of GPVI vary in response to these stimuli forms the basis of what we are trying to understand with this research.

One of the limitations of the work by Bigalke and colleagues was that GPVI-dimer expression was not measured in their AF cohort. Our results demonstrate that patients with AF express significantly higher GPVI-dimer compared to the control group, despite expressing lower total GPVI (Figure 6.3). These results are similar to that seen in obese patients who also demonstrated higher GPVI-dimer expression (as well as total GPVI) compared to controls (Barrachina, 2019). Furthermore, in our AF cohort, the presence of CCF, and pro-thrombotic and inflammatory biomarkers such as serum fibrinogen, BNP and hs-CRP were associated with GPVI expression. As previously described in the literature, AF as well as other cardiovascular diseases involve systemic inflammation and the upregulation of pro-inflammatory biomarkers such as hs-CRP (Galea, 2014; Dawood, 2016). We could hypothesise that the systemic inflammatory state in the setting of AF in these patients, could be driving platelet expression of GPVI-dimer on the platelet surface. In turn, the presence of more GPVI-dimer on the platelet surface may then be key to platelet interactions with collagen and fibrin and drive thrombus formation in AF.

What was an unexpected result was the observation that AF patients express significantly lower total GPVI levels on their platelets compared to controls yet exhibit significantly higher GPVI-dimer expression (Figure 6.3). Whether this represents more GPVI monomer being dimerised in the setting of AF is unknown. Lower total GPVI expression in AF patients has been observed previously by Bigalke et al, with a corresponding higher sGPVI seen in the plasma (Bigalke, 2009). Although this was in the setting of ACS, the conclusion from the authors was that this observation represents increased platelet activation followed by MMP induced preferential shedding of monomeric GPVI from the platelet surface. Certainly, increased MMP levels have been observed in AF patients (Xu, 2012; Li, 2014). Therefore, it may well be that MMPs are causing GPVI to be shed from the platelet surface in AF patients more so than in controls. Our study would have been strengthened by quantifying serum sGPVI in control and AF patients, which would have indicated whether AF patients had higher sGPVI, therefore, higher monomeric GPVI shedding compared to controls.

When the AF cohort is further divided into either permanent or pAF, we see that the pAF patients express even lower total GPVI, but similar GPVI-dimer compared to the

permanent AF group (Figure 6.5). We wondered whether this was because of less circulating thrombotic markers such as fibrinogen and hs-CRP (as well as BNP) within the pAF group, indicating a lower overall systemic inflammatory burden (Table 6.7). However, this may not be the case as both permanent AF and pAF patients still expressed significantly lower total GPVI compared to the control group (both $P<0.0001$), highlighting that lower total GPVI is common to both forms of AF. One of the limitations of this study is that we also did not measure these biomarkers in the control cohort, therefore, have no means of accurate comparison.

Resting P-selectin exposure was significantly higher in the AF cohort compared to controls consistent with circulating platelets in AF patients being more primed for thrombotic activity, this is in keeping with platelets expressing more GPVI-dimer. However, resting fibrinogen binding, as well as P-selectin exposure and fibrinogen binding after ADP or CRP-XL stimulation were significantly lower, despite all AF patients expressing more GPVI-dimer compared to controls (Table 6.6). We hypothesised whether this could be due to the effects of anticoagulants. Certainly, the mechanism by which most anticoagulants act in the body is to reduce the systemic effect of the coagulation system. However, the whole AF cohort were split into patients taking anticoagulation or not, we were unable to see any significant differences in fibrinogen binding or P-selectin exposure in response to ADP or CRP-XL stimulation (Table 6.11). It may indicate that there are variables outside the remits of what was measured in our work, causing an overall reduction platelet function in AF patients compared to controls.

We also observed that patients on FXa inhibitors such as rivaroxaban an apixaban may express more GPVI on their platelets compared to those not taking anticoagulants (Figure 6.6 and 6.7). Previous work has demonstrated that FXa causes GPVI downregulation in vitro, possibly through FXa induced ADAM10 activation and subsequent GPVI shedding, and that rivaroxaban (a factor X inhibitor) but not dabigatran (a direct thrombin inhibitor) blocks this process (Al-Tamimi, 2011). The numbers in each of the cohorts tested are small, so the results must be interpreted with caution. However, being on FXa inhibitors were predictive of both higher GPVI-dimer levels and total GPVI (Table 6.12).

These findings have potential impact on choosing the correct anticoagulant in patients. If FXa inhibitors do indeed inhibit GPVI downregulation, it may lead to increased thrombotic events in patients prescribed FXa inhibitors, as they will express more GPVI on their platelet surface. However, this needs to be evaluated in the context of larger trials investigating GPVI expression and sGPVI with different anticoagulant treatment and again highlights not measuring sGPVI as a potential limitation of this study.

GPVI-dimer expression was also significantly associated with BNP and hs-CRP levels (Table 6.15) and negatively correlated with serum D-dimer levels (Figure 6.9). Our previous work and results from another group demonstrated that D-dimer in solution can inhibit collagen induced platelet aggregation in vitro through GPVI (Onselaer, 2017; Induruwa, 2018). Although we can only speculate, this could either be that D-dimer binds to GPVI-dimer at a location close to where 204-11 Fab binds (used to detect GPVI-dimer) or it be a protective mechanism against thrombosis in AF patients, despite the observation of higher GPVI-dimer levels. In keeping with this previous work has described that soluble fibrin causes a GPVI signalling defect (Lee, 2017).

Finally, our results demonstrate that GPVI-dimer expression an independent predictor of the presence of AF, independent of age, MPV and total GPVI (Table 6.17) suggesting that higher GPVI-dimer expression must have an inherent role in the presence of AF and may explain why patients with AF for over 2 years expressed more total GPVI and GPVI-dimer compared to those with a diagnosis of AF made less than 2 years ago (Table 6.13). To summarise, patients with AF have a pro-thrombotic platelet profile, with higher GPVI-dimer expression and resting P-selectin exposure compared to controls, whether patients have permanent or pAF. Despite this, may questions remain unanswered within the limitations of this study further discussed in section 8.05. Further information on how GPVI expression and platelet function changes from a pre-stroke AF population to those who have just had a stroke will be explored in Chapter VII, which discusses the Glycoprotein VI in Stroke (*GYPSIE*) study and may give us further insights into how GPVI is involved in ischaemic stroke.

6.10 Supplementary Files

Modelling associations between age and MPV inserted as single predictor variables with unadjusted total GPVI and GPVI-dimer expression for the AF population (n=75), was carried out using simple linear regression. Both total GPVI and GPVI-dimer expression and platelet function values were adjusted for age and MPV prior to analysis in this chapter.

	Total GPVI		**GPVI-dimer**	
	Coefficient B	Significance (*P*)	Coefficient B	Significance (*P*)
Age (years)	0.04	0.725	0.04	0.91
MPV (fL)	0.29	0.07	**0.1**	**0.02**

Supplementary Table 6.1 Simple linear regression to determine associations between single predictor variables; age and MPV with unadjusted total GPVI and GPVI-dimer expression values in the AF (population).

CHAPTER VII. PLATELET RECEPTOR GLYCOPROTEIN VI-DIMER IS SIGNIFICANTLY OVER-EXPRESSED IN ISCHAEMIC STROKE PATIENTS

The **Gly**co**p**rotein **S**ix **I**n Strok**E** *(GYPSIE)* Study

Chapter Summary

Stroke patients express significantly more GPVI, both total GPVI and GPVI-dimer, on their platelet surface compared to healthy controls and non-stroke patients. Stroke patients also demonstrate significantly more resting P-selectin exposure as well as P-selectin exposure after exogenous addition of ADP or CRP-XL compared to healthy controls and non-stroke patients. At 'day 90' post-stroke, the GPVI-dimer levels are significantly higher than at admission, but this effect is not observed with total GPVI expression or P-selectin exposure. All ischaemic stroke aetiologies (LAS, CES, SVO) and haemorrhagic strokes demonstrate more total GPVI and GPVI-dimer expression compared to the controls. Current antithrombotic medication does not appear to effect GPVI expression in stroke patients. GPVI-dimer expression was independently predictive of stroke.

7.01 Introduction

Platelet activation and subsequent thromboembolism underpins the pathophysiology of ischemic stroke. Currently used antiplatelet and anticoagulation agents are effective at reducing ischemic events (Rajkumar, 2015) but at the expense of systemic side effects such as bleeding (Lee, 2013). Therefore, there is a need for more specific anti-thrombotic targets which also provide a safer pharmacological profile.

The focus of the *GRAFITE* study, discussed in the previous chapter was to investigate GPVI expression and platelet activity in patients with AF who had not yet had a stroke. Quantitating platelet GPVI expression in a cohort of patients who have had a stroke, therefore, was the next step in delineating the role of GPVI, and especially GPVI-dimer, in stroke.

What makes investigating GPVI most tantalising is its ability to bind to both fibrillar collagen and fibrin (Onselaer, 2017; Induruwa, 2018; Mangin, 2018). It is well established that GPVI-collagen interactions activate platelets but the extent of platelet activation through GPVI-fibrin interactions remains to be fully established. Nevertheless, this suggests an important role for GPVI in the pathophysiology of large artery ischaemic stroke (LAS), where GPVI-dimer activates platelets when it binds to exposed collagen at sites of atherothrombotic plaque rupture and endothelial disruption. The GPVI–fibrin interaction then highlights a role for GPVI in cardioembolic stroke (CES), where endothelial dysfunction within the cardiac LAA causes activation of the coagulation system, leading to platelet activation and thrombus formation through the generation of thrombin and fibrin as discussed earlier.

What is also crucial is that as well as GPVI-dimers being constitutively present on the surface of resting platelets (about 20-29% of total GPVI), dimer expression can further increase on the platelet surface upon platelet activation (Chen, 2002; Jung, 2012) and propel thrombus formation.

The aim of the *GlYcoProtein Six In StrokE (GYPSIE) study* was to investigate whether patients presenting with stroke have inherently more active platelets and whether this was due to a constitutively higher number of GPVI-dimers and/or increased GPVI-dimerization, representing a potentially controllable point of thrombosis in ischemic stroke

7.02 *GYPSIE* study aims

1) Identify whether patients who present with stroke express higher GPVI (total or dimer) on their platelet surface compared to healthy controls
2) Investigate whether these patients have more active platelets as demonstrated by increased P-selectin exposure compared to healthy controls
3) Investigate whether the observed GPVI expression and platelet activity is different at 90 days post stroke
4) Identify if there are any differences in platelet GPVI expression and platelet activity considering:
    d) Any effects exerted by antiplatelet, anticoagulation or hyperacute treatment
    e) Aetiology of stroke; ischaemic vs haemorrhagic and ischaemic sub-categories

7.03 *GYPSIE* study approvals and support

The GYPSIE study was conducted at Cambridge University Hospitals NHS Foundation Trust (CUH) between January 2015 and September 2018. The study protocol was given ethics approval by the East of England – Essex Ethics Committee (ref 14/EE/1062).

7.04 Participant eligibility to recruitment

(a) Inclusion and exclusion criteria

The stroke service at CUH admits around 800 patients with a confirmed stroke, per year. The vast majority of these are seen in the CUH emergency department (ED) and as a result of a coordinated improvement in services over the last ten-years, most patients are

bought over as 'blue light' emergencies by the ambulance service – allowing efficient triage, clerking, imaging and treatment within 4 hours of symptom onset.

Patients with suspected ischaemic or haemorrhagic stroke presenting to ED within 8 hours of symptom onset were invited to participate in the study. Those already on antiplatelets or anticoagulation were included, along with those given rtPA by the time of blood collection. For those unable to provide written informed consent at the time, their designated next of kin were approached, and the patient re-consented if capacity was regained. The recruitment to the study included a number of other strict inclusion and exclusion criteria illustrated in Table 7.1.

Inclusion	Exclusion
Presents to CUH ED with a suspected stroke (ischaemic or haemorrhagic) Time of onset of confirmed stroke symptoms within 8 hours of arrival in ED Patient is able to provide informed consent, or personal/nominated consultee advice is obtained indicating that patient can be enrolled	Stroke symptoms fully resolved in < 8 hours e.g. TIA Age less than 18 years Pregnancy Active or previous history of malignancy Known platelet disorder Haemoglobin <9.5 g\L or platelet count <150 x 10^9/L at the time of blood sampling Known HIV/AIDS Known hepatitis B or hepatitis C infection No active myocardial infarction

Table 7.1 Inclusion and exclusion criteria for the *GYPSIE* study population

(b) Sample size

At the time of commencing the GYPSIE study, the power calculations were based on published work on healthy donor blood carried out by Dr S Jung (Jung, 2012). This work established a mean copy number for GPVI-dimer of 860±215/platelet which then rises to 1175 and 1325 upon activation with CRP-XL or thrombin. Power calculations using the following study parameters: α 0.05, 1-β 0.8, suggested that we can resolve differences in GPVI-dimer expression between two donor populations (P <0.05) using a sample size of less than 20.

After the collection of pilot data, revised power calculations were carried out, as discussed in section 6.04b.

(c) Patient recruitment

As a Hyperacute stroke unit, patients with suspected stroke from within the region are admitted directly into the CUH ED where they are seen directly by a stroke nurse 24h a day. As this study required immediate access to the labs for same day analysis, the stroke nurses would inform the research team of any eligible patients between 0900-1630 on weekdays. Details of medical patients admitted to ED are also available on the CUH electronic patient medical record database.

Any individual who met the criteria for recruitment was therefore approached by the research team. As discussed, consent was obtained from the next of kin where informed consent was not possible and re-consented at a later date if applicable. Participants were consented to provide a venous blood sample to carry out study investigations within 8hours of stroke symptom onset (Figure 7.1).

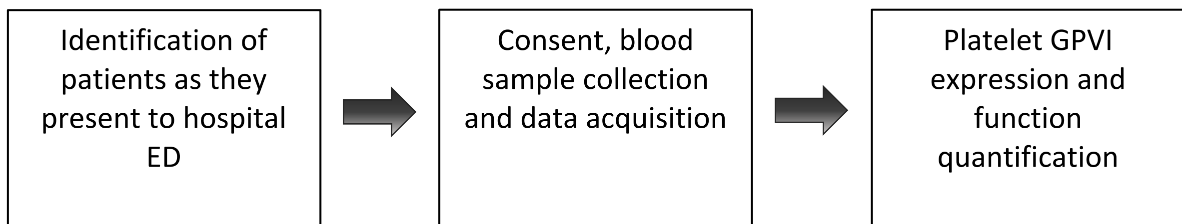


Figure 7.1 Flow diagram of patient approach to recruitment. Assessment, consenting, blood sampling and analysis was all carried out on the same day.

(d) Healthy control recruitment

Healthy donors who were deemed to be at low risk of stroke from the National Institute for Health Research Cambridge BioResource were invited to participate in the study alongside study participants as detailed previously in section 4.4.4. Therefore, the controls used were the same as those in the *GRAFITE* study, except 2 patients with AF were not excluded (n=301). As previously, all control participants were of the common GPVI haplotypes.

The following data were collected from the control cohort.

1. Basic demographic variables
2. Medication history: anti-hypertensive, statin, antiplatelet and anticoagulation
3. Clinical details: relevant vascular risk factors: hypertension, CCF, diabetes, hypercholesterolaemia, IHD and any previous stroke history.

7.05 Study Investigations

(a) Platelet GPVI, function and haematological investigations

Blood was obtained via venepuncture from patients who were willing to participate in the study as well as from the healthy control population as described previously in section 4.5.1. No serum biomarkers were analysed as part of this study. The blood tubes and amounts taken from patients and controls are outlined below.

S-monovette® 3 mL, 0.106 mol/L citrate tube

3. Platelet total GPVI (monomer and dimer) expression and GPVI-dimer expression
4. Platelet function:
   c) P-selectin expression without agonist or with of ADP or CRP-XL agonism
   d) Fibrinogen binding without agonist or with of ADP or CRP-XL agonism

S-monovette® 1.6 mL, 1.6 mg/mL potassium-EDTA tube

1. Full blood count

S-monovette® 3.4 mL, 1.6 mg/mL potassium-EDTA tube

S-monovette® 4.9 mL, 1.6 mg/mL potassium-EDTA tube

S-monovette® 4 mL, 1.6 mg/mL Serum Gel with Clotting Activator

3. Genotyping, gene expression
4. Metabolomic analysis

Done in collaboration with the Ouwehand Laboratory, Department of Haematology, University of Cambridge. Results not yet available, therefore, will not form part of this thesis.

(b) Clinical data collection

After consent was obtained from the patient, further details were gained from the patient's electronic medical notes including:

1. Basic demographic variables
2. Medication history: anti-hypertensive, statin, antiplatelet and anticoagulation
3. Clinical details: admission diagnosis, relevant vascular risk factors: hypertension, CCF, diabetes, AF, hypercholesterolaemia, IHD and any previous stroke history
4. Whether participants were thrombolysed and whether the blood sampling occurred before or after thrombolysis
5. Whether participants underwent thrombectomy
6. NIHSS on admission
7. Pre-admission CHA_2DS_2-VASc score

8. Modified Rankin Scale (mRS) at discharge (collected later)
9. Death within six months of stroke

7.06 Methods

The rationale for flow cytometry analysis of receptor expression and platelet function (section 6.01a) and methods on quantification of GPVI expression (section 6.06a) are preciously described. Fibrinogen binding was not measured in the whole cohort of patients during this study therefore only P-selectin exposure is reported for platelet function.

(a) Platelet function analysis using flow cytometry

To assess platelet activation, 5 μl of aspirinated (100 μM) and hirudinized (4 U/ml) citrated whole blood was added to 45 μl of HBS (after 5 minutes incubation) to make up final volume of 50 μL containing PE-conjugated anti-CD62P (Clone Thromb6, Bristol Institute for Transfusion Science, UK) at a final dilution of 1:50 and either

no agonist

3. HBS

or addition of exogenous agonists

2. ADP (final concentration of 0.5 μM, Sigma-Aldrich) or
4. CRP-XL (final concentration of 4 μg/ml; Farndale Laboratory, University of Cambridge).

For the CRP-XL–induced platelets, apyrase (4 U/mL, final concentration) was added to inhibit activation through the ADP-induced pathway (Sigma-Aldrich) before addition of CRP-XL.

All activation reactions were stopped after 20 minutes by adding 100-fold volume of saline containing 0.2% formyl (37% formaldehyde, 0.85% NaCl; Sigma-Aldrich) (further 10-minute incubation) prior to flow cytometry analysis. Negative controls for the anti-P-selectin were set using a 9E10 isotype control (Bristol Institute for Transfusion Science).

Platelets were identified by light scatter and results were recorded as the percentage of platelets positive (%PP) for the relevant activation marker, calculated as the percentage of platelets expressing the relevant activation marker with MFI greater than 98% of the isotype control.

(b) Stroke sub-classification

Electronic discharge letters from the direct care teams and inpatient medical records, which contain admission information and stroke aetiology, were used to subclassify ischaemic strokes as per the TOAST classification.(Adams, 1993) Ischaemic strokes were therefore classified into

1: large artery atherosclerosis (LAS)

2: cardioembolic stroke (CES)

3: small vessel occlusion (SVO)

4: stroke of other aetiology (Other)

5: stroke of undetermined aetiology (UD).

Haemorrhagic strokes (bleed) were not sub-classified. Electronic records were re-checked six months from discharge to see if stroke aetiology was deemed to have changed from the original classification and our data updated accordingly.

(c) Statistical analysis

A P value <0.05 was taken as statistically significant. Initially, simple linear regression analysis was used to determine association between total GPVI or GPVI-dimer expression with predictor variables. Age and MPV were significantly associated with GPVI expression in the stroke patients. Therefore, GPVI expression and P-selectin exposure results from all cohorts were adjusted for age and MPV and predicted values calculated from adjusted unstandardized residuals were subsequently used when comparing mean values between cohorts.

Data was tested for normality using the Kolmogorov-Smirnov test. Mean comparisons of continuous variables were carried out using either the paired or unpaired t-test for

parametric data or the Wilcoxon signed-rank (paired) or Mann-Whitney U (unpaired) test for non-parametric data. Non-categorical data was analysed using the Chi-squared test. Similarly, analysis of correlation was carried out using Pearson's test for parametric and Spearman testing for non-parametric data, respectively.

Age and MPV adjusted values were also entered into a simple linear regression analysis to determine association between total GPVI or GPVI-dimer expression with other single predictor variables. Significantly associated predictor variables ($P<0.05$) on linear regression were then tested to ensure they fulfil the criteria for regression diagnostics and entered into a multiple regression model to determine independent predictors for GPVI-dimer expression only, as this is the functional form of GPVI, and the molecule of interest for this study. Unstandardized coefficient (B), standard error (SE) and significance (*P*) are reported for each of the significantly associated variables. Furthermore, logistic regression analysis with stroke as the dependent variable and total GPVI, GPVI-dimer, age and MPV was used to see which variables were independently associated with stroke.

Main statistical analysis was carried out using PRISM v8.2 (Graph Pad, USA) and SPSS v.26.0 (IBM, USA) for the regression analyses.

7.07 Results

(a) Recruitment and population details

Results from 301 healthy individuals were included in the study. A total of 247 participants presenting with stroke symptoms were recruited over the study period. This included 186 stroke patients and 61 non-stroke patients, of which 8 had had TIAs and were therefore excluded from the final analysis. Non-stroke patients were defined as patients who presented with a suspected stroke to ED who were initially recruited but then subsequently were given an alternative diagnosis. 83 patients from the stroke and non-stroke cohorts were excluded due to; presence/history of malignancy, blood/platelet disorder or incomplete blood collection, errors in sample analysis or donor request (Figure 7.2). No patients were excluded from the study on the basis of stroke severity or acuity of illness.

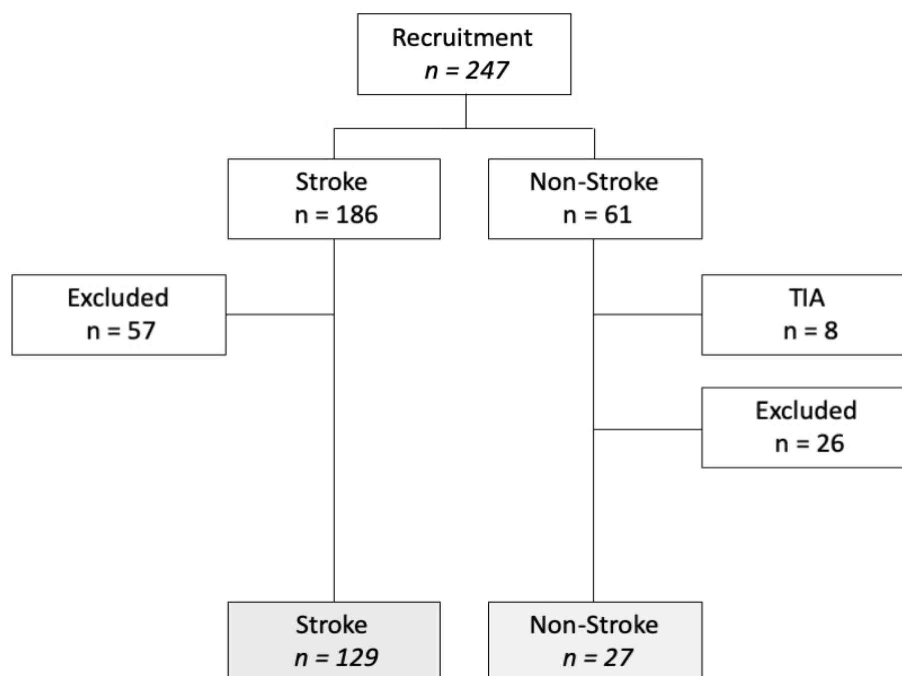


Figure 7.2 Flow diagram of all patients recruited prior to exclusion from study. The boxes shaded in grey depict the final numbers included in the analysis of stroke and non-stroke cohorts. The non-stroke cohort comprised of patients with a range of diagnoses as illustrated in Table 7.2.

Non-stroke diagnosis	Number of patients
Migraine	9
Syncope/Falls	6
Seizure	5
Sepsis	4
Carpal tunnel syndrome	1
Bell’s palsy	1
Non-specific symptoms	1

Table 7.2 The variety in final diagnoses of the included non-stroke patients (*n*=27*)*

Demographic details, blood parameters, comorbidities, admission scores and medication of the control, stroke and non-stroke population discussed below, are included in Table 7.3.

The control population were significantly younger compared to the stroke group and non-stroke groups (median age (IQR): controls, 60 years (47–68); stroke, 79 years (67–85), *P* <0.0001; non-stroke, 70 years (58–84), *P* <0.0001) and included a larger proportion of female participants compared to the stroke cohort (control, 65.8%; stroke, 38.0%, *P* <0.0001). Subsequent analysis however revealed no significant differences between total GPVI (*P*=0.30), GPVI-dimer (*P*=0.91) or resting P-selectin exposure (*P*=0.96) between males or females in the stroke cohort. Full blood count analysis revealed that although there were differences between the control and stroke cohorts in mean haemoglobin and platelet counts, they were well within normal parameters. The stroke population had significantly larger platelets as measured by MPV compared to controls, but not the non-stroke cohort (MPV (fL) $\pm$ SD: stroke 10.80$\pm$0.99, control 10.4$\pm$0.9; *P*=0.007, non-stroke, 10.61$\pm$0.87, *P*=0.35).

The control population also had fewer vascular risk factors compared to both the stroke and non-stroke groups (*P*<0.0001), whereas the risk-factors between the stroke and non-

stroke cohorts were not significantly different apart from a higher proportion of AF (*P*=0.005) and hypertension (*P*=0.03) in the stroke group. As expected, a higher proportion of stroke and non-stroke patients were taking aspirin, clopidogrel and a statin at admission compared to the control group (*P* <0.0001).

At admission, the stroke group had a median NIHSS of 8 (IQR 4–15). 50 patients received thrombolysis with alteplase. 4 of those patients also underwent thrombectomy and a further 2 patients had thrombectomy without prior thrombolysis. 33/50 patients who were thrombolysed had their blood taken after receiving thrombolysis and this is discussed later in this chapter.

The stroke and non-stroke cohorts were evenly matched in terms of quantified markers of illness severity such as C-RP (*P*=0.92) and NEWS score (*P*=0.21). The CHA_2DS_2-VASc scores were not significantly different between the stroke and non-stroke groups (*P*=0.97) but both were significantly higher than the control group (*P*<0.0001) reflecting the higher risk factor burden within the stroke and non-stroke cohorts. 24/129 (18.6%) patients admitted with stroke and 5/27 (18.5%) non-stroke patients died within six months of discharge from hospital.

	Control	Stroke 0-day	Non-Stroke	P^1	P^2	P^3
n	301	129	27			
Age	60 (47-68)	79 (67-85)	70 (58-84)	**<0.0001**	**0.0001**	0.18
Female (%)	65.8	38.0	59.3	**<0.0001**	0.53	0.06
Hemoglobin (g\L) ± SD	136.4±11.4	139.8±17.9	137.3±16.05	**0.02**	0.70	0.51
Platelet Count (10^x9/L)± SD	260.2±57.3	239.6±88.1	269.3±59.8	**0.004**	0.45	0.50
Mean platelet volume (fL)±SD	10.4±0.9	10.80±0.99	10.61±0.87	**0.007**	0.18	0.35
CHA_2DS_2-VASc Score	1 (1-2)	3 (2-4)	3 (1-5)	**<0.0001**	**<0.0001**	0.97
Admission NIHSS	-	8 (4-15)	-	-	-	-
Admission C-RP	NA	3.38 (1-7)	1.76 (1-5)	-	-	0.92
Admission NEWS	NA	1 (0-2)	1 (0-1)	-	-	0.93
Thrombolysed, n (%)	NA	50 (38.8)	NA	-	-	-
Thrombectomy, n (%)	NA	6 (4.7)	NA	-	-	-
Risk factors for thrombotic disease, n (%)						
Atrial Fibrillation	2 (0.66)	61 (47.3)	5 (18.5)	**<0.0001**	**<0.0001**	**0.005**
Congestive Cardiac Failure	0 (0)	9 (7.0)	4 (14.8)	**<0.0001**	**<0.0001**	0.24
Hypertension	27 (9.0)	94 (72.9)	14 (51.9)	**<0.0001**	**<0.0001**	**0.03**
Diabetes	1 (0.33)	24 (18.6)	4 (14.8)	**<0.0001**	**0.0002**	0.79
Ischemic Heart Disease	0 (0)	24 (18.6)	5 (18.5)	**<0.0001**	**<0.0001**	0.99
Cholesterol	24 (8.0)	50 (38.8)	7 (25.9)	**<0.0001**	**0.005**	0.27
Previous stroke	0 (0)	26 (20.2)	7 (25.9)	**<0.0001**	**<0.0001**	0.6
Admission medication, n (%)						
ACE inhibitor or ARB	21 (7.0)	24 (18.6)	3 (11.1)	**0.0006**	0.65	0.51
Aspirin	1 (0.33)	33 (25.6)	5 (18.5)	**<0.0001**	**<0.0001**	0.60
Clopidogrel	0 (0)	7 (5.4)	4 (14.8)	**0.0003**	**<0.0001**	0.18
Apixaban	0 (0)	1 (0.8)	1 (3.7)	0.66	0.13	0.77
Dabigatran	0 (0)	1 (0.8)	0 (0)	0.66	-	0.63
Rivaroxaban	1 (0.33)	3 (2.3)	1 (3.7)	0.44	0.39	0.73
Warfarin	0 (0)	9 (7.0)	0 (0)	**<0.0001**	**-**	0.34
Statin	22 (7.3)	36 (27.9)	10 (37.3)	**<0.0001**	**<0.0001**	0.48

Table 7.3 (previous page). Baseline characteristics of control, stroke and non-stroke groups at admission, including haematological parameters, vascular risk factors and admission details. Means are reported with standard deviation and medians with interquartile range in the parenthesis. Differences between cohorts were calculated using Student's t-test for parametric and Chi-squared test for non-parametric data and are reported as a *P* value.

P^1 = control vs. stroke 0 day, P^2 = control vs. non-stroke, P^3 = stroke 0 day vs. non-stroke

SD = standard deviation, IQR = interquartile range, NIHSS = National Institutes of Health Stroke Scale, C-RP = C-reactive protein, NEWS =National Early warning Score 2, ACE = angiotensin-converting-enzyme, ARB = angiotensin-receptor blocker

(b) General platelet characteristics of control and stroke cohorts

As discussed earlier, the platelet counts between groups were well within normal parameters, and the differences in the counts themselves are not likely to affect the flow cytometric quantification of GPVI expression or P-selectin exposure.

The stroke and non-stroke patients had significantly larger platelets, as measured by MPV (Table 7.3). Age and MPV were not correlated in the control or stroke cohorts (Supplementary Figure 7.3).

As GPVI-dimer constitutes part of the total GPVI measurements, therefore, as expected, the MFI of GPVI-dimer expression was significantly correlated with total GPVI expression in the control (r^2=0.14, *P*<0.0001) and stroke (r^2=0.49, *P*<0.0001) cohorts (Figure 7.3).

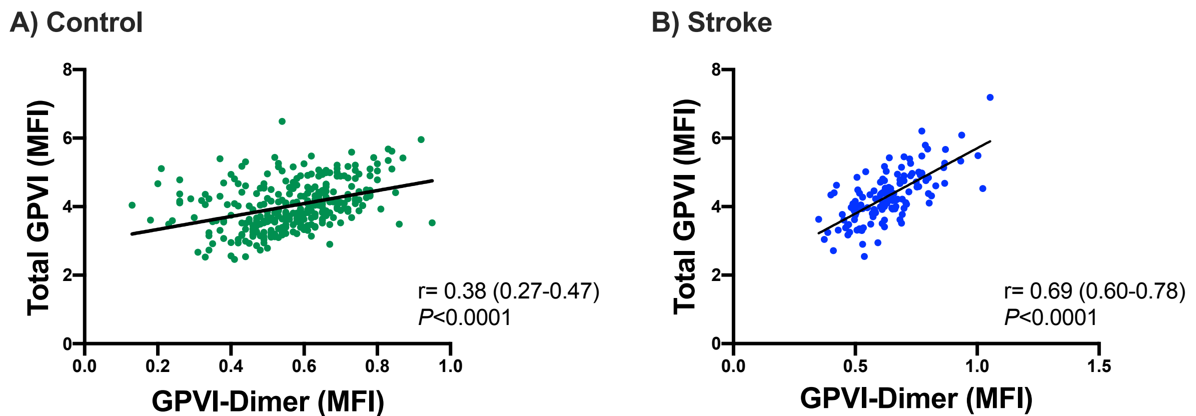
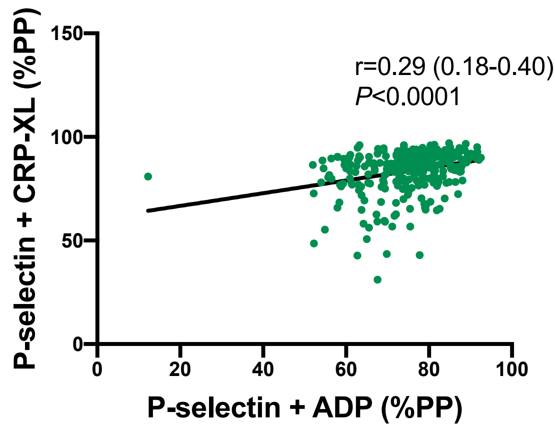


Figure 7.3 Correlation between total GPVI and GPVI-dimer in A) controls **B)** stroke population at day 0, calculated using Pearson correlation coefficient. Black line represents regression line calculated using simple linear regression and Pearson R value and 95% confidence interval is presented within each image.

P-selectin exposure after exogenous addition of ADP was correlated to P-selectin exposure after CRP-XL addition in controls (control, r=0.21, *P*<0.0001) and stroke patients (r=0.41, *P*<0.0001) (Figure 7.4)

A) Control



B) Stroke

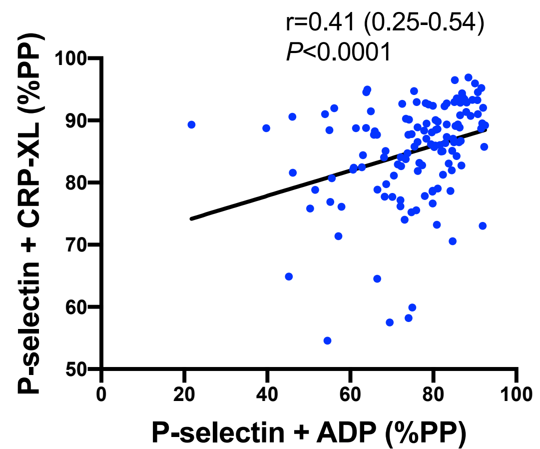


Figure 7.4 Correlation between P-selectin exposure after ADP addition to P-selectin exposure after CRP-XL in A) controls **B)** stroke population at day 0. Correlations calculated using Spearman's rank correlation coefficient and regression line is presented in black.

Interestingly, P-selectin exposure after CRP-XL addition in the stroke population was also not correlated with total GPVI-expression (r=0.07, *P*=0.40) or resting P-selectin exposure (r=0.1, *P*=0.26). GPVI-dimer expression was however correlated with P-selectin exposure after CRP-XL addition (r=0.33, *P*=0.0002).

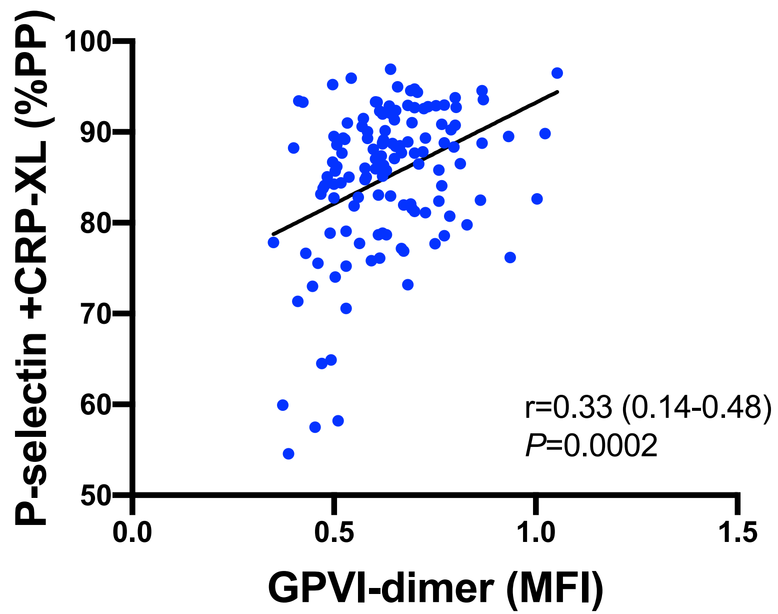


Figure 7.5 Correlation between GPVI-dimer and P-selectin exposure after CRP-XL in the stroke population at day 0. Correlations calculated using Spearman's rank correlation coefficient. Black line represents simple linear regression line.

(c) GPVI expression in control compared with stroke and non-stroke groups

Compared to controls, stroke patients expressed significantly higher GPVI-dimer (MFI±SD; control 0.566± 0.01, stroke 0.633±0.105, *P*<0.0001) and total GPVI (MFI±SD; control 4.027±0.08, stroke 4.294±0.32, *P*<0.0001) at admission.

Compared to the non-stroke group, who had a similar risk factor profile to the stroke cohort, stroke patients again demonstrated higher GPVI-dimer (MFI ± SD; non-stroke 0.543±0.03, *P*<0.0001), and total GPVI expression (MFI ± SD; non-stroke 3.954±0.91, *P*<0.0001).

Neither the total GPVI or GPVI-dimer expression was significantly different between control and non-stroke patients (Table 7.4, Figure 7.6).

	Control (n=301)	**Stroke 0-day (n=129)**	**Non-Stroke (n=27)**	P^1	P^2	P^3
Platelet GPVI expression (MFI)±SD						
Total	4.027±0.08	4.294±0.32	3.952±0.30	**<0.0001**	0.42	**<0.0001**
Dimer	0.566± 0.01	0.633±0.05	0.543±0.03	**<0.0001**	0.20	**<0.0001**

Table 7.4. Total GPVI and GPVI-dimer expression as measured by median fluorescence intensity (MFI) in the control cohort compared to the stroke and non-stroke cohorts. P^1 = control vs stroke 0 day, P^2 = control vs non-stroke, P^3 = stroke 0-day vs non-stroke.

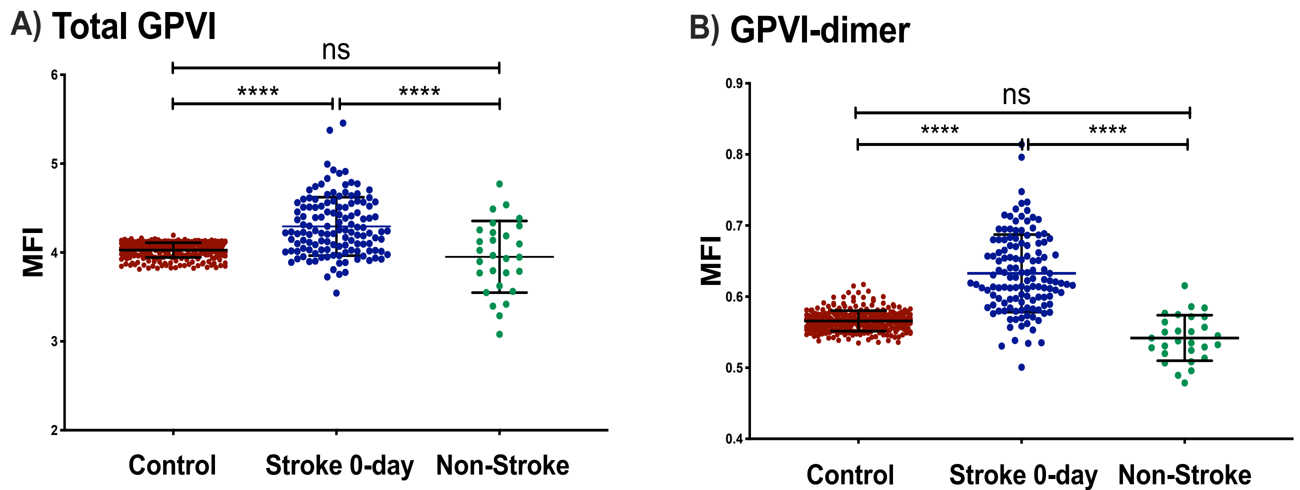


Figure 7.6. GPVI expression between control, stroke and non-stroke cohorts. A) Total GPVI and **B)** GPVI-dimer are both significantly higher in the stroke population at admission compared to the control and non-stroke cohorts (P<0.0001). The error bars represent the mean MFI of each of the cohorts ± SD

(d) Platelet P-selectin exposure in control, stroke and non-stroke groups

Even though GPVI expression was not different between control and non-stroke cohorts, resting platelets from both stroke and non-stroke patients already showed some activation compared to the controls as indicated by higher P-selectin exposure (P<0.0001). Furthermore, stroke patients demonstrated significantly more P-selectin exposure after ADP stimulation compared to non-stroke patients and more P-selectin exposure after CRP-XL stimulation compared to both control and non-stroke patients (Table 7.5 and Figure 7.7).

	Control (n=301)	Stroke 0-day (n=129)	Non-Stroke (n=27)	P^1	P^2	P^3
Median (Q_1-Q_3) P-Selectin exposure (%PP)						
Resting	19.8 (19.7-19.9)	33.2 (28.6-33.2)	29.6 (28.5-30.3)	**<0.0001**	**<0.0001**	**<0.0001**
+ADP	75.4 (73.7-76.4)	75.2 (73.9-77.1)	71.4 (68.7-73.6)	0.18	**<0.0001**	**<0.0001**
+CRP-XL	83.5 (82.5-84.6)	85.4 (84.1-86.2)	82.3 (79.4-85.1)	**<0.0001**	0.07	**<0.0001**

Table 7.5. P-selectin exposure (%PP) in control, stroke and non-stroke populations. P^1 = control vs stroke 0 day, P^2 = control vs non-stroke, P^3 = stroke 0-day vs non-stroke.

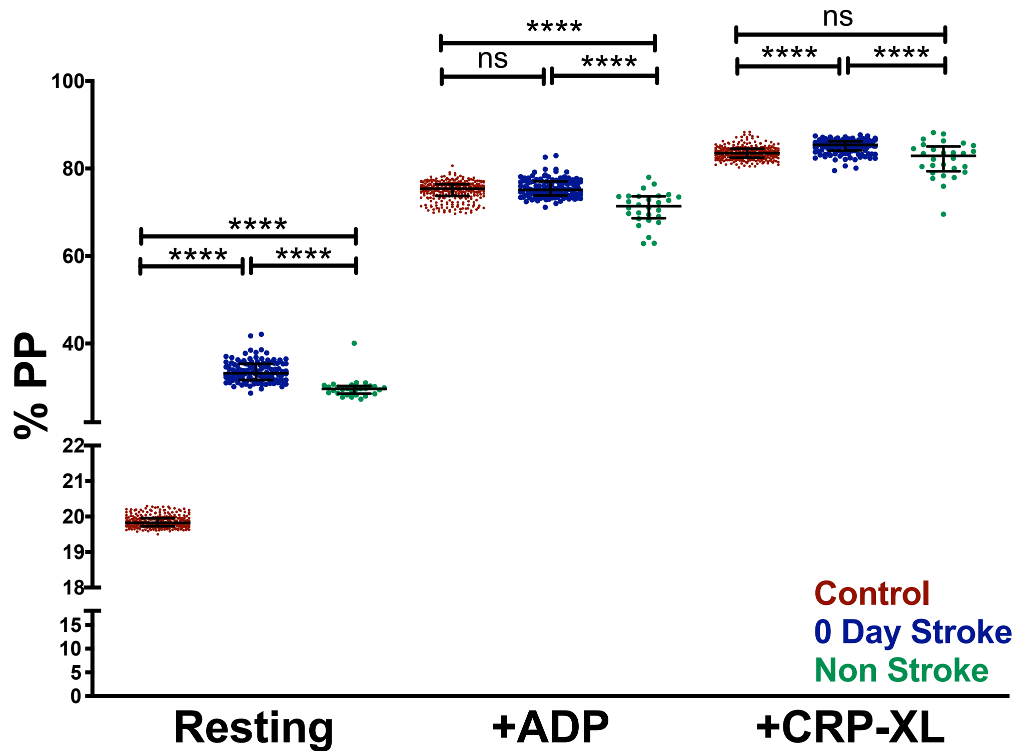


Figure 7.7. P-selectin exposure between control, stroke and non-stroke cohorts. A) P-Selectin exposure in resting platelets in control, stroke and non-stroke cohorts and after ADP or CRP-XL addition. The error bars represent the median (Q_1-Q_3) %PP of each of the cohorts.

(e) GPVI expression and P-selectin exposure and day 0 + 90 in stroke patients

Out of the 129 patients, 114 had had ischaemic strokes of different aetiologies. From those, excluding those who had died, discharged to nursing care, were too frail to attend follow up or those originally from another geographical area, 51 ischaemic stroke patients who attended their 3-month follow up (median days to follow up (IQR): 110 (92-128)) were consented to give a repeat blood sample which was analysed for GPVI expression and platelet function as before. This will be referred to as the 'day-90'sample and was included in the analysis to facilitate comparison of GPVI expression and platelet function at day-0 and day-90 post stroke.

Comparing the 51 patients who were followed up with the 78 who were not (table 7.5) reveals that those who were followed up were significantly younger (P=0.002) and had a greater proportion of male subjects (P=0.003). As expected, those who were not followed up were more disabled on discharge, as measured by mRS (P<0.0001) and a significantly higher proportion had died by six months (P=0.0004). Interestingly, there were no significant differences in admission parameters and comorbidities (except for CCF) between the two groups.

	Day 90 Follow up	No Day 90 follow up	Significance
n	51	78	
Age	71.5 (61-81)	80 (72-80)	**0.002**
Female (%)	11 (21.6)	38 (48.7)	**0.003**
Hemoglobin (g\L) ± SD	143.3±16.8	137.5±18.3	0.07
Platelet Count (10^x9/L)± SD	231.1±74	244.9±96	0.39
Mean platelet volume (fL) ± SD	10.87±1.1	10.82±0.9	0.80
CHA_2DS_2-VASc Score	3 (1-4)	3 (3-5)	0.10
Admission NIHSS	7.5 (4-14)	9 (5-16)	0.22
Admission C-RP	3.2 (1-6)	3.4 (2-8)	0.67
Admission NEWS	1 (0-2)	1 (0-2)	0.19
Discharge mRS	1 (1-2)	3 (1-5)	**<0.0001**
Death within 6 months *n*, (%)	2 (3.9)	22 (28.2)	**0.0004**
Risk factors for thrombotic disease, n (%)			
Atrial Fibrillation	23 (45.1)	48 (48.7)	0.07
Congestive Cardiac Failure	0 (0.0)	9 (11.5)	**0.01**
Hypertension	38 (74.5)	56 (71.8)	0.84
Diabetes	7 (13.7)	17 (21.8)	0.36
Ischemic Heart Disease	8 (15.7)	16 (20.5)	0.64
Cholesterol	23 (45.1)	27 (34.6)	0.27
Previous stroke	12 (23.5)	14 (18.0)	0.50

Table 7.6. Characteristics of the stroke cohort who were followed up at day-90, compared to those who were not; including haematological parameters, vascular risk factors and admission details. Means are reported with standard deviation and medians with interquartile range in the parenthesis. Differences between cohorts were calculated using Student's t-test for parametric and Chi-squared test for non-parametric data and are reported as a *P* value (significance).

Total GPVI expression not different at day 90 compared to day-0 (day-0, 4.262±0.36; day-90, 4.220±0.36, *P*=0.52) however, GPVI-dimer levels were significantly higher (day-0, 0.626±0.056 day-90, 0.686±0.09, *P*<0.0001).

Interestingly, despite an observation of significantly increased GPVI-dimer expression, resting P-selectin is in fact significantly lower at day-90 compared to admission (day-0, 33.5 (32.8-34.2); day-90 30.44 (29.7-31.1), *P*<0.0001). Similarly, P-selectin exposure after ADP (day-0, 75.60 (74.8-76.4; day-90 69.26 (67.5-71.1), *P*<0.0001) and CRP-XL (day 0, 84.65 (84.3-86.0); day-90, 83.37 (83.0-84.0), *P*=0.0001) stimulation at day-90 is also lower compared to day-0 (Figure 7.8).

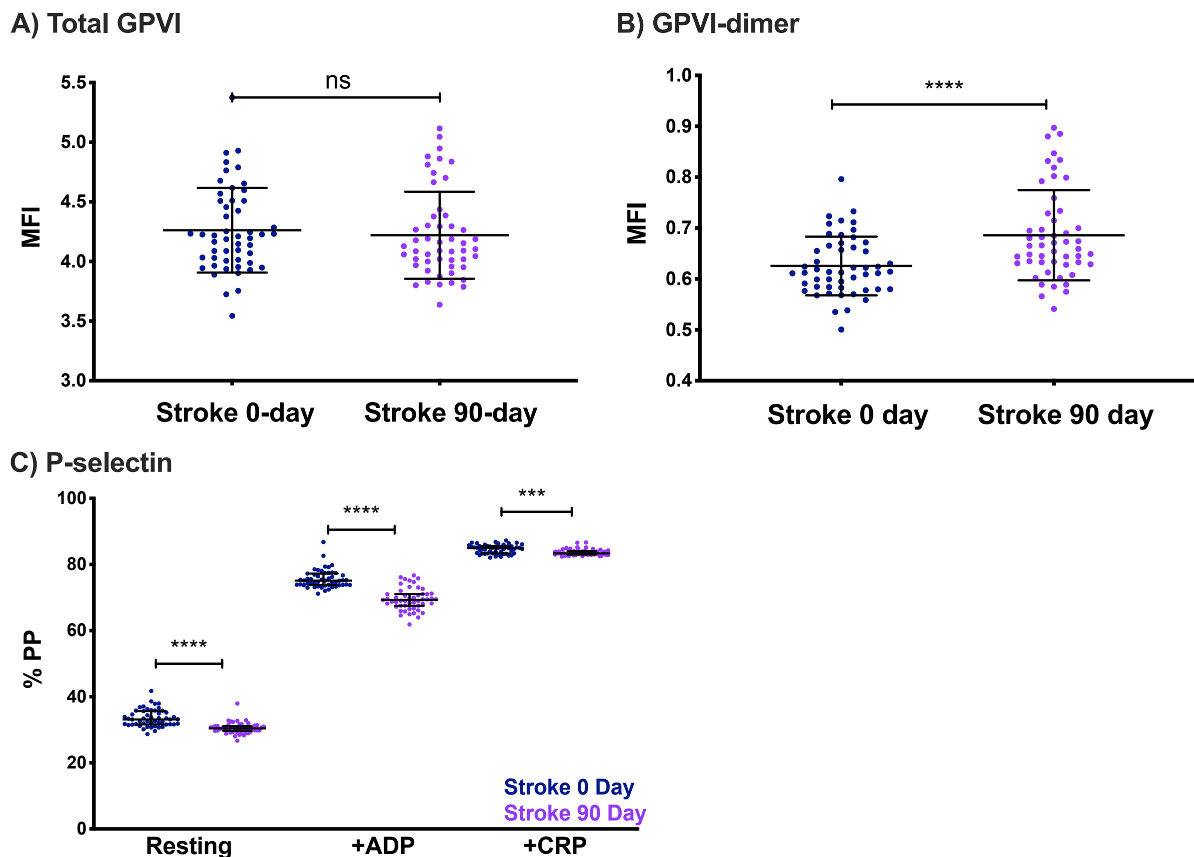


Figure 7.8. Matched GPVI expression and P-selectin exposure between ischaemic stroke patients at day 0 and at day 90 (n=51). A) Total GPVI expression **B)** GPVI-dimer expression at day 0 and day 90 post stroke. The error bars represent the mean MFI of each of the cohorts ± SD. **C)** P-selectin exposure in resting, +ADP or +CRP-XL at day 0 or day 90 post stroke. The error bars represent the median (Q_1-Q_3) %PP of each of the cohorts.

At their follow up appointment, more patients had been started on a DOAC or clopidogrel, compared to when they were admitted (Table 7.6). The fact that the GPVI-dimer levels are higher despite more patients being started on antiplatelets and anticoagulation suggests that conventional antithrombotic medication probably does not impact GPVI-dimer expression. The effect of antiplatelet therapy we do observe is as a significantly impaired P-selectin response after ADP addition in patients on clopidogrel, as expected, at the time of venepuncture at day 90 which is discussed in more detail in section 7.1.7.

Medication	Day 0	Day 90
Aspirin	11	**13**
Clopidogrel	2	**18**
Warfarin	3	1
DOAC	2	**17**

Table 7.7 Number of patients on antithrombotic medication at day 0 compared to day 90

(f) GPVI expression according to stroke aetiology

All ischaemic stroke patients were sub-classified by stroke aetiology as described in the methods. Haemorrhagic strokes (bleed) were not further sub-classified and sub-arachnoid haemorrhages were not recruited.

129 patients were categorized into LAS (n=17), CES (n=56), SVO (n=18), Other (n=2), UD (n=21) and bleed (n=15). Data from stroke patients classified into strokes of Other aetiology were excluded due to low numbers and UD were excluded due to ambiguity in interpreting their aetiological significance.

All remaining stroke subtypes (LAS, CES, SVO, bleed) demonstrated significantly higher P-selectin exposure compared to controls ($P<0.0001$, all categories) suggesting that patients who have had a stroke have more activated circulating platelets compared to controls. Furthermore, all strokes, whether ischaemic or haemorrhagic, demonstrated significantly

higher total GPVI ($P<0.0001$, all categories) and GPVI-dimer expression ($P<0.0001$, all categories) compared controls (Figure 7.9)

There were no significant differences when comparing total GPVI ($P=0.33$) GPVI-dimer ($P=0.29$) or resting P-selectin exposure ($P=0.57$) between all ischaemic (n=91) and haemorrhagic strokes (n=15) (not shown).

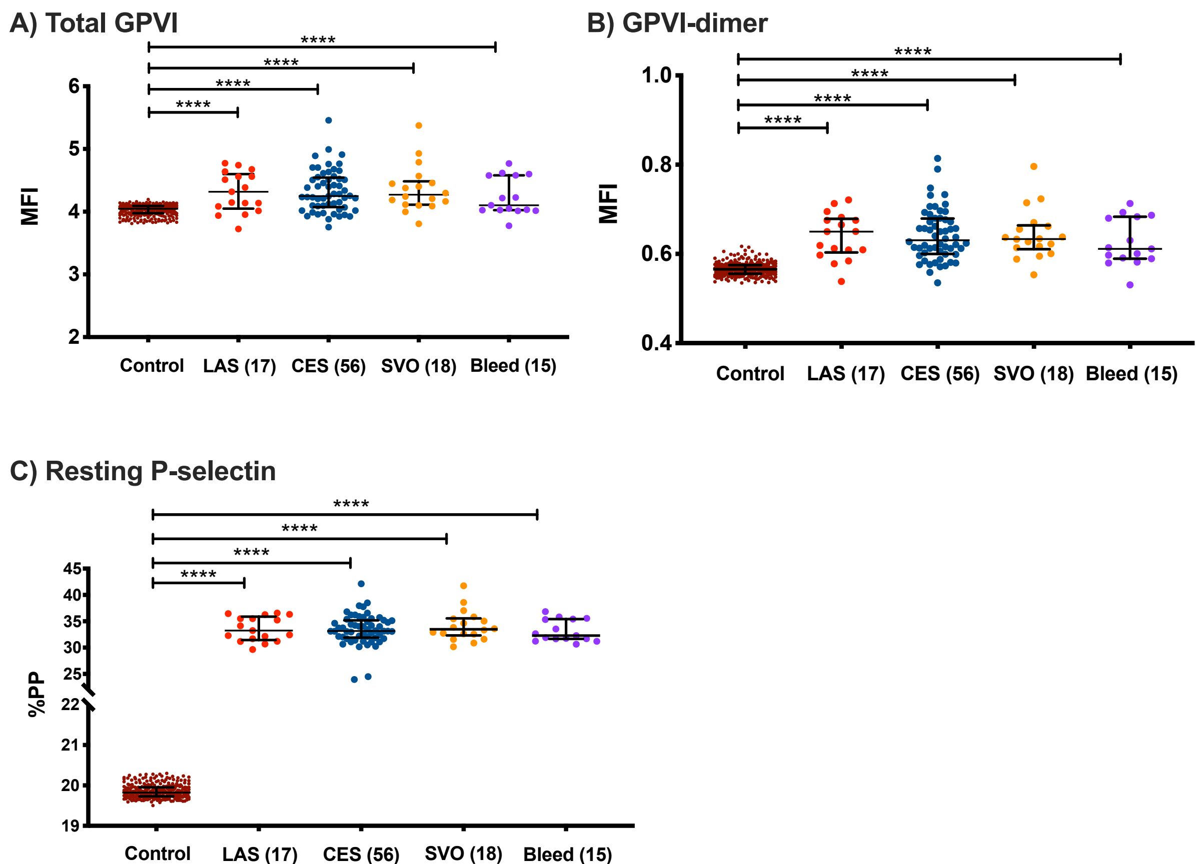


Figure 7.9 A) Total GPVI expression **B)** GPVI-dimer expression **C)** Resting P-selectin exposure between control, subclassified ischaemic strokes and haemorrhagic strokes. Error bars median (Q_1-Q_3) %PP. Numbers in parentheses represent the number of participants within in group.

(g) GPVI expression with antiplatelet, anticoagulation and thrombolysis

One important question is whether antithrombotic medication or receiving thrombolysis effects GPVI expression or platelet function. At admission 33 (25.6%) patients in the stroke cohort were on aspirin, 7 (5.4%) on clopidogrel and 14 (10.8%) on anticoagulation (1 apixaban, 1 dabigatran, 3 rivaroxaban, 9 warfarin). 50 (38.8%) patients received thrombolysis.

Total GPVI (P=0.37), GPVI-dimer (P=0.14) expression and resting P-selectin exposure (P=0.58) were not significantly different in those taking antiplatelets at admission, compared to those not taking antiplatelets or anticoagulation at admission. Similarly, there were no differences in any of the measured parameters between those taking antiplatelets or anticoagulation. 11 patients who were newly started on clopidogrel by their day-90 sample (i.e. were not on clopidogrel on admission) demonstrated significantly lower P-selectin exposure after ADP stimulation at day-90 (P=0.002) (Figure 7.10), although their total GPVI (P=0.46), GPVI-dimer (P=0.11) or resting P-selectin exposure were not different (P=0.12).

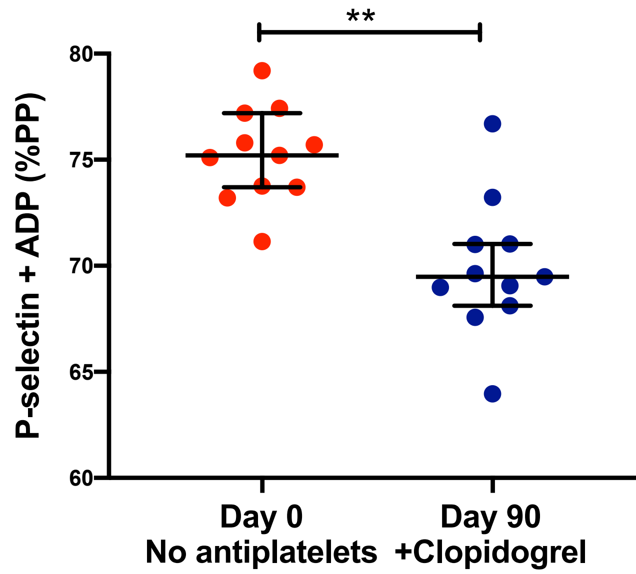


Figure 7.10 P-selectin exposure after exogenous addition of ADP in patients who were on clopidogrel by day 90 but not on antiplatelets at admission. Error bars represent median (Q_1-Q_3) %PP.

There were also no differences in patients who were thrombolysed compared to those who were not in terms of total GPVI (*P*=0.94) or GPVI-dimer expression (*P*=0.74) (Figure 7.11)A.

Similarly, resting P-selectin (*P*=0.82) or P-selectin exposure after addition of ADP (*P*=0.97) or CRP-XL (*P*=0.90) were also not different between these cohorts.

We then analysed whether blood sampling after thrombolysis made any difference to GPVI expression or platelet function. There were no differences observed in total GPVI (*P*=0.62) or GPVI-dimer (*P*=0.51) expression (Figure 7.11B) and as above no differences in P-selectin exposure was observed either resting (*P*=0.74) or after ADP (*P*=0.71) or CRP-XL stimulation (*P*=0.83).

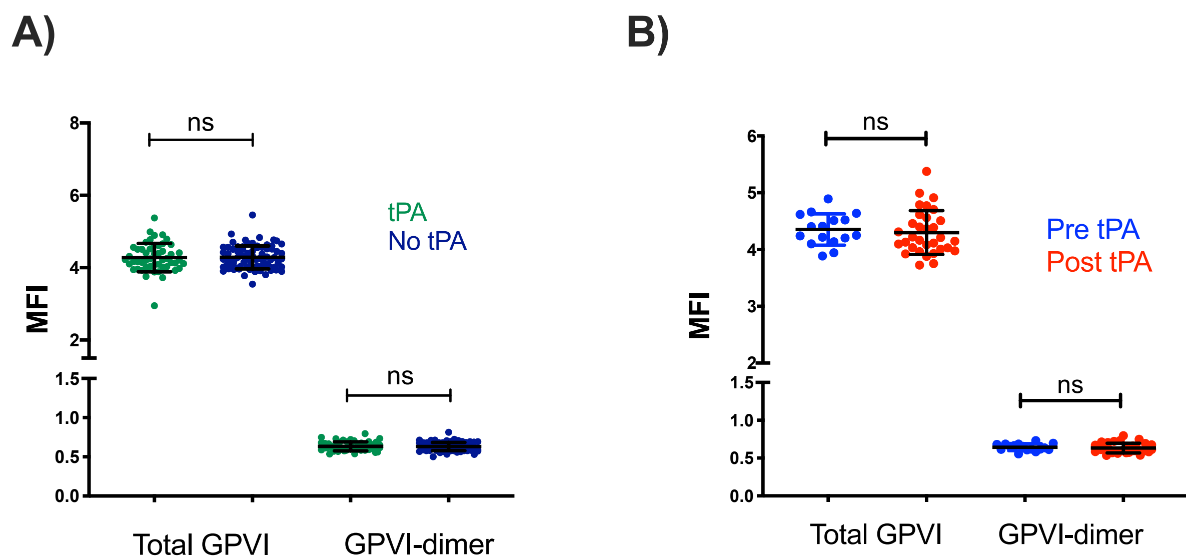


Figure 7.11 Total GPVI and GPVI-dimer expression between **A)** thrombolysed (n=50) compared to not thrombolysed (n=79) and **B)** blood taken pre-thrombolysis (n=16) compared to post-thrombolysis (n=32). The error bars represent the mean MFI of each of the cohorts ± SD.

(h) Variables associated with GPVI expression in stroke patients

Simple linear regression was used in order to determine the relationship between total GPVI or GPVI-dimer expression and a single predictor variable.

In stroke patients, the presence of AF and diabetes were both significantly associated with total GPVI (AF B 0.13, SE 0.06, *P*=0.02; diabetes B 0.2, SE 0.07, *P*=0.008) B and GPVI-dimer (AF B 0.02, SE 0.009, *P*=0.01; diabetes B 0.03, SE 0.01, *P*=0.01) expression. Hypertension was also a significantly associated with GPVI-dimer expression only (B 0.02, SE 0.01, *P*=0.02).

Furthermore, parameters such as the CHA_2DS_2-VASc score of all stroke patients and admission NIHSS and discharge mRS were both associated with total GPVI (CHA_2DS_2-VASc B 0.05, SE 0.02, *P*=0.001; NIHSS B 0.01, SE 0.005, *P*=0.02). and GPVI-dimer expression. (CHA_2DS_2-VASc B 0.01, SE 0.002, *P*<0.0001; NIHSS B 0.002, SE 0.001, *P*=0.009). Discharge mRS was also associated with GPVI-dimer expression (B 0.005, SE 0.002, *P*=0.03), but not total GPVI (Table 7.8). Neither gender, or any of the markers of illness acuity measured on admission within the stroke patients (C-RP and NEWS) were associated with GPVI expression.

	Total GPVI		GPVI-dimer	
	Coefficient B	Significance (P)	Coefficient B	Significance (P)
Female	0.08	0.07	0.02	0.08
Comorbidities				
Atrial Fibrillation	**0.13**	**0.02**	**0.02**	**0.01**
CCF	0.06	0.58	0.01	0.47
Hypertension	0.12	0.07	**0.02**	**0.02**
Diabetes	**0.20**	**0.008**	**0.03**	**0.01**
Ischaemic Heart Disease	0.1	0.18	0.01	0.10
Cholesterol	0.01	0.82	0.002	0.87
Previous Stroke	-0.01	0.91	-0.003	0.81
Admission and discharge parameters				
Antiplatelets at admission	0.00	0.89	0.00	0.78
CHA_2DS_2-VASc score	**0.05**	**0.001**	**0.01**	**<0.0001**
Admission NIHSS score	**0.01**	**0.02**	**0.002**	**0.009**
Admission C-RP	0.00	0.83	0.00	0.65
Admission NEWS score	0.005	0.78	0.01	0.60
Discharge mRS	0.02	0.16	**0.005**	**0.03**
Death within six months	0.08	0.23	0.02	0.12

Table 7.8 Simple linear regression to determine associations between single predictor variables and total GPVI or GPVI-dimer expression.

The variables that were significantly associated with GPVI-dimer expression were then modelled using multiple linear regression (Table 7.8). This analysis revealed that only the CHA_2DS_2-VASc score, a composite score of vascular risk factors (usually reserved for AF patients but in this case calculated for all stroke patients) was independently associated with GPVI-dimer expression.

GPVI dimer	**Coefficient B**	**Standard Error**	**Significance (*P*)**
CHA_2DS_2-VASc score	**0.008**	**0.004**	**0.03**
Atrial Fibrillation	0.018	0.01	0.06
Admission NIHSS score	0.001	0.001	0.11
Diabetes	0.006	0.014	0.67
Hypertension	0.005	0.013	0.72
Discharge mRS	-0.001	0.003	0.75

Model summary: Adjusted R^2 = 0.14, *P*=0.001

Table 7.9 Multiple regression analysis of factors associated with GPVI-dimer expression. There was no multicollinearity between any of the tested variables in this model.

(i) Predictors of stroke in the whole population

The whole population of study participants (control, stroke and non-stroke; n=457) were dichotomised into whether they had had a stroke or not, which was then used as the dependent variable in a multivariable binary logistic regression analysis. Unadjusted values of total GPVI, GPVI-dimer, age and MPV were tested as independent variables.

Parameters	**Odds Ratio**	**95% CI**	**Significance (*P*)**
Age (years)	**1.08**	**1.06 – 1.10**	**<0.0001**
MPV (fL)	**1.42**	**1.08 – 1.83**	**0.01**
GPVI dimer	**10.4**	**1.4 – 76.5**	**0.02**
Total GPVI	1.07	0.74 – 1.55	0.73

Table 7.10 Binary logistic regression analysis of parameters: total GPVI, GPVI-dimer, age and MPV, to assess for predictors of stroke.

The results suggest GPVI-dimer expression is an independent predictor of stroke (Odds Ratio 10.4 (1.4-76.5), *P*=0.02), when modelled together with total GPVI, age and MPV.

7.08 Discussion

Through its unique ability to bind collagen and fibrin, platelet receptor GPVI has been the subject of extensive research in the last decade, especially the consideration of its inhibition as a novel antithrombotic target in clinical studies. Despite this, many questions remain, especially focused around the relationship between GPVI expression and thrombotic disease in vivo, as well as the relationship between the GPVI-monomer and dimer in the setting of thrombotic disease.

Previous clinical studies investigating GPVI in thrombotic disease have either focussed on quantifying soluble GPVI (sGPVI); the metalloproteinase-cleaved ectodomain of GPVI shed from the platelet upon activation (Gardiner, 2004; Rabie, 2007) or the surface expression of platelet total GPVI (Bigalke, 2010; Wurster, 2013). Plasma sGPVI measurements in stroke patients have demonstrated increased levels in LAS, but not SVO and CES subtypes (Al-Tamimi, 2011) and others have reported that sGPVI levels were lower in stroke patients than in TIA (Wurster, 2013). These mixed results in stroke are difficult to translate into clinical use. Conversely, platelet expression of total GPVI has consistently been shown to be increased in ischaemic stroke and TIA patients compared to non-stroke patients (Bigalke, 2010; Wurster, 2013) and this result also extends to patients with acute coronary syndrome (Bigalke, 2010). This is important because the adhesive and signalling abilities of platelets have been shown to be proportional to the GPVI density on the platelet, implying a potential role for it in thrombus formation and stroke (Chen, 2002). However, despite their practicality, none of the previous work has quantified platelet expression of the dimeric active form of GPVI, which would be the target for any pharmacological intervention in thrombotic disease.

<u>The main results from this chapter are as follows</u>

1) Patients with stroke express more total GPVI ($P<0.0001$) and GPVI-dimer ($P<0.0001$) on their platelet surface compared to healthy controls and non-stroke patients.

2) GPVI-dimer expression at 'day-90' post stroke is significantly higher than at admission (P=0.0007), whereas total GPVI is not different (P=0.07) and P-selectin exposure is significantly less (P<0.0001).
3) All stroke patients, whether subclassified by ischaemic stroke categories or into haemorrhagic stroke, express higher GPVI-dimer compared to the control population (P<0.0001).
4) The presence of AF (P=0.01), hypertension (P=0.02) and diabetes (P=0.01) are associated with higher GPVI-dimer expression. Higher GPVI-dimer expression was an independent predictor of stroke within our population (P=0.02).
5) The elevation of GPVI-dimer observed in stroke patients is not reversed by existing anti-thrombotic medication.

This study represents the first piece of work to investigate both total GPVI and GPVI-dimer in a cohort of patients presenting to hospital with stroke. Our results agree with previous work by Bigalke and colleagues who observed higher total GPVI expression in stroke and TIA patients compared to a control population (Bigalke, 2010). Our novel finding however, is that stroke patients also have more GPVI-dimers on their platelet surface at the time of their stroke compared to both a control and non-stroke population (Figure 7.6).

What is yet to be conclusively established is whether the over-expression of GPVI-dimer specifically to stroke patients is due to presence of vascular risk-factors, whether the increased dimer expression is due to the acute stroke itself, or both. There certainly maybe a risk-factor driven element to GPVI-dimerisation. This is evidenced from our results since AF, an inflammatory condition known to modulate platelet activity (Guo, 2012; Harada, 2015), hypertension and diabetes as well as a higher CHA_2DS_2-VASc score, a composite score made up of individual stroke risk-factors, were all significant predictors of higher GPVI-dimer expression (Table 7.8). Therefore, it may be likely that the stroke patients had more GPVI-dimer on the platelets prior to their stroke, inherently increasing their stroke-risk. This is further evidenced as even in the control group, the presence of hypertension was predictive of higher GPVI-dimer (but not total GPVI) expression (B 0.06, SE 0.02, P<0.0001).

It is likely that vascular risk factors also play a role in driving P-selectin exposure, as both the stroke and non-stroke cohorts, who had similar proportion of risk factors demonstrated more resting P-selectin exposure on their circulating platelets (Table 7.5). However, we did not observe higher GPVI-dimer expression in the non-stroke patients compared to the stroke patients (Table 7.4), suggesting that resting platelet P-selectin exposure may not necessarily relate to thrombotic risk through a GPVI pathway. This is further highlighted by the fact that resting P-selectin was not correlated to GPVI expression (Supplementary Figure 7.4) in stroke patients. What we do observe is that GPVI levels could govern platelet response to exogenous agonists. P-selectin exposure after CRP-XL addition strongly correlated to GPVI-dimer but not total GPVI expression in the stroke cohort (Figure 7.5) and even though P-selectin exposure post ADP was not related to GPVI expression, overall P-selectin exposure after CRP-XL and ADP addition were highly correlated (Figure 7.4). This means that whereas GPVI expression and resting platelet activation appear unrelated, a significant proportion of the variability of platelet activation in response to collagen is due to GPVI-dimer levels and the remainder is due to other shared, or non-GPVI related pathways.

Analysis of platelets from patients at day-90 post-stroke reveals that GPVI-dimer expression, but not total GPVI or P-selectin exposure, was significantly higher compared to day-0 (Figure 7.8). This could be because acute stroke conditions on day-0 could actually favour GPVI-dimer shedding, whereas by day-90 this is no longer the case. This could result in an apparently higher GPVI-dimer level at day-90, which in fact may more accurately reflect the constitutive number of dimers present in the patients' platelets prior to stroke. Conversely, P-selectin exposure appears to decline from day-0 to 90 in stroke patients, returning to levels similar to that of the non-stroke group. This suggests an acute rise approximate to time of stroke, before returning to previous levels as described in the literature previously (Marquardt, 2002). Our results do not point to an antiplatelet effect apart from P-selectin exposure after ADP addition in patients taking clopidogrel, however antiplatelet effects on suppressing P-selectin exposure has also been described in the literature (Bath, 2017; Fox, 2019) and may play a role in the significantly lower P-selectin exposure seen at day-90.

At admission, GPVI-dimer expression was higher in all ischemic stroke subtypes and all haemorrhagic stroke patients (Figure 7.9). This is not surprising, as ischaemic and haemorrhagic stroke patients share similar risk-factor profiles, and therefore would be expected to demonstrate higher GPVI expression. Although there were only 15 haemorrhagic strokes, compared to 91 ischaemic ones (excluding UD and Other), there were no observed differences between GPVI expression between the two stroke types. It is likely that those patients who had a haemorrhagic stroke would also have been at risk of ischaemic stroke, and that GPVI expression is unlikely to play a differentiating role here.

Our results finally demonstrate that GPVI-dimer expression was predictive of stroke independent of age, MPV and crucially, total GPVI (Table 7.10) and secondly that a higher admission NIHSS score, which represents a higher stroke severity was associated with higher GPVI expression (Table 7.8).

To further the quest for new anti-thrombotic targets in ischemic cardio- or cerebrovascular disease, we demonstrate that GPVI-dimer, the functional form of the platelet-collagen receptor, is over-expressed in stroke patients and could emerge as a viable and safe option to inhibit platelet activity. GPVI-dimer inhibition, in contrast to drugs targeting other platelet receptors such as integrin αIIbβ3 (Ciccone, 2014), may not cause the unwanted side-effect of bleeding, since GPVI-deficient patients exhibit little bleeding tendency (Sugiyama, 1987; M Moroi, 1989). This is consistent with observations that GPVI inhibition in aged and comorbid mice has proved protective against ischemic stroke without increasing bleeding complications (Kraft, 2015), and with translational studies in healthy volunteers showing early promise (Ungerer, 2011; Voors-Pette, 2019). Further work will need to be carried out in the context of clinical trials to fully assess the risks and benefits of GPVI inhibition in patients with, or at risk of, thrombotic disease.

The purpose of this study was to explore the presence and significance of GPVI-dimer on the platelet surface, and especially its relationship with platelet activation in the presence of cardio- and cerebrovascular risk factors and thrombotic disease. Crucially, we demonstrate that stroke patients overexpress GPVI-dimer at least until day 90 post-stroke,

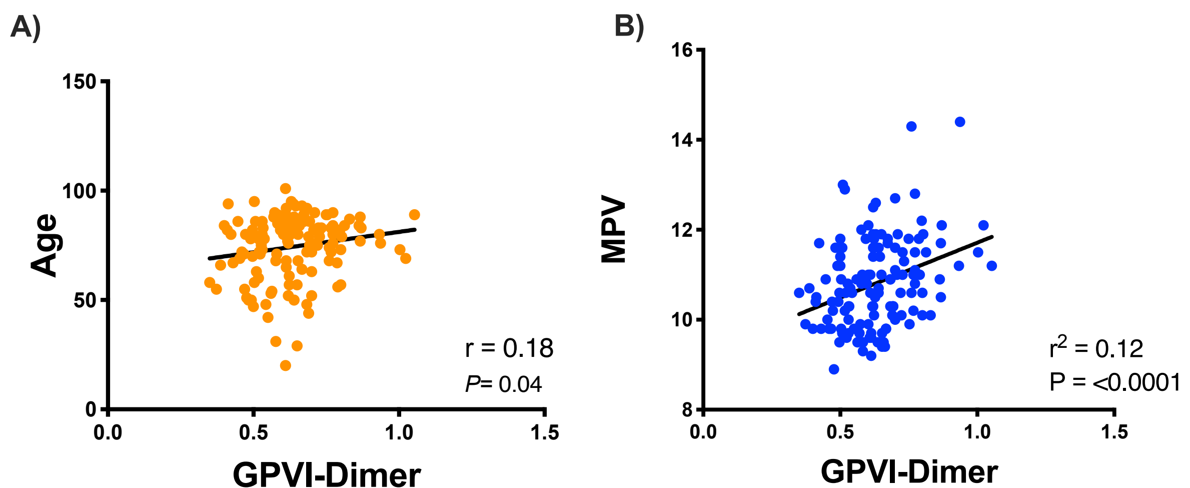
and that anti-thrombotic therapy; including antiplatelet drugs, anticoagulation or thrombolysis do not affect GPVI expression (Figure 7.11).

The ability of GPVI to interact with the two main ligands that drive thrombosis, collagen and fibrin (Alshehri, 2015; Mammadova-Bach, 2015; Induruwa, 2018) cements its role as a central platelet receptor in human thromboembolic disease. Furthermore, our results intimate an important role for GPVI-dimer in thrombosis in both large-artery and cardioembolic stroke and the over-expression of GPVI-dimer in all ischemic stroke patients suggests that direct inhibition of GPVI-dimer would be a promising therapy against ischemic stroke of different sub-types.

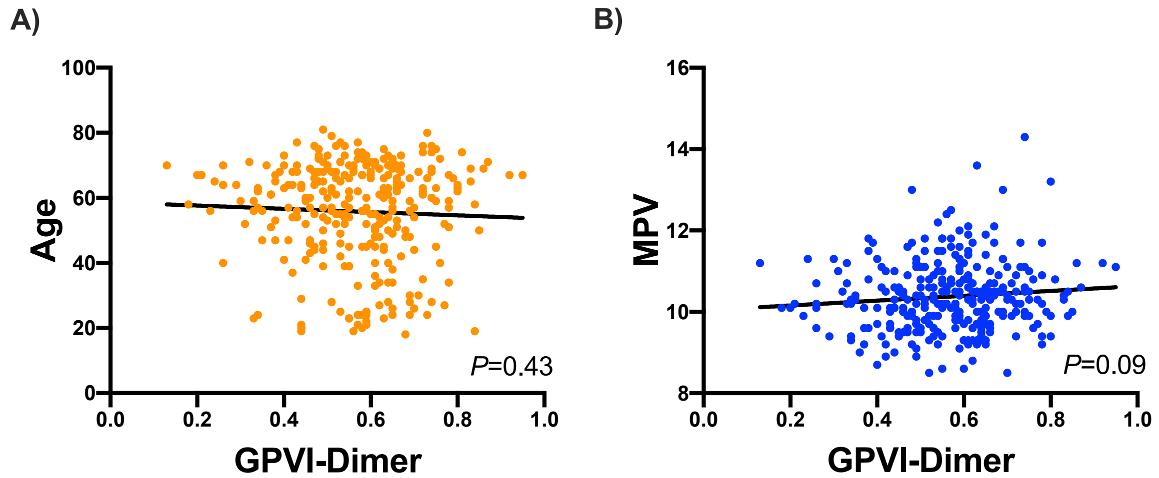
7.09 Supplementary files

Initial statistical analysis revealed that age and MPV were both correlated with GPVI-dimer expression. Linear regression revealed that age (*P*=0.049*)* MPV (*P* <0.0001) were significant associates of GPVI-dimer expression (Supplementary Figure 7.1). MPV was also correlated with total GPVI expression (r^2=0.18, *P*<0.0001) whereas age was not (r =0.08, *P*=0.34).

In the control population, neither age or MPV were related to GPVI-dimer expression (Supplementary Figure 7.2). Resting P-selectin exposure was also associated with MPV (r =0.1, *P*=0.007), but not with age (*P*=0.79).

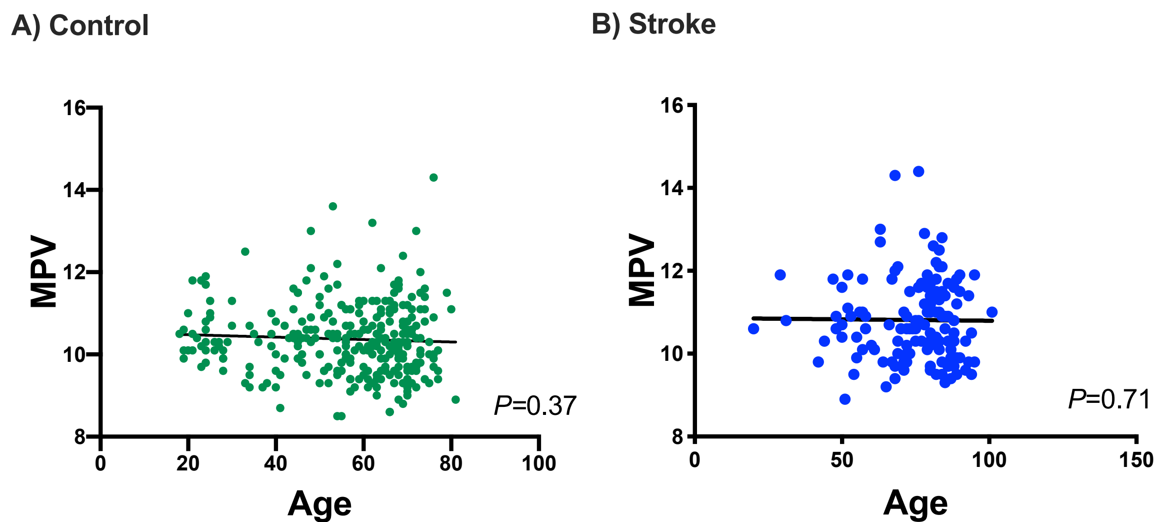


Supplementary Figure 7.1 Correlation between GPVI-dimer (MFI), and **A)** age and **B)** MPV (fL) in the stroke population. A) Calculated using Spearman's rank correlation coefficient and B) using Pearson correlation coefficient.



Supplementary Figure 7.2 Correlation between GPVI-dimer (MFI), and **A)** age and **B)** MPV (fL) in the control population. A) Calculated using Spearman's rank correlation coefficient and B) using Pearson correlation coefficient.

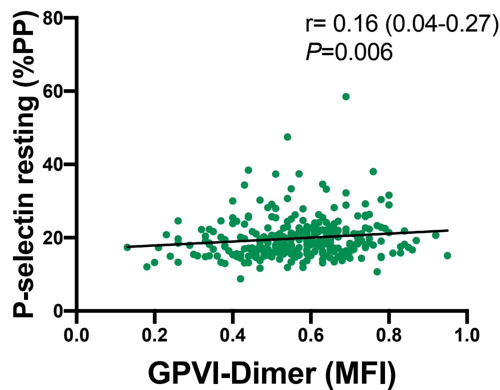
There is no relationship between age and MPV in either the control or stroke populations.



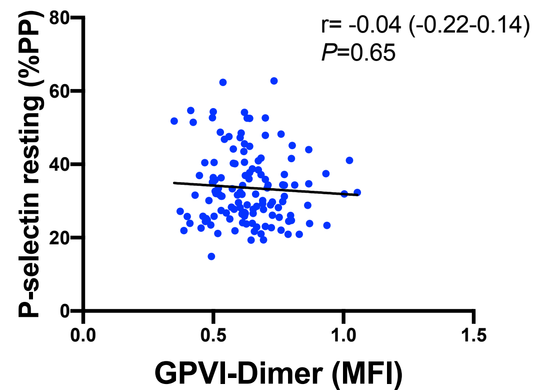
Supplementary Figure 7.3 Correlation between age and MPV (fL) in **A)** control and **B)** stroke 0-day populations. Calculated using Spearman's rank correlation coefficient.

Resting P-selectin exposure in control platelets were correlated to GPVI-dimer expression (r=0.16, *P*=0.006), However, we do not see this in the stroke population where GPVI-dimer and resting P-selectin exposure are not correlated (stroke, r=-0.04, *P*=0.65) (Supplementary Figure 7.4).

A) Control



B) Stroke



Supplementary Figure 7.4 Correlation between resting P-selectin exposure and GPVI-dimer expression in A) controls **B)** stroke population at day 0. Correlations calculated using Spearman's rank correlation coefficient. Black line represents regression line calculated using simple linear regression and Spearman r value and 95% confidence interval is presented within each image.

Modelling associations between age and MPV inserted as single predictor variables with unadjusted total GPVI and GPVI-dimer expression for the stroke population, was carried out using simple linear regression. Both total GPVI and GPVI-dimer expression and platelet function values were then adjusted for age and MPV prior to analysis.

	Total GPVI		**GPVI-dimer**	
	Coefficient B	Significance (*P*)	Coefficient B	Significance (*P*)
Age (years)	0.12	0.19	**0.17**	**0.049**
MPV (fL)	0.42	0.20	**0.36**	**<0.0001**

Supplementary Table 7.1 Simple linear regression to determine associations between single predictor variables; age and MPV with unadjusted total GPVI and GPVI-dimer expression values in the stroke (n=129).

CHAPTER VIII. PLATELET RECEPTOR GLYCOPROTEIN VI IN ISCHAEMIC STROKE

8.01 GPVI binding to fibrin; what we know now

As discussed in chapter III the two seminal papers from Mammadova-Bach et al. and Alshehri et al. in 2015 opened up a new avenue of research in to the possibility of GPVI as a receptor for fibrin.

They established that the activation of platelets through GPVI is stimulated with polymerised fibrin, which suggested the now recognised contribution of GPVI as a receptor for polymerised fibrin. Briefly, the authors described that the presence of fibrin-stimulated signalling caused tyrosine phosphorylation, thrombin generation and platelet PS exposure in order to support thrombus formation under flow. Initially however, whether GPVI was a receptor for fibrin at all was contentious. Much of the platelet research community were dubious from the limited evidence available with regards to this interaction, and some of this debate still remains.

At the time of writing this thesis, a lot more research has been completed on GPVI-fibrin(ogen), which has eased some of the initial concerns from other groups. The main point of contention that remains is about which form of GPVI, the monomer or the dimer, binds to fibrin. Similarly, whether GPVI binds to both fibrinogen and fibrin and whether this is a primary interaction or a collaborative one together with other platelet receptors such as integrin αIIbβ3 are also still debated.

After the initial two papers by Mammadova-Bach and Alshehri, other researchers also became interested in the subject. Our group noticed the potential dual role for GPVI in thrombus formation in LAS and CES, through its interaction with both collagen and fibrin, hence sought to investigate this further in both AF patients at risk of stroke and stroke patients. Despite this only one or two relevant papers were published in the following two years. This was until Onselaer and colleagues in 2017, from the Birmingham platelet group reported in 2017 that recombinant monomeric GPVI, made up of the collagen binding D1 and D2 domains bound to fibrin and not fibrinogen (Onselaer, 2017). They also demonstrated that fibrin was not able to activate GPVI-deficient human (and mouse) platelets and reported that recombinant GPVI-dimer (Revacept) did not bind to fibrin.

The work by Onselear and colleagues, which preceded the publication of our work discussed in chapters IV and V (Induruwa, 2018), further solidified the idea that GPVI could indeed have roles in thrombosis outside of the established GPVI-collagen interaction. However, numerous key differences were present between these results of our work and theirs. Firstly, our GPVI-monomer ($GPVI_{ex}$) did not bind fibrin or D-fragment (probably the GPVI-binding site in fibrinogen). We found that our GPVI-dimer ($GPVI\text{-}Fc_2$) bound saturably, with one-site kinetics to D-fragment of fibrinogen and D-dimer of fibrin. Onselaer did not report whether Revacept bound to fibrinogen or D-fragment but showed that their monomer did not bind to fibrinogen. Both the study by Onselaer et al. and our study demonstrated that neither form of GPVI bound to E-fragment.

Furthermore, in 2018 Ebrahim and colleagues from the Siess group in Munich reported that two forms of their GPVI-dimer ($GPVI\text{-}Fc_1$ (Revacept) and $GPVI\text{-}Fc_2$) did not bind to physiologically prepared fibrin from purified fibrinogen. Nor did they bind to fibrin produced through exposing TF coated surfaces of atherosclerotic plaque slices to arterially flowing blood (Ebrahim, 2018). They also reported no binding of their recombinant dimer to fibrinogen. However, Ebrahim and colleagues did not test a monomeric form of GPVI to demonstrate whether either form of GPVI could bind to their fibrin, which is one of the main limitations of this work.

Therefore, thus far the published research was uncertain about which form of GPVI bound to fibrin, if at all, with only our group establishing that it could also interact with immobilised fibrinogen through the D-domain. The fact that mFAB-F could inhibit platelets binding to and being activated on fibrin coated surfaces under flow, however, suggested a specific role for GPVI-dimer in these interactions.

Initially, why GPVI could bind to fibrin but not fibrinogen as reported by other groups was curious to us. Some of the early work indicated that GPVI did not bind to monomeric fibrin either, which is similar in structure to fibrinogen. Subsequently, Mangin and colleagues reported that human GPVI monomer binds to immobilised fibrinogen which leads to intracellular signalling and platelet activation as well as platelet aggregation under flow

(Mangin, 2018). This is similar to our work, although we reported that it is the GPVI-dimer that bound to the immobilised fibrinogen D-domain. The need for fibrinogen to be immobilised appears to be important certainly through the in vitro results obtained as both groups reported that GPVI was not able to interact with fibrinogen in suspension. This is in keeping with physiological observations that platelets do not form thrombi merely through binding to soluble fibrinogen in the circulation. This also highlights that integrin αIIbβ3 must play a role in platelet-fibrin(ogen) adhesion. What remains unclear is the exact nature of the collaborative relationship between GPVI and integrin αIIbβ3. Our work in patients with GT suggested that GPVI-dimer may have a limited but independent role in the absence of αIIbβ3. However, in normal platelets, whether GPVI propagates αIIbβ3 induced signalling when adhering to fibrin(ogen), much like $\alpha 2\beta 1$ with collagen, or whether GPVI binds to fibrinogen first, leading to signalling that promotes the high-affinity conformation of αIIbβ3 or a mixture of both is not established. It is likely that GPVI has different roles in different sites and stages of thrombus formation.

To explain the discrepant observations by the different laboratories we must consider the methods employed, e.g., ELISA assays with immobilised substrates, a system far from a physiological interaction between platelets and fibrin. Static binding assays cannot mimic how GPVI would bind to fibrin fibres within a clot, particularly as platelets would interact with clots formed from a three-dimensional network of fibrin fibres that have been also been crosslinked via the transglutaminase activity of FXIIIa and Ca^{2+}. A review by Alexandre Slater from the Birmingham platelet group, published in 2019, dissected why the four different groups have obtained discrepant results (Slater, 2019). Their conclusion is that it could be due to 1) the recombinant GPVI constructs, both monomeric and dimeric; 2) different preparations of fibrinogen and fibrin; used and 3) as discussed above, binding assay conditions. I have summarised the results reported by each group in Table 8.1. Despite this, it is clear that GPVI has a role in adhesion to fibrin, and possibly also fibrinogen.

	Mammadova-Bach 2015	Alshehri 2015	Onselaer 2017	Induruwa 2018	Ebrahim 2018	Mangin 2018
Fibrinogen	None	None	None	Dimer not monomer	None but only tested dimer	Monomer not dimer
Fibrin	Dimer only tested	Monomer only tested	Monomer not dimer	Dimer not monomer	None but only tested dimer	Not tested/ reported
Collagen	Dimer only tested	Not tested/ reported	Dimer Monomer binds weakly	Dimer Monomer binds weakly	Dimer	Not tested/ reported

Table 8.1 Published results from different groups investigation the GPVI-fibrin(ogen) interaction.

8.02 GPVI binding to fibrin and relationship with stroke thrombus architecture

GPVI adhering to fibrin as well as immobilised fibrinogen to cause platelet activation opens up a wealth of possibilities when considering the role of GPVI in thrombotic disease, particularly ischaemic stroke. Within a thrombus, many blood cells including platelets, erythrocytes, leukocytes are trapped, associated with fibrin, exposed collagen and other coagulation-related proteins. Therefore, consideration of how platelets and platelet GPVI is organised within the constituents and histology of a thrombus, will help us elucidate the potential roles that GPVI will have in thrombosis through its roles as a collagen and fibrin(ogen) receptor. This in turn will facilitate in understanding of how potential anti-GPVI medication could fit into the current line-up of anti-thrombotics used in stroke.

The advent of thrombectomy for ischaemic stroke enables analysis of excised thrombi from LAS and CES and histological analysis to date has revealed different cellular composition between clots from these two ischaemic stroke subtypes. These studies reveal that non-cardioembolic stroke thrombi are generally erythrocyte rich, and that fibrin-rich thrombi are significantly associated with a cardioembolic source (Boeckh-Behrens, 2016; Sporns, 2017). Furthermore, thrombus histology of ESUS was significantly associated with that of cardioembolic strokes rather than non-cardioembolic ones (Boeckh-Behrens, 2016; Santos, 2016; Sporns, 2017) in keeping with evidence that a proportion of thrombi in ESUS strokes maybe from a cardiac source (Wachter, 2017). This is of particular relevance considering how GPVI is involved in predominantly collagen-driven large artery carotid thromboembolism as well as thrombin and fibrin driven cardioembolism.

The physiological significance of GPVI activity therefore could have multiple roles in the growing thrombus, regardless of stroke aetiology. For example, in LAS, GPVI-dimer would bind to collagen and activate platelets, thereby propagating the release of various platelet agonists to cause nearby platelet activation and thrombus growth. In venous thrombi or CES, it would be endothelial dysfunction, TF release and fibrin deposition on the endothelial layer that would attract platelets. A platelet monolayer would grow on the thrombus through GPVI-collagen adhesion in LAS or GPVI-fibrin adhesion in CES (as well as other platelet receptors including GP1b, $\alpha 2\beta 1$ and $\alpha IIb\beta 3$). As this platelet layer develops into a thrombus, fibrin is actively formed on the surface of activated platelets, triggering divergent coagulation pathways in order to reinforce thrombus growth in both LAS and CES.

We can therefore hypothesise that GPVI must have a role in these platelet-fibrin interactions to help develop, but also stabilise the thrombus. Studies have shown that platelet dependent fibrin formation commences at the base of the thrombus, growing upwards and outwards (Swieringa, 2018). This means that densely packed fibrin fibres are generally found within the core of a thrombus, alongside collagen and activated platelets (Stalker, 2014), whereas loosely packed fibrinogen and fibrin is likely to be present in the outer shell of the thrombus (Macrae, 2018). This suggests a model in which thrombus

stability could be sustained by GPVI with collagen and fibrin in the centre of the thrombotic core but thrombus growth through platelet activation also in association with fibrin(ogen) on the outside of the thrombus; potentially together with integrin $\alpha IIb\beta 3$; relevant in arterial, venous and cardiac-thrombosis. An in vitro thrombus model reported by Lehmann and colleagues demonstrated that TF induced fibrin deposition and then GPVI driven platelet adherence and activity (quantitated through PS expression) drove thrombus growth in venous thrombosis, a mechanism of thrombosis similar to that in CES (Lehmann, 2018).

To further understand the role that GPVI could play in the clot structure, we also sought to determine GPVI binding to fibrin clots formed from thrombin catalysis of near serum concentrations of fibrinogen, which mimics the clots formed from a three-dimensional network of fibrin fibres as seen in vivo. These fibrin clots were either non-crosslinked or crosslinked using FXIII and Ca^{2+}, closely approximating physiological conditions. This set of experiments, designed by Dr M Moroi, are detailed in supplementary section 8.09. The recombinant GPVI constructs we used were 1) our dimer used in previous work (GPVI-Fc_2) 2) Revacept (another GPVI-dimer) or 3) GPVI-His (monomer of Revacept) followed by clot-bound protein analysis.

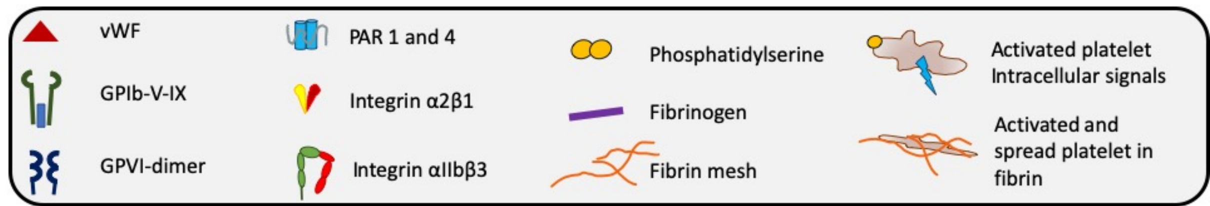
Both GPVI-Fc_2 (Supplementary Figure 8.1) and Revacept (data not shown) bound to non-crosslinked and crosslinked fibrin clots with dose-dependent, saturable one-site binding kinetics; crosslinking of the clots increased the affinity two-fold. The sequence of the dimer constructs were the same as those previously used in ELISA (chapter IV and V) with immobilised substrates and the K_d values determined in this clot assay were consistent with those obtained for D-fragment and D-dimer by ELISA. By contrast, monomeric GPVI binding to either type of clot was non-saturable and nearly linear, indicating very low affinity or non-specific binding (data not shown).

Anti-GPVI-dimer antibody mFAB-F partially but significantly inhibited the GPVI-Fc_2 binding to the clot (100 μg/mL, P=0.02; 150 μg/ml, P=0.001; 200 μg/ml, P=0.002, n=6), indicating a GPVI-dimer–specific interaction. D-fragment and D-dimer weakly inhibited (P=0.0129 and P=0.0235, respectively), supporting the conclusion that GPVI-Fc_2 binds to these

fragments and bind to fibrin clots in close proximity to where D-dimer binds to GPVI (Supplementary Figure 8.2).

We then formed clots from a mixture of platelets and fibrinogen, to examine how platelet-surface GPVI and other receptors [GPIb and CD61 (integrin β3)] contribute to the interaction between platelets and fibrin in the clot. Interestingly, the addition of Integrillin (anti-αIIbβ3) to fibrin clots increased the amount of clot-bound GPVI-dimer (data not shown). This is consistent with our previous work in GT patients and indicates that GPVI-dimer may have an independent role to play in thrombus formation in the absence of the main fibrin receptor, integrin αIIbβ3 on platelets.

Overall these results suggest that GPVI, but more specifically GPVI-dimer and not the monomer, would significantly contribute to the binding of resting platelets to fibrin within a clot. Whether this GPVI-fibrin interaction then induces signalling to assist activation of αIIbβ3 needs to be established.



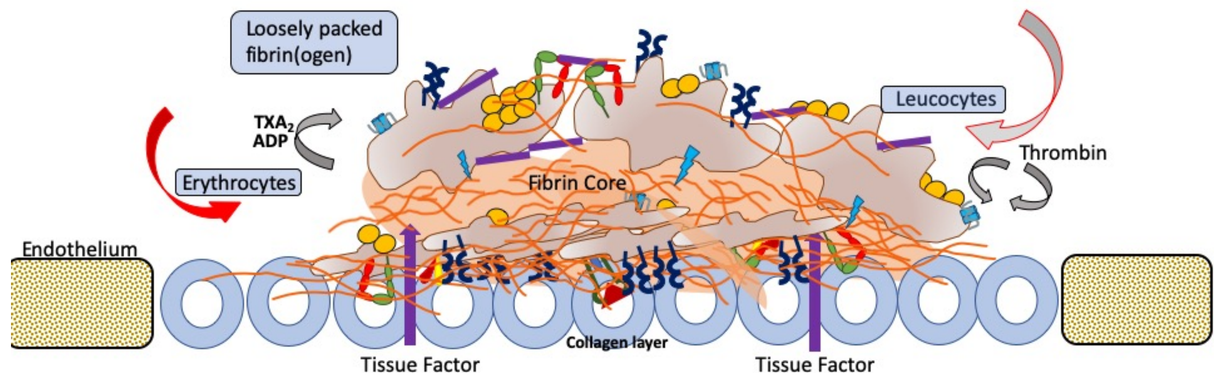


Figure 8.1 Hypothetical model of GPVI-dimer involvement in a stroke thrombus. GPVI-dimer binds to exposed collagen or deposited fibrin, causing platelet activation and spreading. As the thrombus grows, a fibrin core develops, and platelets are packed here using GPVI-dimer and αIIbβ3-based adhesion. Loosely bound fibrin(ogen) on the periphery of the thrombus facilitates growth through GPVI-dimer and αIIbβ3.

8.03 GPVI expression in pre-stroke AF and stroke cohorts

In recent years, platelets have become increasingly recognised as important markers as well as potential clinical targets for several diseases and disease processes. Many are outside the conventionally associated haemostasis and thrombosis; such as inflammation, metastases and neurodegenerative conditions (Yun, 2016). We now have a reasonable understanding of how increased platelet activation has a role in facilitating disease progression, and markers such as pP-selectin or sP-selectin allow us to quantitate and compare this between different disease pathologies. However, little work has been carried on investigating how expression of the key platelet receptors, GP1b-V-IX, GPVI and αIIbβ3 varies in thrombosis and how indeed this relates to the platelet phenotype if we consider a population's thrombotic risk.

The results from the *GRAFITE* study, which measured platelet surface expression of GPVI in an AF population at high stroke-risk as well as the *GYPSIE* study, which measured platelet surface expression of GPVI in a stroke population, suggest that both pre-stroke and post-stroke patients significantly overexpress the functional form of GPVI, the GPVI-dimer, when compared to a healthy control cohort. Since both studies were carried out using the same personnel, in the same lab, with the same materials we are in a strong position to compare the GPVI expression and platelet function results from both studies.

In both studies, age and MPV in the patient cohorts were significantly higher than the control group. MPV is associated with both platelet activation (Park, 2002), AF (Choudhury, 2007; Makowski, 2017) and stroke (Greisenegger, 2004; Ghahremanfard, 2013) and age is a well-established risk-factor for both AF and stroke (Kernan, 2014). Therefore, statistically adjusting all GPVI expression and platelet function measurements by age and MPV allowed us to reduce some of the variability and associations caused by these two factors.

What we observe is a significant rise in GPVI-dimer expression when moving from a healthy, to a pre-stroke diseased (non-stroke form *GYPSIE* and AF from *GRAFITE)* and then

a post-stroke population (Figure 8.2). Total GPVI expression was more variable, where AF patients expressed less total GPVI than both the control and stroke cohorts

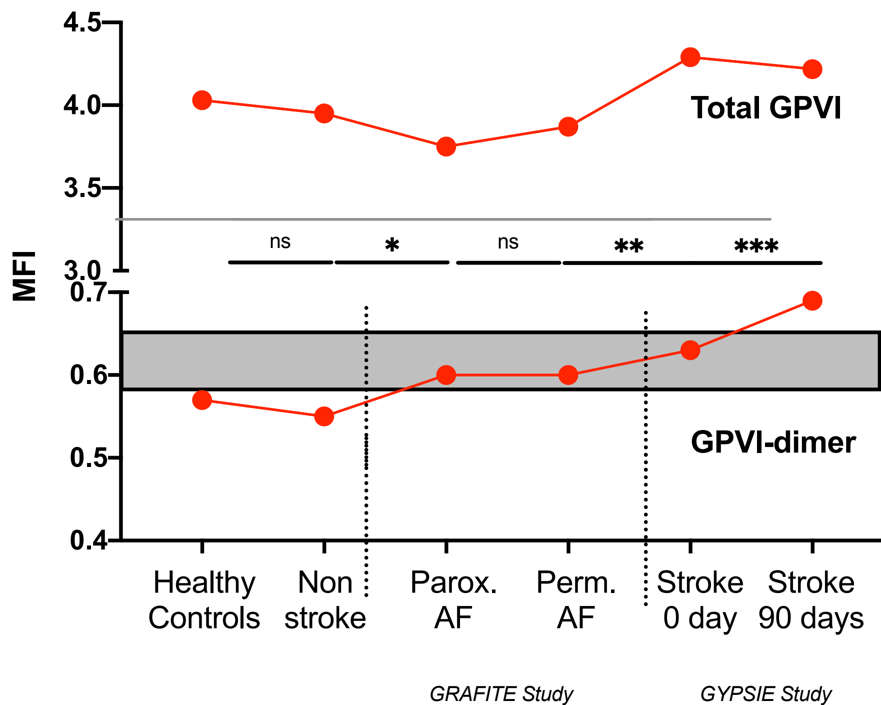


Figure 8.2 Expression of total GPVI and GPVI-dimer in all the cohorts tested. The expression of GPVI-dimer (mean MFI) increases when moving from a healthy control to a pre-stroke, then post-stroke sample, whereas total GPVI expression is more variable. The grey-box indicates the GPVI-dimer level above which stroke-risk increases. ns = not significant, *P<0.05, **P<0.01, ***P<0.001 calculated using an unpaired t-test.

If we compare CES patients in the *GYPSIE* study to the AF patients in the *GRAFITE* study, again, we see that patients who have had an AF-related CES, express significantly higher GPVI (both total and dimer, P<0.0001) compared to the pre-stroke AF cohort (Figure 8.3). There are limitations when analysing this data, as they are not the same patients with AF who have then had a CES. Furthermore, whether some of the AF patients had an in-situ LAA thrombus at the time of sampling, however, was never ascertained and would have strengthened our results.

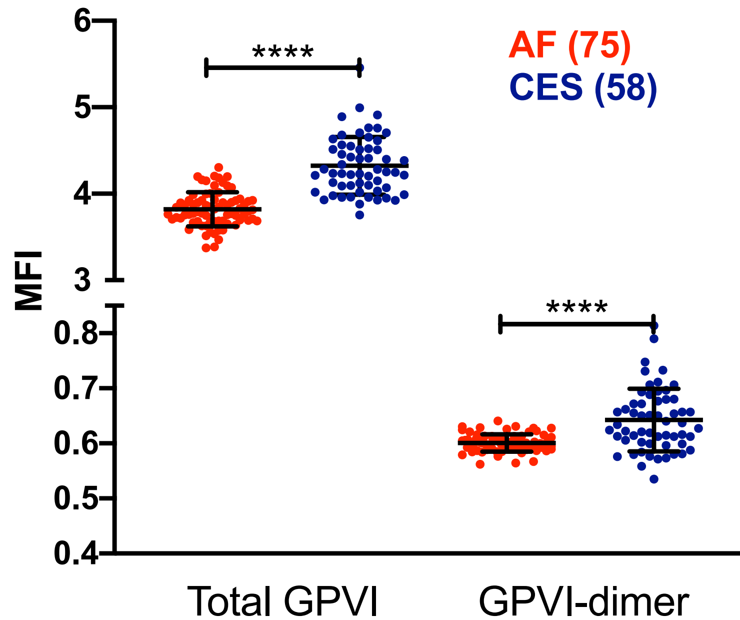


Figure 8.3. Total GPVI and GPVI-dimer expression between AF and CES patients. Total GPVI and GPVI-dimer are both significantly higher in CES compared to AF patients (*P*<0.0001). The error bars represent the mean MFI of each of the cohorts ± SD and significance was calculated using unpaired t-tests.

The GPVI-dimer MFI from all non-stroke patients over both studies were combined (n=402; healthy controls, non-stroke, AF) and plotted against all the stroke patients (n=129) in a receiver operating characteristic (ROC) curve where the binary classifier was defined as stroke yes or no (0 or 1) (Figure 8.4). The area under ROC curve = 0.87 (95% CI 0.82-0.91), *P*<0.0001, suggests that GPVI-dimer MFI is a good test to distinguish between non-stroke and stroke. The cut-off value for GPVI-dimer MFI indicative of stroke was 0.611. This is the value which represents the highest sensitivity (65.12%) and specificity (95.02%) for stroke and was calculated using a the Youden's index.

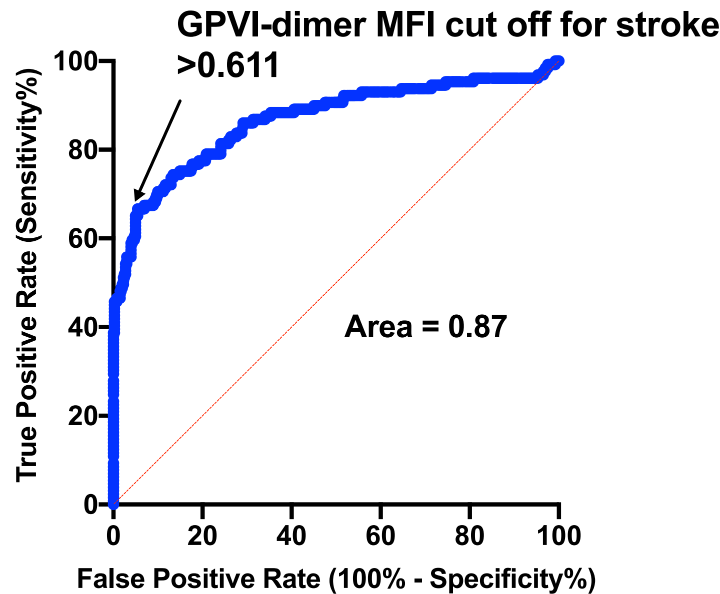


Figure 8.4 GPVI-dimer expression represented as a receiver operative characteristic (ROC) curve. The GPVI-dimer expression (MFI) cut-off value for stroke is observed at 0.611 and the area under the curve = 0.87 (95% CI 0.82-0.91), $P<0.0001$.

Consistent with our findings, previous studies have suggested that a GPVI MFI value (total GPVI) above a certain threshold was sensitive and specific for stroke (area under curve 0.683) (Bigalke, 2010). Bigalke et al demonstrated that these patients with a higher total GPVI MFI had a poorer clinical outcome and increased likelihood of death at three months ($P=0.045$). None of our stroke patients went on to have another cardiovascular event during the six-month period, allowing for the limitations of our data collection. However, 24 of them had died within six-months.

Table 8.2 compares those within the stroke cohort who had a GPVI-dimer MFI of <0.611 to >0.611. The results show that the admission CHA_2DS_2-VASc score is significantly higher in those with an MFI of >0.611 ($P=0.0003$), again suggesting that vascular risk-factors may be related to GPVI-dimer expression and maybe driving dimerization itself, which could result in a stroke. However, no significant differences were seen between the two cohorts when analysing admission NIHSS score or C-RP, discharge mRS or the proportion who had died by six months.

	MFI <0.611	MFI >0.611	Significance (*P*)
n	45	84	
Admission NIHSS	7 (4-15)	8 (4-15)	0.58
Admission CHA_2DS_2-VASc Score	3 (1-4)	4 (3-5)	**0.0003**
Admission C-RP	3.3 (1.6-7.0)	3.4 (1.4-8.2)	0.92
Discharge mRS	2 (1-4)	2 (1-4)	0.27
Death at 6 months (%)	5 (11.1)	21 (25.0)	0.07

Table 8.2 Comparison between stroke patients with a GPVI-dimer MFI <0.611 and >0.611. Statistical analysis between non-parametric continuous data was carried out using a Mann-Whitney U test. Statistical differences between the two cohorts analysing death at 6 months was carried out using a Chi Squared test.

Studies have demonstrated that premature activation of platelets, in the setting of vascular risk-factors, rather than coagulation system activation can cause pathological clot formation (van Rooy, 2015). The results from *GRAFITE* and *GYPSIE* studies suggest that vascular risk factors are related to GPVI-dimer expression and could indeed be a driver for GPVI-dimerization; precipitating a stroke in a pre-stroke AF and stroke population.

Total GPVI expression appears to be more variable and less clear-cut. Whether the reason we see similar dimer levels but lower total GPVI levels when looking at the AF and stroke cohorts is due to rapid exposure of monomeric GPVI from the internal platelet granules at the time of the stroke, or due to factors outside what is measured in these studies is not clear. sGPVI measurements would have enabled us to better ascertain if the reason total GPVI is lower in the AF patients compared to controls and stroke patients is due to increased monomeric GPVI shedding. We are currently re-grouping the collected and stored serum samples from all the patients to see if sGPVI quantitation can be carried out.

What we also cannot fully elucidate from our results, however, is whether the reason GPVI-dimer is higher in the stroke population is due to the stroke itself or whether it was already raised pre-stroke. The fact that GPVI-dimer expression is even higher at day 90 post-stroke suggests that it is unlikely to be an acute effect.

What do the results from the *GRAFITE* and *GYPSIE* studies tell us about how GPVI expression relates to platelet function in response to exogenous agonists? Herein lies another limitation of this work; we do not have results for platelet fibrinogen binding for the stroke cohort, which would have represented an interesting marker to compare between the pre-stroke and stroke cohorts.

We observe a significant rise in resting P-selectin exposure when moving from healthy control to AF to stroke cohorts, indicating that as vascular risk increases, there is also a similar and measurable increase in 'activation' of circulating platelets. This however, appears to be a distinct mechanism to GPVI-dimer expression as we did not see a correlation between GPVI-dimer and P-selectin exposure in either the AF or stroke population.

In the AF patients we see that both P-selectin exposure and fibrinogen binding after CRP-XL are significantly negatively associated with GPVI-dimer expression. We observe the opposite in stroke patients where P-selectin exposure after CRP-XL is significantly positively correlated with GPVI-dimer expression. We initially hypothesised that this could be due to the effect of anticoagulation, at least in the AF cohort. However, if we divide the AF patients into anticoagulation (n=50) or not (n=25), we do not see any difference in either fibrinogen binding or P-selectin exposure with regards to GPVI-dimer expression. We observe a very strong negative correlation between GPVI-dimer and P-Selectin exposure after CRP-XL in both anticoagulated and not anticoagulated groups ($P<0.0001$ for both). A similar result is seen with fibrinogen binding where there was a strong negative correlation between GPVI-dimer and fibrinogen binding, regardless of anticoagulation ($P=0.0003$ and $P<0.0001$, respectively). Therefore, there must be other parameters, such as genetic variation, affecting how platelets in the AF cohort function compared to stroke patients outside of anticoagulation.

Another difference between the AF and stroke cohorts with regards to GPVI-dimer and P-selectin exposure after CRP-XL could be due to GPVI clustering. Poulter et al. demonstrated that GPVI oligomerised, or clustered, after binding to collagenous substrates which in turn could facilitate both platelet activation and function (Poulter, 2017). The clustering observed was platelet-wide, not just in the area that was in contact with collagen fibres. This supports the theory that proximity of GPVI-dimer binding sites increases GPVI avidity by bringing together the necessary signalling components to initiate efficient platelet activation and thrombus formation (Poulter, 2017). GPVI clustering after thrombosis could be a requisite for platelet function in response to agonists such as ADP and collagen (Berlanga, 2007). In AF patients, where no thrombotic event had taken place, this would not have occurred, hence why we do not see an impressive P-selectin response in response to collagen.

However, why overall these parameters were less in the AF patients compared to controls is not clear. None of our AF patients went on to have a stroke during the study period, therefore, we cannot determine how their platelet function and also GPVI expression would change after a thrombotic event.

8.04 Limitations of experimental work

We must take into consideration some of the limitations of the experimental work performed in Chapter IV. All experiments described in this thesis were carried out by me, after appropriate laboratory training. That being said, it does not detract from introduction of a high level of subjectivity and human error.

Generally, working with platelets can be difficult. Once out of blood, the platelet function tends to decrease with time and how quickly this occurs varies between individual to individual. This is not a problem in most static assays but can be cumbrous during time consuming platelet function experiments such as aggregometry and flow adhesion. Accounting for this is difficult and we may have been subject to errors in data produced in experiments carried out towards the end of the day.

We were also encumbered by the amount of healthy volunteer blood used in the laboratory work as our ethics was limited to members of the biochemistry group leading to selection bias. This meant that there was only a small and limited pool of young, healthy blood donors available, who do not represent the wider population. Volunteer-related factors which also have to be taken into account prior to investigating platelet function including, exercise, circadian variations, food and coffee consumption, NSAID use and smoking. To limit these effects, we did exclude anyone who had taken medication which could affect platelet function in the last 7 days and took blood at the same time every day.

Pre-registration of the research question and subsequent research plan would avoid some of the bias associated with the experiments performed and data analysed only by myself. Specific improvements within the experimental methodology could also have accounted for a reduction in experimental errors. For example, the preparation of polymerised fibrin from fibrinogen requires the addition of specific amounts of thrombin as well as the addition of GPRP to then produce monomeric fibrin. Due to time and product constraints we could not check each time using an anti-fibrin/anti D-dimer antibody whether we had successfully created fibrin each time, but this would be a useful addition to the protocol which may have helped to explain some of the negative results seen in the ELISA studies (Figure 5.1 + 5.2).

To enable analysis of thrombus formation under flow adhesion, the operator must define the coverslip plane (see Figure 4.3) in order to quantify both platelet SA and MTH. This can be subject to observer bias, and in these set of experiments the coverslip plane was defined only by me. A way of reducing this would have been to have another member of the group to also come and define the coverslip plane 'blind' each time we were quantifying SA and MTH which may have corrected for unwanted large effect sizes.

8.05 Limitations of translational work

Further work needs to be carried out to evaluate how GPVI-expression leads to platelet function in the setting of thrombotic disease as well as in pre-stroke populations. Although the findings discussed here suggest interesting and plausible pathophysiological processes, we must exercise a degree of caution when attempting to interpret their clinical relevance.

One of the major limitations of the observational case-control studies (Chapters VI and VII) presented in this thesis are the differences between the control and patient populations. Certainly, the overall AF and stroke patient cohorts appear suitably reflective of the broader stroke population, however, they are distinct to the control population recruited with significant differences in age and sex. With little crossover in the ages of our two populations, for example, it was difficult to form matched groups which were comparable as only a small proportion of the two groups overlapped. The GPVI expression and platelet function results were instead adjusted to age, as a major confounding factor. However, adjusting data for GPVI expression and platelet function by age and MPV, as well as the subgroup analyses from there onwards raises the risk of obtaining a type one statistical error. The groups within the study are limited in size, and therefore, it is difficult to know how the results we obtain relates to that from the wider population. Future studies would be designed to recruit healthy controls that are healthy but otherwise represent a sample that is comparative of the patient cohort.

Further patient recruitment would have helped overcome potential issues with insufficient sample size within the subgroup analysis, for example, when attempting to assess if haemorrhagic strokes also significantly express higher GPVI-dimer compared to the controls, similar to what we see with the ischaemic strokes (Figure 7.9) and whether patients taking FXa inhibitors express more GPVI-dimer than those not on anticoagulation (Figures 6.6 and 6.7)

As mentioned in the previous section, platelet function can be affected by numerous factors. Platelets can be activated during venepuncture and we used methods such as taking blood without a tourniquet/releasing the tourniquet prior to collecting the citrate sample to minimise this. Although it was possible to govern this during the sample collection of the *GRAFITE* study (as all venepuncture was only carried out by me), many of the samples for the *GYPSIE* study were taken by different stroke research nurses with varying venepuncture ability. It is entirely possible that during venepuncture, where blood is also being taken for clinical results, the tourniquet was not released every time – leading to unwanted platelet activation. This is also true of platelet activation during specimen transport and processing. To minimise this, during the *GYPSIE* study, a member of the lab team met the team in ED to collect the sample. By the time the GRAFITE study was underway, we had altered the study design so that the samples were being delivered straight after venepuncture with minimal delay. Whether the differences in venepuncture technique explained the difference in the platelet function results. In the AF population ADP and CRP agonism demonstrated lower P-selectin exposure (all samples taken by me) and in the stroke population, ADP and CRP agonism demonstrated significantly higher P-selectin exposure (samples taken by various research nurses) compared to controls in not known.

Flow cytometry analysis of the samples for platelet function and GPVI expression were carried out by one staff member on a particular day. Experimenter bias therefore could have been reduced if one member ran the sample on the flow cytometer and another lab member did the analysis of data. However, due to staff time constraints, this was not possible. Similarly, in the *GYPSIE* study, strokes were subclassified as per the TOAST classification only by me. Involving another stroke physician 'blinded' to do this would have further reduced bias during data analysis.

With regards to the day-90 follow up sample, there were some patients who did not attend follow-up, or who were too frail to attend follow-up. It would have been interesting to quantify GPVI expression in these patients, who we ascertained were older and frailer than the patients followed up. We also do not have the genetic data from the AF or stroke participants to see whether they have a rare GPVI haplotype. This would certainly affect

the population GPVI levels as well as the interpretation of our work and is currently under analysis.

However, we did observe a generally consistent pattern that patients with vascular risk factors had higher resting platelet activity and GPVI-dimer expression. Although this study was not designed as a longitudinal cohort study, the availability of two further measurements would have certainly strengthened our data set. Firstly, a follow up blood sample from all AF patients at three months would have allowed us to compare fluctuations in GPVI-dimer expression in a group who have not had a stroke, to the stroke day-90 measurements. Secondly, a longer follow up period for both AF and stroke patients to see if they have any thrombotic events, with the plans in place to repeat their blood sample would have added to our current knowledge.

Therefore, these limitations need to be considered when planning future work and the findings of these studies need to be verified in larger more focussed studies with a pre-specified primary outcome, sub-group analyses and stringent follow-up procedures in place.

However, there are repeated observations from our translational work that suggests that GPVI-dimer should be the focus of any future work looking at GPVI as an anti-thrombotic target. This is because:

1) Patients with AF, regardless of anticoagulation, express significantly more GPVI-dimer on their platelet surface whereas total GPVI is significantly lower.
2) Stroke patients express significantly more GPVI-dimer on their platelet surface at admission and at 90-days post stroke.
3) After adjusting for age and MPV, both the AF and stroke populations showed independent associations with GPVI-dimer expression and the presence of vascular risk factors and serum biomarkers.
4) Higher GPVI-dimer is a specific and sensitive measure of the presence of stroke

8.06 GPVI-dimer as a potential antithrombotic target

Considering the roles that GPVI-dimer appears to play in binding to fibrin both in static and flow systems using immobilised fibrin and in fibrin clots consisting of larger crosslinked fibrin fibres, as well as the over-expression of GPVI-dimer in both pre-stroke and stroke populations, there are specific areas within stroke treatment where inhibiting the activity of GPVI-dimer could be utilised.

1) GPVI-dimer inhibition as an adjunct to intravenous thrombolysis
2) GPVI-dimer inhibition during intra-arterial thrombolysis or thrombectomy
3) GPVI-dimer inhibition as an anti-thrombotic in primary or secondary prevention of ischaemic stroke

(a) GPVI-dimer inhibition as an adjunct to intravenous thrombolysis

The acute phase of ischaemic stroke is associated with abnormal fibrin properties leading to reduced clot permeability and a lower likelihood of successful lysis (Undas, 2009). Whether all types of ischaemic stroke share these abnormal fibrin properties is unknown, but fibrin constitutes a large part of CES and ESUS thrombi and also to some extent LAS.

Previous studies have shown that more permeable clots (i.e., greater ease of which soluble molecules move through the platelet, fibrin and erythrocyte architecture within the clot) are more amenable to fibrinolysis (Diamond, 1993; Siudut, 2016). Since GPVI-dimer could play a role in clot stability through supporting close platelet interactions with fibrin, GPVI-dimer inhibition could have a part in increasing thrombus permeability and facilitating either recanalization at the time of administering intravenous rtpA.

This has been demonstrated previously in an MCAO mouse model. Reimann and colleagues used 0.1 or 0.35 mg/kg of rtPA either with or without 1mg/kg of Revacept and demonstrated that the addition of the soluble dimeric GPVI-Fc (Revacept) to rtPA significantly increased cerebral reperfusion as well as intracerebral oedema (Reimann, 2016). They also showed that the addition of Revacept to rtPA did not result in any excess bleeding, suggesting that low doses of an anti-GPVI-dimer could safely provide better recanalization rates with the added benefit of requiring a lower dose of rtPA.

(b) GPVI-dimer inhibition during intra-arterial thrombolysis or thrombectomy

Studies analysing thrombus histology have noted that further manoeuvres were needed at thrombectomy to retrieve cardioembolic clots and the authors concluded that this could be due to the higher organisational degree of such thrombi (Boeckh-Behrens, 2016). In keeping with this, clots with a higher erythrocyte and less fibrin content have been shown to be associated with a significantly higher likelihood of successful thrombectomy (Hashimoto, 2016; Shin, 2018). αIIbβ3 inhibitors such as abciximab are used during intra-arterial thrombolysis or to alleviate in-stent thrombosis in both the cardiology- and neuro-interventional settings (Tong, 2000). As an intra-arterial agent aimed directly at the thrombus, a GPVI-dimer inhibiting drug could break up some of the platelet-fibrin and platelet-collagen interactions within a thrombus to assist in recanalization.

Work by Ahmed and colleagues, presented at the International Society on Thrombosis and Haemostasis World Congress 2019, revealed that GPVI blockade with anti-GPVI Fab ACT017 promotes efficient disaggregation of human thrombi pre-formed on collagen or on human atherosclerotic plaque material in an in vitro model (*Unpublished* Ahmed 2019). This implies that impairing GPVI-mediated platelet activation could facilitate thrombus disaggregation in the setting of intra-arterial procedures to facilitate recanalization.

(c) GPVI-dimer inhibition in stroke prevention

The results of the translational work discussed in this thesis supports the idea that GPVI-dimer expression is associated with AF and stroke. Although many questions, including causality, remain unanswered, being a receptor for collagen and fibrin we are able to hypothesise that GPVI plays a key role in thrombus formation. Previous work has shown that GPVI is crucial to the recruitment of platelets to areas of disrupted and dysfunctional endothelium (Massberg, 2003). Furthermore, GPVI-clustering leads to sustained GPVI induced signalling which can increase platelet activity in in vitro thrombotic settings (Poulter, 2017). Therefore, the higher GPVI-dimer expression in the pre-stroke population could relate to a higher likelihood of a thrombotic event.

A recent study revealed that AF was underestimated in ischaemic stroke (Wachter, 2017). The fact that histology from clots from a CES and ESUS stroke were similar raises an important hypothesis that many of those patients with ESUS are likely to have undiagnosed AF. Currently, until AF is proven, these patients remain on antiplatelet therapy - which do not adequately reduce their risk of stroke.

GPVI-dimer inhibition, therefore, could have a role in reducing thrombotic risk in patients with AF pre-stroke as well as those who have had ischaemic stroke due to LAS, CES and SVO and finally in those who have had an ESUS also. In this setting, another advantage could be that those already on GPVI inhibitors could undergo safe thrombolysis (Schuhmann, 2019), as currently patients on warfarin with an INR (usually >1.4) and DOACs are excluded from receiving rtPA.

8.07 Published and planned papers from this thesis

Publications

- **Induruwa I**, Jung S, Warburton EA. *Int J Stroke* 11(6), 618-625.
  Beyond antiplatelets: The role of glycoprotein VI in ischaemic stroke.

- **Induruwa I,** Moroi M, Bonna A, Malcor JD, Howes JM, Warburton EA, Farndale RW, Jung SM. *J Thromb Haemost*. 2018; 16(2): 389-404.
  Platelet collagen receptor Glycoprotein VI-dimer recognizes fibrinogen and fibrin through their D-domains, contributing to platelet adhesion and activation during thrombus formation.

Planned work

- **Induruwa I et al.** Platelet Receptor Glycoprotein VI-dimer is significantly over-expressed in ischaemic stroke patients: The results of the *GYPSIE* study *(Under review).*

- **Induruwa et al.** Fibrin binding platelet receptor Glycoprotein VI-dimer is significantly over expressed in patients with atrial fibrillation.

- Moroi M, **Induruwa I,** Farndale R, Jung S. Dimers of the platelet collagen receptor Glycoprotein VI (GPVI) bind specifically to in-situ fibrin fibres in clots, but not to intact fibrinogen *(Under review).*

8.08 Future work

To fully assess the GPVI-dimer expression, especially in the context of its inhibition, it must be assessed in a variety of different thrombotic pathologies. These would include arterial diseases such as ACS, MI and peripheral vascular disease as well as venous thrombosis in DVT and pulmonary embolism. Further studies, if designed similarly to the translational work here, must be pragmatically planned, for example with a more suitable age and sex matched control population, to avoid the limitations discussed above and include analysis of sGPVI to better understand the relationship between GPVI expression and shedding in thrombotic disease.

Basic sciences work would include investigating GPVI-dimer in fibrin clots in more detail. Some of our results included in section 8.09 suggest that the thrombus architecture and structure, particularly whether they are crosslinked or non-crosslinked (using FXIIIa) effects GPVI-dimer adhesion to fibrin.

Alongside this, histological analysis of thrombi retrieved from thrombectomy will then form the next stage of our work. We are currently starting the *EGRESS* – Emboli and Gene RESearch in Stroke Study within the CUH stroke department in collaboration with the Department of Radiology, Biochemistry and the Addenbrooke's centre for Clinical Investigation, for which I am a named Principal Investigator.

EGRESS will recruit patients eligible for thrombectomy presenting with LAS, CES or ESUS. Histology and immunohistochemistry from collected thrombi will then be analysed to:

a. identify cellular aggregates.
b. identify glycoprotein VI (GPVI) within the thrombus with relationship to distinct fibrin, leukocyte and erythrocyte regions.

c. analyse gene expression, including DNA and non-coding RNA in order to determine different patterns present in CES and LAS.
d. investigate any association between different nucleic acid expression patterns and stroke aetiology.
e. Investigate any association between different nucleic acid expression patterns or SNPs in thrombi and collected peripheral blood samples.

Finally, we have identified that mFAB-F, an inhibitory human antibody specific for GPVI-dimer developed by Drs M Moroi and Dr S Jung, inhibits thrombus formation on fibrinogen, fibrin and collagen under both arterial and venous shear (chapter V). Ideally, we must investigate its use as a future anti-thrombotic target to inhibit GPVI-dimer activity in patients, but its low affinity excludes it from pharmacological use at present. Future work would involve affinity maturation, stable expression, as well as structural, analytical and immunogenic property characterisation of mFAB-F.

Translational work would involve investigating its pharmacodynamics, pharmacokinetics, and overall safety and tolerability in the context of a clinical study involving high-risk TIA or ischaemic stroke patients. The development of mFAB-F therefore represents possibly the most relevant consideration of future work to arise from this piece of work.

8.09 Conclusion

Stroke is one of the leading causes of disability and death in the world. Thrombus formation is a process combining both platelet and blood coagulation system activation. Platelet GPVI-dimer is central to both these processes, by functioning as a collagen and fibrin receptor for platelets. This suggests a central role in thrombus formation in ischaemic stroke, especially in LAS and CES, which currently dichotomises acute stroke treatment into either antiplatelet or anticoagulation.

The work within this thesis demonstrates that firstly, it is the same dimeric, collagen binding form that binds to both fibrinogen (at the D-domain) and fibrin (at the D-dimer region). We observe that patients from both a pre-stroke as well as ischaemic and

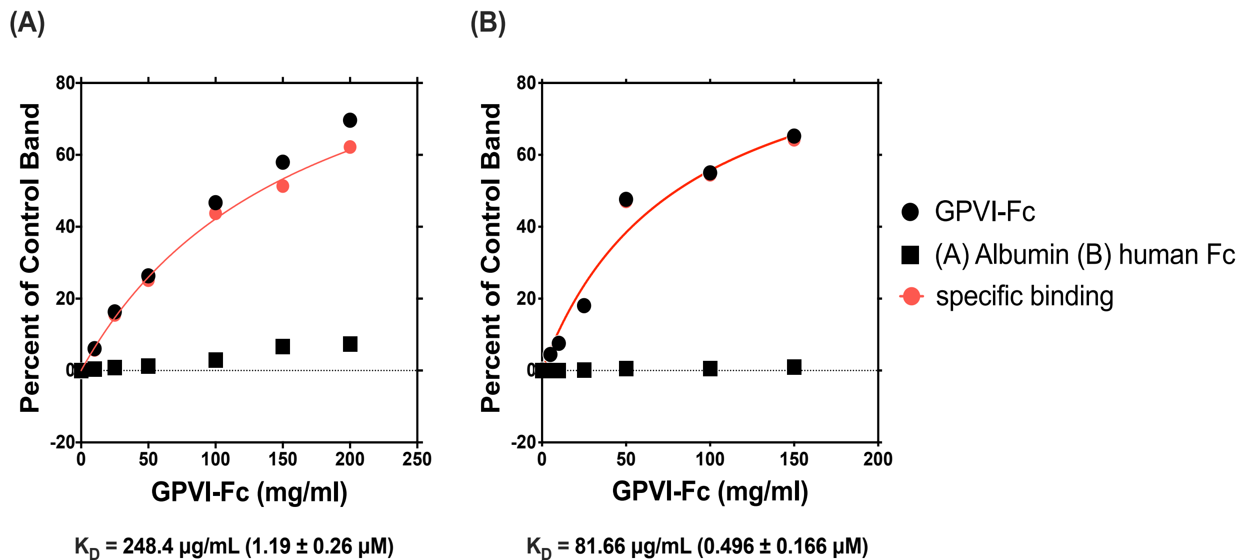
haemorrhagic stroke population, overexpress GPVI-dimer on the platelet surface compared to healthy and diseased counterparts. The over expression of GPVI-dimer is likely linked to platelet response to collagen rather than its circulating activity – in keeping with the fact that GPVI-dimer must encounter its ligands to cause the necessary signalling to cause platelet activation. Nevertheless, the overexpression of GPVI-dimer in the AF (as well as obese (Barrachina, 2019)) and a stroke population suggests that their platelets are more 'primed' to response to vascular injury at sites of thrombus formation.

In a stroke cohort, inhibiting GPVI-dimer to control thrombosis would have little impact on bleeding as it primarily controls thrombus formation at the site of vascular injury, with limited systemic effects on haemostasis. Therefore, GPVI-dimer inhibition represents a valuable and valid pharmacological target to control this platelet response and possibly reduce thrombus formation in different categories of pre- and post-stroke patients.

Especially as the role of GPVI in binding to fibrin becomes clearer and results of the anti-GPVI-dimer agents in healthy and diseased subjects (Revacept and ACT017) are on the horizon, no doubt the next few years will herald an exciting time in GPVI research. Our own Fab, mFAB-F also showed promise in in vitro work and witnessing its development, as well as Revacept and ACT017 into anti-thrombotic agents, and considering how they may shape the way ischaemic stroke patients are managed in the future will prove to be a fascinating time in both platelet and stroke research.

8.10 Supplementary files

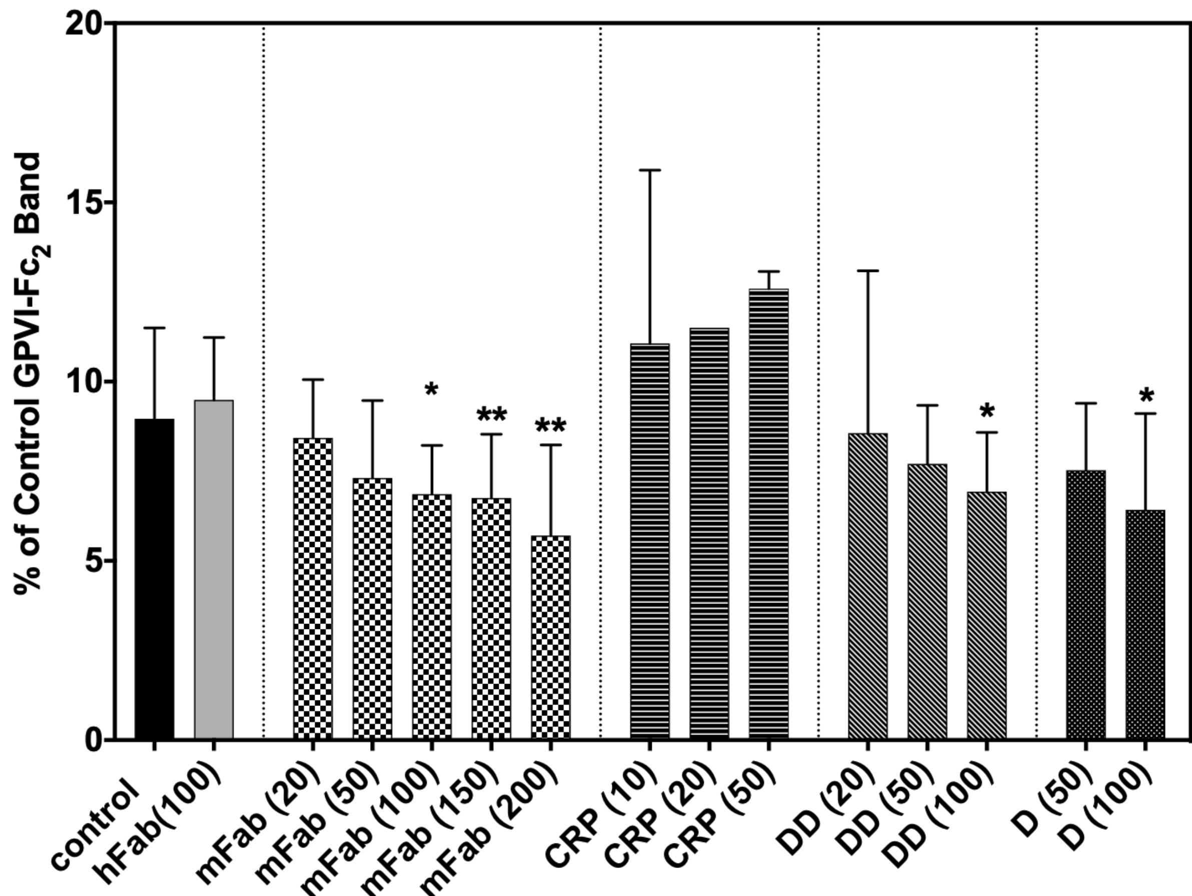
These experiments were done in conjunction with Dr M Moroi and Dr S Jung who designed the experiments. The figures are edited from M Moroi et al., (section 8.06) - manuscript under review.



Supplementary Figure 8.1 GPVI-Fc$_2$ binding to A) non-crosslinked and B) crosslinked fibrin clots. A) the quantitative relationship between the amount of GPVI-Fc$_2$ and band strength; total clot-bound GPVI-Fc$_2$ and albumin (non-specific binding) are expressed as a percent of the normalization standard (clotting mixture containing 25 µg/mL each of GPVI-Fc$_2$ and albumin, but no added thrombin). Specific binding of GPVI-Fc2 (%, red circles) is calculated by subtracting non-specific binding (albumin, %, black squares) from the total binding of GPVI-Fc$_2$ (%, black circles) and fit to a one-site binding model (red curve). The GPVI-Fc$_2$ binding to the clot is dose-dependent and saturable, exhibiting classical receptor binding kinetics.

B) GPVI-Fc$_2$ was reduced, and thus detected as GPVI-Fc with anti-human IgG antibody which detected the Fc portion of GPVI-Fc and the control protein was human Fc. After normalization with the standard, total binding of GPVI-Fc$_2$ (black circles, %) was subtracted by the value for human Fc (black squares, %) to obtain specific binding of GPVI-Fc$_2$ (%, red circles); specific binding was fitted to a one-site model. GPVI-Fc$_2$ binding to the crosslinked

clot is dose-dependent and saturable and had 2.4x higher affinity than its binding to the non-crosslinked clots.



Supplementary Figure 8.2 Effects of inhibitors on GPVI-Fc$_2$ binding to fibrin clots. The effects of the anti-GPVI-dimer–specific antibody mFAB-F; collagen mimetic CRP-XL; fibrinogen fragment D (D), and D-dimer (DD) on GPVI-Fc$_2$ binding to non-crosslinked fibrin were analysed. The concentrations of each antagonist in µg/mL is given in parentheses. mFAB-F significantly inhibits GPVI-Fc$_2$ binding to fibrin clots at concentrations greater than 100 µg/mL and fragment D and D-dimer weakly inhibited the binding at the highest concentration of 100 µg/mL. CRP-XL had no effect. *P<0.05, **P<0.01, compared to the Control (no added inhibitor) were calculated using a paired t-test. This experiment was done by Dr M Moroi and Dr S Jung.

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APPENDIX A. PRIZES, PUBLICAITONS AND PRESENTATIONS DURING PHD

AWARDS AND PRIZES

Young Investigator Award 2017

International Society of Thrombosis and Haemostasis World Congress, Berlin

Healthcare Pioneers Award 2018

Implementation of a secondary care service for screening, optimisation and support for stroke prevention in atrial fibrillation (SOS-AF). AF Association and Arrhythmia Alliance, London

British Association of Stroke Physicians Abdul Majid Basic Neuroscience and Translational Research Prize 2018

Highest scored abstract in translational category, UK Stroke Forum, Telford

British Association of Stroke Physicians - Warlow Prize 2018

Best Oral Presentation, UK Stroke Forum, Telford

Royal College of Physicians Edinburgh, Senior Fellows' Club Prize 2019

Highest scored paper by trainee, Edinburgh

Finalists BMJ Awards April 2019 - Stroke and Cardiovascular Team of the Year

Implementation of a secondary care service for screening, optimisation and support for stroke prevention in atrial fibrillation (SOS-AF), London.

PUBLICATIONS

1. **Induruwa I**, Jung S, Warburton EA
   Beyond antiplatelets: The role of glycoprotein VI in ischaemic stroke. *Int J Stroke* 11 2016 (6), 618-625.

2. **Induruwa I,** Evans NR, Aziz A, et al.
   Clinical frailty is independently associated with non-prescription of anticoagulation in older patients with atrial fibrillation. *Geriatr Gerontol Int* 2017; 17: 2178-2183.

3. **Induruwa I**, Amis E, Hannon N, Khadjooi K.
   The increasing burden of atrial fibrillation in acute medical admissions, an opportunity to optimise stroke prevention. *J R Coll Physicians Edinb*. 2017; 47: 331-5.

4. **Induruwa I,** Moroi M, Bonna A, et al. The platelet collagen receptor GPVI-dimer recognizes fibrinogen and fibrin through their D-domains, contributing to platelet adhesion and activation during thrombus formation. *J Thromb Haemost. 2018*;16(2):389-404.

5. **Induruwa I,** Liu W, Khadjooi K.
   Screening. Optimisation. Support: a call for stroke prevention in atrial fibrillation. *Br J Hosp Med (Lond).* 2018;79(8):424-425.

6. Hinz C, Liggi S, Mocciaro G, Jung SM, **Induruwa I**, et al.
   A comprehensive UHPLC ion mobility QTOF method for profiling and quantification of eicosanoids, other oxylipins and fatty acids. *Anal Chem.* 2019; 91(13):8025-8035.

7. **Induruwa I,** Holland N, Gregory R, Khadjooi K. The impact of misdiagnosing Bell's palsy as acute stroke. *Clin Med*. 2019 *(In press).*

CONFERENCE ORAL PRESENTATIONS

1. The platelet collagen receptor GPVI-dimer binds to the D-domain of fibrinogen and D-dimer and may contribute to platelet adhesion and activation during thrombus formation. **International Society on Thrombosis and Haemostasis World Congress, Berlin, Germany 2017**

2. Platelet receptor glycoprotein VI-dimer is overexpressed in ischaemic but not haemorrhagic stroke; a promising future anti-thrombotic target? **UK Stroke Forum, Telford, 2018**

3. The increasing burden of atrial fibrillation in acute medical admissions, an opportunity to optimise stroke prevention ***(Invited).*** **Royal College of Physicians Edinburgh, Medical Trainees Symposium: New Approaches to Old Problems, Edinburgh, 2019**

4. Patients with atrial fibrillation significantly overexpress fibrin-binding platelet surface receptor GPVI-dimer. A future anti-thrombotic target? **International Society on Thrombosis and Haemostasis World Congress, Melbourne, Australia 2019**

5. Platelet receptor glycoprotein VI-dimer in ischaemic stroke; a promising future anti-thrombotic target? ***(Invited as 2018 Warlow Prize winner).*** **Stroke Society of Australasia, Canberra, Australia 2019**

APPENDIX B. RELEVANT PEPTIDE SEQUENCES AND AMINO ACID NOMENCLATURE

GFOGER	Glycine-Phenylalanine-Hydroxyproline-Glycine-Glutamate-Arginine
GLOGEN	Glycine-Leucine-Hydroxyproline-Glycine-Glutamate-Asparagine
GPO	Glycine-Proline-Hydroxyproline
GPP	Glycine-Proline-Proline
GPR	Glycine-Proline-Arginine
GPRP	Glycine-Proline-Arginine-Proline

APPENDIX C. ILLNESS ACUITY, AF AND STROKE SCORING SYSTEMS

CHA_2DS_2-VASc score

Risk factor	+ Point
Congestive Cardiac Failure	1
Hypertension	1
Age > 75 years	2
Diabetes Mellitus	1
Previous stroke/TIA/systemic embolism	2
Vascular disease	1
Age 65-74 years	1
Sex (Female)	1

Annual Stroke Risk (%)
1 = 1.3
2 = 2.2
3 = 3.2
4 = 4.8
5 = 7.2
6 = 9.7
7 = 11.2
8 = 10.8
9 = 12.2

Modified Rankin Scale

Level	Description
0	No symptoms
1	No significant disability, despite symptoms; able to perform all usual duties and activities
2	Slight disability; unable to perform all previous activities but able to look after own affairs without assistance
3	Moderate disability; requires some help but able to walk without assistance
4	Moderately severe disability; unable to walk without assistance and unable to attend to own bodily needs without assistance
5	Severe disability; bedridden, incontinent and requires nursing care and attention
6	Death

National Early Warning Score 2 (NEWS)

Physiological Parameter	Score						
	+3	+2	+1	0	+1	+2	+3
Respiration rate/min	≤ 8		9-11	12-20		21-24	≥ 25
SpO_2 scale 1 (%)	≤ 91	92-93	94-95	≥ 96			
SpO_2 scale 2 (%)	≤ 83	84-85	86-87	88-92 or ≥ 93 on air	93-94 on oxygen	95-96 on oxygen	≥ 97 on oxygen
Air or oxygen		Oxygen		Air			
Systolic blood pressure (mmHg)	≤ 90	91-100	101-110	111-219			
Pulse/min	≤ 40		41-50	51-90	91-110	111-130	≥ 131
Consciousness				Alert			CVPU
Temperature (°C)	≤ 35.0		35.1 – 36.0	36.1-38.0	38.1-39.0	≥ 39.1	

APPENDIX D. RELEVENT PUBLICATIONS

International Journal of Stroke WSO

Beyond antiplatelets: The role of glycoprotein VI in ischemic stroke

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Abstract

Background: Platelets are essential to physiological hemostasis or pathological thrombus formation. Current antiplatelet agents inhibit platelet aggregation but leave patients at risk of systemic side-effects such as hemorrhage. Newer therapeutic strategies could involve targeting this cascade earlier during platelet adhesion or activation via inhibitory effects on specific glycoproteins, the thrombogenic collagen receptors found on the platelet surface.

Aims: Glycoprotein VI (GPVI) is increasingly being recognized as the main platelet-collagen receptor involved in arterial thrombosis. This review summarizes the crucial role GPVI plays in ischemic stroke as well as the current strategies used to attempt to inhibit its activity.

Summary of review: In this review, we discuss the normal hemostatic process, and the role GPVI plays at sites of atherosclerotic plaque rupture. We discuss how the unique structure of GPVI allows for its interaction with collagen and creates downstream signaling that leads to thrombus formation. We summarize the current strategies used to inhibit GPVI activity and how this could translate to a clinically viable entity that may compete with current antiplatelet therapy.

Conclusion: From animal models, it is clear that GPVI inhibition leads to an abolished platelet response to collagen and reduced platelet aggregation, culminating in smaller arterial thrombi. There is now an increasing body of evidence that these findings can be translated into the development of a bleeding free pharmacological entity specific to sites of plaque rupture in humans.

Keywords
Stroke, platelets, glycoprotein VI, antiplatelets

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Introduction

Ischemic stroke is a worldwide leading cause of disability and death.[1] An important and distinct subtype of ischemic stroke is due to the acute rupture of atherosclerotic plaques seen in large artery disease.[2] The mechanism of cerebral hypoperfusion involves platelets binding to exposed sub-endothelial collagen (adhesion), resulting in platelet activation, aggregation, and thrombus formation. Unstable thrombi can detach and travel to cerebral vessels causing stroke. As a result, the current management of acute stroke involves using antiplatelet agents such as aspirin and clopidogrel that inhibit platelet activation/aggregation, but often at the risk of off-target adverse effects such as hemorrhage.[3,4]

Novel therapeutic strategies could involve targeting this cascade earlier during platelet adhesion or activation via inhibitory effects on specific platelet glycoproteins, the thrombogenic collagen receptors on their surfaces. Glycoprotein VI (GPVI) is one such crucial transmembrane collagen receptor and pharmacological inhibition of GPVI, in order to stop pathological thrombus formation specific to the site of vessel injury, is currently being explored.

Hemostasis – clotting and thrombus formation

Platelets are anucleate cells derived from megakaryocytes. They contain unique cytoplasmic structures, α- and dense granules that can rapidly release their contents upon activation, promoting thrombus formation.

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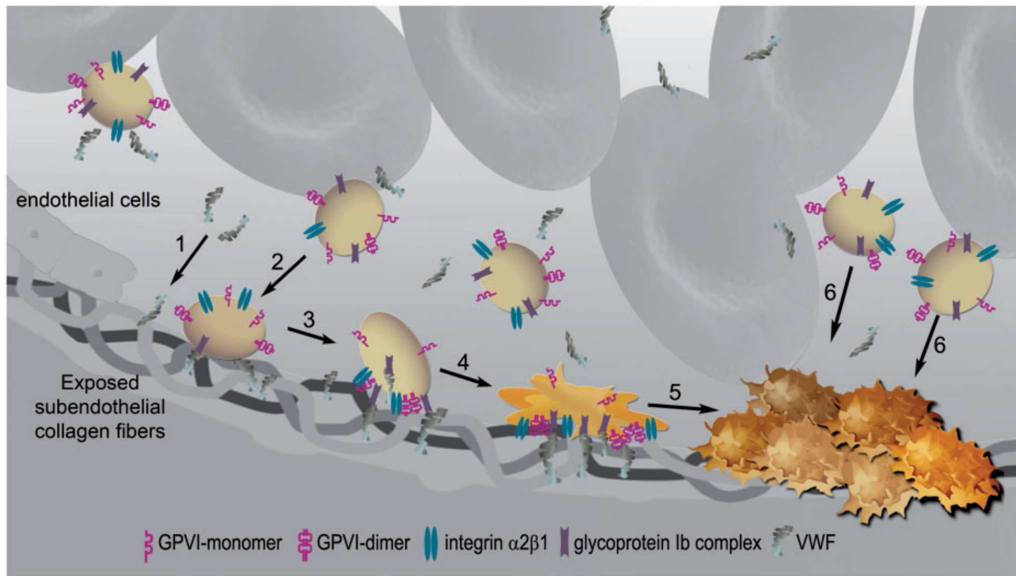
Figure 1. Collagen-binding receptors (GPIb, GPVI, and integrin α2β1) involved in platelet adhesion and activation. Sub-endothelial collagen exposed upon vessel injury binds to von Willebrand factor (vWF) in the blood (step 1). Platelets become tethered to collagen fibers by their vWF receptor GPIb—a weak interaction—so platelets transiently bind and detach, moving along the collagen (step 2). Platelets become firmly attached when their collagen receptors GPVI dimer and integrin α2β1 binds to collagen; signaling through either GPVI or GPIb converts integrin α2β1 to its high affinity form (step 3). GPVI engagement with collagen initiates a signaling cascade that culminates in platelet activation, spreading, and granule contents release (step 4), recruiting other platelets and forming a thrombus (steps 5 and 6). Integrin αIIbβ3 (not shown) becomes activated through inside-out-signaling, enabling it to bind fibrinogen, through which inter-platelet bridges can be formed, allowing thrombus propagation.



Under normal hemostasis numerous protective barriers to thrombus formation exist to contain it to the injured site. This includes the continuous lining of endothelium that prevents platelets coming into contact with the prothrombotic sub-endothelial matrix,[5] expression of ectonucleoside triphosphate diphosphohydrolase (CD39/ENTPD1)[6] and secretion of prostacyclin (PGI2) and nitric oxide.

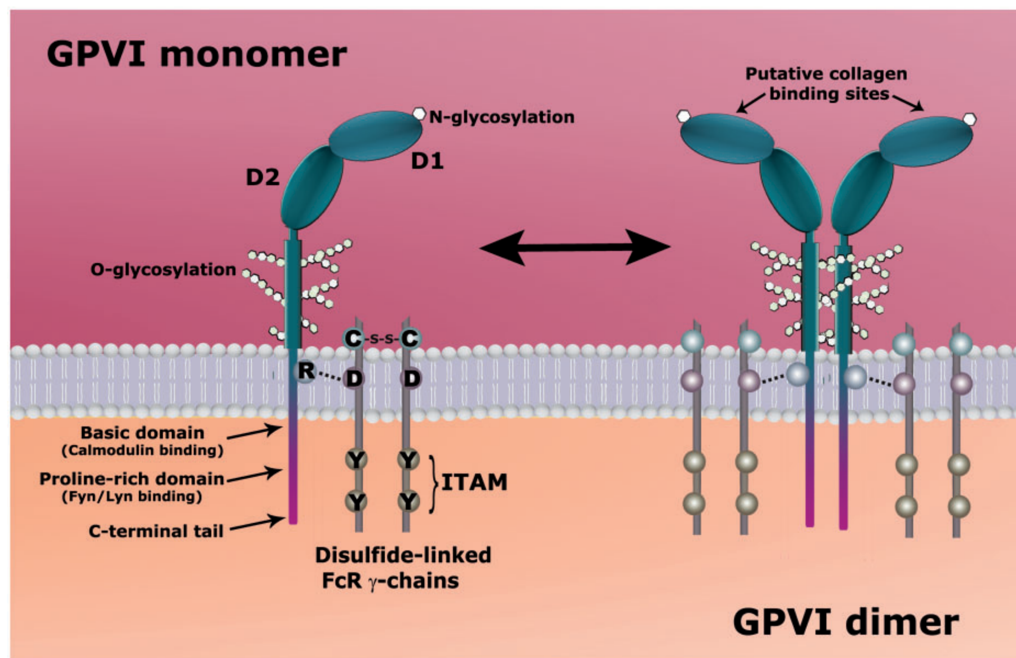
There are four main platelet glycoprotein receptors that participate in the platelet–collagen interaction and facilitate thrombus formation. The GPIb-IX-V complex (GPIbα, GPIbβ, GPIX and GPV) binds to von Willebrand factor (VWF) immobilized on collagen. Both GP Ia/IIa (integrin $\alpha_2\beta_1$) and GPVI bind directly to exposed collagen. GPIIb/IIIa (integrin $\alpha_{IIb}\beta_3$) are converted to their high-affinity forms via inside-out signaling in activated platelets, enabling them to bind free fibrinogen and VWF.

Fibrous collagen is the ligand for both GPVI and integrin α2β1. Each collagen monomer comprises three 1000 amino acid polypeptides arranged in a triple helix, and bundles of these monomers align to form collagen fibers.[7] With atherosclerotic plaque rupture, the sub-endothelial fibrillar collagens (types I and III) are exposed to the blood stream. Under high shear (arterial flow), VWF becomes immobilized on the exposed collagen fibers and binds with the GPIb-IX-V complex[8–10] (Figure 1). Platelets translocate on the collagen surface until firmly arrested through almost simultaneous binding with integrin α2β1 and GPVI. GPVI engagement with collagen (via GPVI dimers) initiates a signaling cascade leading to platelet activation and inside-out signaling (and intracellular calcium mobilization) that converts low affinity forms of integrins $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$ into their active forms, as well as further clustering of GPVI receptors.[8]

Activated platelets rapidly synthesize thromboxane A2 (TxA2) and secrete this, along with the contents of their alpha (fibrinogen, P-selectin, and VWF multimers) and dense granules (ADP).[9] An increase in intracellular calcium in response to ADP and TxA2 induces platelet shape change to an irregular shape with multiple filipoidal surfaces. This facilitates the formation of a close structure of folded platelets in the platelet plug.

Now non-activated platelets are recruited into the growing thrombus, also becoming activated by ADP and TxA2. GPVI-activated platelets provide a pro-coagulant surface for the generation of thrombin—separately through the coagulation cascade by

Figure 2. Structures of GPVI monomer and dimer. The extracellular domain of each monomer comprises two IgG domains (D1 and D2) and a mucin-like Ser/Thr-rich domain connecting D1/D2 to the transmembrane domain. The binding site recognizing the GPO triplets of collagen, resides in D1. Each monomer is non-covalently associated through a salt bridge with FcRγ. FcRγ is a disulphide-linked dimer, each chain containing an ITAM sequence, which when phosphorylated binds to the tyrosine kinase Syk. The phosphorylated Syk now initiates signaling. The short intracellular domain of GPVI contains a basic domain that binds to calmodulin; a proline-rich domain that binds to Src kinases Fyn and Lyn, which participate in phosphorylation of ITAM; and a C-terminal tail.



releasing several substances like FV, FXIII, fibrinogen, and Protein S. This further activates platelets and converts fibrinogen to fibrin, resulting in a strong mesh structure.

Glycoprotein VI and its structure

Glycoprotein VI, a 62-kDA transmembrane glycoprotein exclusively expressed in platelets and megakaryocytes, is associated with an immunoreceptor tyrosine-based-activation motif (ITAM) containing signaling subunit FcRγ (Figure 2).[10] Its gene is mapped to 19q13.4 of the human genome. GPVI contains two IgG-like extracellular domains (D1 and D2) linked by a peptide strand; D2 is connected to the transmembrane domain via a glycosylated stem; and its 51 amino acid cytoplasmic tail is required for signal transmission.[11,12]

GPVI exists in both monomeric and back-to-back dimeric forms on the platelet surface[13] and forms a non-covalently linked complex with the Fc receptor γ-chain (FcRγ) via a salt bridge formed between their transmembrane domains. FcRγ itself is also a dimer, held together by covalent links and is essential for GPVI expression, as FcRγ knockout mice do not express GPVI.[14] Dimerization or multimerization of GPVI was first suggested by Berlanga et al.[15] but proof of the actual existence on platelets was first provided by Jung et al.[16] Later, they found that constitutive dimers of GPVI make up of about 20% of the total GPVI[13,17] in resting platelets of normal individuals and platelet activation increases the number of dimers.[17] Only GPVI dimer has high affinity for collagen, while the monomer binds weakly if at all. Inhibition by dimer specific antibody m-Fab-F markedly inhibited collagen-induced platelet aggregation, highlighting its role as the functional form of GPVI.

Interaction with collagen and signaling

So how does GPVI interact with the fibrous subendothelial collagen exposed upon vessel injury to induce platelet activation? Much of this data has been gained by using snake venom-derived convulxin and CRP (collagen-related peptide) in vitro, which activate platelets in a similar way to collagen.[7,18] CRP, a triple

helical peptide containing 10 glycine–proline–hydroxyproline (GPO) sequences, is an especially potent platelet agonist specific for GPVI. GPVI was suggested to bind to the GPO sequences in collagen. Smethurst et al.[19] later found that the minimum recognition motif for GPVI is one GPO.

Using the crystal structure of GPVI and a docking model with CRP, Horii et al.[20] found that GPVI formed back-to-back dimers, each containing a shallow groove on the D1 domain that is perpendicular to the collagen triple helix, which fits precisely into this 5.5-nm gap.

Loyau et al.[21] proposed that at least two GPVI dimer-FcRγ complexes are required to create a working signaling unit, as the FcRγ chain is also essential for the function of GPVI and each chain contains an ITAM. Upon GPVI–collagen binding, the Tyr residues of the ITAM sequence become phosphorylated, initiating signal transduction. PCLγ2 is subsequently activated, leading to eventual activation of protein kinase C, resulting in calcium mobilization, degranulation and GPIIb/IIIa activation and the start of the next step in the process—platelet aggregation.

GPVI shedding and receptor downregulation

An important consequence of GPVI downstream signaling, which serves to limit thrombus growth, is antibody or metalloproteinase (MMP) induced shedding of a 55 kDa GPVI ectodomain into the blood stream, leaving a 10 kDa remnant that remains platelet-associated.[22–24] This plasma soluble form of GPVI (sGPVI) has been the marker for many quantitative studies on platelet activation through GPVI. The transmembrane metalloproteinases with protease activity, ADAM10 and ADAM17, have been identified as two sheddases that cleave the extracellular portion of GPVI independently of each other under various stimuli including the binding of GPVI ligands.[25] However, when antibody-mediated GPVI downregulation occurs, the catalyst for GPVI shedding appears to be independent of these MMPs, as antibody induced GPVI shedding occurred in mice depleted of ADAM10 and ADAM17.[25] Thus, it is likely that more sheddases participate in GPVI shedding and their role in stroke as a therapeutic target is yet to be determined.[26]

GPVI and ischemic stroke

The importance of GPVI in hemostasis was first reported in a Japanese patient deficient in GPVI who had mild bleeding tendency and whose platelets failed to aggregate in response to collagen.[27] Since then it has been found that platelets treated with GPVI-specific inhibitory antibodies show no interaction with collagen in vivo;[28] In addition, GPVI-deficient individuals exhibit a mild bleeding tendency[28] and GPVI-deficient mice show increased bleeding times.[30] These observations cement GPVI's role as the main signal generator leading to platelet activation, rather than functioning primarily as a platelet adhesion receptor,[29] but suggest that more work is needed to clarify the exact bleeding profile once GPVI is inhibited.

The exact role of GPVI in the different phenotypes of stroke is yet to be elucidated. We know that it plays a role in large artery atherosclerotic infarcts, but its role in lacunar and cardioembloic stroke is unclear. In large artery disease, elevated GPVI expression was shown to be associated with increased risk of stroke development. Enhanced GPVI expression is also seen after ischemic stroke and TIA, with these patients having a poorer clinical outcome at follow-up.[30] Elevated sGPVI levels were found in patients specifically after large artery infarcts and these levels decrease after 3–6 months, which may highlight a role for sGPVI measurements in this stroke substrate, but the evidence in cardioembolic and lacunar types were less convincing.[31]

The pathogenesis of lacunar infarcts is believed to be due to progressive ischemic leukoaraiosis caused by a genetic susceptibility to inflammation-mediated cerebrovascular injury in combination with the classic atherosclerotic risk factors.[32] There is evidence that chronic endothelial dysfunction and activation leading to a prothrombotic environment may cause progression of leukoaraiosis. This is evidenced by higher levels of prothormbotic proteins such as ICAM1, thrombomodulin, fibrinogen, tissue factor in patients with cerebral leukoaraiosis compared to controls.[33–35] Recently, thrombogenic fibrin was shown to be an activator of GPVI in mice.[36] Therefore, we could postulate that GPVI is involved in lacunar stroke and small vessel disease. Nevertheless, further studies are needed looking at GPVI in this subtype of stroke, where currently aspirin and clopidogrel play an important role in secondary prevention.

Recently, functional GPVI has been implicated as a possible receptor for polymerized fibrin, propagating thrombin generation.[37] Further studies may reveal the role of GPVI in cardioembloic stroke as well as thrombus propagation, clot stabilization and infarct growth after ischemic stroke.[38]

Increased GPVI dimerization/multimerization could be one of the earliest measurable steps in platelet activation after plaque rupture. Studies are underway to measure GPVI dimer levels after acute stroke in comparison to healthy controls, which may be a useful diagnostic tool in the future. Since the dimer is the functional receptor form of GPVI, this may partially

explain why the studies discussed above measuring total GPVI (dimers plus monomers) did not find any correlation between its levels and severity of stroke.[30]

Targeting GPVI in ischemic stroke: Beyond antiplatelet therapy

Current antiplatelet therapy acts on this cascade of platelet activation via different methods and is licenced for both acute coronary syndrome (ACS) and ischemic stroke. Aspirin irreversibly inhibits both COX 1 and 2, thus stopping TxA2 formation after platelet activation. Clopidogrel and ticagrelor both irreversibly block the ADP receptor $P2Y_{12}$.[39] Thus both these classes of antiplatelet drugs work by inhibiting platelet aggregation. In comparison with single antiplatelet therapy, dual antiplatelets do convey a reduction in early stroke recurrence, combined transient ischemic attack (TIA), stroke and ACS, and all death[40] but dual antiplatelet therapy is reserved for high-risk individuals after TIA or stroke due to the risk of hemorrhage.

Studies on whether other platelet glycoprotein receptors could be targets for antithrombotic therapy have yielded less promising results. Abciximab, the Fab fragment of a chimeric mouse/human monoclonal antibody, is licenced in ACS for patients awaiting percutaneous coronary intervention. It antagonizes GPIIb/IIIa, which is the final mediator in the pathway to platelet aggregation. Unfortunately it has shown no benefit in functional outcome in acute stroke, coupled with significant increases in fatal or symptomatic intracranial hemorrhage.[41]

Due to the fact that GPIbα (part of the GPIb-IX-V complex) plays a crucial role in platelet adhesion to endothelium in high shear conditions, it has become an attractive target for potential pharmaceutical development. GPIbα and GPVI are closely linked on the platelet surface and are thought to activate similar signaling processes.[42] Much of the knowledge on platelet receptors and ischemic stroke has come from experimentation on animal models, particularly rats and mice, by causing transient middle cerebral artery occlusion (tMCAO). Blockade of GP1bα using Fab fragments, although reducing infarct volume after tMCAO, led to prolonged tail bleeding times compared to those treated with the monoclonal antibody (mAb) anti-GPVI JAQ1.[43]

From similar studies in mice, GPVI was shown to have a huge role in the formation of arterial thrombi. After tMCAO, GPVI-depleted mice (via JAQ1) demonstrate significantly reduced brain infarct volumes, no hemorrhagic transformation, normal platelet counts, and only moderately increased tail bleeding times.[28,43] As platelets are anucleate cells and cannot synthesis protein de novo, injection of rat anti-GPVI antibodies (JAQ 1, 2, and 3) into mice in other studies offered long-term depletion of GPVI from murine platelet surfaces.[28,44] Furthermore, the Fab fragments of antibodies 5C4,[45] OM2,[46] OM4,[47] 9O12,[48] and mFab-F[16] are also potent inhibitors of GPVI-mediated platelet activation (Table 1). The evidence for antibody-driven

Table 1. Antibodies that inhibit GPVI-mediated activation

	Type	Effects
JAQ 1[28]	Rat anti-mouse GPVI	Abolished platelet response to collagen or CRP Significantly reduced infarct size in tMCAO mice *Thrombocytopaenia and moderate bleeding-time prolongation*
9O12 (Fab)[48]	Mouse mAb against human GPVI	Protects against arterial thrombus propagation and collagen-induced aggregation in vitro and ex vivo *Mild bleeding-time prolongation*
5C4 (Fab)[45]	Rat mAb against human GPVI	Almost complete inhibition of platelet aggregation in vivo
OM2 (Fab)[46]	Mouse mAb against human GPVI	Inhibits collagen-induced platelet aggregation *Mild bleeding-time prolongation*
OM4 (Fab)[47]	Mouse mAb against human GPVI	Inhibits collagen-induced platelet aggregation in vitro and ex vivo Reduces in vivo thrombosis in rat carotid artery model *No bleeding-time prolongation*
10B12[58]	Human scFv against human GPVI	Inhibits CRP- and collagen-induced platelet aggregation in vitro
m-Fab-F[16]	Human Fab against human GPVI	GPVI dimer specific Inhibits aggregation and platelet adhesion on collagen

GPVI blockade as a target for stroke therapy is increasing, with further efforts being made to attempt clearer translation into clinical medicine. Recently, Kraft et al.[49] successfully showed that GPVI inhibition using JAQ 1 in adult mice with diabetes and hypertension resulted in smaller cerebral infarcts, better functional outcome, and reduced intracerebral hemorrhage rates compared to mice treated with anti-GPIIb/IIIa.[49]

Patients with autoantibodies to GPVI-FcRγ chain, causing its clearance from the platelet surface are seen to have inefficiently formed thrombi on collagen-coated surfaces.[50] However, their clinical presentation of mild thrombocytopenia, ecchymosis, epistaxis, and prolonged bleeding times highlights the difficulty in translating experimental findings into clinical practice.

Building on the work initially done on animal models,[45] Revacept (PR-15, GPVI-Fc), a recombinant, soluble fusion protein between the human extracellular collagen-binding domain of GPVI and the C-terminal of human Fc, has been developed to competitively inhibit GPVI binding sites on exposed collagen. It was tested in a phase I trial, after intravenous administration to 30 healthy males, in 2011[51] and showed clear dose-dependent inhibition of collagen responsiveness and platelet aggregation. This was done without any significant effect on hemostasis and no significant thrombocytopenia.

Revacept appears to avoid bleeding tendencies by blocking both the GPVI and VWF-mediated platelet activation processes specifically at the site of collagen exposure, thereby avoiding prolongation of bleeding times as described with antibodies to GPVI.[28,43] However, this has only been confirmed on trials in mice after left MCA lesions.[52] Revacept also seems to exert an anti-atherosclerotic effect via vessel endothelial remodelling and a role as a primary preventative medication in the absence of plaque rupture may emerge.[53] It is currently undergoing phase II trials on patients with symptomatic carotid artery stenosis.[54]

Losartan, the angiotensin II receptor blocker has previously shown anti-atherosclerotic effects by blocking inflammogenic mediators and also anti-aggregatory effects independent of its effects on hypertension.[55] EXP3179, its active metabolite, selectively inhibits GPVI function and therefore platelet adhesion and aggregation both in vitro and in vivo.[56] This occurs when losartan binds to the IgG-like domain of GPVI[57] and this interaction, coupled with the fact that losartan has no effect on bleeding profiles, may be the basis of a therapeutic target in the future.

Conclusion

The platelet–collagen interaction, and subsequent platelet activation and thrombus formation, can lead to devastating neurological damage via ischemic stroke. There is a growing body of evidence pointing towards the importance of platelet glycoprotein receptors in stroke due to their requisite role in initiating downstream signaling mechanisms leading to platelet activation and pathological thrombus generation. Current understanding of thrombus formation suggests that GPVI is one such crucial receptor and inhibition of its collagen-induced signaling would be a specific pharmacological target for ischemic stroke. However, most of the research has been carried out in murine experimental models and may not necessarily translate to bleeding-free therapy in humans. The current clinical trials that are underway on Revacept are promising and could lead the way to a better understanding of GPVI antagonism in humans. Smaller studies are being carried out looking at novel small molecule inhibitors of GPVI and if a successful entity is discovered, the next decade should see the development of efficacious and highly specific therapies in ischemic stroke that a have large safety margin.

Declaration of conflicting interests

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ORIGINAL ARTICLE

Platelet collagen receptor Glycoprotein VI-dimer recognizes fibrinogen and fibrin through their D-domains, contributing to platelet adhesion and activation during thrombus formation

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Essentials

- Glycoprotein VI (GPVI) binds collagen, starting thrombogenesis, and fibrin, stabilizing thrombi.
- GPVI-dimers, not monomers, recognize immobilized fibrinogen and fibrin through their D-domains.
- Collagen, D-fragment and D-dimer may share a common or proximate binding site(s) on GPVI-dimer.
- GPVI-dimer–fibrin interaction supports spreading, activation and adhesion involving αIIbβ3.

Summary. *Background:* Platelet collagen receptor Glycoprotein VI (GPVI) binds collagen, initiating thrombogenesis, and stabilizes thrombi by binding fibrin. *Objectives:* To determine if GPVI-dimer, GPVI-monomer, or both bind to fibrinogen substrates, and which region common to these substrates contains the interaction site. *Methods:* Recombinant GPVI monomeric extracellular domain ($GPVI_{ex}$) or dimeric Fc-fusion protein ($GPVI\text{-}Fc_2$) binding to immobilized fibrinogen derivatives was measured by ELISA, including competition assays involving collagenous substrates and fibrinogen derivatives. Flow adhesion was performed with normal or Glanzmann thrombasthenic (GT) platelets over immobilized fibrinogen, with or without anti-GPVI-dimer or anti-αIIbβ3. *Results:* Under static conditions, $GPVI_{ex}$ did not bind to any fibrinogen substrate. $GPVI\text{-}Fc_2$ exhibited specific, saturable binding to both D-fragment and D-dimer, which was inhibited by mFab-F (anti-GPVI-dimer), but showed low binding to fibrinogen and fibrin under our conditions. $GPVI\text{-}Fc_2$ binding to D-fragment or D-dimer was abrogated by collagen type III, Horm collagen or CRP-XL (crosslinked collagen-related peptide), suggesting proximity between the D-domain and collagen binding sites on GPVI-dimer. Under low shear, adhesion of normal platelets to D-fragment, D-dimer, fibrinogen and fibrin was inhibited by mFab-F (inhibitor of GPVI-dimer) and abolished by Eptifibatide (inhibitor of αIIbβ3), suggesting that both receptors contribute to thrombus formation on these substrates, but αIIbβ3 makes a greater contribution. Notably, thrombasthenic platelets showed limited adhesion to fibrinogen substrates under flow, which was further reduced by mFab-F, supporting some independent GPVI-dimer involvement in this interaction. *Conclusion:* Only dimeric GPVI interacts with fibrinogen D-domain, at a site proximate to its collagen binding site, to support platelet adhesion/activation/aggregate formation on immobilized fibrinogen and polymerized fibrin.

Keywords: fibrin; fibrin fragment D-dimer; fibrinogen fragment D; platelet membrane glycoprotein VI; platelet membrane glycoproteins; receptors, collagen.

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Introduction

Myocardial infarction and ischemic stroke, leading causes of disability and death worldwide [1], usually result from atherosclerotic plaque rupture, leading to arterial thrombus formation followed by distal tissue infarction; platelets are crucial for the development of such thrombi. Disruption of the thrombo-protective endothelial layer within blood vessels during plaque rupture exposes collagen and tissue factor (TF). Platelet GPIb-IX-V binds to von Willebrand factor (VWF) deposited on exposed subendothelial collagens, slowing them down so

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Glycoprotein VI (GPVI) can bind to the collagen, which initiates a signaling cascade leading to thrombus formation [2,3]. A dimer consisting of two GPVI-monomers is the functionally active form of this receptor, which is constitutively present (about 20% of the total GPVI) on resting platelets [4]. GPVI-dimers recognize Gly-Pro-Hyp (GPO) peptide repeats on collagen [5,6] and once platelets adhere via GPVI, activation of integrin α2β1 supports firm adhesion to collagen [7].

The subsequent growth and stability of a clot relies on coordinated activation of both the collagen pathway and TF (tissue factor) pathway [8], which can also form thrombi independent of collagen exposure [9], relying on thrombin (factor IIa) generation via the proteolytic extrinsic coagulation cascade. Thrombin generates insoluble fibrin from soluble circulating fibrinogen, which polymerizes and acts to stabilize the growing clot via platelet receptor integrin αIIbβ3 [10,11].

Human fibrinogen molecules are comprised of six disulfide-linked polypeptide chains $(A\alpha, B\beta, \gamma)_2$ organized as outer D-domains containing the C-termini of the three chains, connected to a central E-domain. Thrombin cleaves fibrinopeptides A and B from the N-termini of the α- and β-chains within the E-domain, exposing new binding sites that allow fibrin polymerization through linking to one or two D-domains in an end-to-middle configuration [12]. D-domain γ-chains undergo reciprocal crosslinking mediated by FXIIIa, leading to a strengthened fibrin mesh [13]. The peptide Gly-Pro-Arg-Pro (GPRP), which binds to the fibrin polymerization sites in the D-domain, inhibits its binding to an adjacent E-domain, leading to formation of non-crosslinked, or monomeric fibrin [14]. Plasmin can act on fibrinogen to release D- or E-domains separately, or on polymerized fibrin to release D-dimer, typically consisting of two FXIIIa crosslinked D-domains from an original fibrinogen molecule [15].

Initially, αIIbβ3 is converted to its high-affinity form, enabling binding to fibrinogen and fibrin, leading to platelet aggregation, thrombus growth and formation of a hemostatic plug [16]. The complex role that αIIbβ3 plays in thrombus formation has already been extensively investigated, but translation of its inhibition to an antiplatelet agent has not been successful in conditions such as ischemic stroke [17].

It has been long suspected that GPVI plays a role in thrombus growth independent of collagen exposure through ligands such as thrombin [18,19], laminin [20], fibronectin [21,22] and vitronectin [23]. Recently, it has been reported that GPVI interacts with polymerized fibrin to facilitate thrombus growth [24–26].

The present study provides evidence indicating that the collagen-binding, dimeric form of GPVI, is also able to recognize the D-domain, a region universal to fibrinogen, D-fragment (resulting from proteolytic cleavage of fibrinogen), D-dimer, and monomeric and polymerized fibrin. The binding site on GPVI-dimer for the D-domain is proximate to its collagen-binding site and GPVI-dimer assists αIIbβ3 in thrombus growth and stabilization, suggesting that GPVI-dimer could be the central platelet receptor linking both collagen and TF pathways in thrombosis.

Materials and methods

Fibrinogen/fibrinogen derivatives

Figure S1 summarizes the commercial preparations of human fibrinogen and derivatives used; SDS-PAGE confirmed the manufacturer's stated purity. Preliminary studies showed that all the listed fibrinogens gave similar results. We used Fibrinogen 3 (Fbg-3) in this study because it was plasminogen-, VWF- and fibronectin-depleted, but not factor XIII– depleted.

Recombinant GPVI-dimer and -monomer

$GPVI_{ex}$ is a recombinant protein comprising the extracellular domain of GPVI, including the collagen binding portions D1D2 and most of the highly glycosylated extracellular stem portion. $GPVI\text{-}Fc_2$ is a fusion protein of the GPVI extracellular domain (same amino acid sequence as $GPVI_{ex}$) and the Fc domain of IgG, which spontaneously dimerizes ($[GPVI\text{-}Fc]_2$, abbreviated as $GPVI\text{-}Fc_2$). Both proteins were designed and characterized by M. Moroi and colleagues [4,27].

Collagen substrates (synthesized and crosslinked in-house)

CRP-XL (crosslinked collagen-related peptide; GCO-$(GPO)_{10}$-GCOG-NH_2) is a GPVI-specific agonist [28] and GPP10-XL is its inactive analogue (GCP-$(GPP)_{10}$G-NH_2) [5].

GPVI-dimer-specific antibodies

mFab-F is a GPVI-dimer-specific inhibitory Fab [3] that recognizes the dimers on resting platelets. GPVI-dimer-specific non-inhibitory 204-11 Fab [4] recognizes the GPVI-dimers on resting and activated platelets. Both antibodies were developed by M Moroi and colleagues.

Immobilization of fibrinogen substrates and collagens

For ELISA, static adhesion, confocal microscopy and flow adhesion, respectively, 96-well black optical polymer-bottom ELISA plates (Nunc A/S, Roskilde, Denmark), Maxisorp ELISA plates (Nunc A/S), glass-bottomed dishes (10-mm, No. 0; MatTek, Ashland, MA, USA) or glass slides were incubated with fibrinogen or collagenous substrates in phosphate-buffered saline (PBS; for all substrates except Horm, which was diluted in supplied diluent), overnight at 4 °C; details are provided in the figure legends. The immobilized surfaces were blocked by incubating with 1%

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bovine serum albumin (BSA)/PBS for 1 h, washed twice with PBS, and used for experiments.

Polymerized fibrin (pFibrin) was prepared by adding thrombin (2 U mL^{-1}; Sigma-Aldrich, Dorset, England) to Fbg-3 (10 μg mL^{-1} unless otherwise stated), and incubated for 30 min at RT, aliquoted to the surface to be coated and left overnight at 4 °C. The coated surface was blocked with 1% BSA, treated with hirudin or protease inhibitor cocktail for 10 min to inhibit active thrombin, and washed with PBS. The presence of fibrin strands was confirmed by staining with anti-fibrin (UC45)/Alexa-Fluor-Plus 555 anti-mouse IgG (ThermoFisher Scientific, Warrington, UK); Fig. 4(B) shows a representative image.

Immobilized *monomeric fibrin (mFibrin)* was prepared by adding GPRP (2 mM) to Fbg-3 (10 μg mL^{-1}, unless stated otherwise) and incubated for 30 min at RT before adding thrombin as above; other procedures are the same as for pFibrin.

ELISA analysis of GPVI-dimer and GPVI-monomer binding to fibrinogen and its derivatives

GPVI binding to immobilized substrates was measured by ELISA as described previously [4]. Bound GPVI was measured by reacting the wells with 1G5 (mouse anti-pan GPVI Mab; Biocytex, Marseille, France) [20,29] followed by IRDye®800CW goat anti-mouse IgG (Licor Biosciences, Cambridge, UK) or Alexa-fluor 647-conjugated anti-human Fc (BioLegend, London, UK).

Washed platelets

ACD-anticoagulated blood from healthy volunteers or the Glanzmann thrombasthenic (GT) patient was obtained with informed consent, in accordance with the Treaty of Helsinki. Washed platelets were prepared as described previously [30] and resuspended in HEPES-Tyrode's buffer (HT: 134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 10 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid, 5.5 mM glucose, pH 7.3).

Other platelet assays

Aggregometry was performed with a PAP-8E aggregometer (Bio/Data Corporation, Alpha Laboratories (UK distributor), Hampshire, UK); details are given in the legend for Fig. 5. Flow cytometry was performed with an Accuri C6 flow cytometer (BD, Oxford, UK) on washed platelets; details are given in the legend for Fig. 3. Static platelet adhesion (Fig. 3) experiments were performed as described previously [4] to determine the effects of mFab-F and Hip8 (anti-αIIbβ3) on adhesion of washed platelets to fibrinogen-substrate-coated or collagen-coated ELISA plates.

Confocal imaging

Washed platelets (3×10^7 mL^{-1} in HT/2 mM $MgCl_2$) were allowed to adhere on fibrinogen-substrate-coated MatTek dishes for 1 h at 37 °C and formalin fixed as described previously [31]. Pre- and post-staining details are provided in the figure legends. Stained samples were imaged under an Olympus FV300 IX81 laser-scanning confocal microscope (Olympus, Essex, UK), with a 60x oil immersion objective).

Effect of anti-GPVI-dimer antibodies on platelets under flow

Flow adhesion experiments were performed with normal or GT blood and image acquisition was carried out as described [32]; details are given in the figure legends. After 5 min, Z-stacks (ΔZ = 0.69-μm increments from the coverslip plane) were collected and analyzed by ImageJ1.35 (National Institutes of Health). The coverslip plane was defined as the Z-plane with the largest fluorescent platelet area and used to calculate the surface area coverage (SA; μm^2). Thrombus volume (μm^3) was calculated as the sum of the detected surface areas of all images in the Z-stack, multiplied by ΔZ. Mean thrombus height (MTH) was calculated as thrombus volume/field area.

Data analyses

Non-linear regression (one-site model) to obtain dissociation constants (K_d) and statistical analyses was performed with Prism version 7.2 (GraphPad Software, San Diego, CA, USA).

Results

Dimeric, but not monomeric, GPVI binds to immobilized D-fragment and D-dimer with high affinity

We measured GPVI-Fc_2 binding to immobilized fibrinogen derivatives and collagen III (col III) (Fig. 1A; mean ± SEM, 3 experiments, duplicate replicates). GPVI-Fc_2 showed high levels of binding to D-fragment and D-dimer, but did not bind E-fragment. There was very low but measurable binding to fibrinogen, pFibrin and mFibrin. GPVI-Fc_2 binding to D-fragment or D-dimer was specific and saturable, their K_d values being higher but similar in magnitude to that for col III (Fig. 1E). At all coating concentrations (10–200 μg mL^{-1}, Fig. 1C), fibrinogen, mFibrin or pFibrin binding was too low and scattered to determine K_d. B_{max}, which depends on the amount of immobilized substrate, cannot be determined by ELISA.

Monomeric GPVI, $GPVI_{ex}$ (0–50 μg mL^{-1}), did not bind D-fragment, D-dimer or fibrinogen and exhibited feeble col III binding compared with GPVI-Fc_2, as previously reported [4] (Fig. 1B).

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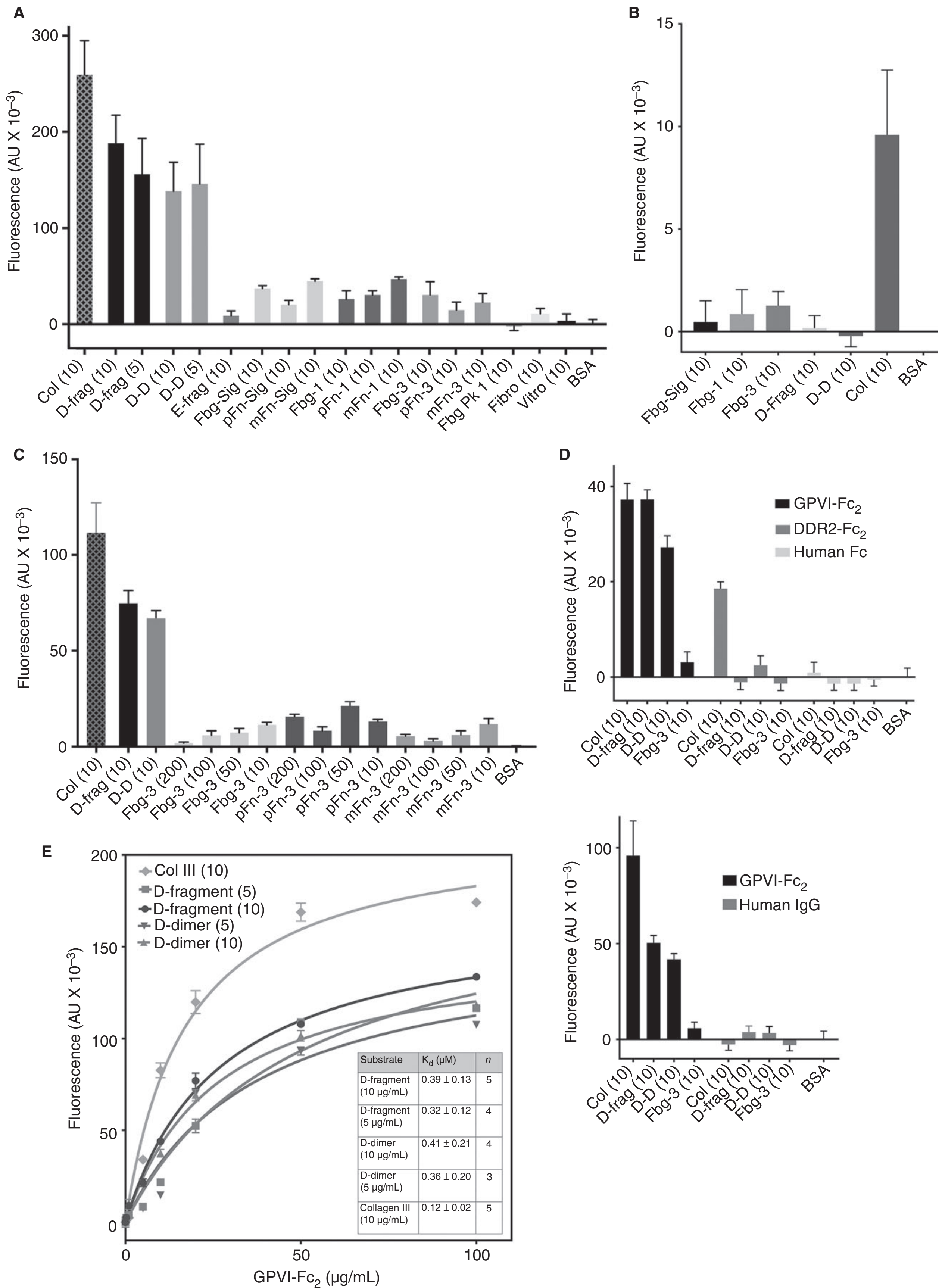

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Fig. 1. Interaction of GPVI-Fc_2 and $GPVI_{ex}$ with immobilized fibrinogen derivatives. The following abbreviations were used in the graphs: collagen type III (Col); D-fragment (D-frag); D-dimer (D-D); E-fragment (E-frag); fibrinogen-Sigma (Fbg-Sig), pFibrin-Sigma (pFn-Sig), mFibrin-Sigma (mFn-Sig); fibrinogen-1 (Fbg-1), pFibrin-1 (pFn-1), mFibrin-1 (mFn-1); fibrinogen-3 (Fbg-3), pFibrin-3 (pFn-3), mFibrin-3 (mFn-3); fibrinogen peak 1 (Fbg Pk1); fibronectin (Fibro); vitronectin (Vitro); BSA (bovine serum albumin). Binding of GPVI-Fc_2 (recombinant GPVI-dimer), $GPVI_{ex}$ (recombinant GPVI monomer), DDR2-Fc_2, human Fc and human IgG to immobilized fibrinogen derivatives was measured by ELISA (enzyme-linked immunosorbent assay) as detailed in the Methods. Collagen type III (Col) was used as a positive control. The numbers in parentheses indicate the concentrations of substrate used to coat the ELISA plate wells in μg mL^{-1} in PBS. Non-specific binding was determined with BSA alone, which was subtracted from the total binding to obtain specific binding. (A) Fibrinogen-Sigma, fibrinogen-1, fibrinogen-3 and fibrinogen peak 1 are the fibrinogens described in Figure S1 and the corresponding polymerized (pFibrin) and monomeric fibrins (mFibrin) produced from thrombin +/− GPRP addition, respectively. Fluorescence values shown are the mean ± SEM, from five different experiments, with duplicate repeats in each. GPVI-Fc_2 (50 μg mL^{-1} shown) shows high levels of binding to D-fragment, D-dimer and col III. In contrast, there is only very low binding to fibrinogen, mFibrin and pFibrin and no binding to E-fragment, fibrinogen peak 1, fibronectin or vitronectin. (B) $GPVI_{ex}$ (50 μg mL^{-1}) showed no binding to immobilized fibrinogen-Sigma, fibrinogen-1, fibrinogen-3, D-fragment or D-dimer with some binding to collagen III, suggesting that the GPVI monomer is unable to recognize the D-domain. (C) No increase in binding to GPVI-Fc_2 is seen with fibrinogen-3 (Fbg-3) or pFibrin-3 and mFibrin-3 produced from it, at higher concentrations (Fbg-3; 10–200 μg mL^{-1}), whereas collagen III, D-fragment and D-dimer bind at 10 μg mL^{-1}. (D) Human Fc shows no binding to the fibrinogen derivatives and no binding to col III. Immobilized DDR2-Fc_2 (20 μg/mL) binds collagen as expected, but neither DDR2-Fc_2, human Fc (20 μg/mL) nor human IgG (25 μg mL^{-1}) bind to fibrinogen, whereas matched concentrations of GPVI-Fc_2 bind well to D-fragment, D-dimer and collagen. (E) Kinetic analyses of the binding of GPVI-Fc_2 to D-fragment and D-dimer to determine K_d (dissociation constant) values. GPVI-Fc_2 exhibits saturable binding to both D-fragment and D-dimer (coated at two different concentrations), with K_d values (data fit to a non-linear regression, one-site model) shown in the inset. The K_d values for D-fragment and D-dimer are similar and they are comparable to the K_d value for col III.

Furthermore, mFab-F significantly reduces GPVI-Fc_2 binding to D-fragment ($P < 0.01$), D-dimer ($P < 0.01$) and collagen type III ($P < 0.05$), compared with a non-specific human Fab (hFab; Jackson ImmunoResearch) control (Fig. 5B). These results suggest that the binding site for GPVI lies in the D-domain, universally present in fibrinogen, D-dimer, mFibrin and pFibrin, for which only GPVI-dimer has good affinity.

Human Fc and DDR2-Fc_2 (recombinant Fc-fusion protein of discoidin domain-containing receptor 2, another collagen-binding protein [33]) and human IgG did not bind to any of the fibrinogen substrates, whereas DDR2-Fc_2 bound to collagen as expected, confirming the specificity of our assay to the GPVI portion of GPVI-Fc_2 (Fig. 1D).

Collagenous substrates inhibit the binding of GPVI-dimer to D-fragment and D-dimer, suggesting proximity of binding sites

To determine if the binding site on GPVI-dimer for D-fragment and D-dimer is close to that for collagen, we incubated GPVI-Fc_2 (20 μg mL^{-1}) with 0–20 μg mL^{-1} of col III (non-fibrous), Horm collagen (fibrous), CRP-XL and GPP10-XL and reacted it with immobilized D-fragment and D-dimer. The converse experiment, where immobilized collagenous substrates were reacted with GPVI-Fc_2 incubated with fibrinogen, fibrins or fibrinogen subdomains, was also performed (described in next section). Col III and Horm showed similar concentration-dependent abilities to inhibit binding of GPVI-Fc_2 to D-fragment, with complete inhibition at ≥ 0.5 μg mL^{-1} (Fig. 2A-i). CRP-XL was effective at lower concentrations, producing complete inhibition at ≥ 0.05 μg mL^{-1}. Higher col III and Horm concentrations were needed to inhibit GPVI-Fc_2 binding to D-dimer, whereas the inhibition curve of CRP-XL was similar to that against D-fragment (Fig. 2A-ii). As expected, GPP10-XL had little effect against GPVI-Fc_2 binding.

Determining if GPVI-Fc_2 and platelet GPVI can interact with fibrinogen or its derivatives in solution

D-fragment, E-fragment, D-dimer, fibrinogen and pFibrin (0–20 μg mL^{-1}) could not inhibit binding of GPVI-Fc_2 (20 μg mL^{-1}) to immobilized col III, Horm or CRP-XL (Fig. 2B i–iii), suggesting that GPVI-Fc_2 cannot bind to them in solution.

Flow cytometry (Fig. 5C) shows that the washed normal platelets bind to fibrinogen and D-dimer, at a low level, but thrombasthenic platelets that lack αIIbβ3 exhibit no binding to either. This means that activation through αIIbβ3 is required for expression of D-dimer and fibrinogen binding; in this case, the platelets may have become slightly activated during the washing procedures.

pFibrin (> 5 μg mL^{-1}) was able to induce platelet aggregation (Figure S2); mFab-F prolonged its lag-time but did not reduce maximum aggregation, whereas 45-μM Eptifibatide (anti-αIIbβ3) abrogated it. This suggests that platelet aggregation through pFibrin in suspension doesn't require GPVI-dimer. Fibrinogen, D-fragment and D-dimer (5–20 μg mL^{-1}) did not induce platelet aggregation (Fig. 5A-i). However, D-dimer concentration-dependently inhibited Horm-induced aggregation of washed platelets (Fig. 5A-ii). In platelets pre-incubated with 100 μg mL^{-1} Horm, lower D-dimer concentrations did not affect mFab-F inhibition of Horm-induced aggregation, but high D-dimer concentration relieves the inhibition, suggesting that D-dimer may bind at a

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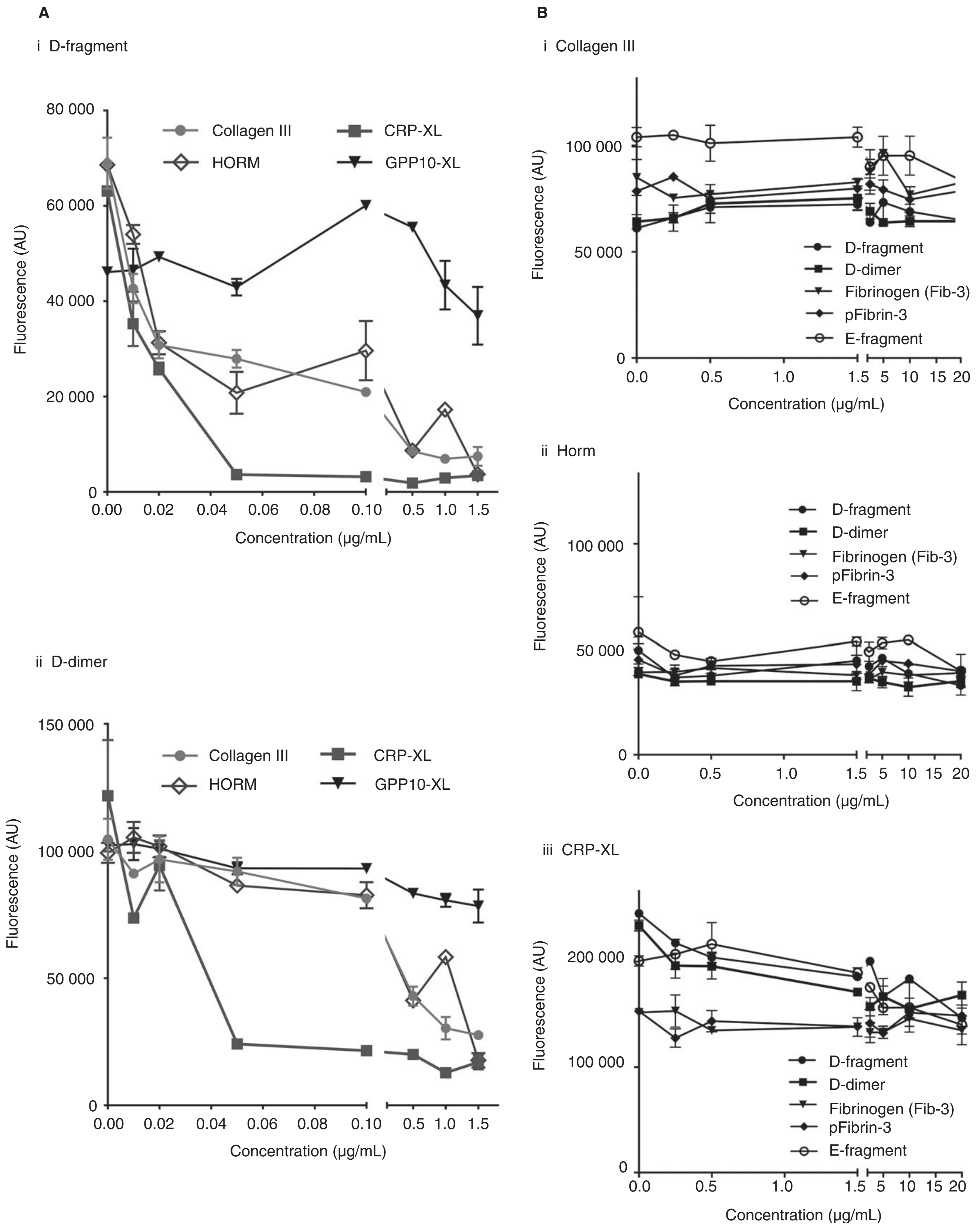

separate but nearby site, perhaps producing a conformational change that may reduce the affinity of mFab (Fig. 5A-iii).

Therefore, fibrinogen derivatives in solution are not able to bind to platelet GPVI-dimer or GPVI-Fc$_2$. Although D-dimer appears to bind to GPVI-dimer in

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Fig. 2. Collagenous substrates compete with GPVI-dimer binding to immobilized D-fragment and D-dimer, but fibrinogen substrates in solution do not displace binding of GPVI-dimer to immobilized collagen substrates. (A) Collagenous substrates inhibit the binding of GPVI-Fc_2 to D-fragment and D-dimer. D-fragment or D-dimer (10 μg mL^{-1}) was immobilized to ELISA wells and left overnight at 4 °C. GPVI-Fc_2 (20 μg mL^{-1}) was incubated with increasing concentrations of collagenous substrate (0–20 μg mL^{-1}) and ELISA performed as described in the Methods. The following collagenous substrates were tested: collagen type III (col III, non-fibrous), Horm collagen (fibrous), CRP-XL and GPP10-XL. (A i) CRP-XL inhibited GPVI-Fc_2 binding to D-fragment, abrogating it at ≥ 0.05 μg mL^{-1} and col III and Horm, at ≥ 0.5 μg mL^{-1}. GPP10-XL, which does not bind to GPVI-Fc_2, did not affect the binding. (A ii) GPVI-Fc_2 binding to D-dimer was similarly inhibited by col III, Horm and CRP-XL, although slightly higher concentrations of col III and Horm were needed to obtain the same degree of inhibition. (B) Col III, Horm and CRP-XL (10 μg mL^{-1}) were immobilized to ELISA wells and left overnight at 4 °C. D-fragment, D-dimer, E-fragment, fibrinogen (Fib-3) and its respective pFibrin (0–20 μg mL^{-1}) incubated with GPVI-Fc_2 (20 μg mL^{-1}) cannot inhibit GPVI-Fc_2 binding to col III (B i), HORM (B ii), or CRP-XL (B iii), suggesting that D-domain in solution does not have sufficient affinity for GPVI-Fc_2. Data are presented as the mean ± SEM obtained from three separate experiments with each data point as a duplicate.

suspension, it does not cause aggregation, and indeed inhibited it, an important physiological barrier to prevent platelets from spontaneous aggregation through GPVI-dimer.

Platelet adhesion to fibrinogen substrates under static conditions and effect of inhibiting antibodies

Under static conditions, washed platelets adhere to fibrinogen, D-fragment, pFibrin, mFibrin and D-dimer (Fig. 3), but not to E-fragment (data not shown in Fig. 3). mFab-F mildly but significantly inhibited platelet adhesion to D-fragment, mFibrin, pFibrin and col III, whereas hFab (human Fab) had no effect. Hip8 dramatically decreased platelet adhesion on fibrinogen, D-fragment, D-dimer, mFibrin and pFibrin. mFab-F + Hip8 further decreased platelet adhesion on D-fragment, D-dimer, mFibrin and pFibrin. Thus, static adhesion to fibrinogen substrates is mainly αIIbβ3-dependent, with a small contribution by GPVI-dimer. In contrast, static

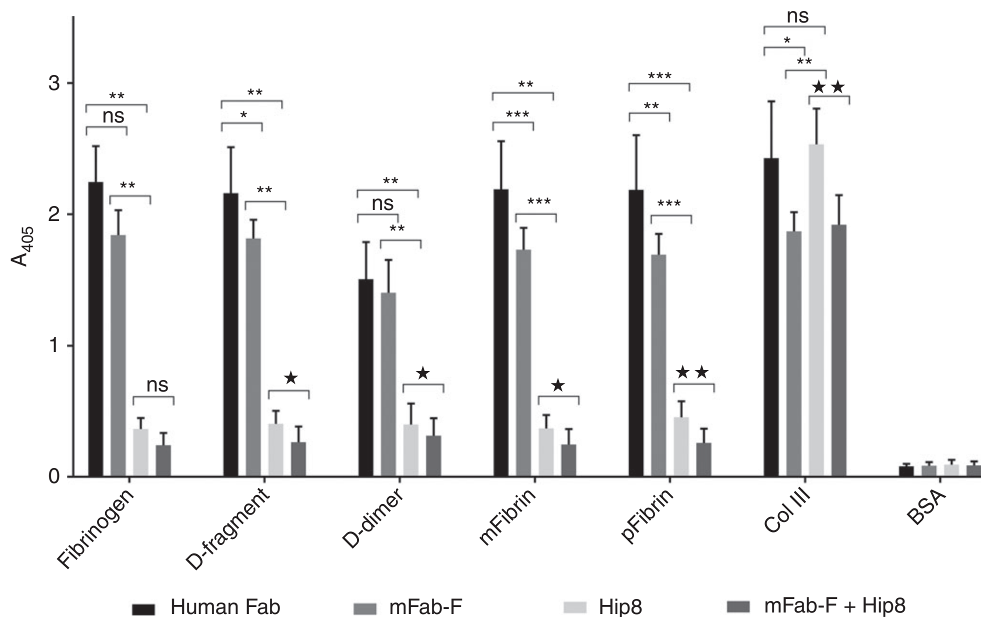

Fig. 3. Platelet adhesion to immobilized fibrinogen substrates under static conditions and effect of antibodies against GPVI-dimer and integrin αIIbβ3. Adhesion of washed platelets pretreated with human Fab (control, 200 μg mL^{-1}), mFab-F (200 μg mL^{-1}), Hip8 (anti-GPIIbα, inhibitory, 10 μg mL^{-1}), or a combination of mFab-F (200 μg mL^{-1}) and Hip8 (10 μg mL^{-1}), was determined as previously described; in this assay, the adhered platelets are lysed and their content of alkaline phosphatase measured in an assay using the chromogenic substrate *p*-nitrophenyl phosphate, which is hydrolyzed by this enzyme to *p*-nitrophenol, which is detectable at 405-nm absorbance. Data shown are the mean ± SD of three separate experiments, each data point run in duplicate, using platelets from three different healthy control individuals. Data were analyzed by the paired *t*-test, with significances shown on the figure: control (human Fab) vs. mFab-F, Hip8 or mFab8 + Hip8 (*$P < 0.05$, **$P < 0.01$,***$P < 0.001$); mFab vs. Hip8 (**$P < 0.01$, ***$P < 0.001$); Hip8 vs. Hip8 + m-Fab-F (a*$P < 0.05$,**$P < 0.01$); ns = not significant. Platelets adhere to fibrinogen, pFibrin (polymerized fibrin made from Fib-3), mFibrin (monomeric fibrin made from Fib-3), D-fragment, D-dimer and collagen type III (col III), the positive control. Platelets do not adhere to E-fragment, so the following discussion of antibody effects only refers to the substrates supporting platelet adhesion. mFab-F mildly but significantly decreased platelet binding to D-fragment, m-fibrin, p-fibrin and collagen. Hip8 markedly decreases platelet adhesion to all fibrinogen substrates, but has no effect on platelet adhesion to collagen, which is mainly supported by integrin α2β1. Adding mFab-F and Hip8 together further diminishes platelet adhesion to all immobilized fibrinogen substrates, suggesting that integrin αIIbβ3 plays the predominant role but GPVI-dimer makes a significant contribution.

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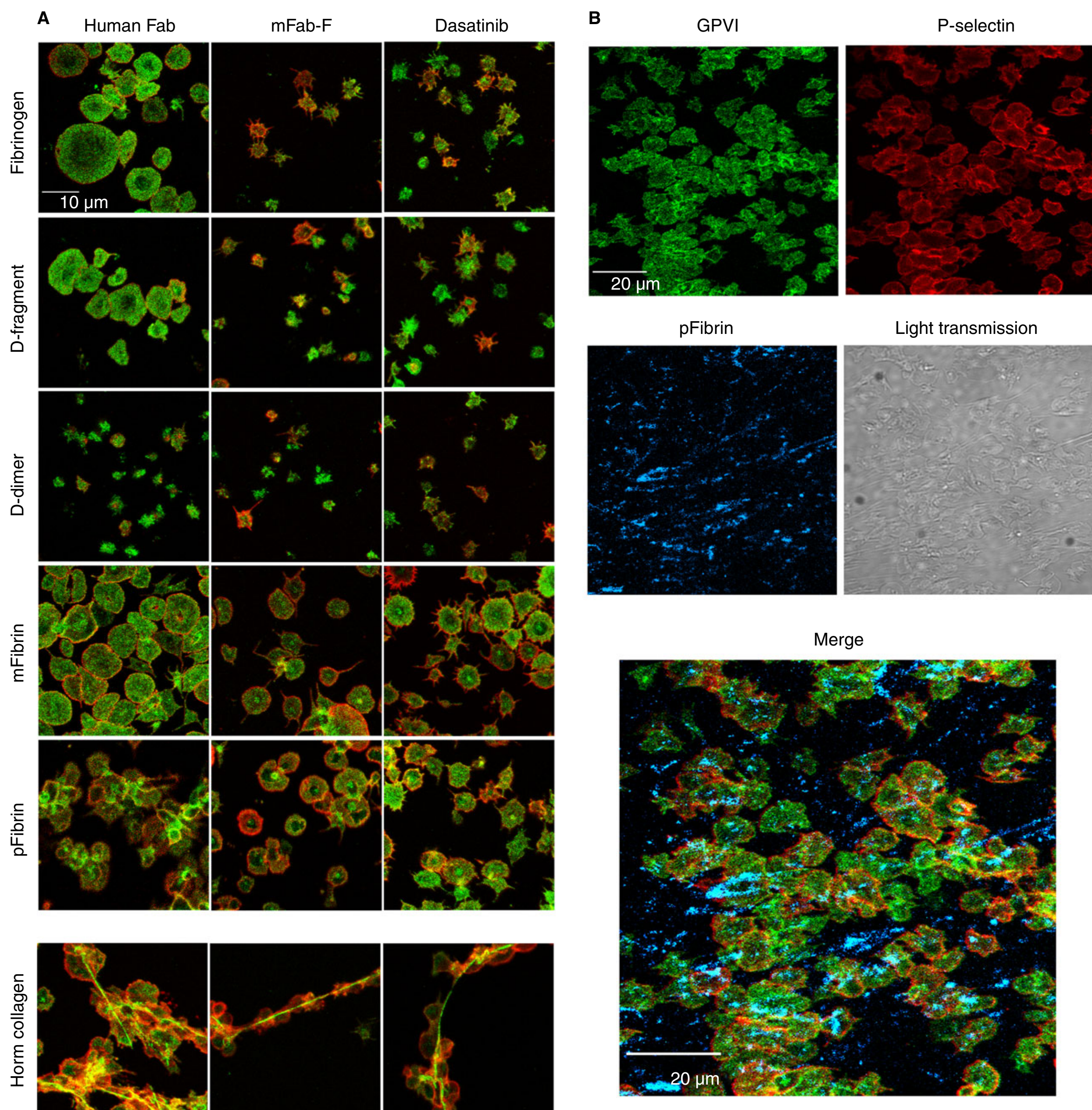

Fig. 4. Confocal images of washed platelets adhered to fibrinogen derivatives and Horm collagen and the effect of inhibitors. Washed platelets suspended in HT buffer were pretreated with human Fab (control, 200 μg mL^{-1}), inhibitory anti-GPVI-dimer mFab-F (200 μg mL^{-1}) or the Src inhibitor Dasatinib (50 nM) for 10 min and then were allowed to adhere to fibrinogen, fibrinogen derivative or Horm collagen immobilized on MatTek glass dishes, followed by fixation and staining with fluorescently labelled antibody (as described in the Methods). (A) Images show platelets stained with Alexa-fluor 488–1G5 (green; pan GPVI) and Alexa-fluor 647–anti-P-selectin (red) to measure platelet activation. Images were obtained with a 60x oil immersion objective on an Olympus confocal microscope. As shown in (A) (left-most column of images), platelets spread well on fibrinogen, D-fragment, mFibrin and Horm collagen, with evident surface expression of P-selectin. Platelet aggregate formation was seen on pFibrin, whereas platelets spread but did not appear aggregated on mFibrin. Horm collagen supported aggregate formation, with the characteristic binding of GPVI along the Horm fibers [31]. In contrast, platelets did not spread well on D-dimer, with only some platelets showing P-selectin expression. Treatment with mFab-F (A, middle column of images) prevented platelet spreading on fibrinogen, D-fragment and Horm collagen, and decreased the extent of spreading, with evident filopodia observed, on mFibrin and pFibrin, whereas there was little change in the less-spread platelets on D-dimer. mFab-F prevented aggregate formation on both pFibrin and Horm collagen, consistent with inhibition of activation through GPVI. Dasatinib (A, right-most column of images) shows effects similar to those of mFab-F. (B) In these experiments, the immobilized pFibrin or mFibrin (data not shown) was stained with anti-fibrin/Alexa-fluor 547–anti-mouse IgG (blue) after the surface was blocked with 1% bovine serum albumin; the dishes were then thoroughly washed and used for the platelet adhesion experiments, which were performed as shown in (A). pFibrin (blue) is clearly evident, whereas there was no staining of the immobilized mFibrin (data not shown). P-selectin (red) expression and aggregate formation is evident on the surface of the adhered platelets. [Color figure can be viewed at wileyonlinelibrary.com]

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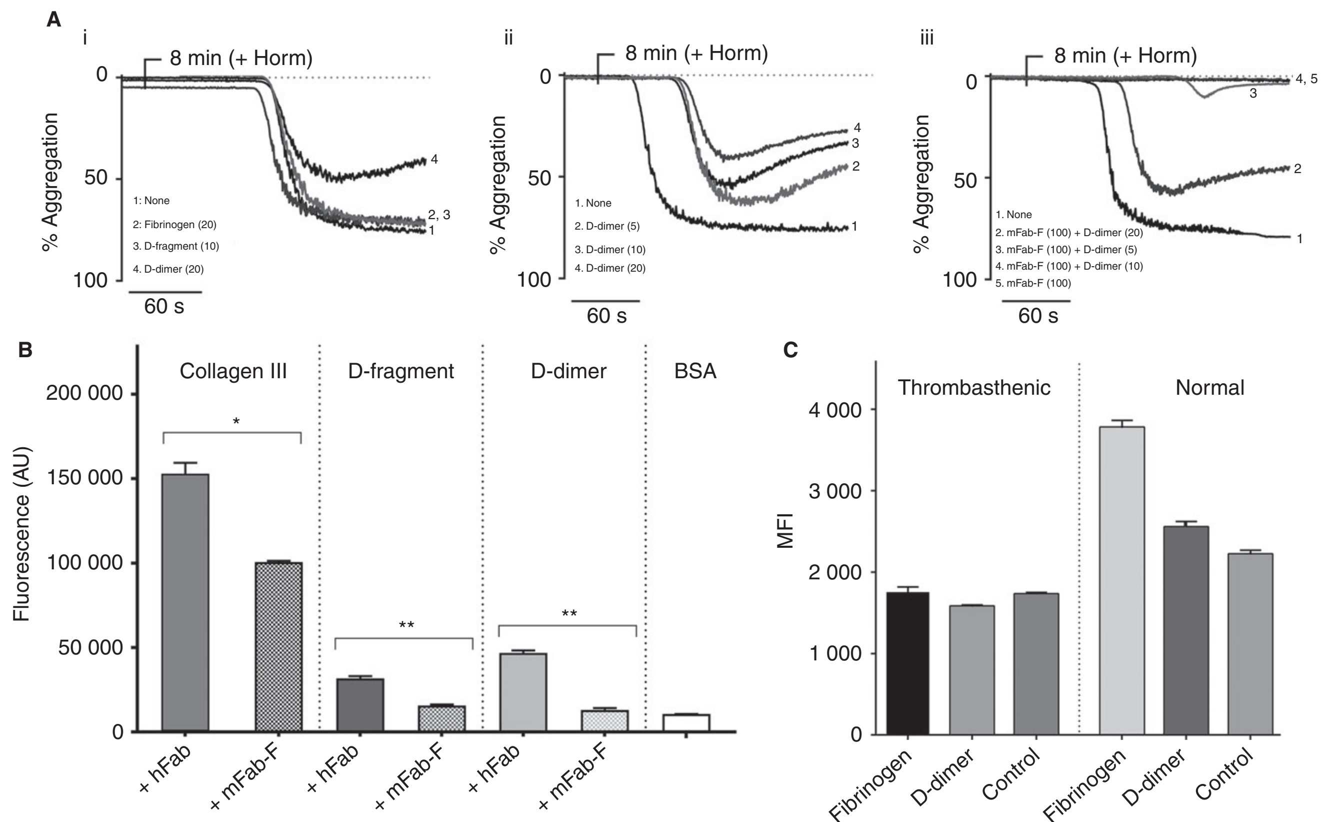

Fig. 5. Binding of GPVI-dimer to D-fragment, D-dimer, fibrinogen and fibrin in solution and effect of mFab-F on static adhesion to D-domain. (A) Aggregometry was performed with washed platelets (2.5×10^8) either untreated or incubated for 5 min with mFab-F (100 μg mL^{-1})). At 3 min, D-fragment, D-dimer or fibrinogen (parentheses indicate concentrations in μg mL^{-1}) was added to the platelets (A i–iii). D-fragment, D-dimer or fibrinogen do not cause aggregation up to the maximum concentration (20 μg mL^{-1}). If no aggregation occurred by 8 min, Horm was added to induce platelet aggregation, verifying that the platelets were active. D-dimer inhibits Horm-induced aggregation (A i) in a concentration-dependent manner (A ii). (A iii) Washed platelets were pre-incubated with 100 μg mL^{-1} of mFab-F for 5 min. D-dimer (5–20 μg mL^{-1}) was added at 3 min and Horm (3 μg mL^{-1}) at 8 min. Horm-induced aggregation is inhibited when mFab-F is present, although at higher concentrations of D-dimer (20 μg mL^{-1}) some aggregation occurs. The images are representative of results from three experiments. (B) ELISA plates were coated with col III, D-fragment or D-dimer (25 μg mL^{-1}) and left overnight at 4 °C. GPVI-Fc_2 (25 μg mL^{-1}) was incubated with either 100 μg mL^{-1} human Fab (hFab), as a control, or 100 μg mL^{-1} mFab-F for 1 h at room temperature and reacted with the coated substrates. Bound GPVI-Fc_2 was detected as detailed in the Methods. Results, presented as the mean ± SEM, confirm that mFab-F significantly reduces GPVI-Fc_2 binding to D-fragment, D-dimer *(*$P < 0.01$) and collagen ($P < 0.05$). (C) Flow cytometry assessment of fibrinogen and D-dimer binding in normal and GT platelets, with isotype control (IgG1). Washed platelets from the GT patient and a normal donor were incubated with 20 μg mL^{-1} of fibrinogen or D-dimer for 30 min; samples were diluted 10-fold with CBS, pelleted by centrifugation and resuspended in HT; then each sample was incubated with anti-human fibrinogen for 10 min, followed by addition of an excess of Alexa fluor-488–anti-mouse Fab. Samples were measured in an Accuri C6 flow cytometer. The normal platelets show low levels of fibrinogen and D-dimer binding, but the GT platelets show neither, suggesting that integrin αIIbβ3 is mainly responsible for binding to fibrinogen and D-dimer.

adhesion on collagen mainly depends on α2β1, with a lesser contribution by GPVI-dimers, under the present non-cation-depleted conditions [4].

Platelet spreading on immobilized fibrinogen substrates requires GPVI-dimer

Confocal images (Fig. 4A) of platelets labeled for pan-GPVI (Alexa-fluor 488–1G5, green) and P-selectin (Alexa-fluor 647–anti-CD 62p, red) indicate that platelets spread well, with P-selectin expression, on fibrinogen, D-fragment, mFibrin and pFibrin, which shows clear aggregate formation. Little spreading occurred on D-dimer. mFab-F and Dasatinib (Src-kinase inhibitor) cause similar inhibition of platelet spreading on fibrinogen and D-fragment, and although platelets still spread on pFibrin, aggregate formation is obviously reduced. As expected, platelets bind through GPVI on Horm fibers, with surface P-selectin expression, as reported before [31]. Figure 4(B) shows a representative image of immobilized pFibrin fibers, pre-stained as described in the Methods, before platelets were added, with adhered

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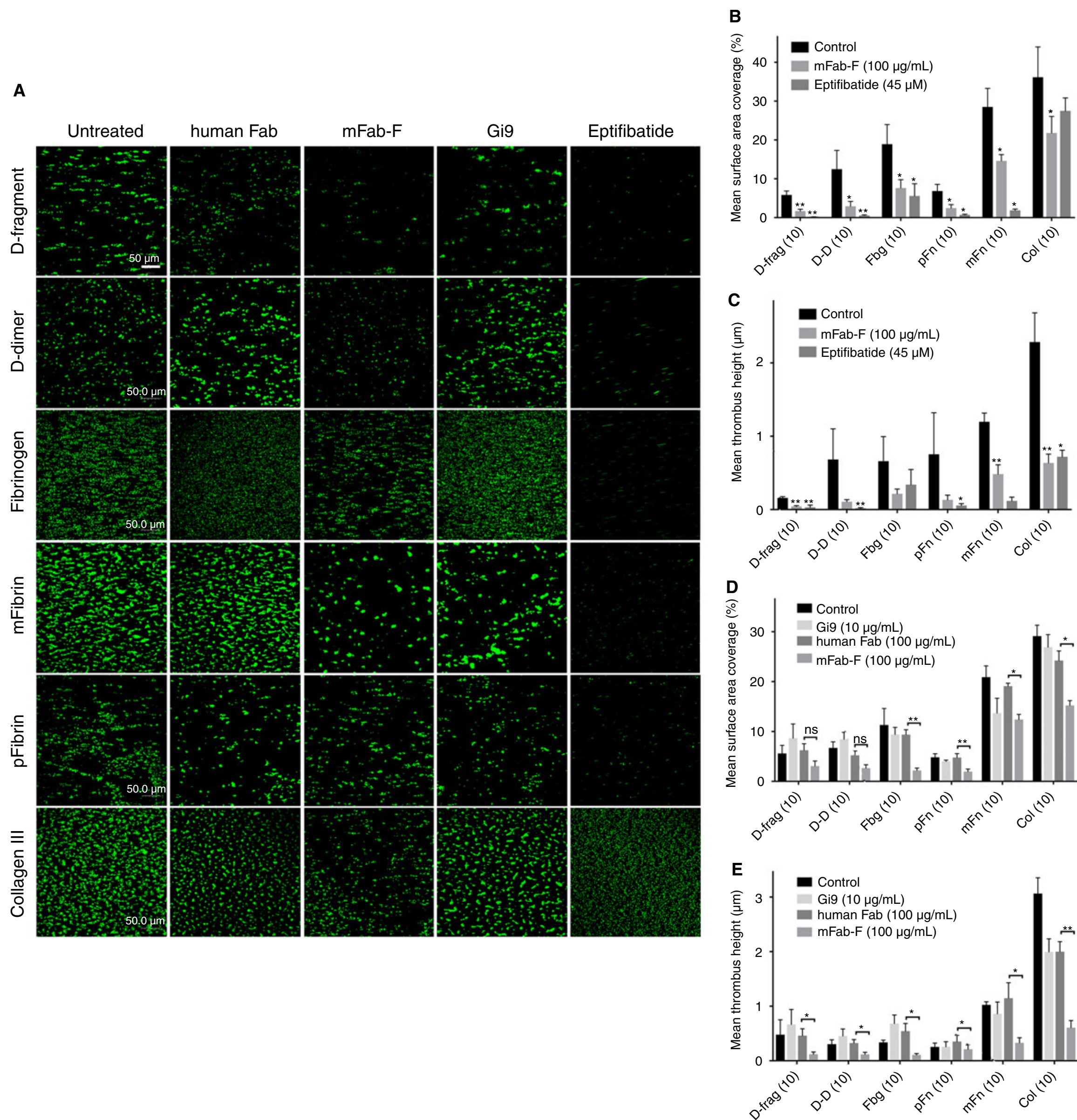

Fig. 6. Adhesion of platelets in whole blood to fibrinogen substrates and collagen under flow conditions. The following abbreviations were used in the graphs (B–E): D-fragment (D-frag), D-dimer (D-D), fibrinogen (Fbg), pFibrin (pFn), mFibrin (mFn) and collagen type III (col). Glass coverslips were coated with fibrinogen (Fbg-3), pFibrin and mFibrin (produced from Fbg-3), D-fragment, D-dimer and collagen type III at 10 µg mL^{-1} in PBS and left overnight at 4 °C. Blood was anticoagulated with 36 µL D-phenylalanyl-prolyl-arginyl chloromethyl ketone (PPACK) and topped up at 1 : 1000 ratio every hour. Platelets in whole blood were fluorescently labelled with 1:4500 DiOC6 (3,3′-dihexyloxacarbocyanine iodide). After 1-min perfusion of flow buffer (136 mM NaCl, 2.7 mM KCl, 5 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 10 mM glucose, 2 mM $MgCl_2$, 2 mM $CaCl_2$, pH 7.4), whole blood was perfused over the coverslips at a shear rate of 350 s^{-1}. Z-stack images were used to calculate surface coverage (SA) of adhered platelets and mean thrombus height (MTH) in panels B–E. (A) Fluorescently labelled whole blood was left untreated (control) or treated with one of the following: human Fab (hFab, 100 µg mL^{-1}), mFab-F (GPVI-dimer specific, inhibitory; 100 µg mL^{-1}), Eptifibatide (αIIbβ3 inhibitor, 45 µM; Tocris Bioscience, UK), Gi9 (anti-α2β1, inhibitory; GeneTex, USA). The images in Panel A are representative of six flow experiments performed with blood from different donors. Statistical significance between control and antibody inhibition (B + C) or human Fab and mFab-F inhibition (D + E) was calculated using paired *t*-tests (*$P < 0.05$, **$P < 0.01$ ***$P < 0.001$). (B + C) Inhibition of αIIbβ3 abrogated SA and MTH to all surfaces. mFab-F reduced platelet adhesion (SA) to all five fibrinogen derivatives (D-fragment, **$P < 0.01$; all others, *$P < 0.05$) and col III (*$P < 0.05$). mFab-F also significantly suppressed MTH on D-fragment, mFibrin and col type III (**$P < 0.01$) and reduced it on D-dimer, fibrinogen and pFibrin compared with the control. This suggests that GPVI-dimer is able to facilitate platelet adhesion and thrombus growth on fibrinogen derivatives, through the D-domain. (C + D) The inhibition of GPVI-dimer with mFab-F compared with the effect of human Fab and anti-α2β1 Gi9 under the same conditions. α2β1 had little to no effect on adhesion to fibrinogen derivatives, and as expected caused some reduction of aggregate formation (MTH) on collagen. mFab-F significantly reduced SA and MTH compared with human Fab on all tested substrates, except SA on D-fragment and D-dimer, which nevertheless tended towards significance ($P = 0.07$ and $P = 0.08$, respectively). [Color figure can be viewed at wileyonlinelibrary.com]

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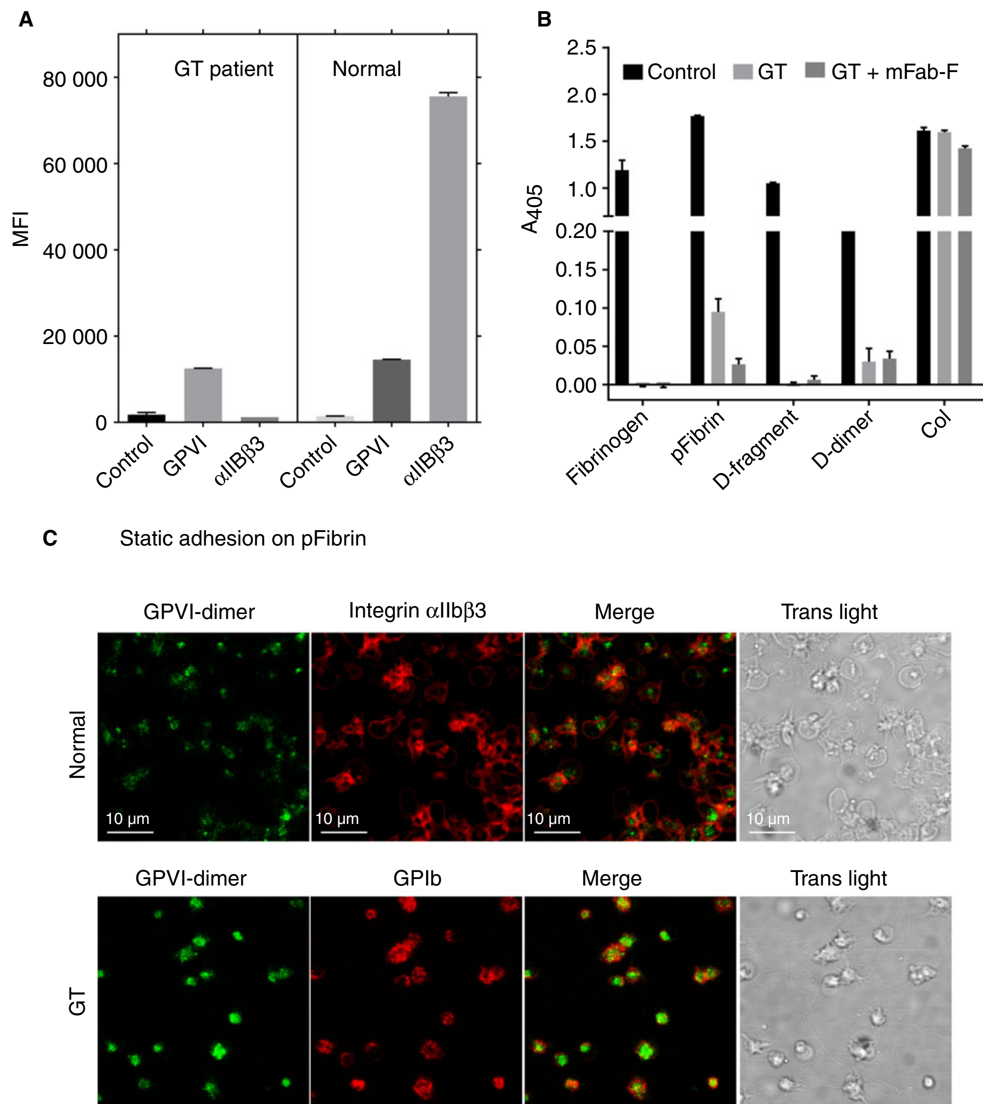

Fig. 7. Static adhesion of platelets from an individual with Glanzmann thrombasthenia (GT) on immobilized fibrinogen substrates. (A) Flow cytometry analysis verified that the GT platelets contain no integrin αIIbβ3, but had normal levels of GPVI. (B) In these experiments, the ELISA wells were coated by adding a 100-μg mL^{-1} solution of each substrate. The GT platelets did not adhere to fibrinogen or D-fragment, but retained the ability to adhere to pFibrin and D-dimer, although this was markedly reduced. mFab-F markedly reduced the adhesion on pFibrin, suggesting that GPVI-dimer may be involved in this interaction. (C) Confocal images of normal platelet and GT platelets adhered to immobilized pFibrin. Both normal and GT platelets were prelabelled with Alexa fluor 488-conjugated 204-11 Fab and allowed to adhere on pFibrin-coated MatTek dishes, for 30 min at 37 °C. The platelets were then fixed and stained with Alexa fluor 647-conjugated antibody specific for integrin αIIbβ3 (normal platelets) or GPIb (GT platelets), as described in the text, before imaging. The GT platelets retain the ability to adhere to pFibrin, although they do not spread; close inspection of the transmitted light images shows that the GT platelets adhere on or close to the fibers of pFibrin. [Color figure can be viewed at wileyonlinelibrary.com]

platelets showing pan-GPVI and surface P-selectin expression.

Both GPVI-dimer and integrin αIIbβ3 contribute to platelet adhesion and thrombus formation on fibrinogen derivatives under flow conditions

Human blood from a healthy donor was perfused over immobilized fibrinogen derivatives or col III (control) at 350 s^{-1} (Fig. 6) and 1000-s^{-1} (Figure S3). Thrombus formation is apparent on all control images (left column, Fig. 6A). Addition of mFab-F (middle column of images, Fig. 6A) decreases both platelet adhesion (SA) (Fig. 6B) and mean thrombus height (MTH) (Fig. 6C) on all tested surfaces. Eptifibatide abrogated platelet adhesion on all four fibrinogen derivatives (right column, Fig. 6A).

Inhibition of α2β1 by mAb Gi9 (10 μg mL^{-1}) only mildly inhibited adhesion on collagen, but not the fibrinogen substrates. Compared with hFab (100 μg mL^{-1}), GPVI-dimer inhibition resulted in reduced SA and MTH

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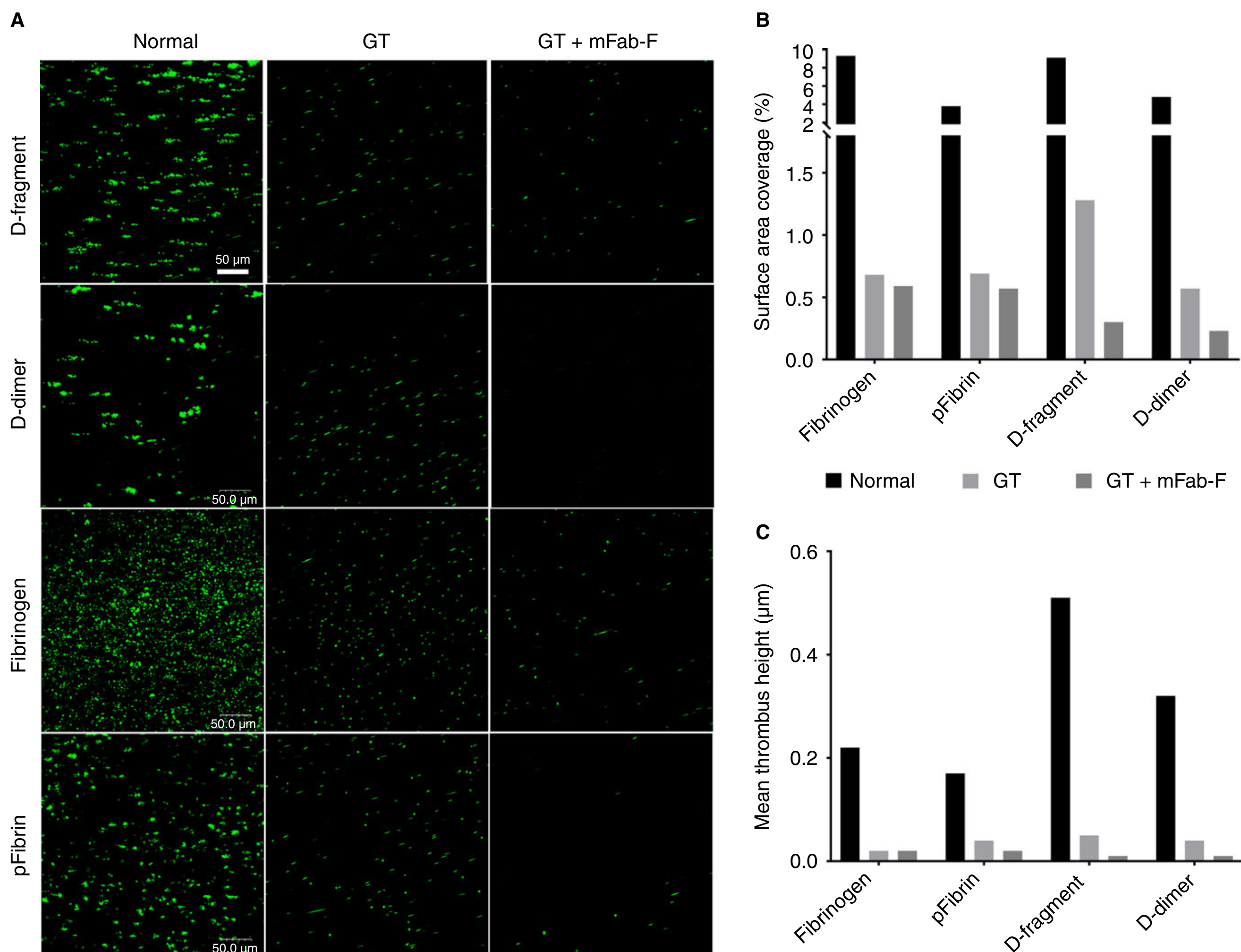

Fig. 8. Analysis of Glanzmann thrombasthenia (GT) platelets under flow conditions. Blood was obtained from an individual with GT, whose platelets lack integrin αIIbβ3. (A) Labelled whole blood was then perfused over surfaces of D-fragment, D-dimer, fibrinogen and pFibrin (slides were coated with 100 μg mL^{-1} of substrate in PBS, overnight at 4 °C). Whole blood was obtained from a healthy volunteer for control measurements. Thrombasthenic blood was perfused with or without the addition of mFab-F (100 μg mL^{-1}) at 350 s^{-1}. GT platelets retain some ability to adhere on all four surfaces, albeit at dramatically reduced levels compared with normal platelets and there is no aggregate formation. mFab-F further reduced the level of adherent GT platelets. Z-stack images were used to calculate SA (B) and MTH (C). For the GT platelets, inhibition of GPVI-dimers by mFab-F causes a further reduction of platelet adherence and activation on D-fragment and D-dimer. [Color figure can be viewed at wileyonlinelibrary.com]

on all fibrinogen derivatives (Fig. 6D and E). All differences were statistically significant apart from mean SA on D-fragment and D-dimer, which was close to reaching significance ($P = 0.07$ and $P = 0.08$, respectively). This confirms that GPVI-dimer contributes to platelet adhesion and thrombus formation on immobilized fibrinogen and fibrin surfaces, although the effect of αIIbβ3 is greater.

Assessing the role of GPVI-dimer in platelets from a patient with Glanzmann's thrombasthenia (GT)

Because platelets contain far fewer GPVI-dimers than integrin αIIbβ3, which may mask the subtler effects of GPVI-dimer, we examined static adhesion and flow adhesion using blood from a GT patient, whose platelets were confirmed to have no αIIbβ3 but a normal GPVI level (Fig. 7A).

Static adhesion of GT platelets: Washed GT platelets showed essentially no adhesion on fibrinogen or D-fragment, but retained a low level of adhesion on pFibrin and D-dimer (Fig. 7B). mFab-F (100 μg mL^{-1}) further diminished the adhesion to pFibrin, suggesting that this low level of interaction involved GPVI-dimer.

Confocal imaging showed that some GT platelets prelabelled with Alexa-fluor 488-conjugated 204-11-Fab (green) and post-labelled with Alexa-fluor 647-conjugated anti-GPIb (red) (Fig. 7C) adhered to pFibrin fibers (Fig. 7C, transmitted light image), but did not spread/aggregate like the normal platelets (post-labelled with Alexa-fluor 647-conjugated anti-αIIbβ3).

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Under flow conditions, adhesion of GT platelets to D-fragment, D-dimer, fibrinogen and pFibrin was dramatically reduced, but platelet adhesion was still evident (Fig. 8A). Quantitation of SA (Fig. 8B) showed that GT platelets retain some ability to adhere to fibrinogen, pFibrin, D-fragment and D-dimer. Their SA on D-fragment and D-dimer was reduced by mFab-F, suggesting GPVI-dimer involvement in this adhesion. MTH for the GT platelets (Fig. 8C) was very low, indicating little platelet activation had occurred. This suggests that the absence of αIIbβ3 does not abolish the interaction between GPVI and fibrinogen substrates, although it appears that its absence impairs the ability of platelets to activate on fibrinogen and fibrin.

Discussion

GPVI-dimer, constitutively present in resting platelets [4], by virtue of its high affinity for collagen, is the functional form of this receptor that initiates signaling leading to thrombogenesis [27]. Recent work has identified GPVI as a fibrin receptor. Mammadova-Bach *et al.* [24] first showed that GPVI promoted thrombin generation through fibrinogen and fibrin-dependent mechanisms and that Fab 9O12 (inhibitory antibody against pan GPVI) [34] reduced platelet adhesion to fibrin and platelet recruitment to formed fibrin-rich thrombi [24]. Subsequently, Alshehri *et al.* concluded that fibrin stimulates tyrosine kinase phosphorylation and platelet spreading through GPVI, independent of αIIbβ3 [25], and Onselaer *et al.* recently reported that GPVI-monomer binds to fibrin [26].

The present study presents the novel finding that GPVI-dimer, not the monomer, binds to immobilized fibrinogen and fibrin through their D-domains. GPVI-dimer binds to the D-domain with high affinity, exhibiting K_d values similar to that for collagen (Fig. 1A, E). We found that GPVI-monomer bound to none of the fibrinogen derivatives (Fig. 1B), confirming a specific interaction between GPVI-dimer and the D-domain. Neither recombinant nor platelet GPVI-dimer binds E-fragment. Interestingly, the ability of collagenous substrates to inhibit GPVI-dimer binding to D-fragment and D-dimer (Fig 2A), as well as the ability of soluble D-dimer to inhibit collagen-induced platelet aggregation (Fig 5A-ii), suggests that the collagen and D-domain binding sites on GPVI-dimer are proximate.

The collagen-binding properties of the GPVI-ectodomain used by the Watson group (Alsheri *et al.* [25]) initially have not been characterized, so we are unable to compare it with our $GPVI_{ex}$. Subsequently, they (Onselaer *et al.* [26]) used recombinant GPVI proteins and concluded that GPVI-monomer selectively binds to fibrin. We found the opposite to be true: only GPVI-dimer binds to the D-domain common to fibrinogen and its derivatives, consistent with the specific binding of the recombinant GPVI-Fc to fibrin reported by Mammadova-Bach *et al.* [24]. These discrepancies would come from the difference between our GPVI recombinant proteins and those used by the Watson group: their recombinant proteins do not include the highly glycosylated, sialic-acid-rich stem portion of GPVI and the lack of such strong anionic charges would influence the binding of their recombinant GPVI to fibrin. To obviate the potential problem caused by the lack of carbohydrate in their constructs, they showed that Revacept, a dimeric GPVI-Fc-fusion protein, also did not bind fibrin. However, Revacept has a different structure from platelet GPVI-dimer and our recombinant dimer because a flexible linker sequence has been inserted between the GPVI-extracellular domain and the Fc portion [35]. This may make its conformation different from that of physiological GPVI-dimer and thereby change its ability to bind to fibrin. It must be highlighted that our antibodies 204-11 Fab and mFab-F, designed against our GPVI-Fc_2, are able to recognize both the constitutive dimers present on resting platelets and those on activated platelets and mFab-F inhibits Horm-induced aggregation, supporting that our GPVI-Fc_2 is similar to physiological platelet GPVI-dimers.

A key finding from our work is that the immobilized nature of the fibrinogen substrates is important for platelet activation through GPVI-dimer. Fibrinogen subdomains in solution with GPVI-dimer cannot inhibit its binding to collagen (Fig. 2B) and GT platelets lacking αIIbβ3 fail to bind fibrinogen and D-dimer in solution (Fig. 5C). Aggregometry confirms that D-fragment, D-dimer and fibrinogen in solution do not cause platelet aggregation (Fig. 5A-i), and pFibrin suspension induces platelet aggregation independent of GPVI-dimer, presumably via αIIbβ3, as mFab-F increased the lag-time, but did not affect the level of maximum aggregation (Figure S2). Together, these results suggest that GPVI-dimer cannot bind to fibrinogen or its subdomains to cause activation unless they are immobilized. This is crucial as platelet aggregation through binding to soluble fibrinogen does not occur in the circulation under normal conditions, unless initial platelet activation has occurred, converting resting αIIbβ3 to a high-affinity state [36].

Although the fibrinogen and its subdomains in solution cannot activate platelets, D-dimer, which is found in plasma at high concentrations under pathological conditions as a fibrinolytic product, is inhibitory towards platelet activation through GPVI. Lee *et al.* [37] reported the ability of soluble, crosslinked fibrin, which contains the D-dimer moiety, to inhibit platelet activation through GPVI-signaling and we and Onselaer *et al.* [26] both found that D-dimer inhibits collagen-induced platelet aggregation.

GPVI-dimer contributes significantly to static platelet adhesion, independently of αIIbβ3, on immobilized D-fragment, pFibrin and mFibrin (Fig. 3). Furthermore, mFab-F inhibition of GPVI-Fc_2 leads to a significant reduction in its binding to D-fragment, D-dimer and col III in ELISA (Fig. 5B). GPVI-dimer not only contributes

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to adhesion but also to spreading on fibrinogen, D-fragment, mFibrin, and pFibrin, and aggregate formation on pFibrin (Fig. 4).

Platelet adhesion to fibrinogen substrates under flow is more complex than measuring direct binding using ELISA, because it involves a dominant role for αIIbβ3 at lower (350 s^{-1}) and higher (1000 s^{-1}) shear, as demonstrated by the abrogation of thrombus formation in the presence of Eptifibatide (Fig. 6, Figure S3). GPVI-dimer, however, appears to play its most significant role at low shear, because mFab-F significantly decreases SA on all tested substrates and MTH is significantly reduced on D-fragment, mFibrin and collagen (Fig. 6, Table S1). At 1000 s^{-1}, GPVI inhibition appears to produce more variable results, which suggests that GPVI-dimer is less able to recognize immobilized D-domain at higher shear. This indicates that αIIbβ3 does play a significant role in platelet interactions with fibrinogen and fibrin in both static (Fig. 3) and shear (Fig. 6) conditions, whereas GPVI-dimer has a secondary but requisite role for inducing platelet adhesion and thrombus formation.

This could be through convergent tyrosine kinase signaling downstream from GPVI and αIIbβ3. For example, the crosslinking of GPVI induces Src-dependent tyrosine phosphorylation of FcRγ [2] and, similarly, signaling mediated by αIIbβ3 also includes tyrosine kinases such as Syk and Src [38,39], with specific blockade reducing the tyrosine phosphorylation of signaling proteins downstream from Syk in both the GPVI and αIIbβ3 signaling pathways [40]. Dasatinib and mFab-F each exert a similar inhibitory effect on platelet adhesion, indicating platelet-adhesion-induced signaling through GPVI-dimer is an important step in platelet spreading and activation on fibrinogen and fibrin (Fig. 4). This is consistent with Onselaer *et al*., who show that fibrin is unable to activate GPVI-deficient human platelets, which is further evidence of shared signaling pathways between GPVI and αIIbβ3 [26].

Under static conditions, αIIbβ3-deficient GT platelets can adhere to pFibrin, but are not activated (Fig. 7). Under flow, the GT platelets show drastically reduced adhesion to D-fragment, D-dimer, fibrinogen and pFibrin, but residual adherent but non-spread platelets remain (Fig. 8). mFab-F reduces GT platelet adhesion on all substrates tested, suggesting that GT platelets can bind independently through GPVI-dimer, even without functional αIIbβ3, but these adhered GT platelets appear neither spread nor activated, suggesting that signaling may not occur without the assistance of αIIbβ3. This means that platelet surface GPVI-dimers may support a weak interaction with immobilized fibrinogen or fibrin, but it is not sufficient to initiate platelet activation. It is more likely that αIIbβ3 and GPVI-dimer have collaborative roles in platelet activation on fibrinogen and fibrin, especially as platelets treated with both mFab-F and Hip8 show a further decrease in adhesion compared with Hip8 alone (Fig. 3).

GPVI antagonism could provide highly specific pharmacological drugs that prevent thrombus formation in acute coronary syndrome [41] and ischemic stroke [42]. This study is the first to describe an interaction between GPVI-dimer and fibrinogen and fibrin. GPVI-dimer binds to the D-domain and is involved in signaling, leading to platelet spreading and activation on fibrinogen substrates, in conjunction with αIIbβ3 and independent of collagen exposure. GPVI-dimer clearly plays a key part in *both* initiation and propagation of thrombosis at sites of atherosclerotic plaque rupture through collagen and fibrin. However, our findings also intimate a role for GPVI-dimer in conditions where blood clots form without collagen exposure, including atrial fibrillation and deep vein thrombosis, making it an even more tantalizing pharmacological target.

Addendum

I. Induruwa designed and performed experiments, including flow adhesion studies, prepared the figures and wrote the manuscript. M. Moroi performed the flow cytometry studies, interpreted data, wrote the manuscript and critically read the manuscript. A. Bonna and J.-D. Malcor synthesized CRP-XL and GPRP. J.-M. Howes performed flow adhesion studies. E. A. Warburton read the manuscript and mentored I. Induruwa. R. W. Farndale discussed the data with us all and critically read the manuscript. S. M. Jung, the corresponding and senior author, designed and performed experiments, interpreted data, prepared the figures and wrote the manuscript.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. The table describes the commercial fibrinogens and fibrinogen derivatives that were examined in our preliminary experiments.

Fig. S2. pFibrin causes platelet aggregation through a mechanism independent of GPVI-dimer.

Fig. S3. Adhesion of platelets in whole blood under flow conditions using a shear rate of 1000 s^{-1}.

Table S1. Comparing thrombus formation at low (350 s^{-1}) and high shear (1000 s^{-1}).

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